

**Effects of Adenosine Receptor Agonists of the A₁, A_{2A} and
A₃ Subtypes on the Proinflammatory Activity of Human
Neutrophils *In Vitro***

by

Susanna Salomina Visser

Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

In

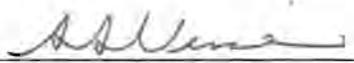
The Department of Immunology
Faculty of Health Sciences
University of Pretoria

April 2002

Declaration

To my knowledge the work contained in this thesis is original, was undertaken by myself with occasional assistance as indicated in the acknowledgements. The interpretation and analysis of data were my primary responsibilities.

It is being submitted for the degree of Doctor of Philosophy at the University of Pretoria. It has not been submitted before for any degree or examination at any other university.

Signed: 

Date : 2002-06-26

Publications

Part of this thesis have been published in the following papers:

1. Visser SS, Theron AJ, Ramafi G, Ker JA & Anderson R. Apparent involvement of the A_{2A} subtype adenosine receptor in the anti-inflammatory interactions of CGS 21680, cyclopentyladenosine, and IB-MECA with human neutrophils. *Biochemical Pharmacology* 2000;60:993-999.
2. Anderson R, Visser SS, Ramafi G & Theron AJ. Accelerated resequestration of cytosolic calcium and suppression of the pro-inflammatory activities of human neutrophils by CGS 21680 *in vitro*. *British Journal of Pharmacology* 2000;130:717-724.

Summary

The apparent insensitivity of neutrophils to the anti-inflammatory effects of corticosteroids underscores the requirement for identifying agents which suppress neutrophil-driven inflammation. In a preliminary study, I was unable to demonstrate an inhibitory effect of glucocorticoids (dexamethasone) on the rapidly-activatable pro-inflammatory functions (superoxide production and elastase release) of human neutrophils activated with FMLP.

My subsequent research was directed at identifying the adenosine receptor (AR) subtypes which down-regulate the pro-inflammatory activities of human neutrophils, as well as the involvement of adenosine 3',5'-cyclic monophosphate (cAMP) and its relationship to cellular handling of Ca^{2+} in mediating these effects. Neutrophils were treated with varying concentrations (0.01 – 1 μ M) of AR agonists operative at A_1 (N^6 -cyclopentyladenosine, CPA), A_{2A} (2(4-[(2-carboxyethyl)phenyl]ethylamino)-5'-N-ethylcarboxamidoadenosine, CGS 21680), and A_3 (N^6 -(3-iodobenzyl-5'-N-methylcarbamoyl)adenosine, IB-MECA) receptors, after which they were activated with the chemoattractant, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 μ M). Intracellular cAMP, superoxide production, and elastase release were assayed using radioimmunoassay, lucigenin-enhanced chemiluminescence (LECL), and colorimetric procedures, respectively, while changes in the concentrations of cytosolic Ca^{2+} were monitored by fura-2-based spectrofluorimetry. CGS 21680, at all concentrations tested, inhibited superoxide production in a dose-related manner, while CPA and IB-MECA were effective only at the highest concentrations tested (0.5 – 1 μ M). The release of elastase from activated neutrophils was also inhibited by all three AR agonists, but was more sensitive to CGS 21680 and IB-MECA than was superoxide production. The inhibitory effects of all 3 agonists on superoxide production and elastase release were associated with accelerated clearance of Ca^{2+} from the cytosol of activated neutrophils, and were effectively neutralized by pretreatment of the cells with the highly selective $A_{2A}R$ antagonist, ZM 241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl)phenol). Increased cAMP was detected in neutrophils treated with CGS 21680 and IB-MECA (1 μ M). These data support the involvement of the $A_{2A}R$ subtype in the suppression

of superoxide production and degranulation by activated human neutrophils, probably by cAMP-mediated alterations in Ca^{2+} handling.

The involvement of the $\text{A}_{2\text{A}}\text{R}$ subtype in regulating the pro-inflammatory activities of neutrophils as well as the biochemical mechanism thereof, were subsequently investigated. Cytosolic Ca^{2+} fluxes, measured by fura-2 spectrofluorimetry in combination with radiometric procedures which distinguish between net efflux and influx of the cation, in FMLP-activated neutrophils in the presence and absence of CGS 21680, were determined. Treatment of neutrophils with CGS 21680 did not affect the FMLP-activated release of Ca^{2+} from intracellular stores, but resulted in dose-related acceleration of the rate of decline in fura-2 fluorescence, as well as decreases in both efflux and store-operated influx of Ca^{2+} , compatible with enhancement of re-sequestration of the cation by the endo-membrane Ca^{2+} -ATPase. These effects on neutrophil Ca^{2+} handling were associated with increased intracellular cyclic AMP and with inhibition of oxidant production and release of elastase. In contrast, treatment of neutrophils with ZM 241385 (2.5 μM), prevented the transient increase in cyclic AMP in FMLP-activated neutrophils which was associated with delayed sequestration of incoming Ca^{2+} during store-operated influx. The CGS 21680-mediated reduction of Ca^{2+} efflux from FMLP-activated neutrophils was also antagonized by pretreatment of the cells with ZM 241385 (2.5 μM), as well as by thapsigargin (1 μM), an inhibitor of the endo-membrane Ca^{2+} -ATPase. ZM 241385 also neutralized the cyclic AMP-elevating and anti-inflammatory interactions of CGS 21680 with neutrophils.

In conclusion, my results are compatible with a role for the $\text{A}_{2\text{A}}\text{R}$ in regulating the pro-inflammatory activities of human neutrophils by promoting cyclic AMP-dependent sequestration of cytosolic Ca^{2+} .

Keywords:

Adenosine; $\text{A}_{2\text{A}}$ receptors; calcium; calcium influx; calcium efflux; CGS 21680; CPA; cyclic AMP; dexamethasone; elastase; IB-MECA; neutrophils; superoxide; ZM 241385.

Samevatting

Die klaarblyklike ongevoeligheid van neutrofiele vir die anti-inflammatoriese effekte van kortikosteroïde beklemtoon die behoefte om agense te identifiseer wat neutrofiel-gedrewe inflammasie onderdruk. Ek was nie in staat om in 'n voorafgaande studie 'n onderdrukkende effek van glukokortikoïde op die snel-aktiveerbare pro-inflammatoriese funksies (superoksied produksie en elastase vrystelling) van menslike neutrofiele wat geaktiveer was met FMLP, aan te toon nie.

My daaropvolgende navorsing was gemik op identifisering van die adenosien reseptor (AR) subtypes wat die pro-inflammatoriese aktiwiteit van menslike neutrofiele af-reguleer, asook die betrokkenheid van adenosien 3',5'-sikliese monofosfaat (sAMF) en sy verhouding tot sellulêre hantering van Ca^{2+} in die bemiddeling van hierdie effekte. Neutrofiele is behandel met verskillende konsentrasies (0.01 – 1 μ M) van AR agoniste wat werkbaar is op A_1 (N^6 -cyclopentyladenosine, CPA), A_{2A} (2-[4-[(2-carboxyethyl) phenyl]ethylamino]-5'-N-ethylcarboxamidoadenosine, CGS 21680), en A_3 (N^6 -93-iodobenzyl-5'-N-methylcarbamoyladenosine, IB-MECA) reseptore, waarna hulle aktiveer is met die chemolokmiddel, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 μ M). Intracellulêre sAMF, superoksied produksie, en elastase vrystelling is bepaal met radio-immuunbepaling, lucigenin-versterkte chemiluminesensie (LECL), en kolorimetriese prosedures, respektiewelik, terwyl veranderinge in die konsentrasies van sitosoliese Ca^{2+} moniteer is met fura-2-baseerde spektrofluorimetrie. CGS 21680, het by alle konsentrasies getoets, superoksied produksie inhibeer op 'n dosis-afhanklike wyse, terwyl CPA en IB-MECA slegs effektief was in die hoogste konsentrasies getoets (0.5-1 μ M). Die vrystelling van elastase vanaf geaktiveerde neutrofiele is ook inhibeer deur al drie AR agoniste, maar was meer gevoelig vir CGS 21680 en IB-MECA as wat superoksied produksie was. Die inhibitoriese effekte van al drie agoniste op superoksied produksie en elastase vrystelling het gepaard gegaan met versnelde opruiming van Ca^{2+} vanaf die sitosol van geaktiveerde neutrofiele, en was doeltreffend neutraliseer deur vooraf behandeling van die selle met die hoogs selektiewe A_{2A} antagonis, ZM 241385 (4-(2-[7-amino-2-(2-furyl) [1,2,4] triazolo [2,3-a] [1,3,5] triazin-5yl amino] ethyl) phenol). Verhoogde sAMF is gevind in

neutrofiële wat behandel was met CGS 21680 en IB-MECA ($1 \mu\text{M}$). Hierdie bevindings ondersteun die betrokkenheid van die $A_{2A}R$ sub tipe in die onderdrukking van superoksied produksie en degranulasie van geaktiveerde neutrofiële, waarskynlik via sAMF-bemiddelde veranderinge in Ca^{2+} hantering.

Die betrokkenheid van die $A_{2A}R$ sub tipe in die regulering van die pro-inflammatoriese aktiwiteite van neutrofiële asook die biochemiese meganisme daarvan, is vervolgens ondersoek. Sitosoliese Ca^{2+} vloeïings, bepaal met fura-2 spektrofluorimetrie in kombinasie met radiometriese bepalinge wat onderskei tussen netto uitvloeïing en invloeïing van die kation, is bepaal in FMLP-geaktiveerde neutrofiële in die teenwoordigheid en afwesigheid van CGS 21680. Behandeling van neutrofiële met CGS 21680 het nie die FMLP-geaktiveerde vrystelling van Ca^{2+} vanaf intrasellulêre store beïnvloed nie, maar het gelei tot dosis-verwante versnelling van die tempo van afname in fura-2 fluoressensie, asook afname in beide uitvloeïing en stoor-beheerde invloeïing van Ca^{2+} , verenigbaar met versterking van hersekwestrasie van die kation deur die endomembraan Ca^{2+} -ATPase. Hierdie effekte op neutrofiel Ca^{2+} hantering het gepaard gegaan met verhoogde intrasellulêre sikliese AMF en met inhibisie van oksidant produksie en vrystelling van elastase. In teenstelling, behandeling van neutrofiële met die selektiewe $A_{2A}R$ antagonis, ZM 241385 ($2.5 \mu\text{M}$), het die verbygaande styging in sikliese AMF in FMLP-geaktiveerde neutrofiële voorkom en het gepaard gegaan met vertraagde sekwestrasie van inkomende Ca^{2+} tydens stoor-beheerde invloeïing. Die CGS 21680-bemiddelde vermindering van Ca^{2+} uitvloeïing vanaf FMLP-geaktiveerde neutrofiële is ook teengewerk deur vooraf-behandeling van die selle met ZM 241385 ($2.5 \mu\text{M}$), asook deur thapsigargin ($1 \mu\text{M}$), 'n inhibitor van die endomembraan Ca^{2+} -ATPase. ZM 241385 het ook die sikliese AMF-verhogende en anti-inflammatoriese interaksies van CGS 21680 met neutrofiële geneutraliseer.

Ten slotte is my resutate verenigbaar met 'n rol vir die $A_{2A}R$ in die regulering van die pro-inflammatoriese aktiwiteite van menslike neutrofiële deur bevordering van sikliese AMF-afhanklike sekwestrasie van sitosoliese Ca^{2+} .

Sleutelwoorde:

Adenosien; A_{2A} reseptore; kalsium; kalsium invloei; kalsium uitvloei; CGS 21680; CPA; dexamethasone; elastase; IB-MECA; neutrofiële; superoksied; sikliese AMP; ZM 241385.

Acknowledgements

I want to express my gratitude to the following people for their assistance with the laboratory research and preparation of this thesis:

My supervisor, Professor Ronald Anderson, Head, Department of Immunology, University of Pretoria, who gave me the opportunity to do this research, inspired me throughout the duration and generously gave of his expertise and time.

My co-supervisor, Professor Annette Theron, Department of Immunology, University of Pretoria, for her guidance, encouragement and support.

Professor James Ker, Department of Internal Medicine, University of Pretoria, who made it possible for me to do the research at the Department of Immunology.

Ms Riana Cockeran for her assistance with the laboratory work (IL-8 measurement).

Dr Grace Ramafi for her assistance with the laboratory work (cAMP determination).

Ms Martie Madgwick for her valuable assistance in the preparation of this manuscript.

The friendly and always helpful librarians, Ms Myleen Oosthuizen and Ms Martie van der Walt, for assisting me in the preparation of the literature study.

Table of Contents

Summary	iv
Samevatting	vi
Acknowledgements	ix
Table of Contents	x
List of Figures	xiv
List of Tables	xvi
List of Abbreviations	xix
Chapter 1: Introduction and Literature Review	1
1.1 Introduction	2
1.2 Literature Review	2
1.2.1 Origins of human neutrophils	2
1.2.2 Neutrophil granules	5
1.3 Neutrophil Functions	8
1.3.1 Extravasation and chemotaxis	10
1.3.2 Adherence to vascular endothelium	10
1.3.3 Tethering and rolling	10
1.3.4 Firm adhesion	12
1.3.5 Transendothelial migration	13
1.3.6 Migration of neutrophils within interstitial tissues	13
1.4 Antimicrobial Mechanisms of Neutrophils	14
1.4.1 NADPH oxidase	14
1.4.2 Nitric oxide synthase	20
1.4.3 Cytokine production by neutrophils	21
1.5 Phospholipase A₂-Derived Mediators of Inflammation	24
1.6 Calcium Fluxes and Restoration of Calcium Homeostasis in Activated Neutrophils	25
1.6.1 Release of calcium from stores	25
1.6.2 Restoration of calcium homeostasis	26
1.7 Anti-Inflammatory Actions of cAMP	28
1.7.1 Cyclic AMP and neutrophils	28

1.8	Neutrophil-Directed, Anti-Inflammatory Chemotherapeutic Strategies	29
1.8.1	Corticosteroids	30
1.8.2	Limitations of corticosteroids	32
1.8.3	Neutrophils and corticosteroids	33
1.9	Adenosine and Neutrophils	39
1.9.1	Adenosine effects on neutrophil function	39
1.9.2	Adenosine and polypeptide mediators of inflammation	43
1.9.3	Adenosine receptors (ARs)	43
1.10	Hypothesis	48
1.11	Objectives	48
 Chapter 2: Effects of Dexamethasone on the Early- and Late-Activatable Pro-Inflammatory Functions of Human Neutrophils		 49
2.1	Introduction	50
2.2	Materials and Methods	51
2.2.1	Chemicals and reagents	51
2.2.2	Neutrophils	51
2.2.3	Oxidant generation	51
2.2.4	Elastase release	53
2.2.5	Interleukin-8 production by neutrophils	54
2.2.6	Statistical analysis	54
2.3	Results	55
2.3.1	Effects of dexamethasone on superoxide production by neutrophils	55
2.3.2	Effects of dexamethasone on the release of elastase by neutrophils	55
2.3.3	Effects of dexamethasone on the release of IL-8 from unstimulated and FMLP-activated neutrophils	55
2.4	Discussion	57
 Chapter 3: Apparent Involvement of the A_{2A} Subtype Adenosine Receptor in the Anti-Inflammatory Interactions of CGS 21680, Cyclopentyladenosine and IB-MECA with Human Neutrophils		 61
3.1	Introduction	61

3.2	Materials and Methods	63
3.2.1	Adenosine receptor agonists	63
3.2.2	Neutrophils	64
3.2.3	Oxidant generation	64
3.2.4	Elastase release	65
3.2.5	Intracellular cAMP levels	65
3.2.6	Spectrofluorimetric measurement of Ca ²⁺ fluxes	66
3.2.7	IL-8 production	66
3.2.8	Statistical analysis	67
3.3	Results	67
3.3.1	Oxidant production	67
3.3.2	Elastase release	68
3.3.3	CAMP	71
3.3.4	Fura-2 fluorescence	73
3.3.5	IL-8 production	76
3.4	Discussion	77
 Chapter 4: Accelerated Resequestration of Cytosolic Calcium and Suppression of the Pro-Inflammatory Activities of Human Neutrophils by CGS 21680 <i>In Vitro</i>		80
4.1	Introduction	81
4.2	Materials and Methods	82
4.2.1	Drugs and reagents	82
4.2.2	Neutrophils	82
4.2.3	Spectrofluorimetric measurement of Ca ²⁺ fluxes	83
4.2.4	Mn ²⁺ quenching of fura-2 fluorescence	83
4.2.5	Radiometric assessment of Ca ²⁺ fluxes	84
4.2.6	Efflux of ⁴⁵ Ca ²⁺ from FMLP-activated neutrophils	84
4.2.7	Influx of ⁴⁵ Ca ²⁺ into FMLP-activated neutrophils	85
4.2.8	Oxidant generation	85
4.2.9	Elastase release	86
4.2.10	Intracellular cAMP levels	86
4.2.11	Statistical analysis	87
4.3	Results	87
4.3.1	Fura-2 fluorescence responses of FMLP-activated neutrophils	87
4.3.2	Influx of Ca ²⁺ using Mn ²⁺ quenching of fura-2 fluorescence	92
4.3.3	Efflux of ⁴⁵ Ca ²⁺ from FMLP-activated neutrophils	92
4.3.4	Influx of ⁴⁵ Ca ²⁺ into FMLP-activated neutrophils	96
4.3.5	Superoxide generation and elastase release	96
4.3.6	Intracellular cAMP	100

4.4 Discussion	101
Chapter 5: Concluding Comments	106
Chapter 6: References	111

List of Figures

	Page
Figure 3.1: The effects of varying concentrations of CGS 21680, CPA and IB-MECA on the production of superoxide by FMLP-activated neutrophils.	69
Figure 3.2: The effects of varying concentrations of CGS 21680, CPA, and IB-MECA on the release of elastase from FMLP/CB-activated neutrophils.	72
Figure 3.3: FMLP-activated fura-2 responses of neutrophils with and without CGS 21680, CPA, or IB-MECA (all at 1 μ M).	74
Figure 4.1: FMLP-activated fura-2 fluorescence responses of control and CGS 21680 (1 μ M)-treated neutrophils.	88
Figure 4.2: FMLP (1 μ M)-activated fura-2 fluorescence responses of control and ZM 241385 (2.5 μ M)-treated neutrophils.	91
Figure 4.3: FMLP (1 μ M)-activated Mn^{2+} quenching of the fura-2 responses of control and CGS 21680 (1 μ M)-treated neutrophils	93
Figure 4.4: Kinetics of efflux of $^{45}Ca^{2+}$ out of unstimulated neutrophils and neutrophils activated with FMLP (1 μ M) in the absence and presence of CGS 21680 (1 μ M).	94
Figure 4.5: Kinetics of influx of $^{45}Ca^{2+}$ into unstimulated neutrophils and neutrophils activated with FMLP (1 μ M) in the absence and presence of CGS 21680 (1 μ M).	97

Figure 4.6: The effects of varying concentrations of CGS 21680 (0.01-1 μM) on superoxide production by FMLP (0.1 μM)-activated neutrophils and on elastase release from FMLP/CB-activated neutrophils. 98

List of Tables

	Page
Table 1.1: Constituents of neutrophil primary and secondary granules	7
Table 1.2: Constituents of neutrophil tertiary granules and secretory vesicles	9
Table 1.3: Cytokines expressed by neutrophils	22
Table 1.4: Agents which trigger cytokine production by neutrophils	23
Table 1.5: Anti-Inflammatory agents which are currently used or are under investigation for the treatment of asthma	30
Table 1.6: Summary of reports on the effects of corticosteroids on early-activatable neutrophil functions <i>in vitro</i>	34
Table 1.7: Summary of reports on the effects of corticosteroids on early-activatable neutrophil functions <i>in vivo</i>	37
Table 2.1: The effects of dexamethasone 1.25, 2.5, 5 and 10 μ M on superoxide generation by FMLP-activated neutrophils	56
Table 2.2: The effects of dexamethasone 10 μ M on superoxide production by PMA activated neutrophils	56
Table 2.3: Superoxide production by the hypoxanthine (1 mM)-xanthine oxidase (18 milliunits/ml) system in the presence and absence of dexamethasone	57
Table 2.4: The effects of dexamethasone on release of elastase from FMLP/CB-activated neutrophils	57

Table 2.5:	The effects of dexamethasone on the spontaneous and FMLP-activated synthesis of IL-8	58
Table 3.1:	The effects of ZM 241385 on CGS 21680-, CPA-, and IB-MECA-mediated inhibition of neutrophil superoxide generation and elastase release.	70
Table 3.2:	The effects of low concentrations (0.1 and 0.25 μ M) of ZM 241385 on CGS 21680-, CPA-, and IB-MECA-mediated inhibition of neutrophil superoxide production.	70
Table 3.3:	The effects of CGS 21680 and IB-MECA individually and in combination with ZM 241385 on cAMP in unstimulated and FMLP-activated neutrophils.	72
Table 3.4:	Peak intracellular calcium concentrations $[Ca^{2+}]_i$ and time taken for these to decline to half peak values in FMLP-activated neutrophils treated with the adenosine receptor agonists.	75
Table 3.5:	The effects of ZM 241385 on peak intracellular calcium concentrations $[Ca^{2+}]_i$ and time taken for these to decline to half peak values in FMLP-activated neutrophils with and without the adenosine receptor agonists.	75
Table 3.6:	The effect of CGS (1 μ M) on IL-8 production by unactivated and FMLP-activated human neutrophils.	76
Table 4.1:	Effects of CGS 21680 on peak cytosolic calcium concentrations ($[Ca^{2+}]_i$) and rates of clearance (half peak values) in FMLP-activated neutrophils.	90
Table 4.2:	Effects of CGS 21680 \pm ZM 241385 on peak intracellular calcium concentrations $[Ca^{2+}]_i$ and rates of clearance in FMLP-activated neutrophils.	90

Table 4.3:	Effects of varying concentrations of CGS 21680 on the efflux of $^{45}\text{Ca}^{2+}$ out of FMLP-activated neutrophils.	95
Table 4.4:	Effects of thapsigargin and ZM 241385 on the CGS 21680-mediated reduction in efflux of $^{45}\text{Ca}^{2+}$ out of FMLP-activated neutrophils.	95
Table 4.5:	Effects of thapsigargin and ZM 241385 on CGS 21680-mediated inhibition of superoxide production by, and elastase release from activated neutrophils.	99
Table 4.6:	The effects of CGS 21680 and ZM 241385 individually and in combination on cAMP levels in unstimulated and FMLP-activated neutrophils.	100

List of Abbreviations

A	adenosine
AA	arachidonic acid
A1	member of family of apoptotic regulators
A ₁ R	adenosine-1 receptor
A _{2A} R	adenosine-2A receptor
A _{2B} R	adenosine-2B receptor
A ₃ R	adenosine –3 receptor
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANCA	anti-neutrophil cytoplasmic antibody
ANOVA	analysis of variance
AP-1	activator protein-1
Apo1	apoptosis receptor-1
AR	adenosine receptor
ARDS	adult respiratory distress syndrome
ARF	ADP-ribosylation factor
ATP	adenosine triphosphate
AV	atrio-ventricular
BH1,2	anti-apoptotic regions in the Mcl-1 gene
BH3	pro-apoptotic region in the Mcl-1 gene
BPI	Bactericidal permeability increasing protein
C	Complement
cAMP	adenosine 3',5'-cyclic monophosphate
([Ca ²⁺] _i)	cytosolic free Ca ²⁺
CB	cytochalasin B
CBP	CREB binding protein
CD30L	CD30 ligand
Ced-9	Caenorhabditis elegans cell death gene
cGMP	cyclic guanosine monophosphate

CGS 21680	2(4-[(2-carboxyethyl)phenyl]ethylamino)-5'-N-ethylcarboxamidoadenosine
CINC	cytokine-induced chemoattractants
COX2	cyclooxygenase 2
CPA	N ⁶ -cyclopentyladenosine
cPLA ₂	cytosolic phospholipase A ₂
CREB	cyclic AMP response element binding factor
DMA	N,N-dimethylacetamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EP receptor	Prostaglandin E receptor
FAD	flavine-adenine-dinucleotide
Fas	CD 95 or Apo1
FasL	Fas ligand
FMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
FURA-2/AM	1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl] -2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N',-tetraacetic acid-acetoxy methylester oil
GC	glucocorticoid
G-CSF	granulocyte-colony stimulating factor
GDP	guanosine diphosphate
GM-CSF	Granulocyte macrophage-colony stimulating factor
gp91 ^{phox}	glycoprotein phagocyte oxidase, 91 kDa molecular weight.
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRO- α	growth-related gene product-alpha
GRO- β	growth-related gene product-beta
GTP	guanosine triphosphate
HAT	histone acyltransferase
HBSS	Hanks' balanced salt solution
HCl	hydrochloric acid
HGF	hepatocyte growth factor
HNP1,2,3	human neutrophil peptides 1,2 and 3

H ₂ O ₂	hydrogen peroxide
HOCl	hypochlorous acid
Hsp	heat shock protein
IB-MECA	N ⁶ -3-iodobenzyl-5'-N-methylcarbamoyladenine
ICAM	intercellular adhesion molecule
IFN-γ	interferon-gamma
IFN-β	interferon-beta
Iκ-B-α	inhibitor protein-kappa B-alpha
IL	interleukin
IL-1Ra	interleukin-1 receptor antagonist
iNOS	inducible nitric oxide synthase
JAM	junctional adhesion molecule
kDa	kiloDalton
LECL	lucigenin-enhanced chemiluminescence
LPC	lysophosphatidylcholine
LPS	lipopolysaccharide
LTB ₄	leukotriene B ₄
LTC ₄	leukotriene C ₄
LTD ₄	leukotriene D ₄
LTE ₄	leukotriene E ₄
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid cell leukaemia gene product, member of family of apoptotic regulators
Mcl-1S/Δ TM	isoform of Mcl-1 with pro-apoptotic characteristics
MCP-1,2,3	monocyte chemotactic protein-1,2,3
M-CSF	macrophage-colony stimulating factor
MIP-1α	macrophage infiltrating protein-1alpha
MIP-1β	macrophage infiltrating protein-1beta
MnCl ₂	Manganese chloride
MPO	myeloperoxidase
mRNA	messenger RNA

NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NADP	nicotinamide adenine dinucleotide phosphate
NECA	5'-N-ethylcarboxamidoadenosine
NF- κ B	nuclear factor-kappa B
nGRE	negative glucocorticoid response element
NO	nitric oxide
NSF	N-ethylmaleimide-sensitive fusion
O ₂ ⁻	superoxide anion
•OH	hydroxyl radical
OSM	oncostatin
p300	300 kDa polypeptide
PAF	platelet-activating factor
PAPA-APEC	2-[4-[2-[2-[phenylmethylcarbonylamino]ethylaminocarbonyl]ethyl]phenyl]ethylamino-5'-N-ethyl-carboxamidoadenosine
PBS	phosphate-buffered saline
PDE	phosphodiesterase
PECAM	platelet endothelial cell adhesion molecule
PGE ₂	prostaglandin E ₂
PKA	protein kinase A
PKC	protein kinase C
PLA ₂	phospholipase A ₂
PLC	phospholipase C
PMA	phorbol-12-myristate 13- acetate
PMN	polymorphonuclear leucocyte
P22 ^{phox}	protein/polypeptide phagocyte oxidase, 22 kDa molecular weight
P40 ^{phox}	protein/polypeptide phagocyte oxidase, 40 kDa molecular weight
P47 ^{phox}	protein/polypeptide phagocyte oxidase, 47 kDa molecular weight
P67 ^{phox}	protein/polypeptide phagocyte oxidase, 67 kDa molecular weight

Rac2	ribosome-associated complex, a member of the Ras superfamily
Raf	a serine kinase linking Ras activation with the nucleus
RANTES	“regulated on activation, normal T-cell expressed and secreted” chemokine
Rap1A	a member of the Ras superfamily
Ras	rat sarcoma gene product, a superfamily of GTP-binding proteins
Rho	a family of G-proteins
RNA	ribonucleic acid
SCF	stem cell factor
SEM	standard error of the mean
SNARES	soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor
SNAP-23	synaptosomal-associated protein of 23 kDa.
STATs	signal transducers and activators of transcription
STZ	serum-treated zymosan
TGF- α , β	tumour growth factor-alpha, beta
TNF- α	Tumour necrosis factor-alpha
TPA	12-O-tetradecanoylphorbol-13-acetate
t-SNARES	target plasma membrane protein-SNARES
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLA-2,5,6,9	very late activation protein-2,5,6,9
v-SNARES	vesicle proteins-SNARES
ZM 241385	4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5yl amino]ethyl)phenol

1.1 Introduction

The laboratory research described in this thesis was undertaken with the primary objective of identifying novel pharmacologic strategies for the control of the pro-inflammatory activities of human neutrophils *in vitro*, with particular emphasis on adenosine receptor agonists. The opening chapter is devoted to a literature review which is focussed on i) the pro-inflammatory activities of human neutrophils (including the recently-documented ability of these cells to synthesise pro-inflammatory cytokines *de novo*) ii) the involvement of calcium (Ca^{2+}) in initiating these activities iii) the anti-inflammatory spectrum, molecular mechanisms of action and limitations of corticosteroids and iv) the sub-types and distribution of adenosine receptors, particularly with respect to human neutrophils and their functions. This is followed by two chapters in which the effects of corticosteroids (chapter 2), as well as those of adenosine receptor agonists operative at the level of the A_1 , A_{2A} and A_3 adenosine receptor sub-types (chapter 3) on pro-inflammatory activities of neutrophils are described. In chapter 4, the effects of an adenosine receptor agonist (CGS 21680) operative at the level of the sub-type A_{2A} receptor on Ca^{2+} fluxes and restoration of Ca^{2+} homeostasis in activated neutrophils are described, while an integrated assessment of the data is presented in the final chapter. Each chapter of results is a distinct entity with its own introduction, materials and methods, results and conclusions sections.

1.2 Literature Review

1.2.1 Origins of human neutrophils

Blood is the most readily obtained source of neutrophils and serves as the vehicle for their delivery to the various tissues from the bone marrow where they are produced. Neutrophils evolve from pluripotent stem cells under the influence of cytokines and colony stimulating factors. It is estimated that 400-500 stem cells are necessary to support haematopoiesis. The haematopoietic system not only produces enough neutrophils (1.3×10^{11} cells per 80 kg person per day) to carry out physiologic functions, but also has large reserve stores in the marrow which can be mobilised in

response to inflammation and infection. Approximately 8-14 days are required for a cell to move through the sequence of 4-6 cell divisions and complete maturation. After cell division is completed there are 3-4 days of neutrophil maturation. During this time the maturing cells can be released from the bone marrow into the blood under conditions of sufficient stress. These cells comprise the marrow neutrophil reserve and are 10 times as numerous as the neutrophils in the blood (Bainton, 1992).

Specific signals, including IL-1, IL-3, TNF- α , colony stimulating factors, the complement factors C3e, C5a and other cytokines mobilise neutrophils from the bone marrow which circulate in the unstimulated state. Under normal conditions 90% of the neutrophil pool is in the bone marrow, 2-3% in the circulation and the rest in the tissues. Up-regulation of the production of these signals during inflammatory stress increases the tempo of production and release of neutrophils from the bone marrow (Bainton, 1992; Goldstein, 1992; Greene, 1992).

The myeloblast is the first recognisable precursor cell and is followed by the promyelocyte which is characterised by the appearance of the classical lysozomal granules, known as primary or azurophilic granules. The promyelocyte divides and differentiates into the myelocyte, which, in addition to the primary granules, also contains secondary or specific granules (Bainton, 1992).

Following the myelocyte stage no further division takes place and during the final stages of maturation the cell passes through the metamyelocyte and then the band neutrophil phases. On maturation of the band form the nucleus becomes lobulated, consisting of up to four segments. The physiologic role of the multilobed nucleus is unclear, but may allow greater deformability during migration into tissue at sites of inflammation.

The circulating neutrophil pool exists in two dynamic compartments, freely flowing and marginated. The freely flowing pool consists of half of the neutrophils in the basal state and is composed of those cells that are in the blood and not in contact with the endothelium. Marginated neutrophils are in close physical contact with the endothelium. In the pulmonary circulation margination occurs because the

capillaries have about the same diameter as the mature neutrophils. Neutrophil fluidity and deformability are necessary for transit through the pulmonary bed. In the systemic post capillary venules, margination is mediated by chemoattractants and adhesion molecules by mechanisms which are described in later sections (1.3.1 and 1.3.2).

The lifespan of the neutrophil is estimated to be 24-48 hours (Bainton, 1992), after which the neutrophils undergo apoptosis (programmed cell death) and removal by the mononuclear phagocyte system, a process which is dependent on interactions between phosphatidylserine translocated from the inner to the outer plasma membrane of the apoptotic neutrophil and phosphatidylserine receptors on monocytes/macrophages (Fadok *et al.*, 1998). Interactions between Fas ligand (FasL) on macrophages, as well as soluble FasL released by these cells, and the Fas "death receptor" on neutrophils may represent a mechanism of resolution of inflammation (Brown & Savill, 1999). Prolongation of the lifespan of the neutrophil occurs following migration of these cells out of the circulation to sites of inflammation and exposure to anti-apoptotic cytokines such as granulocyte/macrophage colony stimulating factor (GM-CSF) (Watson *et al.*, 1999).

Members of the ced-9-Bcl-2 family of apoptotic regulators have been shown to play a pivotal role in the regulation of apoptosis across multiple cell types and between species. Mediators that prolong the lifespan of neutrophils both *in vivo* and *in vitro* may exert these effects by induction of specific anti-apoptotic members of this family. Two anti-apoptotic members, Mcl-1 and A1, are abundantly expressed in neutrophils. An isoform of Mcl-1, (Mcl-1 S/ Δ TM) is rendered similar to BH3 (containing only pro-apoptotic proteins) by deletion of BH1, BH2 and transmembrane regions. Both forms are expressed in neutrophils and thus co-expression of both pro- and anti-apoptotic isoforms of Mcl-1 may be an important regulatory event in the control of neutrophil apoptosis and interference with this balance may provide a strategy to control neutrophil mediated tissue injury (Bingle *et al.*, 2000).

1.2.2 Neutrophil granules

Notwithstanding the dynamic outer membrane and multilobed nucleus, the abundant cytoplasmic granules are the most striking structural features of neutrophils. These neutrophil granules have emerged as an extremely heterogeneous group of organelles and, according to the simplest classification, consist of four different groups distinguished on the basis of protein content, size and density. These are the primary (azurophil), secondary (specific) and tertiary (gelatinase) granules, and secretory vesicles (Witko-Sarsat *et al.*, 2000). As mentioned previously, primary and secondary granules are formed during the promyelocyte and myelocyte/metamyelocyte stages respectively, while tertiary granules develop at the metamyelocyte/band cell stage (Le Cabec *et al.*, 1996). Secretory vesicles are the last to appear becoming evident in band and segmented cells (Borregard & Cowland, 1997). These different granule sub-types vary with respect to efficiency of mobilisation during neutrophil activation. The order of mobilisation is negatively correlated with the size and density of the granules. Secretory vesicles represent the most mobilisable of the neutrophil granule sub-types (Witko-Sarsat *et al.*, 2000), while the order of exocytosis observed in calcium ionophore (A 23187)-activated neutrophils was secretory vesicles, tertiary, secondary and primary granules, which was correlated with progressive increases in cytosolic Ca^{2+} (Sengelov *et al.*, 1993).

In addition to functioning as a mobilisable reservoir of membrane constituents, these various neutrophil granules contain an array of proteases and antimicrobial peptides and polypeptides (including several proteases) which participate in the migratory and antimicrobial activities of the neutrophil. The fact that several granule polypeptides are shared by the different granule sub-types (eg lysozyme and proteinase 3) is indicative of overlap between these (the granules), which may represent a continuum as opposed to clearly demarcated granule types (Witko-Sarsat *et al.*, 2000).

Primary granules

The majority of neutrophil proteases and antimicrobial peptides/polypeptides are stored in the primary granules. In contradistinction to the other granule sub-types, primary granules, perhaps reflecting their relative resistance to exocytosis, do not function as reservoirs of membrane receptors and polypeptides, although CD63 and

CD68 are present on the membranes of these granules, but the functional significance of this remains to be established (Saito *et al.*, 1991; Cham *et al.*, 1994). Myeloperoxidase and the defensins (a family of at least four peptides), also known as human neutrophil peptides (HNP1 to HNP4), small cationic, broad-spectrum antimicrobial peptides that contain six cysteines in disulphide linkage, are present in extremely high concentrations in primary granules (Elsbach & Weiss, 1992; Witko-Sarsat *et al.*, 2000). The constituents of neutrophil primary granules and their distribution are listed in Table 1.1 (page 7).

Although primary granules have not been considered to act as reservoirs of membrane components involved in the migratory, phagocytic and oxidant-generating activities of neutrophils, a very recent report describing translocation of Sialyl Lewis x from the membranes of primary granules to the plasma membrane during activation of these cells with chemoattractants suggests that this may not be the case (Suzuki *et al.*, 2000). These authors contend that primary granules may contribute to the up-regulation of Sialyl Lewis x, the ligand for the endothelial adhesion molecules, E- and P-selectins, on the cell surface following activation of neutrophils (Suzuki *et al.*, 2000).

Secondary granules

Lactoferrin and vitamin B₁₂-binding protein are unique to secondary granules, which are also notable for their high content of membrane-associated adhesion molecules, receptors for chemoattractants, cytokines and adhesion molecules, and cytochrome b558 (an integral component of the phagocyte superoxide-generating system, NADPH-oxidase). During neutrophil activation, mobilisation of secondary granules to the outer membrane is thought to cause sustained neutrophil activation by augmenting and/or replenishing these various pro-adhesive, -migratory and -oxidative polypeptides. The constituents of neutrophil secondary granules and their distribution are shown in Table 1.1 (page 7).

Tertiary and secretory granules

The tertiary and secretory granules also act as a reservoir for membrane polypeptides involved in neutrophil activation and function, albeit to a considerably lesser extent than secondary granules. Their major contribution to neutrophil

Table 1.1: Constituents of Neutrophil Primary and Secondary Granules

Primary Granules		Secondary Granules	
<i>Membrane</i>	<i>Matrix</i>	<i>Membrane</i>	<i>Matrix</i>
CD63	myeloperoxidase	cytochrome b558	lactoferrin
CD68	elastase	CD11b	vitamin B ₁₂ binding protein
Sialyl Lewis x	cathepsin G	FMLP receptor	lysozyme
	proteinase 3	TNF receptor	collagenase
	defensins	G-protein α -subunit	gelatinase
	azurocidin	laminin receptor	histaminase
	BPI*	fibronectin receptor	heparinase
	secretory PLA ₂	thrombospondin receptor	β_2 -microglobulin
	lysozyme	vitronectin receptor	urokinase plasminogen activator
	β -glucuronidase	urokinase plasminogen activator receptor	
	acid β -glycerophosphatase		
	acid mucopolysaccharide		
	α -mannosidase		
	heparin-binding protein		
	sialidase		
	α_1 -antiprotease inhibitor		
	ubiquitin protein		

* Bactericidal permeability increasing protein

function is thought to be achieved through extracellular release of gelatinase which cleaves types IV (basement membrane) and V (interstitial tissues) collagen, thereby facilitating movement of the cells through basement membranes and into underlying tissue (Witko-Sarsat *et al.*, 2000). Secretory vesicles are thought to be endocytic in origin because they contain plasma proteins such as albumin. Interestingly, proteinase 3, a serine proteinase present in neutrophil primary granules, is also localised in the membrane of secretory vesicles, which are the most mobilisable compartment of neutrophils (Witko-Sarsat *et al.*, 1999). Secretory vesicles may also act as a reservoir of membrane components. The constituents of neutrophil tertiary granules and secretory vesicles are shown in Table 1.2 (page 9).

Secretory phospholipase A₂, a 14-kDa group II PLA₂, is also released by activated human neutrophils, and although of probable granule origin, its exact intracellular location remains to be established (Seeds *et al.*, 1998).

The mechanisms involved in membrane docking and fusion between cytoplasmic granules and the plasma/phagosome membrane appear to involve SNARE (soluble N-ethylmaleimide-sensitive fusion [NSF] factor attachment protein receptor) proteins (Martin-Martin *et al.*, 2000). These proteins have been found to mediate vesicle secretion in essentially all organisms investigated. According to the SNARE hypothesis, docking and fusion of vesicles with the plasma membrane is modulated by the specific interaction of vesicle proteins (v-SNARES) with target plasma membrane proteins (t-SNARES). Secretion of secondary granules appears to involve interaction between SNAP-23 (v-SNARE) on the granule membrane and syntaxin 6 (t-SNARE) on the plasma/phagosome membrane, while mobilisation of primary granules involves interaction of syntaxin 6 with an unidentified v-SNARE (Martin-Martin *et al.*, 2000).

1.3 Neutrophil Functions

With respect to protection against microbial pathogens, neutrophil activation is characterised by adhesion to vascular endothelium, trans-endothelial migration,

Table 1.2

Constituents of Neutrophil Tertiary Granules and Secretary Vesicles

Tertiary Granules		Secretary Vesicles	
Membrane	Matrix	Membrane	Matrix
cytochrome b558	gelatinase	cytochrome b558	plasma proteins
CD11b	lysozyme	CD11b	
FMLP receptor	β_2 -microglobulin	FMLP receptor	
diacylglycerol deacylating enzyme	acetyl transferase	urokinase plasminogen activator receptor	
urokinase plasminogen activator receptor			

chemotaxis, engulfment and intraphagocytic eradication of micro-organisms. Poorly regulated activation of these processes, such as that which may occur during inappropriate, hyperacute or chronic inflammatory responses does, however, present the potential hazard of neutrophil-mediated injury to bystander host cells and tissues. This is due to the largely indiscriminate, albeit highly effective, strategies used by neutrophils to eliminate microbial pathogens.

Recently, neutrophils have been shown to produce a range of pro-inflammatory cytokines, particularly IL-8 and TNF- α , raising the possibility of autocrine activation of these cells during inflammatory processes (Cassatella, 1999; Witko-Sarsat *et al.*, 2000).

1.3.1 Extravasation and chemotaxis

The purposeful movement of neutrophils out of the circulation and subsequent migration to sites of infection is dependent upon a complex, highly coordinated series of events involving localised activation of vascular endothelium, adherence of neutrophils, transendothelial migration and chemotaxis.

1.3.2 Adherence to vascular endothelium

The rapid transition of circulating neutrophils from a non-adherent state to a localised adherent state involves the sequential appearance of several families of adhesion molecules on vascular endothelium which initially retard and then immobilise neutrophils. These events are known as tethering and firm adhesion respectively and precede trans-endothelial migration and chemotaxis (Springer, 1994).

1.3.3 Tethering and rolling

This is the initial event in localised immobilisation of neutrophils on activated vascular endothelium and is mediated by L-selectin on neutrophils and by P- and E-selectins on activated endothelial cells (Witko-Sarsat *et al.*, 2000). L-selectin is constitutively expressed on neutrophils and its binding capacity is rapidly and transiently increased, possibly due to receptor oligomerisation, after activation of these cells (Li *et al.*, 1998).

Although the counter-receptor for L-selectin on vascular endothelium awaits precise characterisation, it has recently been reported that clustering of L-selectin on the neutrophil membrane during activation of these cells leads to recruitment of p38 mitogen-activated protein kinase (MAPK) with resultant alterations in cell shape, activation of β_2 -integrins, reactive oxidant production and release of secondary, tertiary and secretory granules (Smolen *et al.*, 2000).

P-selectin is contained in the Weibel-Palade bodies of vascular endothelial cells and is rapidly (within a few minutes) and transiently mobilised to the endothelial cell surface following stimulation with thrombin, histamine, PAF and reactive oxidants (Witko-Sarsat *et al.*, 2000). P-selectin interacts with Sialyl Lewis x-containing structures on the neutrophil plasma membrane (Suzuki *et al.*, 2000), and these interactions augment β_2 -integrin-mediated adhesion and increase the production of reactive oxidants (Ruchaud-Sparagano *et al.*, 2000).

E selectin appears on endothelial cells only 1-2 hours after exposure to the cytokines IL-1 and TNF- α and interacts with Sialyl Lewis x-containing counter-receptors on the neutrophil surface (Witko-Sarsat *et al.*, 2000). Engagement of E-selectin by neutrophils has been reported to increase β_2 -integrin-mediated adhesion, as well as reactive oxidant production by these cells, possibly by sensitising neutrophils for PAF-mediated mobilisation of intracellular Ca^{2+} (Ruchaud-Sparagano *et al.*, 2000).

The transient nature of selectin-mediated adhesive interactions is crucial because it allows neutrophils to sample the endothelium for the presence of triggers that can activate β_2 -integrins and allow the cascade to proceed. These triggers include chemoattractants such as IL-8, PAF and leukotriene B₄ (LTB₄) which are immobilised on proteoglycans on the luminal surface of endothelial cells (Witko-Sarsat *et al.*, 2000). If the intensity of these signals is weak, then the neutrophils will disengage from the endothelium. High intensity signalling, on the other hand, will result in activation of β_2 -integrins and firm adhesion, resulting in the cells coming to a complete halt and changing shape within seconds, acquiring a flattened, adherent morphology.

1.3.4 Firm adhesion

Firm adhesion of neutrophils to endothelial cells appears to involve exclusively the interaction of integrins of the β_2 sub-group (CD11a, CD11b, CD11c/CD18) on neutrophils with the endothelial adhesion molecules, intercellular adhesion molecules-1 and -2 (ICAM-1 and ICAM-2), in contradistinction to monocytes, eosinophils, and lymphocytes which utilise the α_4/β_1 integrin to bind to vascular cell adhesion molecule-1 (VCAM-1) (Witko-Sarsat *et al.*, 2000). ICAM-2 is constitutively expressed, while ICAM-1 expression is increased on activation with IL-1 and TNF α and predominates in binding to inflamed endothelium.

β_2 -integrins (also known as CR3) are present on the surface of resting neutrophils, but are unable to bind to their physiological ligands, the ligand-binding capacity being acquired upon exposure to activation signals (eg chemoattractants such as PAF, FMLP, LTB $_4$, C5a and IL-8, as well as TNF α and GM-CSF). These chemoattractants and cytokines promote clustering and transition of a sub-population of β_2 -integrins to a high affinity state (Stewart & Hogg, 1996). These various signalling pathways that cause integrins to switch from an inactive to an active conformation differ with the type of pro-inflammatory agonist and are still incompletely characterised. Regulation of avidity of β_2 -integrins involves interaction of both α and β chain cytoplasmic tails with the cytoskeleton (Van Kooyk *et al.*, 1999) and the membrane expression of cytohesin-1, a guanine nucleotide exchange protein that binds to the cytoplasmic portion of CD18 and up-regulates β_2 -integrin avidity (Kolanus *et al.*, 1996; Nagel *et al.*, 1998). In addition, the binding of chemoattractants and pro-inflammatory cytokines to neutrophils sets in motion a cascade of signalling events which ultimately leads to phosphorylation of serine/threonine residues in the cytoplasmic tail of CD18, also causing integrin activation (Capocidi *et al.*, 1998; Ghamberg *et al.*, 1998; Jones *et al.*, 1998; Blouin *et al.*, 1999).

The binding of activated β_2 -integrins to their counter-receptors on vascular endothelium, ICAM-1 and ICAM-2, mediates firm adhesion between neutrophils and endothelium. Firm adhesion is a transient event, and following weakening of integrin-mediated adhesion, neutrophils migrate into the interstitium.

1.3.5 Transendothelial migration

Migration of adherent neutrophils out of the vascular space is most evident at the borders of endothelial cells where discontinuities of tight junctions occur. Several types of adhesive interactions have been implicated in transendothelial migration.

These are:

- P-selectin/Sialyl Lewis x interactions. P-selectin has been demonstrated to be highly expressed along endothelial borders and may focus neutrophil adhesion onto these regions (Burns *et al.*, 1999).
- CD31 (also known as PECAM-1, which, like ICAM-1 and ICAM-2, is a member of the Ig-superfamily) is expressed on the neutrophil surface and at the endothelial junction, and mediates neutrophil transendothelial migration by homophilic (CD31/CD31) interactions. Ligation of CD31 also causes activation of β_2 -integrins (Berman & Muller, 1995).
- junctional adhesion molecule (JAM, also a member of the Ig-superfamily) is also concentrated at inter-endothelial junctions, but is not present on neutrophils. Antibodies to JAM inhibit leukocyte transmigration *in vitro* (Martin-Padura *et al.*, 1998). The counter-receptor for JAM on neutrophils has not yet been characterised.
- induction of β_1 -integrin (CD49/CD29) expression in neutrophils has also recently been shown to be associated with transendothelial migration (Werr *et al.*, 2000a; Werr *et al.*, 2000b). The α_2/β_1 -integrin (CD49b/CD29, VLA-2), which binds to collagen and laminin, is up-regulated following activation of β_2 -integrins (Werr *et al.*, 2000a; Werr *et al.*, 2000b).

1.3.6 Migration of neutrophils within interstitial tissues

Neutrophils migrate in tissues by haptotaxis, which is a process of directed movement along a gradient of immobilised, as opposed to soluble, chemoattractants (Witko-Sarsat *et al.*, 2000). These chemoattractants are bound to extracellular matrix components because of their negative charge, facilitating presentation to corresponding counter-receptors on neutrophils. Locomotion of the cells requires the continuous formation of new adhesive contacts at the cell front, while the rear of the cell detaches from the adhesive substrate (Lauffenburger & Horwitz, 1996). Although β_2 -integrins, acting in concert with β_1 - and β_3 -integrins, have been proposed to be the

major adhesins involved in neutrophil migration through the extracellular matrix (Witko-Sarsat *et al.*, 2000), others have reported that the α_2/β_1 -(VLA-2) integrin is the principal receptor used by neutrophils for locomotion in extravascular tissue (Werr *et al.*, 2000a). This may, however, be an over-simplification, since the α_9/β_1 -(VLA-9) integrin has been reported to be the dominant adhesin involved in the migration of neutrophils through human lung and synovial fibroblast barriers, implying a degree of tissue selectivity with respect to utilisation of neutrophil integrins (Shang *et al.*, 1999).

Interestingly, laminin-, fibronectin- and vitronectin receptors such as α_5/β_1 (VLA-5), α_6/β_1 (VLA-6) and α_v/β_3 are mostly stored in neutrophil granules and are rapidly expressed on the plasma membrane following exposure of the cells to chemoattractants and during transendothelial migration (Roussel & Gingras, 1997).

1.4 Antimicrobial Mechanisms of Neutrophils

Neutrophils utilise oxygen-dependent and -independent mechanisms to eradicate microbial pathogens. The latter have been mentioned briefly elsewhere (1.2.1) and this section will focus only on oxygen-dependent antimicrobial systems.

1.4.1 NADPH oxidase

The superoxide (O_2^-)-generating system of phagocytes, NADPH oxidase, is a multi-component, membrane-associated electron transporter. NADPH oxidase comprises resident membrane components and translocatable cytosolic components which come together in the membrane (outer membrane and phagosome membrane) during neutrophil activation to form the fully functional oxidase. Over the past decade the membrane-bound and cytoplasmic components of the phagocyte NADPH oxidase have been identified, cloned and sequenced and the molecular/biochemical mechanisms by which they are assembled into a functional electron-transporting complex have been partly elucidated.

To date six oxidase components have been described *viz* cytochrome b_{558} which is a membrane-bound heterodimer consisting of $gp91^{phox}$ and $p22^{phox}$ (where p and gp =

protein/polypeptide and glycoprotein respectively and *phox* = phagocyte oxidase, while the numbers = molecular weight in kiloDaltons) which is associated with the GTP-binding protein, Rap1A, and four cytosolic components (p40^{phox}, p47^{phox}, p67^{phox} and Rac2, the latter being a member of the Ras superfamily of GTP-binding proteins of molecular weight approximately 21 kDa as is also the case with Rap1A) (Leusen *et al.*, 1996; Clark, 1999; Segal *et al.*, 2000).

Upon activation of the oxidase by receptor-mediated (chemoattractants, opsonized particles, TNF α) and non-receptor-mediated (phorbol esters, long chain unsaturated fatty acids, calcium ionophores) stimuli of membrane-associated oxidative metabolism, the cytoplasmic components of the oxidase, p40^{phox}, p47^{phox} and 67^{phox} translocate *en bloc* to the plasma/phagosome membrane where they interact with membrane-bound cytochrome b₅₅₈ inducing a conformational change in the oxidase which promotes interaction of NADPH with gp 91^{phox} (Segal *et al.*, 2000). Rac2 translocates independently (Segal *et al.*, 2000). NADPH is then oxidised to NADP⁺ and electrons are transported down a reducing potential gradient to flavin adenine dinucleotide (FAD) and then apparently to the two non-identical haem groups of cytochrome b₅₅₈. The final step in the electron transport chain occurs when oxygen accepts an electron and is converted to the superoxide radical according to the following reaction:



Cytochrome b₅₅₈

The membrane-bound gp91^{phox}/p22^{phox} heterodimer is designated cytochrome b₅₅₈ because of its optical spectrum with an absorbance peak at 558 nm; this cytochrome also has an unusually low mid-potential at -245 mV which enables the direct transfer of electrons to molecular oxygen (Leusen *et al.*, 1996). A flavin (FAD) and two haem moieties (redox centres) are contained within the heterodimer such that the catalytic core to transfer electrons from NADPH to molecular oxygen is contained entirely within cytochrome b₅₅₈. In resting neutrophils the cytochrome in association with Rap1A is localised in the membranes of secondary and tertiary granules, as well as in those of secretory vesicles. During neutrophil activation, coalescence of these granules with the plasma membrane results in redistribution of the oxidase (Leusen

et al., 1996). Although the exact nature of the interaction between Rap1A and cytochrome b_{558} during oxidase activation remains to be established, it does appear that translocation and binding to cytochrome b_{558} of Rap1A in its GTP-bound form positively regulates NADPH oxidase activity, while the GDP-bound form negatively affects oxidase activity (Segal *et al.*, 2000).

Cytosolic components of NADPH oxidase

As is the case with cytochrome b_{558} , the cytosolic components of the oxidase are functionally inert in unstimulated neutrophils. Three of the cytosolic components *viz* $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ exist as a cytoplasmic trimolecular complex in the resting state. These interactions are achieved in part through non-covalent links between SH3 and proline rich domains. $P47^{phox}$ and $P67^{phox}$ each possess two SH3 domains, while at least one is present on $p40^{phox}$ (Leusen *et al.*, 1996; Segal *et al.*, 2000). The SH3 domain of $p40^{phox}$ interacts with $p47^{phox}$ and its last 36 amino acids interact with $p67^{phox}$, while the C-terminal SH3 domain of $p67^{phox}$ is crucial for the interaction with both $p40^{phox}$ and $p47^{phox}$ (Leusen *et al.*, 1996). Interestingly, in unstimulated phagocytes $p47^{phox}$ appears to exist in a closed, inactive form in which the SH3 domains are masked via an intra-molecular interaction with the C-terminal region of the polypeptide. As described below, conformational alterations in $p47^{phox}$ result in unmasking of these SH3 regions, which is a key event in activation of NADPH oxidase (Shiose & Sumimoto, 2000).

Activation of NADPH oxidase

Activation of NADPH oxidase through receptor-mediated signalling by chemoattractants involves modification of the GDP-binding state of G-proteins, thus the activated receptor catalyses exchange of GDP for GTP by both the G-protein α subunit and low molecular weight G-proteins of the Ras, Rho and ARF (ADP-ribosylation factor) families. This process leads to the serial activation of phospholipases (PL) C and D and the generation of lipid second messengers. Several converging pathways (protein tyrosine kinases/phosphatidylinositol 3-kinase; Ras/Rho; PLD) activate the serine kinase Raf, which together with the serine/threonine kinase, protein kinase C (activated by PLC/PLD-derived diacylglycerol), activates MAP kinases which in turn cause the phosphorylative

activation of cytosolic PLA₂ (reviewed by Alonso *et al.*, 1998).

Signalling triggered by cross-linking and activation of Fcγ receptors on the other hand involves receptor-mediated, early activation of Src-protein tyrosine kinases and recruitment of various downstream effectors including phosphatidylinositol 3-kinases, PLC and the MAP kinase cascade, resulting in activation of cytosolic PLA₂ and PKC, as well as other kinases (Alonso *et al.*, 1998).

With respect to activation of NADPH oxidase in neutrophils and other phagocytes, the key events are activation of kinases, particularly protein kinase C, and cytosolic PLA₂ (Shiose & Sumimoto, 2000). Phosphorylation of p47^{phox} at several serine residues in the SH3-containing C-terminal region alters the conformation of the polypeptide. This in turn leads to unmasking of the SH3 domains enabling weak interactions of p47^{phox} with p22^{phox} (Segal *et al.*, 2000; Shiose & Sumimoto, 2000). However, this event alone is insufficient for efficient activation of NADPH oxidase, a second complementary/synergistic mechanism being required. This is provided by low concentrations of arachidonic acid, generated during cleavage of membrane phosphatidylcholine by cytosolic PLA₂. Arachidonic acid, by mechanisms which remain to be fully elucidated, maximises the interactions of phosphorylated p47^{phox} with p22^{phox}, resulting in complete activation of the oxidase (Shiose & Sumimoto, 2000). Phosphorylation and arachidonic acid-mediated unmasking of SH3 domains in p47^{phox} also intensify the interactions of this polypeptide with p67^{phox}. Phosphatidic acid, generated during hydrolysis of membrane phospholipids by PLD, also activates p47^{phox}, presumably by the same mechanism as does arachidonic acid (Erickson *et al.*, 1999). However, whether phosphatidic acid also synergises with phosphorylation of p47^{phox} in activation of the oxidase remains to be established.

Although the exact mechanism by which p47^{phox} (in combination with p67^{phox}/p40^{phox}) interacts with p22^{phox} and initiates the electron-transporting activity of the oxidase is incompletely understood, it has been proposed that the interaction of the trimolecular complex of cytosolic polypeptides with cytochrome b₅₅₈ on the cytoplasmic side of the membrane induces a conformational change in the cytochrome that permits binding

of NADPH to gp 91^{phox} and initiation of electron flow in the cytochrome (Leusen *et al.*, 1996; Segal *et al.*, 2000).

With respect to the other cytosolic components of the oxidase, p40^{phox} does not appear to be involved in oxidase activation/activity, but rather contributes to maintaining the latent trimolecular complex in an inactive state. Activated (GTP-bound) Rac 2 binds to p67^{phox}, an interaction which is essential for the activity of NADPH oxidase, and is essential for translocation of p47^{phox} and p67^{phox} (Leusen *et al.*, 1996). Unlike p47^{phox}, which undergoes phosphorylation at multiple (9-10) serine residues in the distal portion of the C-terminal region, probably involving several kinases, p67^{phox} undergoes discrete MAP kinase-mediated phosphorylation of a single amino acid (threonine-233) which is followed by translocation to the membrane during oxidase activation (Forbes *et al.*, 1999).

p47^{phox} and p67^{phox}, both of which are essential for NADPH oxidase activation, have distinct roles in the regulation of electron flow in cytochrome b₅₅₈. p67^{phox} facilitates electron flow from NADPH to the flavin centre resulting in the reduction of FAD, while p47^{phox} is required for electron flow to proceed beyond the flavin centre to the haem groups in cytochrome b₅₅₈ and then to molecular oxygen (Leusen *et al.*, 1996).

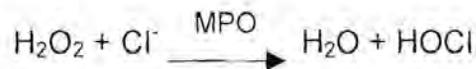
Superoxide-derived antimicrobial oxidants

Although the primary consequence of assembly of the NADPH oxidase complex is the univalent reduction of molecular oxygen to O₂⁻, this oxygen-derived radical is a weak and unstable antimicrobial oxidant. Importantly, however, O₂⁻ functions as the precursor of a series of potent microbicidal oxidants.

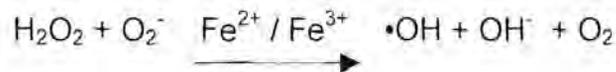
Hydrogen peroxide (H₂O₂) is formed by spontaneous or enzymatic dismutation (by superoxide dismutase) of O₂⁻:



The antimicrobial potential of H₂O₂ is dramatically potentiated by the granule enzyme, myeloperoxidase (MPO), which utilises this oxidant to oxidise chloride to the extremely potent oxidising agent hypochlorous acid (HOCl):



It has also been proposed that neutrophils transform $\text{H}_2\text{O}_2/\text{O}_2^-$ to hydroxyl radical ($\cdot\text{OH}$, the most potent oxidant known in biological systems) by the iron-catalysed Haber-Weiss reaction:

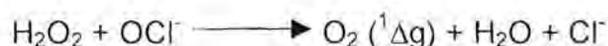


However, production of $\cdot\text{OH}$ by neutrophils via this mechanism has only been demonstrated *in vitro* in the presence of added iron, and appears to be of little or no biological relevance given that under physiological conditions, iron in and around neutrophils is tightly complexed to binding proteins (Ramos *et al.*, 1992). An alternative, transition metal-independent pathway of $\cdot\text{OH}$ generation has, however, been described in neutrophils and involves the interaction of O_2^- and HOCl (Ramos *et al.*, 1992):



Although described several years ago, to my knowledge the biological significance of this pathway has not yet been resolved.

Neutrophils have also been reported to generate significant amounts of singlet oxygen [$\text{O}_2 (^1\Delta\text{g})$], a highly reactive, diffusible and long-lived electronically excited state of molecular oxygen. These cells apparently transform up to 20% of oxygen consumed by NADPH oxidase to singlet oxygen by a MPO-dependent pathway involving interaction of H_2O_2 and HOCl (Steinbeck *et al.*, 1992):



These various phagocyte-derived oxidants acting directly or through more stable intermediates (eg chloramines and chloramides in the case of HOCl) are powerful antimicrobial agents. They are, however, indiscriminate, and, if released

extracellularly during hyperacute and/or chronic activation of phagocytes, they pose the potential threat of oxygen toxicity to bystander host cells and tissues in the vicinity of inflammatory reactions. In this regard they are potentially cytotoxic for eukaryotic cells, as well as being potential carcinogens, pro-proteolytic, pro-adhesive and immunosuppressive (reviewed by Anderson, 1995).

Other activities of NADPH oxidase

Apart from its primary role in generating superoxide, the NADPH oxidase of activated phagocytes also regulates membrane potential in these cells as a consequence of the vectorial, outward transport of electrons across the plasma membrane. The oxidase also functions as a H^+ channel. These activities are important in restoring Ca^{2+} homeostasis in activated neutrophils and are discussed in more detail in section 1.6.2 (page 26).

1.4.2 Nitric oxide synthase

The production of nitric oxide (NO) within phagocytes is an important component of host defences against microbial infection. Although NO *per se* is only weakly antimicrobial, its microbicidal activity is considerably enhanced by reaction with O_2^- to yield the highly reactive anion, peroxynitrite (Koppenol, 1998).

While the role of NO in rodent phagocytes is well-established, the involvement of inducible NO synthase (iNOS) in the intrinsic antimicrobial function of human neutrophils is less clear with many investigators having failed to detect NO production by these cells. Recently, however, Webb *et al.* (2001) have reported that neutrophils isolated from the peripheral blood of healthy adult humans are not primed for iNOS activity. Production of NO by these cells requires β_2 -integrin/ $Fc\gamma$ receptor-independent adhesion together with exposure to the cytokines IL-1, IFN- γ and TNF- α for extended incubation times (3 hours). Under these conditions iNOS was detectable in up to 36% of neutrophils (Webb *et al.*, 2001).

1.4.3 Cytokine production by neutrophils

Until fairly recently neutrophils, largely because of their perceived limited biosynthetic capability, were not considered to be cytokine-producing cells. This perception was compounded by the relatively short lifespan of neutrophils *in vitro*, difficulties in identifying the origins of cytokines (measured by enzyme-based immunoassays) due to the presence of major cytokine-producing, contaminating cells such as monocytes in "pure" neutrophil suspensions, as well as the potential of neutrophils to passively acquire cytokines from other cell types during neutrophil purification procedures. However, with the acquisition of procedures which enable acquisition of ultra-pure neutrophil suspensions (flow cytometry), as well as intracellular detection of cytokine messenger RNA (mRNA), a considerable body of evidence has accumulated which clearly demonstrates that the human neutrophil is a source of various pro-inflammatory cytokines, chemokines and growth factors. The concept that neutrophils can be a source of cytokines has only recently emerged and has been reviewed in considerable detail by Cassatella (1999) and Witko-Sarsat *et al.* (2000).

Although the production of cytokines by neutrophils is striking in its diversity (as shown in Table 1.3, page 22), IL-8 and TNF- α appear to be the major cytokines produced by these cells, suggesting the potential for autocrine regulation of neutrophil function (Cassatella, 1999). TNF- α enhances neutrophil adhesion, sensitises (primes) for and activates reactive oxidant production by adherent cells, and induces release of granule enzymes by these cells (Witko-Sarsat, 2000).

Interleukin-8, a potent chemoattractant for neutrophils and inducer of degranulation, is the most abundantly secreted cytokine by neutrophils (Witko-Sarsat, 2000). Agents which activate cytokine production by neutrophils are shown in Table 1.4 (page 23).

Neutrophils, as is the case with other types of inflammatory cells, are also subject to negative modulation by cytokines. Those cytokines which suppress the pro-inflammatory activities of neutrophils include IL-4, IL-10 in particular, and IL-13 (Witko-Sarsat, 2000).

Table 1.3: Cytokines Expressed by Neutrophils

Cytokines that are expressed by neutrophils <i>in vitro</i>	Cytokines that are not expressed by neutrophils <i>in vitro</i>
Pro-inflammatory cytokines TNF- α IL-1- α and - β IL-12	T-cell-derived cytokines IL-10 IL-13
Anti-inflammatory cytokines IL-1 receptor antagonist (IL-1Ra)	Other cytokines RANTES MCP-2, MCP-3
Chemokines IL-8 Growth-related gene product- α (GRO- α) Macrophage infiltrating protein-1 α (MIP-1 α), MIP-1 β Cytokine-induced chemoattractants (CINC)	
Other cytokines and growth factors Interferon- α (IFN- α), IFN- β Granulocyte colony-stimulating factor (G-CSF) Fas ligand (FasL), CD30 ligand (CD30L) Vascular endothelial growth factor (VEGF) Hepatocyte growth factor (HGF)	
Release under certain conditions Macrophage-CSF (M-CSF), IL-3, GRO- β IL-18 (IFN- γ inducible factor) TGF- α Oncostatin (OSM) and neurotrophins	
Secretion still debated IL-6, monocyte chemotactic protein-1 (MCP-1), granulocyte-macrophage CSF (GM-CSF), stem cell factor (SCF), and IFN- γ	

Adapted from Cassatella, 1999

Table 1.4: Agents which Trigger Cytokine Production by Neutrophils

Cytokines and growth factors

TNF- α , IL-1 α , IL-1 β ,
IL-4, IL-13, IL-10,
GM-CSF,
TGF- β

Chemoattractants

FMLP

Surface molecules

Anti-CD32 (Fc γ RII) and anti-CD16 (Fc γ RIII) antibodies

Particulate agents

Calcium microcrystals
Urate microcrystals

Other agents

Calcium ionophores
PMA, concanavalin A
ANCA
Matrix protein (fibronectin, laminin)

Bacteria and related products

LPS
Staphylococcus aureus
Yersinia enterocolitica
Listeria monocytogenes

Fungi and related products

Candida
Saccharomyces cerevisiae
Zymosan

Protozoa

Plasmodium falciparum

Viruses

Epstein-Barr virus

Adapted from Cassatella, 1999

The discovery that neutrophils are cytokine-producing cells may have important implications for neutrophil-directed anti-inflammatory chemotherapy, suggesting that combinations of those agents which suppress protein synthesis-independent, early activatable pro-inflammatory activities (such as oxidant production, synthesis of eicosanoids/prostanoids and granule enzyme release) together with agents which suppress protein synthesis-dependent, later-occurring activities (such as cytokine production) may be maximally effective.

1.5 Phospholipase A₂-Derived Mediators of Inflammation

Neutrophil activation is accompanied by Ca²⁺-dependent activation of PLA₂ with resultant cleavage of the integral membrane phospholipid, phosphatidylcholine, to the primary hydrolysis products arachidonic acid and lysophosphatidylcholine (LPC). As mentioned above, arachidonic acid, through its direct interaction with P47^{phox}, mediates activation of NADPH oxidase, while LPC possesses a range of pro-inflammatory activities (pro-oxidative, pro-adhesive, induction of degranulation) which result from activation of PKC by this lysophospholipid (Oishi *et al.*, 1988). In addition to pro-inflammatory activity LPC, and to a lesser extent arachidonate, as well as saturated fatty acids, are microbicidal for Gram-positive bacteria (Kondo & Kanai, 1985; Steel *et al.*, 1999), suggesting a role for both cytosolic and secretory PLA₂ in neutrophil antimicrobial function (Weinrauch *et al.*, 1996).

While LPC is converted to biologically "inert" glycerophosphocholine and saturated fatty acid (usually palmitate) by phospholipase A₁, arachidonic acid in neutrophils is converted by cyclooxygenase 1/cyclooxygenase 2 to prostaglandin E₂ (PGE₂) and by 5'-lipoxygenase to leukotriene B₄ (Alonso *et al.*, 1998). PGE₂ possesses a range of pro-inflammatory activities (increase of blood flux, potentiation of oedema, haemorrhagic necrosis, activation of transcription and initiation of the acute phase response - Alonso *et al.*, 1998). In addition, PGE₂ acting via adenylyl cyclase-linked EP₂ receptors and adenosine 3',5'-cyclic monophosphate (cAMP) prolongs the lifespan of activated neutrophils by inhibiting apoptosis (Rossi *et al.*, 1995). LTB₄

also possesses a range of pro-inflammatory actions (mobilisation of intracellular Ca^{2+} , pro-adhesive, chemotactic – Alonso *et al.*, 1998). While neutrophils possess membrane receptors for LTB_4 , they do not synthesise the cysteinyl leukotrienes (LTC_4 , LTD_4 , LTE_4), neither do they possess membrane receptors for these pro-inflammatory lipids (Lam & Austen, 1992).

Neutrophils also produce PAF by the so-called, PLA_2 -dependent, remodelling pathway during which 1-0-alkyl-2 acyl-sn-glycero-3-phosphocholine is converted by PLA_2 to lyso-PAF, which in turn is converted by acetyltransferase to PAF (Zimmerman *et al.*, 1992). PAF is undoubtedly the most potent and versatile of the biologically active lipids produced by neutrophils and other inflammatory cells, and when released extracellularly can amplify inflammatory responses by interacting with G-protein/PLC-coupled PAF receptors on target cells, particularly eosinophils and neutrophils (Zimmerman *et al.*, 1992; Prescott, 1999).

1.6 Calcium Fluxes and Restoration of Calcium Homeostasis in Activated Neutrophils

Transient elevations in cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) precede and are a prerequisite for the receptor-mediated activation of many neutrophil functions, including activation of β_2 -integrins and adhesion to vascular endothelium, superoxide production, granule enzyme release and activation of pro-inflammatory cytosolic nuclear transcription factors, including NF- κB (Lew *et al.*, 1986; Thelen *et al.*, 1993; Pettit & Hallet, 1996; Dolmetsch *et al.*, 1997).

1.6.1 Release of calcium from stores

Intracellular Ca^{2+} in neutrophils is reportedly stored in specialised storage vesicles termed calciosomes (Favre *et al.*, 1996). This may be somewhat of an oversimplification, however, since there are two distinct cellular locations for Ca^{2+} stores in neutrophils which may have differential involvement in activation of pro-inflammatory functions and which may utilise different molecular/biochemical mechanisms of Ca^{2+} mobilisation (Pettit & Hallet, 1996). One site is located

peripherally under the plasma membrane and appears to be involved in activation of β_2 -integrins, while the other (probably calciosomes) is localised in the juxtannuclear space and is mobilised by the chemoattractant, FMLP (Pettit & Hallet, 1996).

Because differences between the molecular/biochemical mechanisms of mobilisation of Ca^{2+} from these two different storage sites have not yet been established, I will focus on those utilised by FMLP. Occupation of neutrophil membrane receptors for this chemotactic tripeptide results in receptor-G-protein coupling with consequent activation of PLC and generation of inositol triphosphate by hydrolysis of phosphatidylinositol 4,5-biphosphate (Favre *et al.*, 1996; Alonso *et al.*, 1998). Inositol triphosphate then interacts with Ca^{2+} -mobilising receptors on calciosomes, resulting in discharge of the cation into the cytosol. These events are extremely rapid, occurring within less than 1 second after the ligand-receptor interaction (Favre *et al.*, 1996; Anderson & Goolam Mahomed, 1997). In the case of neutrophils, the abrupt increase in cytosolic Ca^{2+} following exposure to FMLP, results exclusively from release of the cation from intracellular stores with little or no contribution at this early stage (within the first 30-60 seconds) from extracellular Ca^{2+} and results in an increase in the basal $[\text{Ca}^{2+}]_i$ from around 100 nM to $\pm 1 \mu\text{M}$ (Favre *et al.*, 1996; Anderson & Goolam Mahomed, 1997; Geiszt *et al.*, 1997). Influx of extracellular Ca^{2+} is delayed, being detectable at around 1 minute after the addition of FMLP and terminating at around 5 minutes. This type of influx is characteristic of a store-operated Ca^{2+} influx (ie primarily involved in re-filling of stores as opposed to activation of neutrophils) and is operative in a large variety of cell types, including neutrophils.

1.6.2 Restoration of calcium homeostasis

Peak cytosolic $[\text{Ca}^{2+}]_i$ is achieved abruptly in FMLP-activated neutrophils, peaking at around 10-20 seconds, and subsiding rapidly thereafter to close to base-line values within 3-5 minutes. Restoration of Ca^{2+} homeostasis in activated neutrophils is essential to prevent Ca^{2+} overload and hyperactivity of these cells. This is achieved by rapid clearance of Ca^{2+} from the cytosol of the cells in the setting of carefully regulated influx of extracellular cation, which is efficiently diverted into stores. Clearance of cytosolic Ca^{2+} is accomplished through the action of the plasma

membrane and endo-membrane Ca^{2+} -ATPases operating in unison, while extracellular cation is excluded from the cells through the membrane depolarising activity of NADPH oxidase.

The plasma membrane Ca^{2+} -ATPase of neutrophils is modulated by calmodulin which shifts the pump to a higher affinity state for Ca^{2+} , resulting in enhanced maximal velocity (Lagast *et al.*, 1984). A dramatic and transient (over a 30 second time course) increase in efflux of Ca^{2+} , coincident with the release of the cation from stores, is observed in FMLP-activated neutrophils which results in the extrusion of about 50% of cell-associated cation (Anderson & Goolam Mahomed, 1997). Activation of neutrophils with the chemoattractant (and several other G-protein/PLC-coupled receptor-mediated stimuli) also results in immediate activation of adenylate cyclase (Snyderman & Uhing, 1992), which appears, at least in part, to be linked to activation of cAMP-dependent protein kinase (PKA) and up-regulation of the sarcoplasmic reticulum (calciosome), endo-membrane Ca^{2+} -ATPase (Tao *et al.*, 1992; Villagrasa *et al.*, 1996; Anderson *et al.*, 1998). The endo-membrane Ca^{2+} -ATPase contributes to Ca^{2+} clearance from the cytosol of activated neutrophils by promoting re-sequestration of the cation. Operating in harmony, these two Ca^{2+} pumps (plasma membrane and endo-membrane) are the major, if not the sole contributors to clearance of cytosolic Ca^{2+} in activated neutrophils, since the $\text{Na}^+/\text{Ca}^{2+}$ exchanger does not appear to mediate Ca^{2+} efflux in these cells (Simchowicz & Cragoe, 1988).

Efficient Ca^{2+} clearance by these systems is greatly facilitated by the membrane depolarising actions of NADPH oxidase which limit influx of extracellular Ca^{2+} . The dramatic decrease in membrane potential which accompanies activation of the oxidase following exposure of FMLP, and which is coincident with release of Ca^{2+} from granulocyte intracellular stores and activation of superoxide production, has been attributed to the electrogenic activity of the oxidase (Henderson *et al.*, 1988; Schrenzel *et al.*, 1998; Jankowski & Grinstein, 1999), as well as to the action of a rapidly activated H^+ conductance with resultant influx of H^+ (Bánfi *et al.*, 1999). This type of abruptly-occurring depolarisation, which accompanies activation of various types of inflammatory cells, including basophils, mast cells and neutrophils, has been shown to limit the influx of extracellular Ca^{2+} (Di Virgilio *et al.*, 1987; Mohr & Fewtrell,

1987; Penner *et al.*, 1988; Geiszt *et al.*, 1997). It has been proposed that when the cells are depolarised the driving force for entry of Ca^{2+} is abolished because the electrical component of the electrochemical gradient for Ca^{2+} is markedly reduced.

Recovery of membrane potential in FMLP-activated neutrophils becomes evident at around 1 minute after exposure to the chemoattractant and proceeds gradually over a 5-10 minute time course. Interestingly, the kinetics of influx of extracellular Ca^{2+} (for store-refilling) are virtually superimposable on those of membrane repolarisation (Anderson & Goolam Mahomed, 1997; Geiszt *et al.*, 1997; Tintinger *et al.*, 2001). Repolarisation appears to be achieved through the action of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger operating in reverse mode i.e. Na^+ efflux/ Ca^{2+} influx (Simchowicz & Cragoe, 1988), and by a slowly activatable H^+ conductance which allows only H^+ extrusion and repolarisation (Schrenzel *et al.*, 1998). This H^+ conductance appears to be a function of $\text{gp91}^{\text{phox}}$ (Bánfi *et al.*, 2000). Carefully regulated influx of Ca^{2+} probably ensures efficient diversion by the endo-membrane Ca^{2+} -ATPase of incoming cation into stores, thereby preventing flooding of the cytosol with Ca^{2+} and possible hyperactivation of the cells.

1.7 Anti-Inflammatory Actions of cAMP

The broad-spectrum anti-inflammatory potential of physiologic and pharmacologic cAMP-elevating agents, which spans many different types of immune and inflammatory cells, including neutrophils, has been recognised for more than two decades (Moore & Willoughby, 1995). These agents suppress the pro-inflammatory activities of human neutrophils, eosinophils, monocytes and lymphocytes by PKA-dependent mechanisms which are both dependent and independent of gene transcription (Moore & Willoughby, 1995; Barnes & Adcock, 1997).

1.7.1 Cyclic AMP and neutrophils

Receptor-mediated activation of human neutrophils with Ca^{2+} mobilising stimuli (chemoattractants, opsonised particles) is associated with an immediate, transient

(peaks within 30 seconds in the case of chemoattractants) increase in intracellular cAMP (Snyderman & Uhing, 1992; Goolam Mahomed & Anderson, 2000). This transient increase in cAMP appears to be due to the release of adenosine, an endogenous anti-inflammatory agent (Iannone *et al.*, 1989; Cronstein, 1994), by activated neutrophils. Adenosine in turn causes autocrine activation of G-protein/adenylate cyclase-linked subtype A_{2A} adenosine receptors (which are reviewed in a later section, 1.9.3, page 43) on neutrophils, resulting in transiently elevated levels of cAMP. Cyclic AMP in turn may fulfil an anti-inflammatory function by restoring Ca^{2+} homeostasis in activated neutrophils, resulting in down-regulation of the pro-inflammatory activities of these cells (Anderson *et al.*, 1998).

1.8 Neutrophil-Directed, Anti-Inflammatory Chemotherapeutic Strategies

Anti-inflammatory chemotherapeutic agents can be classified into two groups, those which have selective targets in immune and inflammatory cells and those with broad-spectrum activity affecting several different molecular targets. There are many selective anti-inflammatory chemotherapeutic agents, some of which are shown in Table 1.5 (page 30). With the possible exception of the leukotriene receptor antagonists in the treatment of mild-to-moderate bronchial asthma, monotherapy with these agents is either ineffective or of limited value (and in some cases impractical) in the treatment of patients with acute and chronic inflammatory conditions. On the other hand, the elite group of broad-spectrum anti-inflammatory agents currently consists of only one member, the corticosteroids. These agents are the mainstay of anti-inflammatory chemotherapy in bronchial asthma. The value of inhaled corticosteroids in the treatment of this chronic inflammatory disorder is captured in the following quotation:

“If ever there was a magic potion that should resolve the symptoms of an affliction, it is the use of corticosteroids in asthma” (McFadden, 1998).

Table 1.5: Anti-inflammatory agents which are currently used or are under investigation for the treatment of asthma

Selective	Broad-spectrum
Cyclooxygenase (Cox 1/Cox 2) inhibitors	Corticosteroids
5'-Lipoxygenase inhibitors	
Leukotriene receptor antagonists	
PAF receptor antagonists/degrading enzymes	
Cytokine receptor antagonists	
Chemokine receptor antagonists	
Anti-oxidants	
Anti-proteases	
Adhesion molecule antagonists	

1.8.1 Corticosteroids

These agents have a unique triple mechanism of anti-inflammatory action resulting from their interactions with cytosolic glucocorticoid (GC) receptors (GR). GR are members of the nuclear receptor superfamily that includes other steroids (oestrogen, progesterone), receptors for vitamins (vitamins A and D) and thyroid hormone (Barnes & Adcock, 1998). GR are expressed in most types of cells in an inactive form bound to a protein complex that includes two molecules of a 90 kDa heat shock protein (hsp90) and an immunophilin, which act as nuclear chaperones, protecting the nuclear localisation site. GC bind to GR in the cytoplasm, resulting in the dissociation of the inactive complex and rapid translocation to the nucleus of the GC/GR complex (reviewed in detail by Barnes & Adcock, 1998).

Interestingly, GC/GR interactions result in at least three different types of anti-inflammatory actions (Barnes & Adcock, 1998). These are:

- activation of genes encoding anti-inflammatory polypeptides
- activation of histone deacetylase activity, preventing the interaction of transcription factors with binding regions on target genes, leading to gene repression

- direct protein-protein interactions between the activated GR and activated, pro-inflammatory cytosolic-nuclear transcription factors.

With respect to the first of these, activated GR form homodimers to interact with GC response elements (GRE) in the promoter regions of target genes, resulting in increased gene transcription. This type of interaction results in the increased transcription of several anti-inflammatory polypeptides, including lipocortin-1 (antagonist of PLA₂), serum leukoprotease inhibitor, the IL-1 receptor antagonist (Barnes & Adcock, 1998), and possibly IκB-α, the antagonist of NF-κB (Scheinman *et al.*, 1995). In addition, this type of interaction also results in the increased transcription of β₂-adrenoreceptors (Mak *et al.*, 1995), which although of primary importance in maintaining the sensitivity of the airways to β₂-receptor agonists, may also contribute to anti-inflammatory activity since inflammatory cells, including neutrophils, contain adenylate cyclase-linked β₂-adrenoreceptors (Mueller *et al.*, 1988).

Interestingly, however, relatively few genes possess GRE, indicating that an alternative mechanism, or mechanisms, must account for the major anti-inflammatory actions of corticosteroids. It is now accepted that the major anti-inflammatory effects of corticosteroids are achieved via repression of immune and inflammatory genes and, although it was thought that these were likely to be mediated through negative GRE, resulting in gene repression, none of the immune and inflammatory genes that are switched off by steroids appears to have nGRE in their promoter sequences (Barnes & Adcock, 1998).

There has recently been increasing evidence that corticosteroids may affect the chromatin structure of DNA, thereby preventing the interaction of pro-inflammatory cytosolic nuclear transcription factors with their target genes (Barnes & Adcock, 1998). The association of transcription factors with their target genes is facilitated by interaction with large co-activator molecules such as cyclic AMP response element binding factor (CREB) binding protein (CBP) and p300, which bind to the basal transcription factor apparatus. These co-activator molecules have an intrinsic histone acetyltransferase (HAT) activity, resulting in acetylation of histone proteins

around which DNA is wound in the chromosome (Ogryzko *et al.*, 1996; Wolffe, 1997). This leads to unwinding of DNA and facilitates binding of transcription factors, resulting in increased gene transcription. Activated GR bind to a steroid receptor co-activator that is bound to CBP, interfering with the activation of CBP and leading to deacetylation of histone residues. This results in tighter coiling of DNA, excluding transcription factors such as NF- κ B and AP-1 from binding to DNA (Kamei *et al.*, 1996). The consequence is interference with the synthesis of a range of pro-inflammatory polypeptides including cytokines, chemokines, COX2, iNOS, adhesion molecules and immunoreceptors (Barnes, 1997; Lane *et al.*, 1998).

The third, and perhaps somewhat more controversial mechanism of anti-inflammatory action of corticosteroids, is by direct protein-protein interactions between activated GR and several different types of pro-inflammatory cytosolic nuclear transcription factors, resulting in functional inactivation of the latter. Activated GR have been reported to bind to activated NF- κ B, AP-1 and signal transduction-activated transcription factors (STATs) and prevent their binding to promoter regions on target genes (Barnes, 1997; Barnes & Adcock, 1998).

1.8.2 Limitations of corticosteroids

In spite of their clinical efficacy, enthusiasm for corticosteroids must be tempered by an awareness of their limitations. These include concerns about their long-term safety (including small, but nevertheless significant effects of inhaled corticosteroids on the growth of children), steroid-resistance (especially in asthma), slow onset of action, and the relative insensitivity of neutrophils to these agents (McFadden, 1998). Corticosteroid resistance may be due to decreased affinity and/or numbers of GR in the cytosol of immune and inflammatory cells, or to excessive production of AP-1 by these cells (Lane *et al.*, 1998), while the slow onset of action of these agents may reflect the time required for *de novo* synthesis of anti-inflammatory polypeptides, as well as the time required to suppress biosynthetic processes already initiated by pro-inflammatory, cytosolic nuclear transcription factors.

1.8.3 Neutrophils and corticosteroids

The reported insensitivity of the rapidly-activatable functions of neutrophils to therapeutically-relevant concentrations of corticosteroids *in vitro* is due to the independence of these pro-inflammatory activities on *de novo* protein synthesis. The insensitivity of oxidant production, adhesion, chemotaxis and phagocytosis to corticosteroids has been described by many investigators and these reports are summarised in Table 1.6 and 1.7 (pages 34-36 and 37-38 respectively). Although some authors have described inhibitory effects of corticosteroids on rapidly-activatable neutrophil functions (summarised in Table 1.6), it is possible that these may be due to non-GR-mediated effects such as membrane stabilisation.

Somewhat worryingly, there are also reports that corticosteroids may potentiate the pro-inflammatory activities of neutrophils by delaying apoptosis in these cells (Cox, 1995; Meagher *et al.*, 1996; Daffern *et al.*, 1999). This contrasts with the apoptosis-inducing effects of corticosteroids in other types of immune and inflammatory cells, including eosinophils (Meagher *et al.*, 1996). The differences between neutrophils and other types of inflammatory cells have been attributed not only to the paucity of mitochondria in the former, but also to differences in the structure of these organelles.

The insensitivity of neutrophils to corticosteroids may account for the low therapeutic efficacy of these agents in the treatment of conditions associated with hyperactivity of neutrophils. These include ARDS, chronic obstructive pulmonary disease, cystic fibrosis, bronchiectasis and certain categories of asthmatics. On the other hand, corticosteroids (which would be expected to inhibit slowly-activatable neutrophil pro-inflammatory functions such as cytokine synthesis), may be most effective in controlling neutrophil-mediated inflammation when used in combination with agents which inhibit rapidly-activatable neutrophil functions. This contention is supported by the observation that glucocorticoids inhibit IL-8 production by neutrophils, while superoxide production by these cells was unaffected (Cox, 1995)

Table 1.6: Summary of Reports on the Effects of Corticosteroids on Early-Activatable Neutrophil Functions *In Vitro*

Functions Investigated:					
Adhesion	Oxidant Production	Chemotaxis	Phagocytosis	Degranulation	Author
0 (stimulated), slight ↓ (spontaneous)		0		0	Schleimer <i>et al.</i> , 1989
0		↓			Hirata <i>et al.</i> , 1980
		↓			Kurihara <i>et al.</i> , 1984b
0 (bovine)		↑ (bovine)			Jayappa & Loken, 1983
		↓ (rabbit)			Ward, 1966
		↓			Rivkin <i>et al.</i> , 1976
	0	↓			Freischlag <i>et al.</i> , 1992
				↓	Burnett <i>et al.</i> , 1989
	↓				Feunfer <i>et al.</i> , 1979
	0	↓ (suprapharm. concentr)			Lomas <i>et al.</i> , 1991
	0	↓		↓	Llewellyn-Jones <i>et al.</i> , 1994
	↓				Goldstein <i>et al.</i> , 1976
	↓				Jones <i>et al.</i> , 1983
	↓				Umeki & Soejima, 1990
				0	Røshol <i>et al.</i> , 1995
		0 (rabbit)			Borel 1973
		0			Rhinehart <i>et al.</i> , 1974
	0 (bovine)				Phillips <i>et al.</i> , 1987
	0/↑ (suprapharm.concentr)				Horan <i>et al.</i> , 1982

Table 1.6 (continued)

Adhesion	Oxidant Production	Chemotaxis	Phagocytosis	Degranulation	Author
0/↓ (canine suprapharm concentration)	0	0	0 (↑ at suprapharm concentration)		Van Dyke <i>et al.</i> , 1979 Trowald-Wigh <i>et al.</i> , 1998
	↓ (high concentr)				Levine <i>et al.</i> , 1981
	0 (except Dex)				Niwa <i>et al.</i> , 1987
	0		0		Olds <i>et al.</i> , 1974
	0 (bovine) /↓ (suprapharm concentr ex Betameth)			0	Hoeben <i>et al.</i> , 1998
	0				Humphreys <i>et al.</i> , 1993
				0	Mandell <i>et al.</i> , 1970
				↓	Wright & Malawista, 1973
				0	Persellin & Ku, 1974
	0	0		↓	Gadaleta <i>et al.</i> , 1994
↓					Pichyangkul <i>et al.</i> , 1988
0		0			MacGregor <i>et al.</i> , 1974
↓	↓				Bratt & Heimburger, 1999
0 (unstim. PMN) /↓ (stim. PMN)				0	Filep <i>et al.</i> , 1997
↓					Liu <i>et al.</i> , 2000
↓	↓				Heimburger <i>et al.</i> , 2000
↓					Zouki <i>et al.</i> , 2000
↓					Suzuki <i>et al.</i> , 2000

Table 1.6 (continued)

Adhesion	Oxidant Production	Chemotaxis	Phagocytosis	Degranulation	Author
↓	↓				<i>Filep et al., 1999</i>
↓ (bovine)+ human N's					<i>Shimoyama et al., 1997</i>
↓ (rat)					<i>Sakamoto et al., 1997</i>
↓					<i>Wheller & Perretti, 1997</i>
↓ (activated N's)				0	<i>Filep et al., 1997</i>
↓		↓			<i>Yoshida et al., 1997</i>

Table 1.7: Summary of Reports on the Effects of Corticosteroids on Early-Activatable Neutrophil Functions *In Vivo*
Functions Investigated:

Adhesion	Oxidant Production	Chemotaxis	Phagocytosis	Degranulation	Author
↓ (suprapharm concentr)		0			Clark <i>et al.</i> , 1979
↓ (suprapharm concentr)		↓			MacGregor <i>et al.</i> , 1974
↓		↓			Ackerman <i>et al.</i> , 1982
	↓				Dandona <i>et al.</i> , 1999
	↑				Kuzniar <i>et al.</i> , 1991
	0	↓			Lomas <i>et al.</i> , 1991
	0	↓			Fleming <i>et al.</i> , 1991
	↓	↓		↓	Adams <i>et al.</i> , 1989
	↓				Røshol <i>et al.</i> , 1995
	↑ (bovine)				Phillips <i>et al.</i> , 1987
	↓ (bovine)				Roth & Kaeberle, 1981
	0	0		0	Llewellyn-Jones <i>et al.</i> 1996
↓ (canine)	↑	↑	↑		Trowald-Wigh <i>et al.</i> , 1998
		↑ (canine, pharm. conc)			Guelfi <i>et al.</i> , 1985
		↑ (equine, pharm. conc) ↑			Morris <i>et al.</i> , 1988
				0	Persellin & Ku, 1974
		↑ (unstim) ↓ (stim. ster. sens. asthma)			Zak-Nejmark <i>et al.</i> , 1996
		↑ (stim. ster. resist. asthma)			

Table 1.7 (continued)

Adhesion	Oxidant Production	Chemotaxis	Phagocytosis	Degranulation	Author
0					Oda & Katori, 1992
		↓			Davis <i>et al.</i> , 1991
↓ (methyl-pred) 0 (Dex)					Bassaris <i>et al.</i> , 1987
↓	↑			↑	Hetherington & Quie, 1985
	↓				Macconi <i>et al.</i> , 1993
↓					Nakagawa <i>et al.</i> , 1999
↓		↓			Crockard <i>et al.</i> , 1998
	0		0	0	Dale <i>et al.</i> , 1998
0				0	Tailor <i>et al.</i> , 1997
↓					Van Overveld <i>et al.</i> , 2000
↓		0			Lim <i>et al.</i> , 1998
↓ (rat)		↓			Davenpeck <i>et al.</i> , 1998
↓					Youssef <i>et al.</i> , 1996
↓					Perretti <i>et al.</i> , 1996
↓ (bovine)					Burton & Kehrl, 1995
↑ (β ₂ -integrin)	↑	↓			Barton <i>et al.</i> , 2000
↓ (L-selectin)					
↓	↓	↓		↓	Goulding <i>et al.</i> , 1998
↓					Hill <i>et al.</i> , 1994

Second generation type 4 phosphodiesterase inhibitors (Torphy, 1998; Wang *et al.*, 1999) and adenosine receptor agonists operative at the level of the subtype A_{2A} receptor (Cronstein, 1994; Hannon *et al.*, 1998; Underwood *et al.*, 1998) are promising pharmacologic inhibitors of early activatable, pro-inflammatory functions of neutrophils.

1.9 Adenosine and Neutrophils

Adenosine is a ubiquitous autocoid with a broad-spectrum of biological activities, including modulation of leucocyte function. It is a physiologic anti-inflammatory agent released by many cell types and is a normal constituent of all body fluids in which its levels are raised by hypoxia and ischaemia. Adenosine is released by endothelial cells and neutrophils in response to physiologic stimuli (Fredholm, 1997).

Adenosine was first administered to humans on an experimental basis in 1930 and was found to induce transient sinus bradycardia and AV nodal block. Its clinical usefulness was only recognised in the 1980s and it is now one of the most commonly used agents in the diagnosis and treatment of supraventricular arrhythmias, as well as in the diagnosis of narrow complex tachycardias, while its ability to produce coronary vasodilation is utilised in combination with myocardial perfusion scintigraphy to assess whether perfusion defects occur as a consequence of coronary artery stenosis. A potential therapeutic use is to provide protection to the heart during ischaemia or infarction, a process known as preconditioning. This approach may be applied to unstable patients about to undergo complex angioplasty or coronary bypass surgery (Olah & Stiles, 1995). Reperfusion injury may be reduced by adenosine-mediated inhibition of neutrophil activation (Jordan *et al.*, 1997).

1.9.1 Adenosine effects on neutrophil function

Adenosine, of both exogenous and endogenous (neutrophil-derived) origin, is a potent modulator of several neutrophil functions including superoxide production,

adhesion to vascular endothelium, migration and phagocytosis (Iannone *et al.*, 1989; Cronstein *et al.*, 1993).

Superoxide production

In 1983 Cronstein *et al.* reported that adenosine inhibited neutrophil O_2^- generation stimulated by the chemoattractants (FMLP) and C5a, as well as the Ca^{2+} ionophore A23187, but not by phorbol myristate acetate (a direct activator of protein kinase C). Adenosine appeared to mediate these effects extracellularly since blockade of purine uptake did not reverse the effect of added adenosine on neutrophil function. Adenosine inhibited O_2^- generation via occupancy of specific adenosine receptors (AR). It was also found that removal of endogenously released adenosine (by addition of adenosine deaminase) resulted in enhanced O_2^- generation by stimulated neutrophils. This observation led to the hypothesis that adenosine is an endogenously released anti-inflammatory agent. Confirmation of this was subsequently derived from both *in vitro* and *in vivo* studies (Church & Hughes, 1985; Cronstein *et al.*, 1986; Gunther & Herring, 1991).

After exposure to agents such as endotoxin, phorbol myristate acetate, platelet-activating factor and $TNF-\alpha$, neutrophils become primed to generate greater quantities of H_2O_2 and O_2^- after stimulation with chemoattractants. Adenosine has been reported to inhibit $TNF-\alpha$ -mediated priming of adherent neutrophils, but not neutrophils in suspension, although it did inhibit PAF-mediated priming of neutrophils in suspension (Steward & Harris, 1993). The biochemical basis of priming and the mechanism by which adenosine inhibits priming remain to be clarified.

Chemotaxis

Rose *et al.* (1988) found that adenosine and its analogues augmented neutrophil chemotaxis to FMLP and C5a at concentrations two to three orders of magnitude below those which inhibited O_2^- generation. This was ascribed to occupancy of A_1 receptors. However, when primed, neutrophils demonstrated a reduced chemotactic response to chemoattractants. The effect of $TNF-\alpha$ on neutrophil chemotaxis was reversed by adenosine (Takenawa *et al.*, 1986).

Degranulation

In 1980 Marone *et al* reported that adenosine had no effect on degranulation by stimulated neutrophils. These results were widely accepted and for several years no further research was performed in relation to the effect of adenosine on neutrophil degranulation. In 1988 Cronstein *et al* confirmed that adenosine at best poorly inhibited granule release from stimulated neutrophils, while McGarrity *et al.*, (1989) also reported only modest effects of adenosine on secretion of granule proteins from neutrophils.

In contrast to these findings, Bouma *et al.*, (1997) demonstrated that adenosine dose-dependently inhibited human neutrophil degranulation in whole blood activated with either LPS or TNF- α , and that it attenuated the release of three different azurophil granule proteins, neutrophil elastase, bactericidal/permeability-increasing protein and defensin, to approximately the same extent. The inhibitory effect was also of comparable magnitude when the neutrophils were stimulated with agents which activate different signal transduction pathways, i.e. FMLP and STZ (serum-treated zymosan). The reported lack of adenosine effects on degranulation demonstrated in previous *in vitro* studies (Cronstein *et al.*, 1988; McGarrity *et al.*, 1989), had been attributed to the use of cytochalasin B to enhance neutrophil secretion in suspension. It had also been suggested that neutrophil adherence to biological surfaces might be required for mediation of the inhibitory effects of adenosine on the respiratory burst, as well as degranulation. However, Bouma *et al.*, (1997) in their study observed no major difference in the magnitude of the inhibitory effects of adenosine on degranulation in either whole blood or isolated, cytochalasin-B-pretreated neutrophils in suspension, using LPS or TNF- α as activators, both of which are very potent and selective inducers of azurophilic granule release.

Aggregation

Cronstein *et al.* (1983) reported that adenosine did not inhibit leucocyte aggregation (neutrophil-neutrophil adhesion).

Adherence and phagocytosis

The stable adenosine analogue 2-chloroadenosine has been reported to inhibit adhesion of stimulated neutrophils to vascular endothelial cells (Cronstein *et al.*, 1986), whereas low concentrations of compounds specific for the adenosine A₁-receptor promote adhesion of stimulated neutrophils to cultured endothelial cells and other surfaces. Higher concentrations of adenosine and adenosine A₂-receptor specific agonists inhibited the adherence of activated neutrophils to endothelial cells (Cronstein *et al.*, 1992).

Low concentrations of adenosine A₁-receptor agonists promote phagocytosis of immunoglobulin-coated red blood cells, while higher concentrations of adenosine A₂-receptor agonists have been reported to inhibit phagocytosis of Ig-coated red blood cells (Salmon & Cronstein, 1990).

Leukotriene production

Adenosine accumulates in leucocyte suspensions as a consequence of the extracellular breakdown of ATP. This accumulation of adenosine results in attainment of levels of this agent that exert suppressive effects on neutrophil functions, including LTB₄ synthesis. Thus, endogenous adenosine is an efficient modulator of LTB₄ biosynthesis by neutrophils and in inflammatory exudates rich in neutrophils, accumulation of adenosine may play an important role in modulating the local generation of important lipid mediators of inflammation (Krump *et al.*, 1997).

Calcium

Adenosine does not interrupt the early PLC-mediated signals (the early wave of diacylglycerol and inositol 1,4,5-triphosphate production, as well as Ca²⁺ mobilisation observed 10-20 seconds after chemoattractant receptor occupancy), but decreases by 50% the sustained increase in diacylglycerol synthesis in these cells (Walker *et al.*, 1990; Cronstein & Haines, 1992), as well as the late increment in [Ca²⁺]_i, presumably by attenuating the influx of extracellular Ca²⁺ (Cronstein, 1994).

1.9.2 Adenosine and polypeptide mediators of inflammation

Adenosine has been reported to inhibit the synthesis of pro-inflammatory cytokines, such as TNF- α by LPS-treated monocytes and macrophages (Parmely *et al.*, 1993), as well as the secretion of complement C2 by stimulated monocytes (Lappin & Whaley, 1984). In lymphocytes, adenosine inhibits the synthesis of immunoglobulins (Moroz & Stevens, 1980), as well as lymphocyte-mediated cytotoxicity (Wolberg *et al.*, 1975).

1.9.3 Adenosine receptors (ARs)

When acting on cell surface receptors, adenosine elicits a large number of responses throughout several organ systems. Activation of adenosine receptors can occur in response to endogenous adenosine or upon administration of adenosine (exogenous) or its more stable pharmacologic analogues. The anti-inflammatory and immunosuppressive potential of adenosine is, however, restricted by several factors *viz* the short half-life (2 seconds) in the circulation, receptor promiscuity, and potent cardiovascular effects (hypotension and bradycardia). These limitations have been overcome by the development of pharmacologic adenosine receptor agonists which are resistant to adenosine deaminase and which have receptor specificity (Cronstein, 1994).

Four different types of adenosine receptors have been identified, A₁, A_{2A}, A_{2B} and A₃. There is indirect evidence for the presence of A₁ and A₃ receptors on granulocytes (Fredholm *et al.*, 1996; Bouma *et al.*, 1997; Walker *et al.*, 1997) and fairly compelling evidence for the presence of A_{2A} receptors on neutrophils (Varani *et al.*, 1998). However, the exact involvement of these receptors in mediating the anti-inflammatory effects of adenosine remains to be conclusively established (Bouma *et al.*, 1997; Fredholm *et al.*, 1996), as does the role of cAMP (Cronstein, 1994; Varani *et al.*, 1998).

A₁R

This receptor is a single polypeptide, consisting of 326 amino acids with a molecular weight of ~ 36 kDa and is expressed in brain, spinal cord, fat, testis, heart and kidney. The A₁R has been classically associated with the inhibition of adenylyl cyclase (i.e. decreased production of cAMP) and is linked to G_i signal transduction proteins. The chemoattractant receptors of neutrophils are similarly linked to these pertussis toxin-sensitive signal transduction systems and amplification of G_i-stimulated signals by A₁ agonists may account for their enhancement of chemotaxis and phagocytosis. Alternatively, occupancy of A₁ receptors may promote more efficient recycling of chemoattractant and Fc receptors to effect more rapid chemotaxis and phagocytosis (Cronstein, 1994).

A_{2A}R

This is also a single polypeptide of 410-412 amino acids and molecular weight 45 kDa. A_{2A} receptors are present in human brain, heart, lung, kidney, liver and platelets. Occupation of A_{2A}R is associated with activation of adenylyl cyclase via G_s-protein and increased intracellular cAMP. Resultant generation of cAMP is responsible for A_{2A}R-mediated inhibition of platelet aggregation, modulation of neutrophil function as described below and possibly vasodilation of certain vascular beds. The receptor demonstrates high affinity binding of several pharmacologic adenosine mimics such as CGS 21680, PAPA-APEC and NECA.

Interestingly, sustained elevations in circulating adenosine concentrations, leading to uncontrolled interaction with A_{2A} receptors on T- and B-lymphocytes is thought to underpin the immunologic hyporesponsiveness of these cells in patients with the adenosine deaminase deficiency variant of severe combined immunodeficiency disease.

Adenosine, via occupancy of A_{2A}R on neutrophils, inhibits their adherence to endothelial cells, as well as generation of superoxide and phagocytosis (Cronstein, 1994). A_{2A} Receptor activation exerts cardioprotection, primarily during reperfusion, by inhibiting neutrophil function (superoxide radical production) and reducing the adherence of these cells to vascular endothelium (Jordan *et al.*, 1999; Harada *et al.*, 2000). A_{2A} Receptor activation has also been reported to protect against

inflammation-mediated renal injury by preventing neutrophil adhesion (Okusa *et al.*, 2000). The mechanism by which adenosine inhibits neutrophil function is not fully understood. However, it is evident that the $A_{2A}R$ is involved and that engagement of this receptor results in elevated intracellular cAMP, which has long been recognised as a cellular event leading to inhibition of the functional responses of neutrophils (Iannone *et al.*, 1989). Some studies do, however, cast doubt on the hypothesis that cAMP is the intracellular messenger for inhibition of O_2^- generation via activation of $A_{2A}R$. It has been reported for example that treatment of neutrophils with cell-soluble analogues of cAMP, inhibits O_2^- generation which is completely reversed by inhibitors of cAMP-dependent protein kinase (protein kinase A) (Cronstein *et al.*, 1992). Supporting a role for cAMP is the finding of synergistic effects of $A_{2A}R$ agonists and type IV phosphodiesterase inhibitors (Sullivan *et al.*, 1995). The inhibitory effects of $A_{2A}R$ occupancy on O_2^- generation are, however, reported to be unaffected, or only partially counteracted, by protein kinase A inhibitors, indicating that adenosine does not utilise the cAMP-protein kinase A system to inhibit O_2^- generation (Cronstein *et al.*, 1992). It is possible, however, that other neutrophil functions are affected as a result of $A_{2A}R$ -mediated increases in intracellular cAMP concentrations (Cronstein, 1994). Nevertheless, the possible involvement of cAMP in $A_{2A}R$ -mediated inhibition of superoxide production by activated neutrophils requires additional investigation.

As mentioned above, coupling of the $A_{2A}R$ to adenylyl cyclase results in enhanced cAMP levels in neutrophils. Other agents that cause elevation of intracellular cAMP e.g. PGE_2 , the β -adrenergic agonist isoproterenol and the type 4 phosphodiesterase inhibitor rolipram all inhibit LT biosynthesis and/or AA release in activated neutrophils (Ham *et al.*, 1983; Fonteh *et al.*, 1993). Indirect, but compelling evidence indicates that elevation of intracellular cAMP levels results in profound inhibition of AA release in activated neutrophils and that cAMP is an intracellular mediator of the inhibitory effect of $A_{2A}R$ agonists on LTB_4 biosynthesis in human neutrophils.

Flamant *et al.*, (2000) have reported that CGS 21680, an analogue of adenosine expressing high selectivity for the $A_{2A}R$, potently inhibits LTB_4 biosynthesis, both in heparinised whole blood stimulated with FMLP and in isolated, agonist-stimulated

neutrophils. Studies with adenosine analogues selective for the A_1 and A_{2A} receptors, as well as with selective A_{2A} receptor antagonists, clearly established that the inhibitory effect of adenosine on LT biosynthesis involves occupancy of the $A_{2A}R$ on neutrophils (Krump *et al.*, 1996). The rapid rise in the release of AA by neutrophils primed with LPS and TNF- α and stimulated with PAF was almost completely abolished by the $A_{2A}R$ agonist CGS 21680. CGS 21680 was also shown to completely inhibit AA release in thapsigargin-activated neutrophils (Krump *et al.*, 1996; Flamant *et al.*, 2000).

As the release of AA in activated neutrophils is a Ca^{2+} -dependent process, a mechanism to account for the inhibition of this release by CGS 21680 was ascribed to the inhibitory effect of adenosine on agonist-induced Ca^{2+} influx in neutrophils. However, neutrophils activated by PAF in the absence of extracellular Ca^{2+} consistently produced 5-lipoxygenase products which were inhibitable by CGS 21680. The activation of cPLA₂, a cytosolic enzyme in resting neutrophils, involves two events: a Ca^{2+} -dependent translocation to nuclear structures on cell activation and phosphorylation of Ser-505 which results in increased catalytic activity (Leslie, 1997). Neither of these events was affected by CGS 21680 (Flamant *et al.*, 2000). Thus, engagement of the $A_{2A}R$ causes inhibition of AA release in activated neutrophils by an as yet unknown mechanism.

$A_{2B}R$

These receptors mediate a theophylline-sensitive, NECA-induced stimulation of adenylyl cyclase. $A_{2B}R$ transcripts are expressed in caecum, large intestine, bladder, brain, spinal cord and lung. Examples of coupling of the $A_{2B}R$ to phospholipase C stimulation are limited and receptor-mediated increases in inositol phosphate have not been reported. $A_{2B}R$ -mediated stimulation of calcium channel activity has been described and this receptor subtype may mediate the secretory action of adenosine on mast cells.

A_3R

This is a polypeptide of 320 amino acids, which has been isolated in testis, kidney, heart, lung and brain. Stimulation of the receptor results in inhibition of adenylyl

cyclase activity. The abundant levels of A₃R mRNA in the lung may be significant in mediating allergic responses in the airways. The A₃ receptor exhibits large differences in structure, tissue distribution and pharmacologic properties between species, hampering the extrapolation of properties of this receptor between species (Linden, 1994). The relevance of non-human models to human A₃ function should, however, be reviewed with caution. Whereas A₃R activation inhibits degranulation of human neutrophils, it facilitates degranulation of rat mast cells (Fozard *et al.*, 1996). The mechanism involved in A₃R-mediated responses in the rat include activation of phospholipase C with elevation of intracellular inositol 1,4,5-triphosphate and Ca²⁺ levels, generation of nitric oxide and subsequent elevation of cGMP (Ramkumar *et al.*, 1993). In contrast, in human macrophages, A₃R-mediated inhibition of TNF- α expression has been shown to be independent of phospholipase C activation (Sajjadi *et al.*, 1996). The underlying mechanisms involved in A₃R-mediated inhibition of neutrophil degranulation remain to be defined.

Much work has focused on the ability of A₃Rs to promote preconditioning, and recently the involvement of this receptor in myocardial ischaemia and reperfusion injury has been investigated. It was demonstrated that pre-treatment with the selective A₃R agonist, IB-MECA, protected isolated rabbit hearts from injury induced by 30 min of regional ischaemia and crystalloid reperfusion (Jordan *et al.*, 1999). Cardioprotection has been shown to be elicited through stimulation of myocardial K_{ATP} channels. The mechanisms involved were investigated by Jordan *et al.*, (1999) who found that IB-MECA, at concentrations of 0.1-100nM, did not regulate superoxide production or the release of myeloperoxidase (degranulation) from isolated canine PMNs. This is in contrast to what was suggested by Bouma *et al.*, (1997) who demonstrated that >1 μ M IB-MECA inhibited release of bacterial permeability-increasing protein, elastase and defensins from human PMNs in whole blood in response to cytokine and endotoxin stimulation. It is possible that the higher concentrations used by Bouma *et al.*, (1997) may have resulted in inhibition of degranulation by influencing other adenosine receptor subtypes, notably the A_{2A} receptor. Alternatively, the disparate observations may be ascribed to species differences, measurement of different granule proteins, or differences in the stimulants used to activate the PMNs.

It was speculated that the inhibitory effect of A_3R agonists on neutrophil adherence may be mediated by regulation of surface expression of adhesion molecules. Bouma *et al.*, (1997), demonstrated that adenosine reduced the surface expression of E-selectin and VCAM-1 in human endothelial cell cultures stimulated with $TNF-\alpha$. Preservation of endothelial function was observed when adherence of neutrophils was blocked with antibodies to endothelial adhesion molecules.

1.10 Hypothesis

The hypothesis to be tested is that adenosine receptor agonists possess anti-inflammatory properties which, unlike those of corticosteroids, enable these agents to suppress the harmful, pro-inflammatory, pro-oxidative and proteolytic activities of activated human neutrophils and that these are mediated predominantly through $A_{2A}R$ and increases in intracellular cAMP.

1.11 Objectives

The primary objectives of my study were as follows:

- to investigate the anti-oxidative and anti-inflammatory interactions of adenosine receptor agonists operative at the level of A_1 , A_{2A} and A_3 receptors with human neutrophils *in vitro*.
- to identify which receptor subtype(s) are predominantly involved in mediating anti-inflammatory activity.
- to investigate the relationship, if any, between the anti-inflammatory effects of occupation of A_1 , A_{2A} and A_3 receptors on neutrophils and alterations in intracellular cAMP.

- to investigate the effects of AR occupancy on Ca^{2+} fluxes and restoration of Ca^{2+} homeostasis in activated neutrophils as possible mechanisms of anti-inflammatory activity.
- to investigate and compare the effects of dexamethasone with those of an $\text{A}_{2\text{A}}\text{R}$ agonist (CGS 21680) on the early-activatable (superoxide production, elastase release) and late-activatable (interleukin-8 production) pro-inflammatory functions of human neutrophils *in vitro*.

Chapter 2

Effects of Dexamethasone on the Early- and Late-Activatable Pro-Inflammatory Functions of Human Neutrophils

2.1 Introduction

Glucocorticoids are the most well-known and potent anti-inflammatory agents in use today to suppress inflammatory and immunologic responses in conditions such as bronchial asthma, cerebral oedema, inflammatory bowel disease and collagen vascular disease. Their mechanism of action has been a topic of intense study and speculation and it has become evident that a vast array of cell types are targets of their action. Neutrophils play a prominent role in the inflammatory response and therefore the effects of glucocorticoids on neutrophil function are of considerable importance.

Rapidly-activatable pro-inflammatory neutrophil functions include adhesion to vascular endothelium, oxidant production, chemotaxis, phagocytosis and degranulation (Witko-Sarsat *et al.*, 2000). Neutrophils were previously thought to be devoid of transcriptional activity with little or no capacity for protein synthesis. However, convincing molecular evidence has now revealed that the neutrophil is not only a target, but also a source of cytokines, chemokines and growth factors (Cassatella, 1999). Interestingly, IL-8 is the most abundantly secreted cytokine by neutrophils and neutrophils are also the primary cellular target of IL-8, which is the most powerful chemoattractant for these cells (Gainet *et al.*, 1998).

Glucocorticoids require considerable time to mediate their anti-inflammatory actions both *in vivo* and *in vitro* and receptor-mediated effects occur at submicromolar concentrations. Most glucocorticoids used experimentally saturate the glucocorticoid receptor at concentrations of 1 μ M or less (Ballard *et al.*, 1975). The use of higher concentrations yields results which are probably not relevant to glucocorticoid receptor-mediated effects. Dexamethasone is a commonly used glucocorticoid and is known to be approximately 25 times as potent as hydrocortisone.

The direct modulatory effects of corticosteroids on early-activatable neutrophil functions such as adhesion to vascular endothelium, superoxide production, granule enzyme release and phagocytosis are controversial as already outlined in section 1.8.3 (page 33), while there is some evidence to suggest that these agents may

suppress the later-onset production of pro-inflammatory cytokines such as IL-8 by these cells (Cox, 1995).

The experiments described in this chapter were designed to investigate the effects of a potent glucocorticoid, dexamethasone, on the rapidly-activatable (superoxide production and elastase release), as well as the late-activatable pro-inflammatory functions (IL-8 production) of human neutrophils *in vitro*.

2.2 Materials and Methods

2.2.1 Chemicals and reagents

Unless indicated these were purchased from the Sigma Chemical Co, St Louis, MO, USA.

2.2.2 Neutrophils

Purified neutrophils were prepared from heparinised (5 units of preservative-free heparin/ml) venous blood of healthy adult human volunteers and separated from mononuclear leucocytes by centrifugation on Histopaque®-1077 (Sigma Diagnostics) cushions at 400 g for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatine to remove most of the erythrocytes. Residual erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (> 90%) and viability (>95%), were resuspended to 1×10^7 /ml in PBS and held on ice until used. Purity of isolated neutrophils was assessed microscopically and assessment of viability was done by dye-exclusion using 0.1% methylene blue.

2.2.3 Oxidant generation

This was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) method (Minkenbergh & Ferber, 1984). Neutrophils ($1 \times$

10^6 /ml final) were preincubated for 15 min in 900 μ l indicator-free Hanks' balanced salt solution (HBSS, pH 7.4, Highveld Biological, Johannesburg, South Africa) containing 0.2 mM lucigenin in the presence and absence of the glucocorticoid, dexamethasone (1.25, 2.5, 5 and 10 μ M, kindly provided by Dr M Johnson, Glaxo Smith Kline, Stockley Park, London, UK), prior to activation with the synthetic chemotactic tripeptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 μ M final). Spontaneous and FMLP (1 μ M)-activated LECL responses were then recorded using a LKB Wallac 1251 chemiluminometer after the addition of the stimulant (100 μ l). LECL readings were integrated for 5 sec intervals and recorded as mV x seconds⁻¹ (mV.sec⁻¹). FMLP was used as stimulant because it causes activation of measurable Ca²⁺-dependent pro-inflammatory responses. Additional experiments were performed in the same manner to investigate the effect of dexamethasone (10 μ M) on the LECL responses of neutrophils activated with PMA (phorbol 12-myristate 13-acetate 25 ng/ml, 100 μ l) and the superoxide-scavenging properties of dexamethasone (10 μ M) using a cell-free hypoxanthine (1 mM)-xanthine oxidase (17 milliunits/ml) superoxide-generating system.

2.2.4 Elastase release

Neutrophil degranulation was measured according to the extent of release of the primary granule-derived protease, elastase. Neutrophils suspended in HBSS in the presence or absence of dexamethasone (1.25, 2.5, 5 and 10 μ M) were incubated for 10 min at 37°C. The stimulant FMLP (0.1 μ M) in combination with CB (cytochalasin B, 1 μ M final) was then added to the cells which were incubated for 10 min at 37°C. The final neutrophil concentration in each tube was 2×10^6 in a volume of 1 ml HBSS. The tubes were then transferred to an ice-bath, followed by centrifugation at 400 g for 5 min to pellet the cells. The neutrophil-free supernatants were then decanted and assayed for elastase activity using a micro-modification of a standard spectrophotometric procedure (Beatty *et al.*, 1982). Briefly, 125 μ l of supernatant was added to 125 μ l of the elastase substrate, N-succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide, 3 mM in 0.3% dimethyl sulphoxide in 0.05 M Tris-HCl (pH 8.0). Elastase activity was assayed at a wavelength of 405 nm and the results for dexamethasone-treated systems expressed as the mean percentage of the amount of enzyme released by the corresponding FMLP/CB-activated, drug-free control

systems. Absolute concentrations of the protease were determined from a standard curve constructed using varying concentrations (0.015 – 15.5 units/ml of porcine pancreatic elastase type III).

2.2.5 Interleukin-8 production by neutrophils

Neutrophils (2×10^6) were preincubated for 10 min with and without dexamethasone (10 μ M) in HBSS supplemented with bovine serum albumin (1 mg/ml) followed by the addition of FMLP to activate synthesis of IL-8 (Witko-Sarsat *et al.*, 2000). The final volume in each tube was 2 ml. The remaining tubes were then incubated for 0 and 6 hours at 37°C (a predetermined incubation time) after which total and extracellular IL-8 were measured using an antigen capture ELISA procedure (Roche Diagnostics Corp, Indianapolis, USA). Extracellular cytokine was measured in cell-free supernatants following removal of the neutrophils by centrifugation, while total IL-8 was measured in the lysates of neutrophils which had been treated with the detergent, lysophosphatidylcholine (0.01%), following removal of cellular debris by centrifugation. Quantitatively the lower limit of sensitivity for the IL-8 assay is 12.3 pg/ml. Systems (unstimulated and FMLP-activated) containing cycloheximide (10 μ g/ml) were also included to distinguish between pre-existing and newly-synthesised cytokine. The results of these investigations are expressed as picograms IL-8/ 10^6 neutrophils.

2.2.6 Statistical analysis

The results of each series of experiments are expressed as the mean values \pm SEM. Levels of statistical significance were calculated by paired Student's *t*-test when comparing two groups, or by analysis of variance (ANOVA) with subsequent Tukey-Kramer multiple comparisons test for multiple groups. A computer-based software system (Instat II®) was used for analysis.

2.3 Results

2.3.1 Effects of dexamethasone on superoxide production by neutrophils

The results of these experiments are shown in Tables 2.1 and 2.2 (page 55) for cells activated with FMLP and PMA respectively. In the case of FMLP-activated neutrophils, superoxide production was modestly, but significantly increased by dexamethasone at concentrations of 2.5 and 5 μM and unaffected by this agent at concentrations of 1.25 and 10 μM . In the case of PMA-activated cells dexamethasone, at the highest concentration tested (10 μM) caused slight inhibition of superoxide production by neutrophils.

Dexamethasone at the highest concentration tested (10 μM) did not possess superoxide-scavenging properties. These results are shown in Table 2.3 (page 56).

2.3.2 Effects of dexamethasone on the release of elastase by neutrophils

These results are shown in Table 2.4 (page 56). Dexamethasone at all concentrations tested did not significantly inhibit the release of elastase from FMLP/CB-activated neutrophils. At a concentration of 10 μM it significantly increased elastase release from FMLP/CB-activated neutrophils.

2.3.3 Effects of dexamethasone on the release of IL-8 from unstimulated and FMLP-activated neutrophils

These results are shown in Table 2.5 (page 57). Relative to the time 0 values, the concentrations of IL-8, both extracellular and total, increased after 6 hours for the system containing unstimulated (no FMLP) cells and were not significantly affected by dexamethasone. Activation of the cells with FMLP resulted in a slight increase in the concentration of extracellular IL-8, while the total concentration was unaffected.

Inclusion of dexamethasone in FMLP-activated systems was not accompanied by significant alterations in either the total or extracellular IL-8 concentrations. The

increase in both extracellular and total IL-8 during the 6 hour incubation period for both resting and FMLP-activated systems was attenuated by inclusion of cycloheximide (2.5 μM), confirming that the observed increases in IL-8 following 6 hours of incubation at 37°C are due to increased synthesis of the cytokine.

Table 2.1: The effects of dexamethasone 1.25, 2.5, 5 and 10 μM on superoxide generation by FMLP-activated neutrophils.

System	Superoxide Production	
	Absolute values (mV.sec ⁻¹)	Percentage of control
Background	190 ± 13	
FMLP control	968 ± 100	
FMLP + Dex 1.25 μM	964 ± 99	96 ± 3
FMLP + Dex 2.5 μM	1055 ± 105	112 ± 5 *
FMLP + Dex 5 μM	1133 ± 97	116 ± 6 *
FMLP + Dex 10 μM	1056 ± 116	110 ± 4

The results of 14 experiments are presented as the mean percentages ± SEMs of the corresponding control systems for which the absolute peak values, observed at 10 min after the addition of FMLP, were expressed as mV.sec⁻¹. *P<0.05 for comparison with the control.

Table 2.2: The effects of dexamethasone 10 μM on superoxide production by PMA-activated neutrophils.

System	Superoxide production (mV.sec ⁻¹)	Percentage of control
FMLP + Control	6503 ± 370	
FMLP + Dex 10 μM	6523 ± 319	86.8 ± 3

The results of 10 experiments are presented as the mean values ± SEM of the Corresponding control system for which the absolute peak values observed at 10 min after the addition of PMA were expressed as mV.sec⁻¹.

Table 2.3: Superoxide production by the hypoxanthine (1 mM)-xanthine oxidase (18 milliunits/ml) system in the presence and absence of dexamethasone.

System	Superoxide production (mV.sec ⁻¹)
Control	1519 ± 193
Dex 10 µM	1476 ± 153

The results of 12 experiments expressed as the mean values ± SEMs in mV.sec⁻¹.

Table 2.4: The effects of dexamethasone on release of elastase from FMLP/CB-activated neutrophils.

System	Elastase release	
	absolute values (milliunits enzyme/10 ⁷ cells)	Percentage of control
Control	80 ± 15	
Dex 1.25 µM	77 ± 14	98 ± 3
Dex 2.5 µM	79 ± 16	95 ± 4
Dex 5 µM	83 ± 16	106 ± 5
Dex 10 µM	90 ± 17	113 ± 5 *

The results of 20 experiments are presented as the mean percentages ± SEMs of the corresponding control systems for which the absolute values were expressed as millinunits enzyme/10⁷ cells. *P<0.05 for comparison with the control.

2.4 Discussion

The results presented in this chapter are those of experiments which were designed with the primary objective of identifying the effects, if any, of dexamethasone, considered to be a potent anti-inflammatory corticosteroid operating at the level of gene transcription, on several pro-inflammatory functions of human neutrophils *in vitro*. Even at extremely high concentrations dexamethasone did not interfere with

(but rather slightly potentiated) the production of superoxide by, as well as the release of elastase from, FMLP-activated neutrophils, underscoring the independence of these rapidly-occurring events (maximal within 1 minute) on *de novo* protein synthesis (Snyderman & Uhing, 1992). These observations also appear, at least in the case of dexamethasone, to exclude the existence of non-classical/non-conventional mechanisms (eg. membrane-stabilising actions) of anti-inflammatory activity which may be evident at high, therapeutically improbable concentrations of this agent. These observations are in agreement with the findings of Cox (1995) who reported that coincubation of neutrophils with glucocorticoids for up to 24 hours did not interfere with, but rather moderately increased chemoattractant-activated production of superoxide by these cells. This effect after prolonged exposure of neutrophils and glucocorticoids was attributed to the inhibitory effects of these agents on spontaneous apoptosis. Although not included, I have found similar results with fluticasone to those observed with dexamethasone i.e. no inhibitory effects on the production of superoxide by and release of elastase from activated human neutrophils.

Table 2.5: The effect of dexamethasone on the spontaneous and FMLP-activated synthesis of IL-8.

System	IL-8 concentrations (pg/10 ⁶ cells)	
	Extracellular	Total
Unstimulated time 0	0	100 ± 18
*Unstimulated control	434 ± 79	793 ± 9
Unstimulated control + dexamethasone (10 µM)	232 ± 26	653 ± 87
FMLP-activated control	557 ± 78	629 ± 303
FMLP-activated + dexamethasone (10 µM)	469 ± 36	1083 ± 33
Unstimulated + cycloheximide (2.5 µM)	32 ± 2	213 ± 7

The results of 4 experiments with triplicate determinations for each are presented as the mean values ± SEMs.

*With the exception of the time 0 value, all the other values shown are for systems incubated for 6 hours at 37°C.

Likewise the production of IL-8 by unstimulated and FMLP-activated neutrophils following 6 hours of incubation at 37°C was unaffected by dexamethasone. This observation differs from the findings of Cox (1995) who reported that dexamethasone inhibited the production of IL-8 by unstimulated neutrophils (activated systems were not included). There are, however, some notable differences between my study and that of Cox (1995). Most importantly, Cox (1995) used an incubation period of 24 hours in comparison with the 6 hour incubation used in the current study. I did not go beyond 6 hours because of the onset of spontaneous apoptosis (observations made by my colleague Ms R Cockeran which are a component of her PhD thesis) in neutrophils cultured *in vitro*, which complicates interpretation of results. I do concede, however, that extended exposure of neutrophils may have resulted in decreased synthesis of IL-8 by these cells. Interestingly, dexamethasone appeared to inhibit release of IL-8 in resting neutrophils without affecting synthesis of the chemokine.

In support of my contention that neutrophils are insensitive (at least over the first 6 hours of exposure) to the inhibitory effects of corticosteroids on the biosynthesis of the pro-inflammatory cytokine IL-8, which appears to be the most abundant of the neutrophil-derived cytokines (Witko-Sarsat *et al.*, 2000), it has recently been reported that these cells have relatively low levels of the α -isoform of the glucocorticoid receptor (Strickland *et al.*, 2001). This is the biologically active form of the receptor. Instead, neutrophils have high levels of the GR β isoform which lacks the steroid binding domain and antagonises GR α through the formation of CR α /GR β heterodimers.

Indirect modulatory effects of corticosteroids on neutrophils achieved through inhibition of the production of pro-inflammatory cytokines such as TNF- α , IL-1 and IL-8, as well as bioactive phospholipids such as PAF are not excluded by the current study and their potential importance is acknowledged. However, the apparent insensitivity of the early-activatable, and possibly also the late activatable pro-inflammatory functions of human neutrophils to the direct, inhibitory actions of corticosteroids does underscore the requirement for novel, neutrophil-directed anti-

inflammatory chemotherapeutic strategies and is the topic of the detailed studies presented in the remainder of this thesis.

Chapter 3

**Apparent Involvement of the A_{2A} Subtype Adenosine Receptor in
the Anti-Inflammatory Interactions of CGS 21680,
Cyclopentyladenosine and IB-MECA with Human Neutrophils**

3.1 Introduction

The wide-ranging, receptor-mediated, physiologic activities of adenosine involve interactions of this agent with at least four types of plasma membrane receptors, designated A_1 , A_{2A} , A_{2B} and A_3 . These vary with respect to ligand binding properties, tissue distribution and transductional mechanisms utilized in intracellular signalling (Stiles, 1992; Cronstein, 1994). Although adenosine is an important regulator of many physiologic processes, including immune and inflammatory responses, its chemotherapeutic potential is limited by an extremely short half life *in vivo* and by receptor promiscuity (Stiles, 1992; Cronstein, 1994). These problems have resulted in the development of synthetic agonists which are selective for the different types of AR.

The broad spectrum anti-inflammatory properties of adenosine and its analogues are well-recognized and span many different types of immune and inflammatory cells, including neutrophils (Cronstein, 1994; Hannon *et al.*, 1998) and eosinophils (Walker *et al.*, 1997; Ezeamuzi & Philips, 1999). There is compelling evidence for the presence of A_{2A} receptors on human neutrophils (Fredholm *et al.*, 1996; Varani *et al.*, 1998), while indirect evidence supports the existence of A_1 and A_3 receptors on these cells, as well as on eosinophils (Rose *et al.*, 1988; Bouma *et al.*, 1997; Fredholm, 1997; Walker *et al.*, 1997; Ezeamuzie & Philips, 1999). With respect to neutrophils, A_1 and A_{2A} receptors have been reported to exert opposing effects on the pro-inflammatory activities of these cells. Interaction of adenosine or its analogues with A_1 receptors on neutrophils has been reported to potentiate adherence to vascular endothelium and chemotaxis (Cronstein *et al.*, 1985; Cronstein *et al.*, 1992), while activation of A_{2A} receptors results in suppression of the production of reactive oxidants by these cells (Cronstein *et al.*, 1985; Hannon *et al.*, 1998), as well as decreased expression of β_2 -integrins and adherence to vascular endothelium (Cronstein *et al.*, 1992; Nolte *et al.*, 1992). Neutrophil degranulation on the other hand has been reported to be either insensitive to adenosine (Cronstein *et al.*, 1985), or to be inhibited by mechanisms involving both A_2 and A_3 receptors (Bouma *et al.*, 1997). In the case of eosinophils, interaction of adenosine or its analogues with A_3

receptors appears to promote down-regulation of the pro-inflammatory activities of these cells (Walker *et al.*, 1997; Ezeamuzie & Philips, 1999).

Although adenosine and its analogues acting via A_{2A} receptors suppress some of the pro-inflammatory activities of activated neutrophils, there are several aspects of this relationship, including the involvement of cAMP and the apparent insensitivity of degranulation, which require clarification. With this in mind, the current study was undertaken to identify the AR types involved in regulating the reactive oxidant-generating and degranulation responses of activated human neutrophils, as well as the dependence of these anti-inflammatory activities on receptor-mediated increases in intracellular cAMP.

3.2 Materials and Methods

3.2.1 Adenosine receptor agonists

N^6 -cyclopentyladenosine (CPA, A_1 R agonist), 2(4-[(2-carboxyethyl)phenyl]ethylamino)-5'-N-ethylcarboxamido adenosine - CGS 21680, A_{2A} R agonist) and N^6 -(3-iodobenzyl)-5'-N-methylcarbamoyladenosine (IB-MECA, A_3 R agonist) and rolipram, a selective inhibitor of type 4 phosphodiesterase, the predominant type found in human neutrophils (Torphy, 1998), were kindly provided by Dr Malcolm Johnson, GlaxoWellcome plc, Stockley Park West, London, UK. These agents were dissolved to stock concentrations of 10 mM in 0.05 N HCl (CPA and IB-MECA), 0.1 N NaOH (CGS 21680) or dimethylsulfoxide (rolipram) and diluted thereafter in indicator-free Hanks' balanced salt solution (HBSS, pH 7.4) and used in the various assays described below at a final concentration range of 0.01-1 μ M. ZM 241385, a highly selective antagonist of A_{2A} receptors (Poucher *et al.*, 1995), was purchased from Tocris Cookson Ltd, Bristol, UK and dissolved to 10mM in 0.1 N NaOH and used at concentrations of 0.1- 2.5 μ M. Unless indicated all other chemicals and reagents were purchased from the Sigma Chemical Co.

3.2.2 Neutrophils

Purified neutrophils were prepared from heparinised (5 units of preservative-free heparin/ml) venous blood of healthy adult human volunteers and separated from mononuclear leucocytes by centrifugation on Histopaque®-1077 (Sigma Diagnostics) cushions at 400 g for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatine to remove most of the erythrocytes. After centrifugation, erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), were resuspended to 1×10^7 /ml in PBS and held on ice until used.

3.2.3 Oxidant generation

This was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) method (Minkenbergh & Ferber, 1984). Neutrophils (1×10^6 /ml, final) were pre-incubated for 15 min in 900 μ l HBSS containing 0.2 mM lucigenin in the presence and absence of the AR agonists (0.01-1 μ M) prior to activation with the synthetic chemotactic tripeptide FMLP (1 μ M). Spontaneous and FMLP (1 μ M)-activated LECL responses were then recorded using a LKB Wallac 1251 chemiluminometer after the addition of the stimulant (100 μ l). LECL readings were integrated for 5s intervals and recorded as $\text{mV} \times \text{seconds}^{-1}$ (mVs^{-1}). Additional experiments were performed to investigate the following : i) the effects of ZM 241385 (2.5 μ M) added during preincubation at 37°C, 5 min before the AR agonists on the CGS 21680, CPA and IB-MECA (1 μ M)-mediated inhibition of the LECL responses of FMLP-activated neutrophils; adenosine (1 μ M) was also included in these experiments to monitor the activity of ZM 241385, ii) the effects of low concentrations (0.1 and 0.25 μ M) of ZM 241385 on the inhibition of FMLP-activated neutrophil superoxide production mediated by CGS 21680, CPA and IB-MECA (all at 1 μ M) and iii) the superoxide-scavenging activity of CGS 21680, CPA, IB-MECA and ZM 241385 using a cell-free hypoxanthine (1 mM)-xanthine oxidase (17 milliunits/ml) superoxide-generating system.

3.2.4 Elastase release

Neutrophil degranulation was measured according to the extent of release of the primary granule-derived protease, elastase. Neutrophils were incubated at a concentration of 2×10^6 /ml in HBSS in the presence or absence of the AR agonists (0.01-1 μ M) with and without ZM 241385 (0.1-2.5 μ M) for 10 min at 37°C. The stimulant FMLP (0.1 μ M) in combination with CB (1 μ M) was then added and the reaction mixtures incubated for 10 min at 37°C. The tubes were then transferred to an ice-bath, followed by centrifugation at 400 g for 5 min to pellet the cells. The neutrophil-free supernatants were then decanted and assayed for elastase activity using a micro-modification of a standard spectrophotometric procedure (Beatty *et al.*, 1982). Briefly, 125 μ l of supernatant was added to 125 μ l of the elastase substrate N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide, 3 mM in 0.3% dimethyl sulfoxide in 0.05 M Tris-HCl (pH 8.0). Elastase activity was assayed at a wavelength of 405 nm and the results expressed as the mean percentage of the amount of enzyme released by the corresponding FMLP/CB-activated, drug-free control systems.

3.2.5 Intracellular cAMP levels

Neutrophils at a concentration of 2×10^6 /ml in HBSS were preincubated for 10 min at 37°C with CGS 21680, CPA or IB-MECA (1 μ M) with and without ZM 241385 (2.5 μ M). Following preincubation, the cells were activated with 1 μ M FMLP (stimulated cells), or an equal volume of HBSS (unstimulated cells), in a final volume of 1 ml, and the reactions terminated and the cAMP extracted by the addition of ice-cold ethanol (65% v/v) at 20 sec, 1 min, 3 min and 5 min after addition of the stimulant. The resultant precipitates were washed twice with ice-cold ethanol and the supernatants pooled and centrifuged at 2000g for 15 min at 4°C. The supernatants were then transferred to fresh tubes and evaporated at 60°C under a stream of nitrogen. The dried extracts were reconstituted in assay buffer (0.05 M acetate buffer, pH 5.8) and assayed for cAMP using the Biotrak cAMP [125 I] scintillation proximity assay system (Amersham International plc.), which is a competitive binding radioimmunoassay procedure. These results are expressed as pmoles cAMP/ 10^7 neutrophils. Because cAMP is rapidly hydrolysed in neutrophils by phosphodiesterases, these experiments were performed in the presence of 1 μ M rolipram.

3.2.6 Spectrofluorimetric measurement of Ca²⁺ fluxes

Fura-2/AM (Calbiochem Corp), was used as the fluorescent, Ca²⁺-sensitive indicator for these experiments. Neutrophils (1×10^7 /ml) were pre-loaded with fura-2 (2 μ M) for 30 min at 37°C in phosphate-buffered saline (PBS, 0.15 M, pH 7.4), washed twice and resuspended in HBSS. The fura-2-loaded cells (2×10^6 /ml) were then pre-incubated with CPA, CGS 21680 or IB-MECA (0.01-1 μ M) at 37°C for 10 min after which they were transferred to disposable reaction cuvettes, which were maintained at 37°C in a Hitachi 650-10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 nm and 500 nm respectively. After a stable baseline was obtained (1 min), the neutrophils were activated by addition of FMLP (1 μ M) and the subsequent increase in fura-2 fluorescence intensity monitored over a 5 min period. The final volume in each cuvette was 3 ml containing a total of 6×10^6 neutrophils. Cytoplasmic Ca²⁺ concentrations were calculated as described previously (Grynkiewicz *et al.*, 1985).

Additional experiments were performed to investigate the effects of pre-treatment with the selective A_{2A} receptor antagonist ZM 241385 at 2.5 μ M on CGS 21680, CPA, and IB-MECA (1 μ M)-mediated alterations in the fura-2 fluorescence responses of FMLP-activated neutrophils.

3.2.7 IL-8 Production

Neutrophils (1×10^6 /ml) were preincubated for 10 min with and without CGS (1 μ M) in HBSS prior to the addition of the synthetic chemotactic tripeptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 μ M, final). FMLP-free control systems received an equal volume of HBSS. The final volume in each tube was 2 ml. Total and extracellular IL-8 were measured using antibody capture ELISA procedures (Roche Diagnostics Corp, Indianapolis) after 6 hours of incubation at 37°C following the addition of FMLP to the cells. Total IL-8 was measured in the lysates of neutrophils which had been treated with 0.01% lysophosphatidylcholine followed by centrifugation at 300 g for 5 min to remove cell debris, while extracellular cytokines

were measured in cell-free supernatants following the removal of the cells by centrifugation.

Calculations were performed by using the standards provided with the kit to prepare a six point calibration curve and a standard curve was plotted correlating the mean absorbance values of the standards (y-axis) to the analyte concentrations of the standard (x-axis). The lot-specific concentration of each standard is listed on its bottle label. Analyte concentrations were determined by locating the mean sample absorbance on the y-axis and reading from the x-axis the analyte concentration that corresponds to the specific absorbance value (h-Interleukin-8 ELISA, cat. No 1 967 932, Boehringer Mannheim).

3.2.8 Statistical analysis

The results of each series of experiments are expressed as the mean values \pm SEM. Levels of statistical significance were calculated by paired Student's *t* test when comparing two groups, or by analysis of variance (ANOVA) with subsequent Tukey-Kramer multiple comparisons test for multiple groups. A computer-based software system (Instat II®) was used for analysis.

3.3 Results

3.3.1 Oxidant production

The effects of CGS 21680, CPA and IB-MECA on superoxide production by neutrophils activated with FMLP are shown in Figure 3.1 (page 67). Superoxide production was inhibited by CGS 21680 at all concentrations tested (0.01-1 μ M) with maximal inhibition observed at concentrations of 0.1-1 μ M. IB-MECA was less effective, causing significant inhibition of superoxide production only at concentrations of 0.5 and 1 μ M, while CPA was the least effective, causing inhibition only at 1 μ M.

The effect of pre-treatment of neutrophils with ZM 241385 (2.5 μM) on the inhibition of the production of superoxide by FMLP-activated neutrophils mediated by 1 μM CGS 21680, CPA and IB-MECA are shown in Table 3.1 (page 68). The A_{2A} receptor antagonist *per se* slightly increased superoxide production, and also neutralized the inhibitory effects of all 3 AR agonists. ZM 241385 (2.5 μM) also inhibited the effects of adenosine (1 μM) on superoxide production by FMLP-activated neutrophils, with the responses of neutrophils exposed to adenosine only or to adenosine + ZM 241385 being $57 \pm 4 \%$ ($p < 0.05$) and $109 \pm 7 \%$ of the corresponding drug-free control system respectively.

The effects of low concentrations of ZM 241385 (0.1 and 0.25 μM) on CGS 21680-, CPA- and IB-MECA (all at 1 μM)-mediated inhibition of superoxide production by FMLP-activated neutrophils are shown in Table 3.2 (page 68). The inhibitory effects of CPA and IB-MECA on neutrophil superoxide production were completely attenuated by ZM 241385 at both concentrations used, while those of CGS 21680 were completely neutralised only at 0.25 μM ZM 241385.

In experiments designed to evaluate the superoxide-scavenging potential of CGS 21680, CPA and IB-MECA, all 3 AR agonists at the highest concentration tested (1 μM), as well as ZM 241385 (2.5 μM) did not possess superoxide-scavenging properties. LECL values for the control system and for systems containing CGS 21680, CPA, IB-MECA and ZM 241385 were 1464 ± 51 , 1419 ± 159 , 1451 ± 75 , 1412 ± 165 and $1443 \pm 131 \text{ mV}\cdot\text{s}^{-1}$ respectively (results of 12 experiments).

3.3.2 Elastase release

The effects of the 3 AR agonists on elastase release from FMLP/CB-activated neutrophils are shown in Figure 3.2 (page 70). CGS 21680 and IB-MECA caused dose-related inhibition of elastase release which was evident at 0.01 μM , while CPA exerted inhibitory effects at 1 μM .

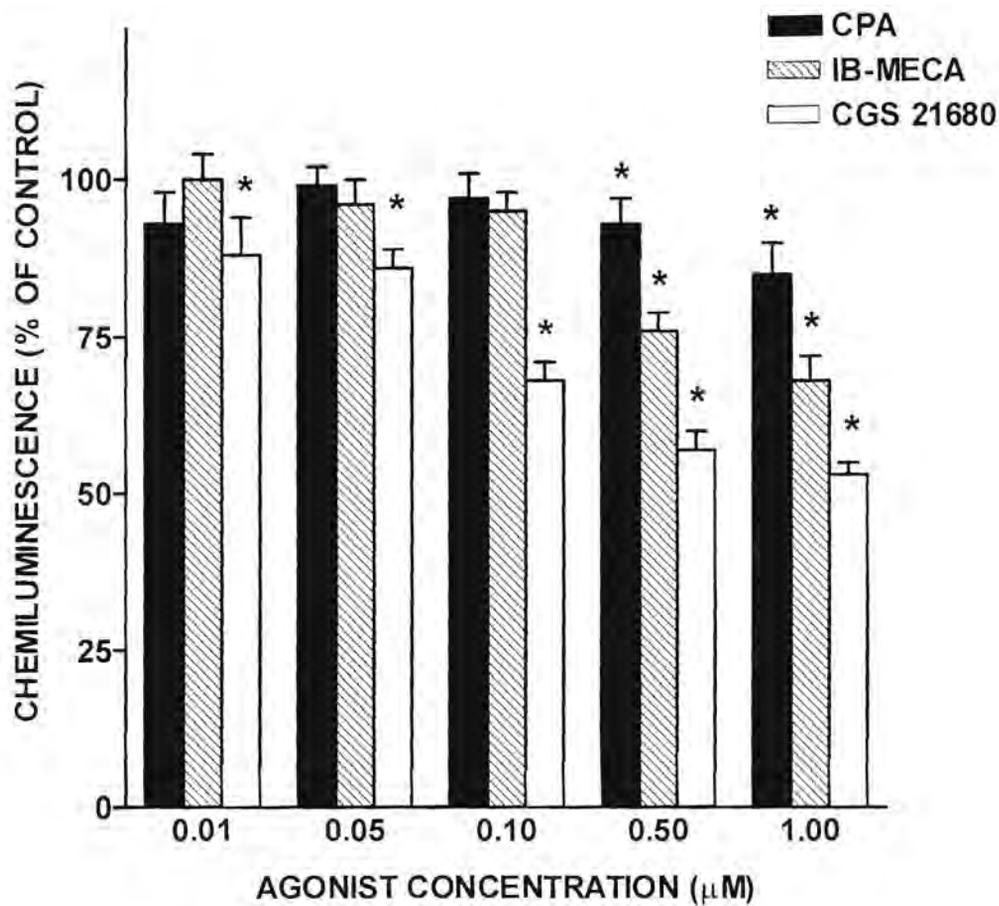


Figure 3.1: The effects of varying concentrations of CGS 21680, CPA, and IB-MECA on the production of superoxide by FMLP-activated neutrophils. The results of 6-23 experiments are presented as the mean percentages \pm SEMs of the control systems. The absolute values for unstimulated and FMLP-activated control neutrophils were 390 ± 112 and 1064 ± 79 mV.sec⁻¹, respectively. *p<0.05.

Table 3.1 : The effects of ZM 241385 on CGS 21680-, CPA-, and IB-MECA-mediated inhibition of neutrophil superoxide generation and elastase release.

System	Superoxide production (% control)	Elastase release (% control)
ZM 241385 2.5 μ M only	108 \pm 8	126 \pm 3
CGS 21680 1 μ M only	56 \pm 3	49 \pm 4
ZM 241385 + CGS 21680	100 \pm 5	126 \pm 10
CPA 1 μ M only	71 \pm 11	70 \pm 1
ZM 241385 + CPA	108 \pm 8	127 \pm 2
IB-MECA 1 μ M only	63 \pm 6	47 \pm 1
ZM 241385 + IB-MECA	96 \pm 7	102 \pm 3

The results of 6-12 experiments are presented as the mean percentages \pm SEMs of the corresponding control systems for which the absolute values were 1358 \pm 57 $\text{mV}\cdot\text{s}^{-1}$ and 600 milliunits enzyme/ 10^7 cells for superoxide production and elastase release respectively.

Table 3.2 : The effects of low concentrations (0.1 and 0.25 μ M) of ZM 241385 on CGS 21680-, CPA-, and IB-MECA-mediated inhibition of neutrophil superoxide production.

System	Superoxide production (% control)
ZM 241385 0.1 μ M only	104 \pm 2
ZM 241385 0.25 μ M only	103 \pm 3
CGS 21680 1 μ M only	59 \pm 1
CGS 21680 + ZM 241385 0.1 μ M	87 \pm 2
CGS 21680 + ZM 241385 0.25 μ M	100 \pm 1
CPA 1 μ M only	72 \pm 2
CPA + 0.1 μ M ZM 241385	108 \pm 7
CPA + 0.25 μ M ZM 241385	109 \pm 3
IB-MECA 1 μ M only	61 \pm 3
IB-MECA + 0.1 μ M ZM 241385	101 \pm 5
IB-MECA + 0.25 μ M ZM 241385	110 \pm 3

Data from 6 experiments are presented as the mean percentages \pm SEMs of the corresponding control system for which the absolute value was 1032 \pm 55 $\text{mV}\cdot\text{s}^{-1}$

The effects of ZM 241385 (2.5 μ M) pre-treatment of neutrophils on the inhibition of FMLP/CB-activated release of elastase mediated by 1 μ M CGS 21680, CPA and IB-MECA are shown in Table 3.1 (page 68). ZM 241385 *per se* potentiated the release of elastase from stimulated neutrophils and completely (CGS 21680 and CPA) or almost completely (IB-MECA) antagonised the inhibitory actions of the AR receptor agonists. Similar effects were observed when ZM 241385 was combined with lower concentrations of CGS 21680 and IB-MECA (data not shown).

3.3.3 cAMP

The effects of CGS 21680 and IB-MECA on intracellular cAMP in unstimulated and FMLP-stimulated neutrophils are shown in Table 3.3 (Page 70). CGS 21680 increased cAMP in resting cells, while IB-MECA had minimal effects. Activation of neutrophils with FMLP resulted in an increase in cAMP which was augmented to a similar extent by both CGS 21680 and IB-MECA at 1 μ M. ZM 241385 caused a drop in cAMP levels in resting neutrophils in both the absence and presence of CGS 21680 and IB-MECA. The A_{2A} receptor antagonist also attenuated the increase in cAMP in FMLP-activated neutrophils in both the absence and presence of the AR agonists.

Treatment of neutrophils with CPA did not significantly affect intracellular cAMP in either unstimulated or stimulated neutrophils. In the case of resting neutrophils, concentrations of intracellular cAMP were 30 ± 2 and 28 ± 20 pmol/ 10^7 cells in the absence and presence of 1 μ M CPA, while the corresponding values for FMLP-activated cells were 127 ± 6 and 85 ± 35 pmol/ 10^7 cells.

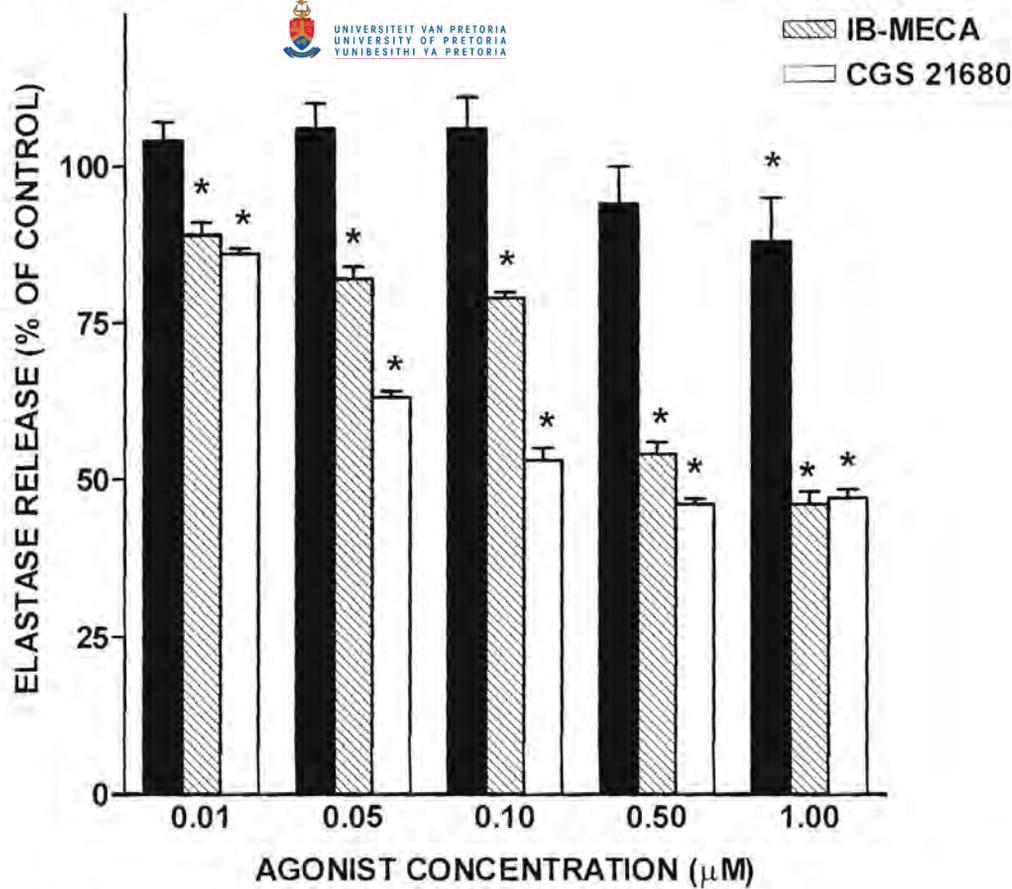


Figure 3.2: The effects of varying concentrations of CGS 21680, CPA, and IB-MECA on the release of elastase from FMLP/CB-activated neutrophils. The results of 15 experiments are presented as the mean percentages \pm SEMs of the control systems. The absolute values for unstimulated and FMLP/CB-activated neutrophils were 3 ± 0.8 and 593 ± 4 milliunits enzyme/ 10^7 cells, respectively. * $p < 0.05$.

Table 3.3 : The effects of CGS 21680 and IB-MECA individually and in combination with ZM 241385 on cAMP in unstimulated and FMLP-activated neutrophils

System	Intracellular cAMP (pmol/ 10^7 cells) on :	
	Unstimulated cells	FMLP-activated cells
Control	30 ± 2	127 ± 6
CGS 21680 1 μ M	59 ± 3	240 ± 15
IB-MECA 1 μ M	39 ± 6	264 ± 16
ZM 241385 2.5 μ M	12 ± 1	41 ± 4
CGS 21680 + ZM 241385	14 ± 1	38 ± 4
IB-MECA + ZM 241385	11 ± 4	68 ± 4

The results of 5 different experiments are presented as the mean values \pm SEMs measured at 1 min after the addition of FMLP.

3.3.4 Fura-2 fluorescence

The results shown in Figure 3.3 (page 72) are traces from a single representative experiment which depict the effects of the 3 AR agonists at 1 μM on the fura-2 responses of FMLP-activated neutrophils. Addition of FMLP to neutrophils was accompanied by the characteristic, abrupt increase in fura-2 fluorescence due to the transient elevation in the concentration of cytosolic Ca^{2+} . This abrupt increase in fluorescence intensity was unaltered by the AR agonists, demonstrating that these agents do not affect the release of Ca^{2+} from cellular stores. However, treatment of neutrophils with CGS 21680 and IB-MECA at 1 μM , and to a lesser extent with CPA, hastened the rate of the subsequent decline in fluorescence intensity, indicative of accelerated clearance of Ca^{2+} from the cytosol.

The results shown in Table 3.4 (page 73) are those from a larger series of experiments and show peak cytosolic Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$), as well as the time taken for fluorescence intensity to decline to half peak ($t_{1/2}$) values for neutrophils activated with FMLP in the presence and absence of varying concentrations of CGS 21680, CPA and IB-MECA. As indicated above, none of the AR agonists affected the abruptly occurring increase in $[\text{Ca}^{2+}]_i$ following activation of the cells with FMLP. However, CGS 21680 (0.01-1 μM) and IB-MECA (0.5-1 μM) caused dose-related acceleration in the rate of decline in peak fluorescence, while CPA was effective only at 1 μM .

The effects of ZM 241385 on CGS 21680-, CPA- and IB-MECA-mediated enhancement of the clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils, as well as on peak $[\text{Ca}^{2+}]_i$ are shown in Table 3.5 (page 73). The $\text{A}_{2\text{A}}$ receptor antagonist prevented CGS 21680-, CPA- and IB-MECA-mediated acceleration of clearance of Ca^{2+} from the cytosol, but did not affect peak $[\text{Ca}^{2+}]_i$.

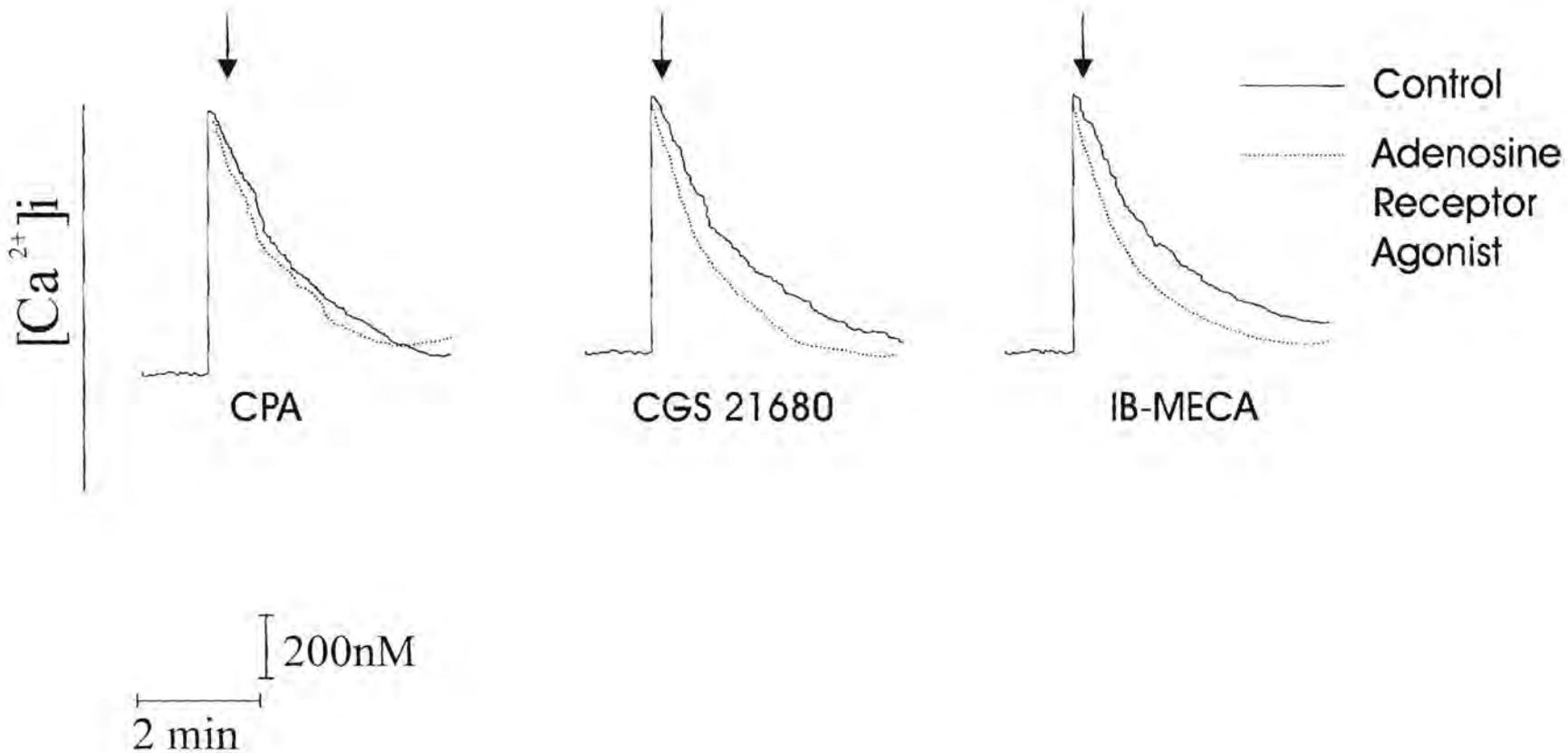


Figure 3.3: FMLP-activated fura-2 responses of neutrophils with and without CGS 21680, CPA, or IB-MECA (all at 1 μ M). FMLP was added as indicated (∇) when a stable base line was obtained (\pm 1 min). Traces are from a single representative experiment.

Table 3.4 : Peak intracellular calcium concentrations $[Ca^{2+}]_i$ and time taken for these to decline to half peak values in FMLP-activated neutrophils treated with the adenosine receptor agonists.

System	Peak $[Ca^{2+}]_i$ values (nM)	Time taken to decline to half peak values (min)
Control	497 ± 28	1.5 ± 0.1
CGS 21680 0.1 µM	442 ± 22	0.9 ± 0.1*
CGS 21680 0.5 µM	425 ± 28	0.9 ± 0.1*
CGS 21680 1 µM	456 ± 39	0.8 ± 0.1*
CPA 0.1 µM	480 ± 55	1.5 ± 0.1
CPA 0.5 µM	461 ± 27	1.4 ± 0.2
CPA 1 µM	486 ± 28	1.2 ± 0.1*
IB-MECA 0.1 µM	485 ± 55	1.3 ± 0.1
IB-MECA 0.5 µM	458 ± 27	1.1 ± 0.1
IB-MECA 1 µM	483 ± 25	1.0 ± 0.1*

The results of 5-12 experiments are presented as the mean values ± SEMs.

* $p < 0.05$ for comparison with the control.

Table 3.5 : The effects of ZM 241385 on peak intracellular calcium concentrations $[Ca^{2+}]_i$ and time taken for these to decline to half peak values in FMLP-activated neutrophils with and without the adenosine receptor agonists

System	Peak $[Ca^{2+}]_i$ values (nM)	Time taken to decline to half peak values
Control	496 ± 9	1.5 ± 0.10
ZM 241385 2.5 µM only	497 ± 6	1.5 ± 0.07
CGS 21680 1 µM only	492 ± 18	1.1 ± 0.04*
ZM 241385 + CGS 21680	485 ± 15	1.5 ± 0.06
CPA 1 µM only	494 ± 6	1.2 ± 0.06*
ZM 241385 + CPA	464 ± 18	1.5 ± 0.07
IB-MECA 1 µM only	497 ± 9	1.1 ± 0.07*
ZM 241385 + IB-MECA	500 ± 9	1.5 ± 0.07

The results of 4 experiments are presented as the mean values ± SEMs.

* $p < 0.05$ for comparison with the control.

3.3.5 IL-8 production

The effect of CGS (1 μ M) on IL-8 production by unactivated and FMLP-activated neutrophils following 6 hours of incubation at 37°C are shown in Table 3.6.

Activation of the cells with FMLP resulted in modest, but statistically significant increases in both extracellular ($p < 0.0002$) and total ($p < 0.008$) IL-8 (relative to the corresponding values for unstimulated cells). Inclusion of CGS 21680 did not significantly affect the synthesis of IL-8 by either unstimulated or FMLP-activated cells.

Table 3.6: The effect of CGS (1 μ M) on IL-8 production by unactivated and FMLP-activated human neutrophils.

System	IL-8 production (pg/10 ⁶ cells)		
	Mean	SEM	N(number)
T=0:			
Control (extracellular IL-8)	0	0	6
Control total (intra- & extracellular IL-8)	99.8	17.8	7
T=6h:			
Unactivated cells:			
Control (extracellular IL-8)	394.2	30.6	9
Control total (intra- + extracellular IL-8)	1099.1	83.4	11
CGS (extracellular IL-8)	361.7	70.3	8
CGS total (intra- + extracellular IL-8)	1153.0	152.5	8
T=6h:			
Activated cells:			
FMLP (extracellular IL-8)	861.5	66.1*	9
FMLP total (intra + extracellular IL-8)	1876.1	312.6**	6
FMLP CGS (extracellular IL-8)	667.1	110.2	8
FMLP CGS total (intra- + extracellular IL-8)	1492.7	298.5	6

The results of these experiments are expressed as the mean values \pm SEMs.

* $P < 0.0002$ for comparison with control (unactivated cells)

** $P < 0.008$ for comparison with control total (unactivated cells)

3.4 Discussion

The results of the present study support the contention that subtype A_{2A} Rs down-regulate the pro-inflammatory activities of human neutrophils (Hannon *et al.*, 1998; Sullivan & Linden, 1998; Varani *et al.*, 1998). Although CPA and IB-MECA were found to suppress the oxidant-generating and degranulation responses of neutrophils, the inhibitory effects of these agents were less than those of CGS 21680, and, in the case of superoxide production, were observed only at concentrations at which receptor selectivity is diminished (Hannon *et al.*, 1998; Sullivan & Linden, 1998). While IB-MECA appeared to be more effective in suppressing elastase release, as opposed to superoxide production by activated neutrophils, apparently consistent with a role for A_3 receptors in regulating neutrophil degranulation (Bouma *et al.*, 1997), this differential sensitivity of the two responses was also observed with CGS 21680. The order of agonist potencies to inhibit the pro-inflammatory activities of neutrophils is the same as that reported by others (Hannon *et al.*, 1998), and also identical to the order of potency to inhibit the A_{2A} receptor defined by radioligand binding to rat and human brain (Jarvis *et al.*, 1989; Wan *et al.*, 1990). Moreover, the inhibitory effects of IB-MECA, as well as those of CGS 21680 and CPA, on both elastase release and superoxide production were neutralised by pre-treatment of the cells with the highly selective A_{2A} R antagonist, ZM 241385. These observations suggest that the concentration-dependent anti-inflammatory interactions of all three AR agonists with neutrophils are mediated through interactions with A_{2A} Rs. However, due to the absence of complete specificity of the various agonists for their respective AR subtypes (Hannon *et al.*, 1998), the data needs to be interpreted cautiously.

The interaction of ZM 241385 with adenosine receptors on human neutrophils was confirmed by the observation that the receptor antagonist completely attenuated the inhibitory effects of adenosine on FMLP-activated superoxide production by these cells. The selectivity of ZM 241385 for the A_{2A} receptor subtype was supported by the observation that at relatively low concentrations (0.1 and 0.25 μ M), the receptor antagonist completely neutralised the anti-oxidative interactions of CPA and IB-

MECA (both at 1 μM) with neutrophils, while complete antagonism of CGS 21680 (1 μM) was only observed with 0.25 μM ZM 241385.

The transient increase in cAMP which accompanies exposure of neutrophils to FMLP is well-recognized (Anderson *et al.*, 1998). In the present study CGS 21680 and IB-MECA, but not CPA, increased cAMP in both unstimulated and FMLP-activated neutrophils, indicative of a relationship between elevated intracellular concentrations of this cyclic nucleotide and suppression of the pro-inflammatory activities of these cells. Failure to detect enhancement of cAMP by CPA may be related to the counteracting effects of this agent on adenylate cyclase, with inhibition and stimulation resulting from interactions with A_1 and A_{2A} receptors respectively (Cronstein, 1994; Hannon *et al.*, 1998). The enhancing effects of both CGS 21680 and IB-MECA on cAMP were attenuated by pre-treatment of neutrophils with ZM 241385, supporting the involvement of A_{2A} receptors.

Not only did ZM 241385 attenuate the increase in neutrophil cAMP mediated by CGS 21680 and IB-MECA, this selective A_{2A} receptor antagonist also decreased cAMP in AR agonist-free, unstimulated cells and abolished the transient increase in cAMP on exposure of these cells to FMLP. These observations support the contention that endogenously generated adenosine released from human neutrophils causes autocrine activation of adenylate cyclase by interacting with A_{2A} receptors, a process which is amplified in response to FMLP (Iannone *et al.*, 1989).

Researchers from my group and elsewhere have previously reported that the anti-inflammatory interactions of cAMP-elevating agents with human neutrophils are achieved through accelerated clearance of Ca^{2+} from the cytosol of activated neutrophils as a result of up-regulation of the activity of the cAMP-dependent protein kinase-modulated, Ca^{2+} -sequestering endo-membrane Ca^{2+} -ATPase (Villagrasa *et al.*, 1996; Anderson *et al.*, 1998). In the present study, CGS 21680, CPA and IB-MECA did not affect the immediately occurring elevation in cytosolic Ca^{2+} following activation of neutrophils with FMLP. However, all three AR agonists, at the same concentrations which inhibited superoxide production and elastase release, accelerated the clearance of Ca^{2+} from the cytosol of the cells. These effects of the AR agonists on neutrophil Ca^{2+} handling were antagonised by pre-treatment of the

cells with ZM 241385, consistent with the involvement of A_{2A} receptors and cAMP. Taken together, these observations are compatible with a mechanism of anti-inflammatory activity involving increased efficiency of the endo-membrane Ca^{2+} -ATPase.

Exposure of neutrophils to FMLP was accompanied by modest, but nevertheless significant increases in both total and extracellular IL-8 following 6 hours of incubation at 37°C. This observation with respect to the total concentration of IL-8 contrasts with the findings reported in Table 2.5 (Chapter 2) in which the corresponding increases in FMLP-activated cells did not achieve statistical significance. This difference may be attributable to the larger series of experiments performed and reported on in the current chapter. Irrespective of these differences, I was unable to detect any significant inhibition of spontaneous and FMLP-activated synthesis of IL-8 by neutrophils treated with CGS 21680. This may be a true effect of CGS 21680. Alternatively, exposure times in excess of 6 hours may be required to detect meaningful effects of CGS 21680 on IL-8 synthesis by neutrophils.

In conclusion, the results of the current study underscore the apparent role of A_{2A} receptors as opposed to A_1 or A_3 receptors, in down-regulating the pro-inflammatory activities of human neutrophils. If safe and highly selective pharmacologic agonists of A_{2A} receptors can be developed, these agents may prove to be particularly useful in the anti-inflammatory chemotherapy of corticosteroid-insensitive, neutrophil-mediated disorders, particularly chronic inflammatory diseases of the airways, including chronic obstructive pulmonary disease, cystic fibrosis, bronchiectasis and certain categories of asthmatics.

Chapter 4

Accelerated Re-sequestration of Cytosolic Calcium and Suppression of the Pro-Inflammatory Activities of Human Neutrophils by CGS 21680 *In Vitro*

4.1 Introduction

In spite of their undisputed clinical efficacy, concerns about the long-term safety of inhaled corticosteroids in patients with bronchial asthma, as well as the apparent insensitivity of neutrophils to the anti-inflammatory effects of these agents, has underscored the requirement for novel anti-inflammatory chemotherapies (Cox, 1995; Meagher *et al.*, 1996; McFadden, 1998). The anti-inflammatory potential of physiologic and pharmacologic cAMP-elevating agents, which spans many different types of immune and inflammatory cells, including neutrophils, has been recognised for more than two decades (Moore & Willoughby, 1995). However, the development of clinically useful cAMP-based, anti-inflammatory chemotherapeutic agents has, until recently, enjoyed limited success due to lack of selectivity of these for immune and inflammatory cells. Recent innovations include the second generation type 4-phosphodiesterase (PDE) inhibitors (Torphy, 1998; Underwood *et al.*, 1998) and adenosine receptor (AR) agonists operative at the level of the A_{2A} receptor subtype (Ongini & Fredholm, 1996).

Novel second generation inhibitors of type 4 PDE, the predominant type found in human neutrophils being PDE 4B2 (Wang *et al.*, 1999), have been designed to maximise anti-inflammatory efficacy in the setting of decreased gastrointestinal toxicity (Torphy, 1998). The anti-inflammatory effects of type 4 PDE inhibitors are mediated by cAMP-dependent mechanisms (Underwood *et al.*, 1998), which, in the case of neutrophils, involve accelerated clearance of cytosolic Ca^{2+} by apparent enhancement of the activity of the endo-membrane Ca^{2+} -ATPase (Anderson *et al.*, 1998).

Subtype A_{2A} receptors have recently been demonstrated on human neutrophils (Varani *et al.*, 1998). Occupation of these by adenosine or adenosine agonists has been reported to suppress the pro-inflammatory activities of human neutrophils, which in some (Hannon *et al.*, 1998; Sullivan & Linden, 1998; Varani *et al.*, 1998), but not all (Cronstein *et al.*, 1985; Iannone *et al.*, 1989) studies has been attributed to a cAMP-dependent mechanism.

In the present study we have investigated the effects of the prototype A_{2A}R agonist, CGS 21680 (Phillis *et al.*, 1990), as well as those of the highly selective A_{2A}R antagonist, ZM 241385 (Poucher *et al.*, 1995), on the pro-inflammatory activities of FMLP-activated human neutrophils *in vitro*, and related changes in these to alterations in Ca²⁺ fluxes and intracellular cAMP.

4.2 Materials and methods

4.2.1 Drugs and reagents

CGS 21680 and rolipram were kindly provided by Dr Malcolm Johnson, (GlaxoWellcome plc, Stockley Park West, London, UK), while thapsigargin and ZM 241385 were purchased from the Sigma Chemical Co and Tocris Cookson Ltd, (Bristol, UK) respectively. Rolipram and thapsigargin were dissolved in DMSO to give a stock concentration of 10 mM for each and diluted in the same solvent. The final concentration of DMSO in all assay systems in which rolipram was used was 0.5% or less and appropriate solvent systems were included. CGS 21680 and ZM 241385 were dissolved in 0.1 N NaOH to give stock solutions of 10 mM, and diluted thereafter in HBSS. Unless indicated, all other chemicals and reagents were purchased from the Sigma Chemical Co.

4.2.2 Neutrophils

Purified neutrophils were prepared from heparinised (5 units of preservative-free heparin/ml) venous blood of healthy adult human volunteers and separated from mononuclear leucocytes by centrifugation on Histopaque®-1077 (Sigma Diagnostics, St Louis, MO, USA) cushions at 400 g for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatine to remove most of the erythrocytes. After centrifugation, erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), were resuspended to 1 x 10⁷/ml in PBS and held on ice until used.

4.2.3 Spectrofluorimetric measurement of Ca^{2+} fluxes

Fura-2/AM (Calbiochem Corp, La Jolla, California, USA) was used as the fluorescent, Ca^{2+} -sensitive indicator for these experiments. Neutrophils ($1 \times 10^7/\text{ml}$) were pre-loaded with fura-2 ($2 \mu\text{M}$) for 30 min at 37°C in phosphate-buffered saline (PBS, 0.15 M, pH 7.4), washed twice and resuspended in indicator-free Hanks' balanced salt solution (HBSS, pH 7.4) containing 1.25 mM CaCl_2 , referred to hereafter as Ca^{2+} -replete HBSS. The fura-2-loaded cells ($2 \times 10^6/\text{ml}$) were then pre-incubated with CGS 21680 (0.01 – $1 \mu\text{M}$) at 37°C for 10 min after which they were transferred to disposable reaction cuvettes, which were maintained at 37°C in a Hitachi 650-10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 and 500 nm respectively. After a stable base-line was obtained (1 min), the neutrophils were activated by addition of the synthetic, chemotactic tripeptide, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, used at a final concentration of $1 \mu\text{M}$) and the subsequent increase in fura-2 fluorescence intensity monitored over a 5 min period. At this concentration ($1 \mu\text{M}$) FMLP activates secretion and superoxide production by neutrophils and has maximal effects on the release of Ca^{2+} from intracellular stores (Snyderman & Uhing, 1992). The final volume in each cuvette was 3 ml containing a total of 6×10^6 neutrophils. Cytoplasmic Ca^{2+} concentrations were calculated as described previously (Grynkiewics *et al.*, 1985).

Additional experiments were performed to investigate the effects of pre-treatment with the selective A_{2A} receptor antagonist ZM 241385 (Poucher *et al.*, 1995) at $2.5 \mu\text{M}$ on CGS 21680-mediated alterations in the fura-2 fluorescence responses of FMLP-activated neutrophils. ZM 241385 was added during preincubation of the cells at 37°C , 5 min prior to CGS 21680.

4.2.4 Mn^{2+} quenching of fura-2 fluorescence

In a limited series of experiments cells loaded with fura-2/AM as described above were activated with FMLP ($1 \mu\text{M}$) in the presence and absence of CGS 21680 ($1 \mu\text{M}$) in HBSS supplemented with $300 \mu\text{M}$ MnCl_2 (added 5 min prior to FMLP) and fluorescence quenching as a measure of Ca^{2+} influx was determined at an excitation

wavelength of 360 nm, which is an isosbestic wavelength, and at an emission wavelength of 500 nm (Geiszt *et al.*, 1997).

4.2.5 Radiometric assessment of Ca^{2+} fluxes

$^{45}\text{Ca}^{2+}$ (Calcium-45 chloride, specific activity 25.4 mCi/mg, Du Pont NEN Research Products, Boston, MA, USA.) was used as tracer to label the intracellular Ca^{2+} pool and to monitor Ca^{2+} fluxes in resting and activated neutrophils. In the assays of Ca^{2+} efflux and influx described below, the radiolabelled cation was always used at a fixed, final concentration of 2 $\mu\text{Ci/ml}$ containing 50 nmol cold carrier Ca^{2+} (as CaCl_2). The final assay volumes were always 5 ml containing a total of 1×10^7 neutrophils. The standardisation of the procedures used to load the cells with $^{45}\text{Ca}^{2+}$, as well as a comparison with silicone oil-based methods for the separation of labelled neutrophils from unbound isotope, have been described elsewhere (Anderson & Goolam Mahomed, 1997).

4.2.6 Efflux of $^{45}\text{Ca}^{2+}$ from FMLP-activated neutrophils

Neutrophils ($1 \times 10^7/\text{ml}$) were loaded with $^{45}\text{Ca}^{2+}$ (2 $\mu\text{Ci/ml}$) for 30 min at 37°C in HBSS which was free of unlabelled Ca^{2+} . The cells were then pelleted by centrifugation, washed once with, and resuspended in ice-cold Ca^{2+} -replete HBSS and held on ice until use, which was always within 10 min of completion of loading with $^{45}\text{Ca}^{2+}$. The $^{45}\text{Ca}^{2+}$ -loaded neutrophils ($2 \times 10^6/\text{ml}$) were then preincubated for 10 min at 37°C in Ca^{2+} -replete HBSS, in the presence and absence of 1 μM CGS 21680, followed by activation with FMLP (1 μM) and measurement of the kinetics (10, 20, 30 and 60 sec) of efflux of $^{45}\text{Ca}^{2+}$. A fixed incubation time of 60 sec was used for dose-response experiments. The reactions were terminated by the addition of 10 ml ice-cold, Ca^{2+} -replete HBSS to the tubes which were then transferred to an ice-bath (Anderson & Goolam Mahomed, 1997). The cells were then pelleted by centrifugation at 400 g for 5 min followed by washing with 15 ml ice-cold, Ca^{2+} -replete HBSS and the cell pellets finally dissolved in 0.5 ml of triton X-100/0.1 M NaOH and the radioactivity assessed in a liquid scintillation spectrometer. Control, cell-free systems (HBSS and $^{45}\text{Ca}^{2+}$ only) were included for each experiment and these values were subtracted from the relevant neutrophil-containing systems. The results

are presented as the amount of cell-associated radiolabelled cation ($\text{pmol } ^{45}\text{Ca}^{2+}/10^7$ cells).

In an additional series of experiments the effects of thapsigargin, a highly specific inhibitor of the endo-membrane Ca^{2+} -ATPase (Lytton *et al.*, 1991), as well as those of ZM 241385 on CGS 21680 ($1 \mu\text{M}$)-mediated modulation of FMLP-activated efflux of $^{45}\text{Ca}^{2+}$ from neutrophils were investigated over a 60 sec time course. Thapsigargin was used at a final, predetermined concentration of $1 \mu\text{M}$ and was added simultaneously with FMLP to $^{45}\text{Ca}^{2+}$ -loaded neutrophils which had been pre-incubated for 10 min with CGS 21680, while ZM 241385 ($2.5 \mu\text{M}$) was added to the cells 5 min before the adenosine receptor agonist.

4.2.7 Influx of $^{45}\text{Ca}^{2+}$ into FMLP-activated neutrophils

To measure the net influx of $^{45}\text{Ca}^{2+}$ into FMLP-activated neutrophils, uncomplicated by concomitant efflux of the radiolabelled cation, the cells were loaded with cold, Ca^{2+} -replete HBSS for 30 min at 37°C after which they were pelleted by centrifugation, then washed once with, and resuspended in ice-cold Ca^{2+} -free HBSS and held on ice until used. Pre-loading with cold Ca^{2+} was undertaken to minimise spontaneous uptake of $^{45}\text{Ca}^{2+}$ (unrelated to FMLP activation) in the influx assay. The Ca^{2+} -loaded neutrophils ($2 \times 10^6/\text{ml}$), were then incubated for 10 min in the presence and absence of CGS 21680 at 37°C in Ca^{2+} -free HBSS followed by simultaneous addition of FMLP and $^{45}\text{Ca}^{2+}$ ($2 \mu\text{Ci}/\text{ml}$), or $^{45}\text{Ca}^{2+}$ only to control, unstimulated systems. The kinetics of influx of $^{45}\text{Ca}^{2+}$ into FMLP-activated neutrophils were then monitored over a 5 min period and compared with those of influx of the radiolabelled cation into the identically-processed, unstimulated cells.

A fixed time interval (5 min) was used for experiments in which the effects of varying concentrations of CGS 21680 (0.1 and $1 \mu\text{M}$) on the influx of $^{45}\text{Ca}^{2+}$ into FMLP-activated neutrophils were investigated.

4.2.8 Oxidant generation

This was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) method (Minkenbergh & Ferber, 1984). Neutrophils ($1 \times$

10^6 /ml, final) were pre-incubated for 15 min in 900 μ l HBSS containing 0.2 mM lucigenin in the presence and absence of CGS 21680 (0.01 – 1 μ M). Spontaneous and FMLP (1 μ M)-activated LECL responses were then recorded using a LKB Wallac 1251 chemiluminometer (Turku, Finland) after the addition of the stimulant (100 μ l). LECL readings were integrated for 5 sec intervals and recorded as mV x seconds⁻¹ (mV.s⁻¹). Additional experiments were performed to investigate the following: i) the effects of ZM 241385 (2.5 μ M), and thapsigargin (1 μ M) on the CGS 21680 (1 μ M)-mediated inhibition of the LECL responses of FMLP-activated neutrophils and ii) the superoxide-scavenging potential of CGS 21680, thapsigargin and ZM 241385 using a cell-free hypoxanthine (1 mM)-xanthine oxidase (17 milliunits/ml) superoxide-generating system.

4.2.9 Elastase release

Neutrophil degranulation was measured according to the extent of release of the primary granule-derived protease, elastase. Neutrophils were incubated at a concentration of 1×10^7 /ml in HBSS in the presence or absence of CGS 21680 (0.01 – 1 μ M) with and without ZM 241385 (2.5 μ M) or thapsigargin (1 μ M) for 10 min at 37°C. The stimulant FMLP (0.1 μ M) in combination with cytochalasin B (1 μ M) was then added and the reaction mixtures incubated for 10 min at 37°C. The tubes were then transferred to an ice-bath, followed by centrifugation at 400 g for 5 min to pellet the cells. The neutrophil-free supernatants were then decanted and assayed for elastase activity using a micro-modification of a standard spectrophotometric procedure (Beatty *et al.*, 1982). Briefly, 125 μ l of supernatant was added to 125 μ l of the elastase substrate N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide, 3 mM in 0.3% dimethyl sulphoxide (DMSO) in 0.05 M Tris-HCl (pH 8.0). Elastase activity was assayed at a wavelength of 405 nm and the results expressed as the mean percentage of the amount of enzyme released by the corresponding FMLP/CB-activated, drug-free control systems.

4.2.10 Intracellular cAMP levels

Neutrophils at a concentration of 1×10^7 /ml in HBSS were preincubated for 10 min at 37°C with CGS 21680 (1 μ M) with and without ZM 241385 (2.5 μ M). Following

preincubation, the cells were activated with 1 μ M FMLP (stimulated cells), or an equal volume of HBSS (unstimulated cells), in a final volume of 1 ml, after which the reactions were terminated and the cAMP extracted by the addition of ice-cold ethanol (65% v/v) at 20 sec, 1 min, 3 min and 5 min after addition of the stimulant. The resultant precipitates were washed twice with ice-cold ethanol and the supernatants pooled and centrifuged at 2000 *g* for 15 min at 4°C. The supernatants were then transferred to fresh tubes and evaporated at 60°C under a stream of nitrogen. The dried extracts were reconstituted in assay buffer (0.05 M acetate buffer, pH 5.8) and assayed for cAMP using the Biotrak cAMP [¹²⁵I] scintillation proximity assay system (Amersham International plc., Buckinghamshire, UK), which is a competitive binding radioimmunoassay procedure. These results are expressed as pmol cAMP/10⁷ neutrophils. Because cAMP is rapidly hydrolysed in neutrophils by phosphodiesterases, these experiments were performed in the presence of 1 μ M rolipram, a selective inhibitor of type 4 phosphodiesterase, the predominant type present in human neutrophils (Torphy, 1998).

4.2.11 Statistical analysis

The results of each series of experiments are expressed as the mean values \pm SEM. Levels of statistical significance were calculated by paired Student's *t* test when comparing two groups, or by analysis of variance (ANOVA) with subsequent Tukey-Kramer multiple comparisons test for multiple groups. A computer-based software system (Instat II®) was used for analysis. Significance levels were taken at a *p* value of <0.05.

4.3. Results

4.3.1 Fura-2 fluorescence responses of FMLP-activated neutrophils

The results shown in Figure 4.1 (page 86) are traces from 3 different experiments which depict the effects of 1 μ M CGS 21680 on the fura-2 responses of FMLP-activated neutrophils. Addition of FMLP to neutrophils in each experiment was

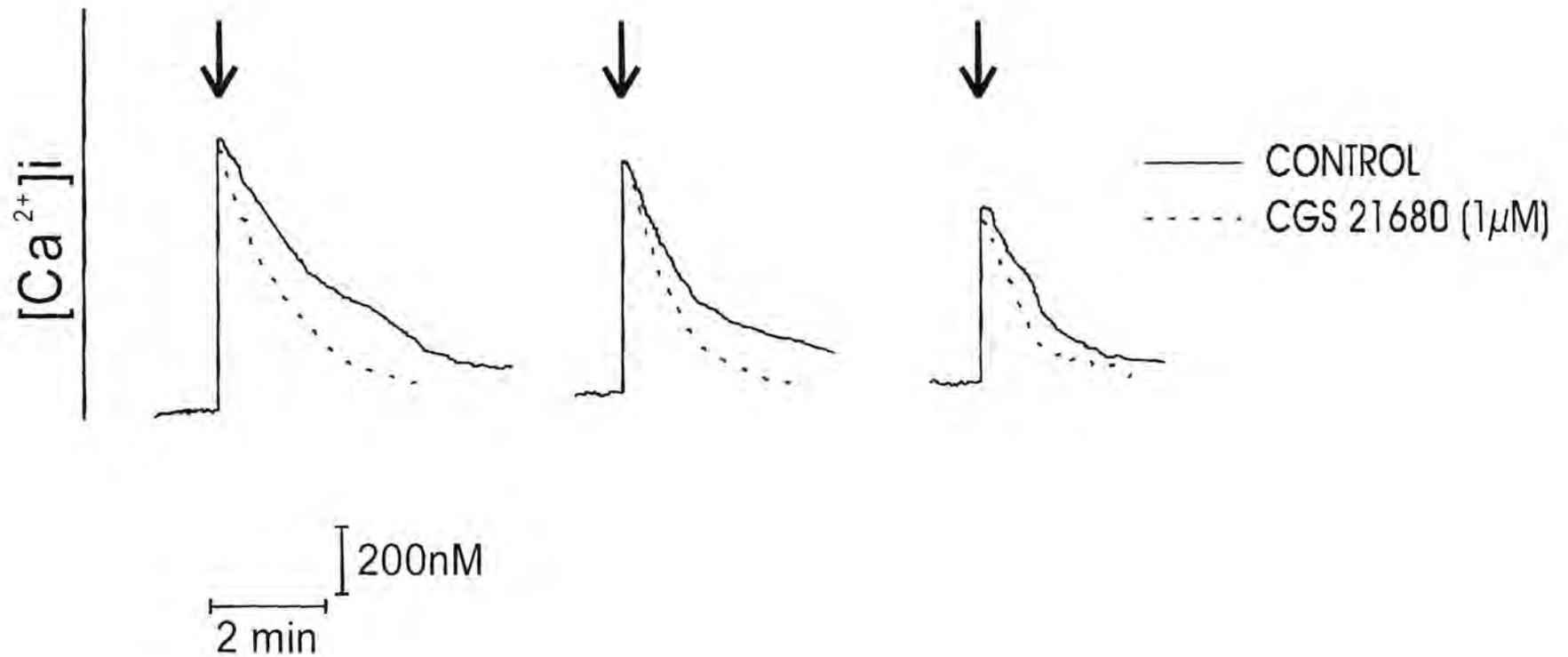


Figure 4.1: FMLP-activated fura-2 fluorescence responses of control and CGS 21680 (1 μ M)-treated neutrophils. FMLP (1 μ M) was added as indicated (\downarrow) when a stable base-line was obtained (\pm 1 min). The traces shown are from 3 different experiments.

accompanied by the characteristic, abrupt increases in fura-2 fluorescence due to elevated concentrations of cytosolic Ca^{2+} . CGS 21680 did not alter this abrupt increase in fluorescence intensity, demonstrating that this agent does not affect the release of Ca^{2+} from cellular stores. However, treatment of the cells with the AR agonist hastened the rate of the subsequent decline in fluorescence intensity, indicative of accelerated clearance of Ca^{2+} from the cytosol.

The results shown in Table 4.1 (page 88) are those from a larger series of experiments and show peak cytosolic Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$), as well as the time taken for fluorescence intensity to decline to half peak ($t_{1/2}$) values, for neutrophils activated with FMLP in the presence and absence of varying concentrations of CGS 21680. As indicated above, CGS 21680 did not affect the abruptly occurring increase in $[\text{Ca}^{2+}]_i$ following activation of the cells with FMLP. However, the $\text{A}_{2\text{A}}$ agonist caused dose-related acceleration in the rate of decline in peak fluorescence intensity, which at all 4 concentrations tested differed significantly from the drug-free control system.

The effects of ZM 241385 (2.5 μM) alone on the fura-2 responses of FMLP-activated control neutrophils are shown in Figure 4.2 (page 89). ZM 241385 had no effects on either peak fluorescence intensity or the subsequent decline in fluorescence during the 2 min period following the addition of FMLP to the neutrophils. Thereafter however, there was a slight transient increase in fluorescence intensity in ZM 241385-treated cells, but not in control neutrophils, which coincided with the store-operated influx of extracellular Ca^{2+} . This lasted for 1-2 min after which fluorescence again subsided, but at a slower rate than that of the control cells. These effects of ZM 241385 on the fura-2 responses of FMLP-activated neutrophils were observed in 12 consecutive experiments using neutrophils from 4 different donors, and are compatible with interference with the sequestration of incoming Ca^{2+} into storage organelles. The effects of ZM 241385 (2.5 μM) alone and in combination with CGS 21680 on the peak cytosolic Ca^{2+} concentrations of FMLP-activated neutrophils, as well as on the time taken for fluorescence intensity to decline to half peak values are shown in Table 4.2 (page 88). Importantly, the half peak point of the decline of fura-2

fluorescence was reached before the effects of ZM 241385 *per se* on Ca^{2+} sequestration were evident. Pre-treatment of neutrophils with ZM 241385 antagonised ($p < 0.05$) CGS 21680-mediated acceleration of the clearance of cytosolic Ca^{2+} from activated neutrophils without affecting peak fluorescence intensity.

Table 4.1 Effects of CGS 21680 on peak cytosolic calcium concentrations ($[Ca^{2+}]_i$) and rates of clearance (half peak values) in FMLP-activated neutrophils

Agent	Peak $[Ca^{2+}]_i$ values (nM)	Time taken to decline to half peak values (min)
Control	474 ± 53	1.1 ± 0.1
CGS 21680 0.01 µM	418 ± 24	0.87 ± 0.1*
CGS 21680 0.1 µM	414 ± 13	0.68 ± 0.1*
CGS 21680 0.5 µM	415 ± 17	0.65 ± 0.1*
CGS 21680 1 µM	417 ± 14	0.60 ± 0.1*

The results of 10 experiments are expressed as the mean values ± SEM. The $[Ca^{2+}]_i$ value for control, unstimulated neutrophils was 111 ± 9 nM.

* $P < 0.05$ for comparison with the control system.

Table 4.2 Effects of CGS 21680 ± ZM 241385 on peak intracellular calcium concentrations $[Ca^{2+}]_i$ and rates of clearance in FMLP-activated neutrophils

Systems	Peak $[Ca^{2+}]_i$ values (nM)	Time taken for peak $[Ca^{2+}]_i$ to decline to half peak values (min)
Control	500 ± 14	1.25 ± 0.1
CGS 21680 1 µM	495 ± 18	0.83 ± 0.1*
ZM 241385 2.5 µM	458 ± 24	1.15 ± 0.1
CGS 21680 + ZM 241385	488 ± 15	1.16 ± 0.1**

The results of 6 experiments are expressed as the mean values ± SEM.

* $p < 0.05$ for comparison with the control system.

** $p < 0.05$ for comparison with CGS 21680 alone.

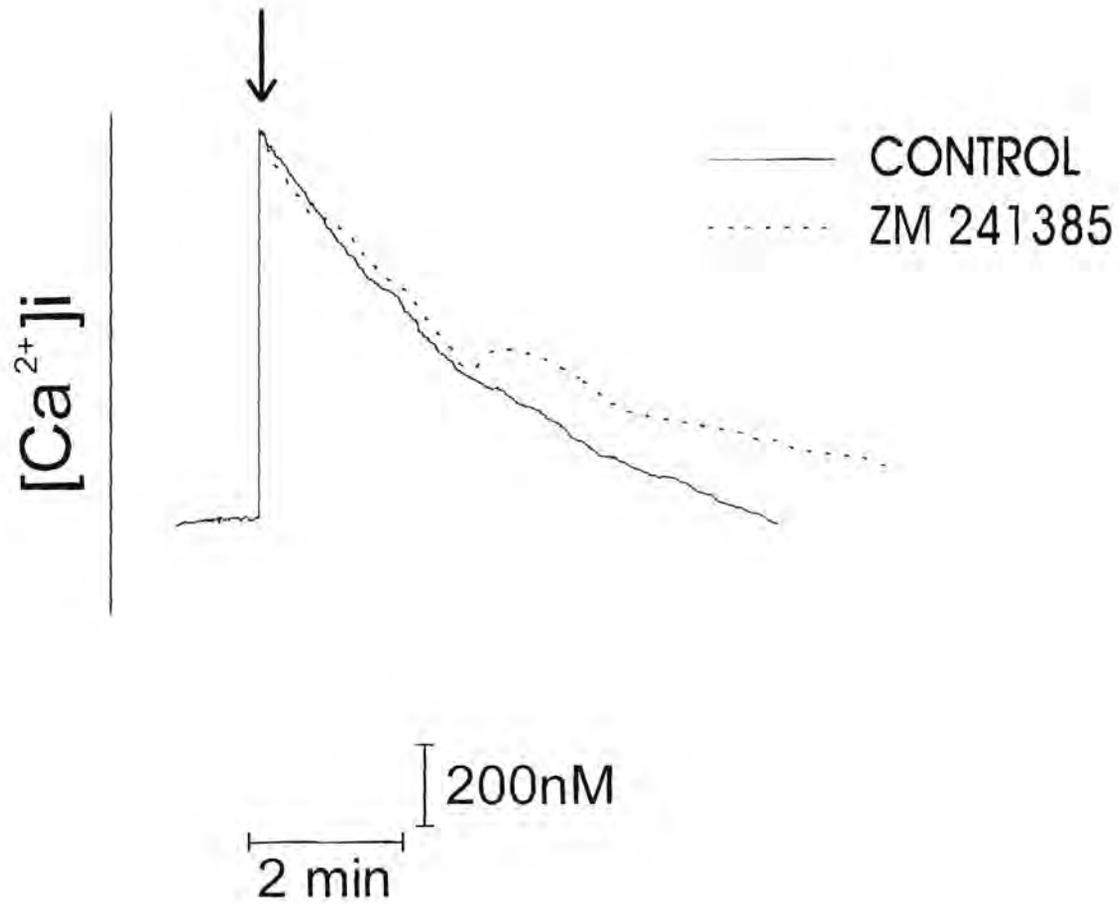


Figure 4.2: FMLP ($1 \mu\text{M}$)-activated fura-2 fluorescence responses of control and ZM 241385 ($2.5 \mu\text{M}$)-treated neutrophils. FMLP was added as indicated (\downarrow) when a stable baseline was obtained (± 1 min). This is a typical trace from 12 different experiments.

4.3.2 Influx of Ca^{2+} using Mn^{2+} quenching of fura-2 fluorescence

The results of the indirect measurement of Ca^{2+} influx into FMLP-activated control and CGS 21680 (1 μM)-treated neutrophils are shown in Figure 4.3 (page 91). The traces shown are from a typical experiment (4 in each series) and clearly show a decrease in both the rate and extent of influx of Ca^{2+} into CGS 21680-treated neutrophils. Because of the reported limitations of this method (Favre *et al.*, 1996), measurement of influx of Ca^{2+} by radiometric procedures, as described below, was identified as the preferred procedure.

4.3.3 Efflux of $^{45}\text{Ca}^{2+}$ from FMLP-activated neutrophils

In these experiments, neutrophils which had been pre-loaded with $^{45}\text{Ca}^{2+}$ and then washed and transferred to Ca^{2+} -replete HBSS (to minimise re-uptake of radiolabelled cation) were activated with FMLP in the presence and absence of CGS 21680 (1 μM) followed by measurement of the amount of cell-associated $^{45}\text{Ca}^{2+}$. The results in Figure 4.4 (page 92) show that exposure of the drug-free, control neutrophils to FMLP resulted in an abrupt efflux of the radiolabelled cation from the neutrophils, which terminated approximately 30 sec after the addition of the stimulant and resulted in the loss of 44% of cell-associated $^{45}\text{Ca}^{2+}$, while there was no detectable loss of the cation from unstimulated cells over the 60 sec time course of the experiment. Treatment of neutrophils with CGS 21680 (1 μM) caused a significant decrease ($p < 0.05$ at 60 sec) in the efflux of $^{45}\text{Ca}^{2+}$ following activation of the cells with FMLP (Figure 4.4, page 92). The results of a series of experiments in which the effects of CGS 21680 at concentrations of 0.01, 0.1 and 1 μM on the efflux of $^{45}\text{Ca}^{2+}$ from FMLP-activated neutrophils using a fixed 60 sec incubation period are shown in Table 4.3 (page 93). The $\text{A}_{2\text{A}}$ agonist caused dose-related inhibition of efflux of $^{45}\text{Ca}^{2+}$.

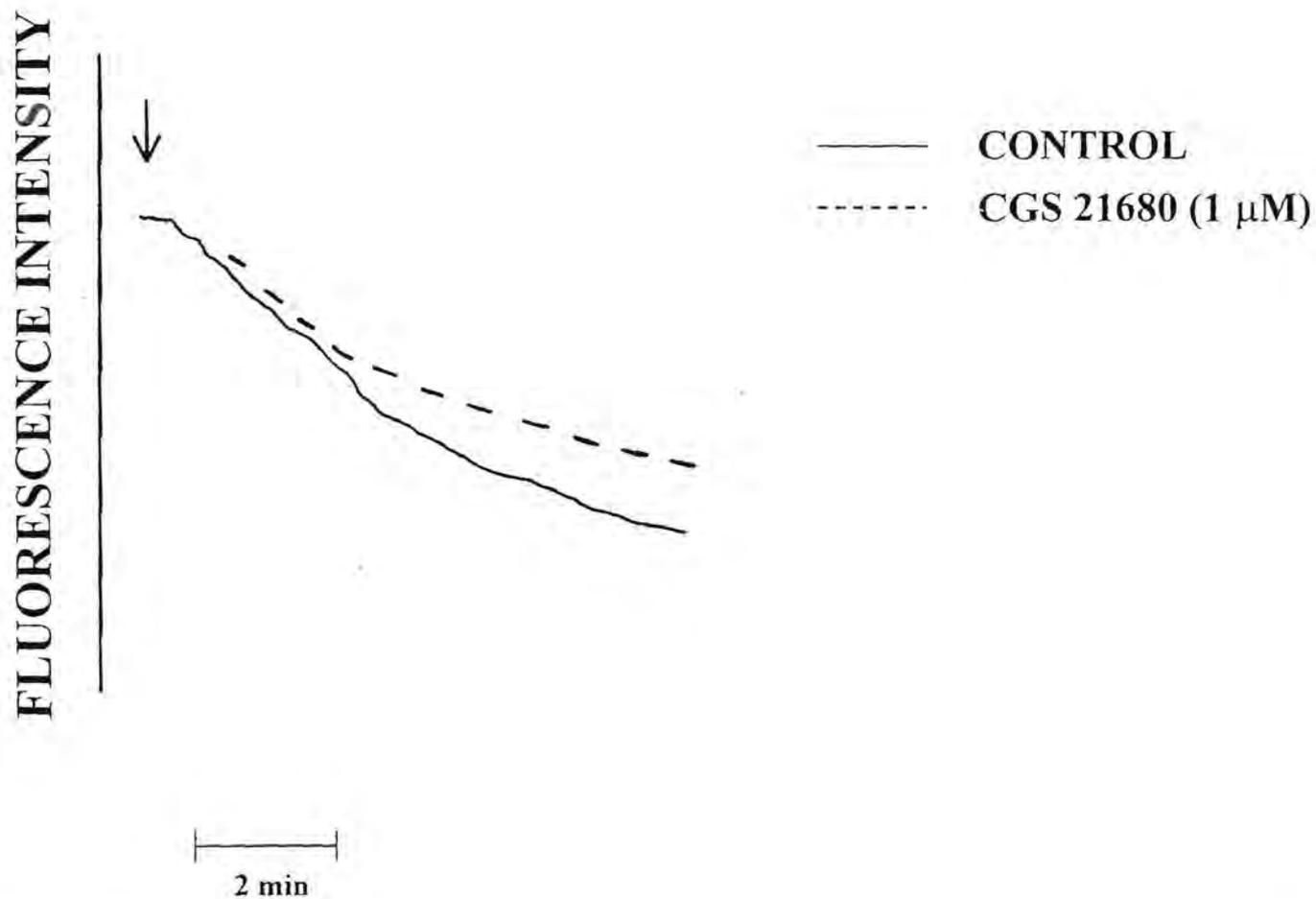


Figure 4.3: FMLP (1 μM)-activated Mn²⁺ quenching of the fura-2 responses of control and CGS 21680 (1 μM)-treated neutrophils. FMLP was added as indicated (↓) and the results shown are typical traces from 4 experiments. The decline in fluorescence intensity represents influx of Ca²⁺.

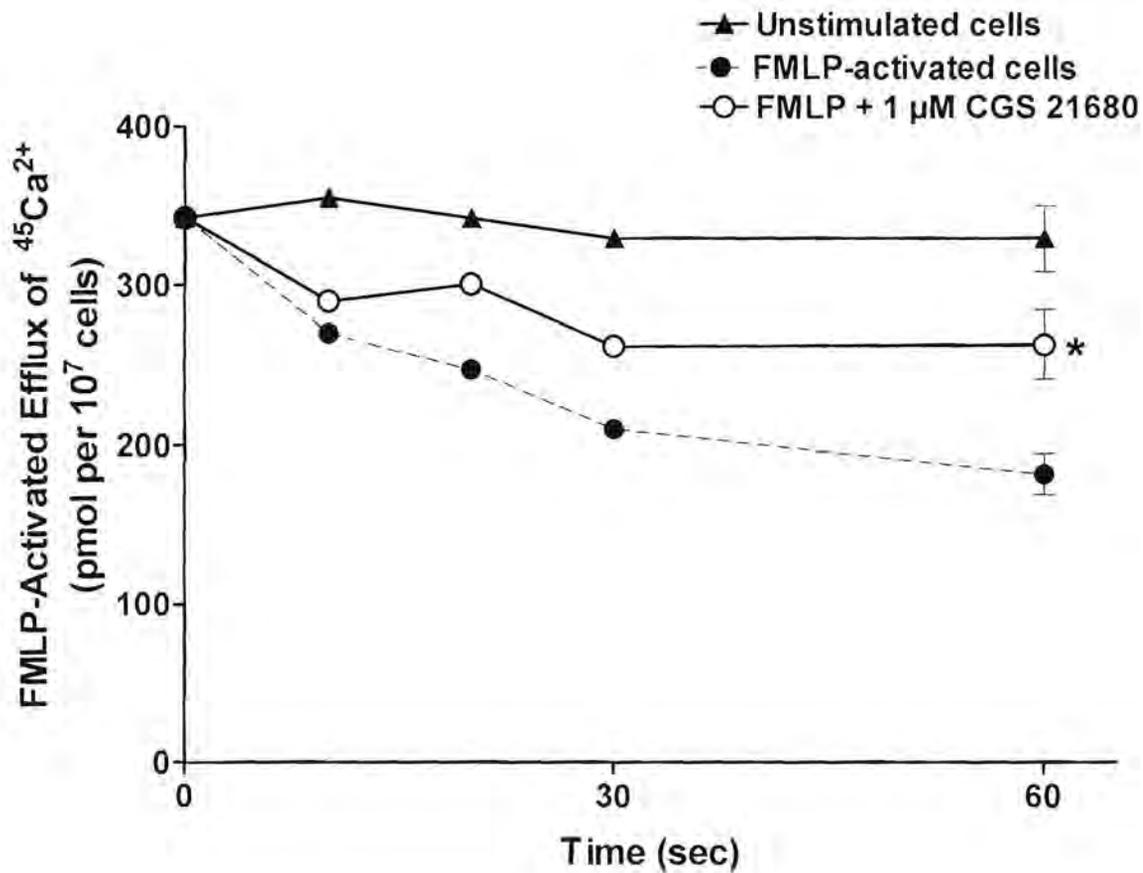


Figure 4.4: Kinetics of efflux of $^{45}\text{Ca}^{2+}$ from unstimulated neutrophils and neutrophils activated with FMLP (1 μM) in the absence and presence of CGS 21680 (1 μM). The results of 11 different experiments are expressed as the mean amount of cell-associated $^{45}\text{Ca}^{2+}$ (pmol per 10^7 cells) and vertical lines show SEM.

* $p < 0.05$ for comparison with the FMLP-activated, CGS 21680-free control system.

Table 4.3: Effects of varying concentrations of CGS 21680 on the efflux of $^{45}\text{Ca}^{2+}$ out of FMLP-activated neutrophils

Agent	Amount of $^{45}\text{Ca}^{2+}$ released from neutrophils 60s after the addition of FMLP (pmol per 10^7 cells)
FMLP only	162 ± 12
FMLP + CGS 21680 0.01 μM	132 ± 14
FMLP + CGS 21680 0.1 μM	83 ± 9
FMLP + CGS 21680 1 μM	65 ± 5

The results of 3 experiments are expressed as the mean values \pm SEM.

The effects of thapsigargin and ZM 241385 on the CGS 21680-mediated decrease in efflux of $^{45}\text{Ca}^{2+}$ out of FMLP-activated neutrophils are shown in Table 4.4.

Thapsigargin (inhibitor of the endo-membrane Ca^{2+} -ATPase) and ZM 241385 attenuated the effects of CGS 21680 on efflux of $^{45}\text{Ca}^{2+}$. Over the short time-course of these experiments (60 sec) the antagonist *per se* caused modest, statistically insignificant increases in the efflux of $^{45}\text{Ca}^{2+}$ from FMLP-activated neutrophils.

Table 4.4: Effects of thapsigargin and ZM 241385 on the CGS 21680-mediated reduction in efflux of $^{45}\text{Ca}^{2+}$ out of FMLP-activated neutrophils.

System	Amount of $[\text{Ca}^{2+}]_i$ released from neutrophils 60 sec after the addition of FMLP (pmol per 10^7 cells)
FMLP only	159 ± 11
FMLP + CGS 21680 1 μM	$76 \pm 10^*$
FMLP + thapsigargin 1 μM	177 ± 10
FMLP + CGS 21680 + thapsigargin 1 μM	152 ± 12
FMLP + ZM 241385 2.5 μM	173 ± 11
FMLP + CGS 21680 + ZM 241385 2.5 μM	163 ± 10

The results of 6 experiments are expressed as the mean values \pm SEM. * $P < 0.05$ for comparison with the drug-free (FMLP only) control system.

4.3.4 Influx of $^{45}\text{Ca}^{2+}$ into FMLP-activated neutrophils

For these experiments neutrophils were pre-loaded with cold Ca^{2+} then transferred to Ca^{2+} -free HBSS prior to activation with FMLP, which was added simultaneously with $^{45}\text{Ca}^{2+}$. This step (loading with cold Ca^{2+}) was undertaken to minimise spontaneous uptake of $^{45}\text{Ca}^{2+}$ by neutrophils (Anderson & Goolam Mahomed, 1997). The results of these experiments are shown in Figure 4.5 (page 95). Activation of control, drug-free neutrophils with FMLP under these experimental conditions resulted in a delayed influx of $^{45}\text{Ca}^{2+}$, which occurred after a lag phase of 30-60 sec. Influx of $^{45}\text{Ca}^{2+}$ appeared to be a true consequence of activation of neutrophils with FMLP, since the influx of the radiolabelled cation over the same time-course into control, identically-processed neutrophils, which received $^{45}\text{Ca}^{2+}$ only in the absence of FMLP, was considerably less. Pre-treatment with CGS 21680 (1 μM) resulted in decreased influx of $^{45}\text{Ca}^{2+}$ into FMLP-activated neutrophils ($p < 0.05$ for the 5 min value).

In dose-response experiments the net influx of $^{45}\text{Ca}^{2+}$ 5 min after exposure to FMLP was 143 ± 3 , 124 ± 3 ($p < 0.05$) and 104 ± 3 ($p < 0.05$) pmol per 10^7 cells for control neutrophils and for cells pre-treated with 0.1 and 1 μM CGS 21680 respectively ($n=7$).

ZM 241385 (2.5 μM) did not affect the store-operated influx of $^{45}\text{Ca}^{2+}$ into FMLP-activated neutrophils. The net influx of $^{45}\text{Ca}^{2+}$ 5 min after exposure to FMLP was 189 ± 6 and 179 ± 6 pmol per 10^7 cells for control and ZM 241385-treated neutrophils respectively ($n=6$).

4.3.5 Superoxide generation and elastase release

The effects of CGS 21680 on oxidant production by, and release of elastase from, neutrophils activated with FMLP and FMLP/CB respectively are shown in Figure 4.6 (page 96). The $\text{A}_{2\text{A}}\text{R}$ agonist caused dose-related inhibition of both oxidant production and elastase release which achieved statistical significance ($p < 0.05$) at concentrations of 0.01 μM and upwards. In both cases the degrees of inhibition caused by 0.1 μM and 1 μM CGS 21680 did not differ significantly. The effects of ZM 241385 and thapsigargin on CGS 21680-mediated inhibition of oxidant production by,

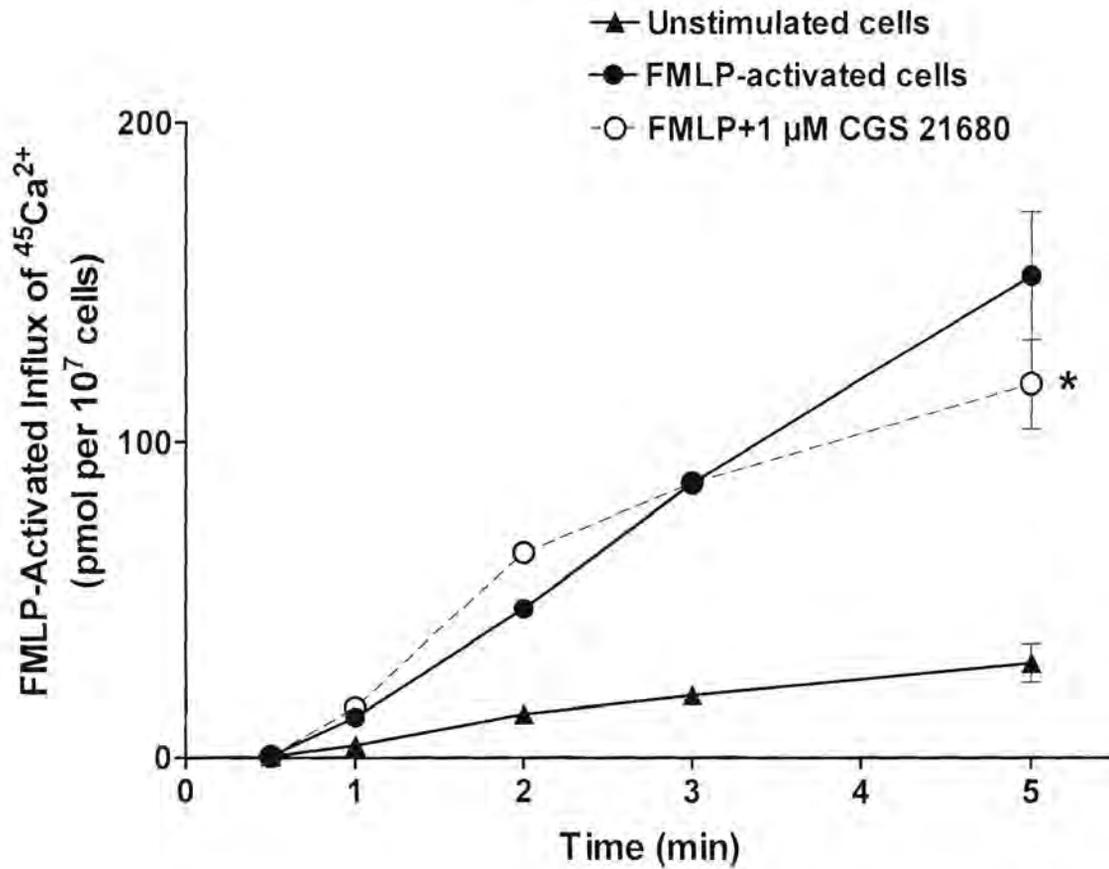


Figure 4.5: Kinetics of influx of $^{45}\text{Ca}^{2+}$ into unstimulated neutrophils and neutrophils activated with FMLP (1 μM) in the absence and presence of CGS 21680 (1 μM). The results of 8 different experiments are expressed as the mean amount of cell-associated $^{45}\text{Ca}^{2+}$ (pmol per 10^7 cells) and vertical lines show SEM.

* $p < 0.05$ for comparison with the FMLP-activated, CGS 21680-free control system.

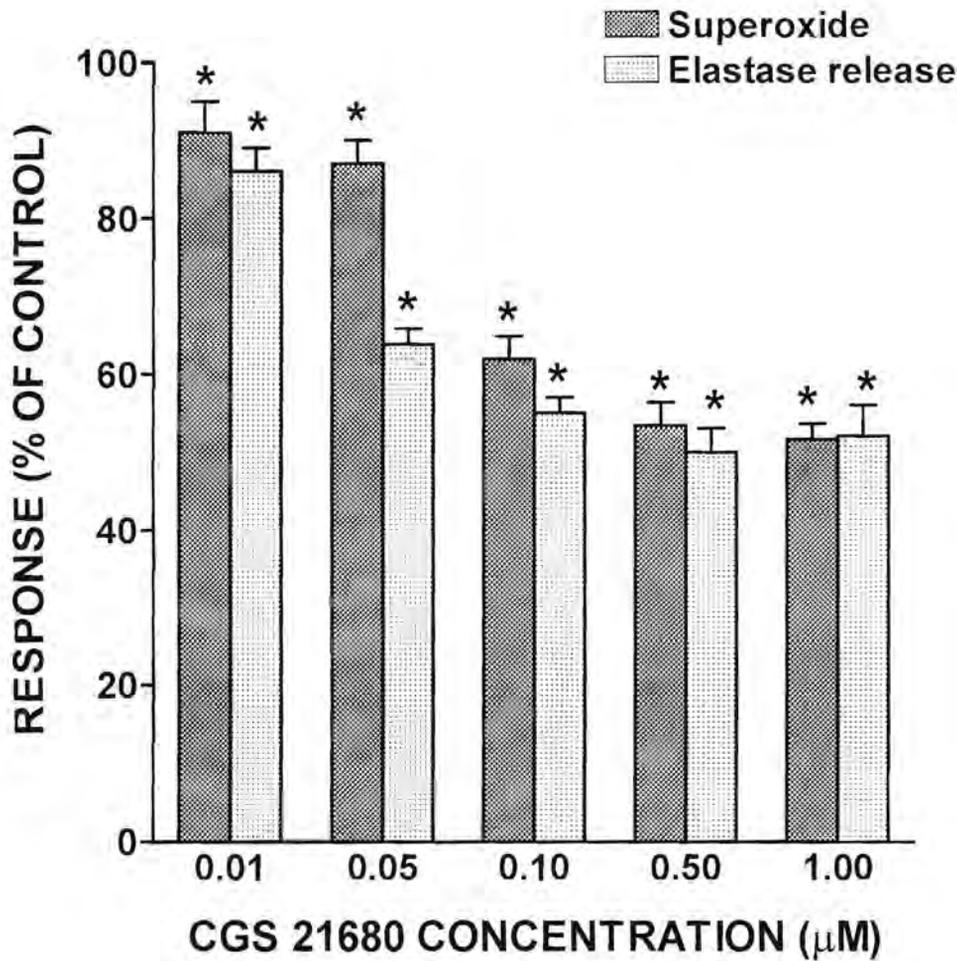


Figure 4.6: The effects of varying concentrations of CGS 21680 (0.01 –1 µM) on superoxide production by FMLP (0.1 µM)-activated neutrophils and on elastase release from FMLP/CB-activated neutrophils. The results of 8 (superoxide), measured by lucigenin-enhanced chemiluminescence (LECL) and 10 (elastase) different experiments are presented as the mean percentage of the drug-free control systems and vertical lines show SEM. In the case of superoxide production, the absolute values for resting and FMLP-activated neutrophils were 256 ± 23 and 911 ± 84 millivolts/sec respectively. The corresponding values for elastase release were 40 ± 2 and 598 ± 70 milliunits enzyme per 10^7 cells respectively.

and elastase release from, activated neutrophils, are shown in Table 4.5. Both thapsigargin and to a lesser extent ZM 241385 potentiated the release of elastase from FMLP/CB-activated neutrophils, in the absence of CGS 21680, but caused no significant enhancement of superoxide production. Pre-treatment of the cells with ZM 241385 completely antagonised the anti-inflammatory effects of the A_{2A}R agonist, while thapsigargin was only slightly less effective.

At the highest concentration used (1 µM), CGS 21680, as well as thapsigargin (1 µM) and ZM 241385 (2.5 µM) did not possess superoxide-scavenging activity according to results obtained with the cell-free hypoxanthine-xanthine oxidase system (not shown).

Table 4.5: Effects of thapsigargin and ZM 241385 on CGS 21680-mediated inhibition of superoxide production by, and elastase release from activated neutrophils.

System	Superoxide Production (% control)	Elastase release (% control)
CGS 21680 1 µM	56 ± 3*	40 ± 1*
Thapsigargin 1 µM	118 ± 8	142 ± 2
ZM 241385 2.5 µM	108 ± 8	126 ± 3
CGS 21680 + thapsigargin	101 ± 6	114 ± 2
CGS 21680 + ZM 241385	100 ± 5	126 ± 10

The results of 6 (superoxide) and 8-12 (elastase) different experiments are presented as the mean percentages of the drug-free control systems ± SEM. In the case of superoxide production, the absolute values for resting and FMLP-activated neutrophils were 282 ± 28 and 1197 ± 50 mV.s⁻¹ respectively. The corresponding values for elastase release from FMLP/CB-activated were 40 ± 2 and 598 ± 10 milliunits enzyme per 10⁷ cells respectively.

*p<0.05 for comparison with the drug-free (FMLP or FMLP/CB only) control system.

4.3.6 Intracellular cAMP

The effects of CGS 21680 (1 μ M) on cAMP in unstimulated and FMLP-activated neutrophils in the presence and absence of ZM 241385 (2.5 μ M) are shown in Table 4.6. Exposure of neutrophils to FMLP was accompanied by an increase in intracellular cAMP which was inhibited by ZM 241385. Treatment of both stimulated and unstimulated neutrophils with CGS 21680 resulted in significant increases in intracellular cAMP concentrations which were completely abolished by pre-treatment of the cells with ZM 241385 prior to the addition of the A_{2A}R agonist.

Table 4.6: The effects of CGS 21680 and ZM 241385 individually and in combination on cAMP levels in unstimulated and FMLP-activated neutrophils.

System	Intracellular cAMP (pmol per 10 ⁷ cells)
Unstimulated neutrophils	30 ± 0.2
Unstimulated neutrophils + CGS 21680 1 μ M	59 ± 3*
Unstimulated neutrophils + ZM 241385 2.5 μ M	12 ± 1.2
Unstimulated neutrophils + CGS 21680 + ZM 241385	14 ± 1.2
FMLP-activated neutrophils	127 ± 6*
FMLP-activated neutrophils + CGS 21680	240 ± 1.5**
FMLP-activated neutrophils + ZM 241385	41 ± 4
FMLP-activated neutrophils + CGS 21680 + ZM 241385	38 ± 0.4

The results of 5 different experiments are expressed as the mean intracellular concentration of cAMP ± SEM measured at 1 min after the addition of the stimulant, FMLP.

*p<0.05 for comparison with the unstimulated, CGS 21680-free control system and

**p<0.05 for comparison with FMLP-activated neutrophils in the absence of CGS 21680.

4.4. DISCUSSION

Transient elevations in cytosolic Ca^{2+} precede, and are a prerequisite, for receptor-mediated activation of neutrophil adhesion to vascular endothelium, superoxide production and granule enzyme release (Lew *et al.*, 1986; Thelen *et al.*, 1993; Pettit & Hallett, 1996). Hyperactivation of neutrophils is prevented by the action of the calmodulin-dependent plasma membrane (Lagast *et al.*, 1984) and cAMP-dependent protein kinase (PKA)-activated endo-membrane Ca^{2+} -ATPases (Schatzmann, 1989; Tao *et al.*, 1992), and possibly by a $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Simchowitz *et al.*, 1990) operating in unison to promote rapid clearance of cytosolic Ca^{2+} . The transient elevation in cAMP which accompanies the exposure of human neutrophils to chemoattractants (Anderson *et al.*, 1976) has been reported to be mediated by endogenously-generated adenosine acting via adenylate-cyclase coupled $\text{A}_{2\text{A}}\text{R}$ and may be involved in the restoration of Ca^{2+} homeostasis in these cells and down-regulation of their pro-inflammatory activities (Iannone *et al.*, 1989) by up-regulating the activity of the endo-membrane Ca^{2+} -ATPase.

This contention is supported by a previous study which reported that rolipram, the prototype selective PDE4 inhibitor, and dibutyryl cAMP decrease the pro-inflammatory activities of human neutrophils *in vitro* by cAMP-dependent enhancement of the activity of the endo-membrane Ca^{2+} -ATPase (Anderson *et al.*, 1998). Rolipram- and dibutyryl cAMP-mediated enhancement of the activity of this Ca^{2+} sequestering/re-sequestering pump resulted in accelerated clearance of the cation from the cytosol of FMLP-activated neutrophils and was accompanied by decreased pro-inflammatory activity of these cells.

Although the anti-inflammatory properties of adenosine and $\text{A}_{2\text{A}}\text{R}$ agonists are well-recognised (Cronstein *et al.*, 1985; Hannon *et al.*, 1998; Sullivan & Linden, 1998; Varani *et al.*, 1998), the exact molecular/biochemical mechanisms by which these effects are achieved, as well as the involvement of cAMP, remain uncertain. In the present study, we investigated the effects of the $\text{A}_{2\text{A}}\text{R}$ agonist, CGS 21680, on intracellular cAMP and Ca^{2+} handling by activated human neutrophils and compared these with alterations in pro-inflammatory activity.

Using the fura-2 spectrofluorimetric procedure, treatment of neutrophils with CGS 21680 did not affect the abruptly-occurring peak fluorescence responses of FMLP-activated neutrophils. Since no detectable influx of extracellular Ca^{2+} coincident with peak fluorescence intensity in FMLP-activated neutrophils was detected with the radiometric procedure, the increase in the cytosolic concentration of the cation appears to originate through its release from intracellular stores. These observations demonstrate that CGS 21680 does not affect the activation of phospholipase C and generation of inositol triphosphate in FMLP-activated neutrophils, nor does it interfere with the interaction of this second messenger with Ca^{2+} -mobilizing receptors on calciosomes, or the subsequent release of the cation from these stores (Prentki, *et al.*, 1984; Krause *et al.*, 1989).

Although CGS 21680 did not affect the release of Ca^{2+} from intracellular stores, this agent, at concentrations of 0.01 – 1 μM , hastened the rate of decline in peak fluorescence intensity, indicative of accelerated clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils. Accelerated clearance of cytosolic Ca^{2+} could result from several different mechanisms, including enhancement of efflux and/or re-sequestration of the cation, or inhibition of influx. To identify which of these was altered by CGS 21680 we used radiometric procedures, which facilitate distinction between net efflux and influx of the cation, in combination with the fura-2 fluorescence method (Anderson & Goolam Mahomed, 1997).

Using the radiometric procedures, exposure of neutrophils to FMLP was accompanied by an abrupt efflux of $^{45}\text{Ca}^{2+}$. This efflux coincided with the peak in fura-2 fluorescence intensity and terminated at about 30 sec after the addition of the stimulant, resulting in extrusion of 44% of cell-associated cation. This observation suggests that not all of the intracellular Ca^{2+} pool is mobilised during exposure of neutrophils to the chemoattractant, or that rapid re-sequestration of cytosolic Ca^{2+} , as a result of activation of the Ca^{2+} sequestering/re-sequestering endo-membrane Ca^{2+} -ATPase also contributes to removal of the cation from the cytosol (Schatzman, 1989). As previously reported (Anderson & Goolam Mahomed, 1997; Anderson *et al.*, 1998), during the period of efflux there was no detectable influx of $^{45}\text{Ca}^{2+}$ into FMLP-stimulated neutrophils. Net influx of the cation occurred only after efflux had

ceased, being detected at 30-60 sec after addition of FMLP and terminating at around 5 min after addition of the stimulant. This delayed influx of Ca^{2+} is characteristic of a store-operated influx, which is operative in many cell types, including neutrophils (Montero *et al.*, 1991; Favre *et al.*, 1996; Anderson & Goolam Mahomed, 1997).

Treatment of neutrophils with CGS 21680 significantly reduced the amount of Ca^{2+} released from FMLP-activated neutrophils in the setting of a decrease in the magnitude of the subsequent store-operated influx of the cation. CGS 21680-mediated reduction in store-operated influx of Ca^{2+} in FMLP-activated neutrophils was also observed using the Mn^{2+} quenching of fura-2 fluorescence procedure. The usefulness of this method has, however, been questioned because store-operated channels are Ca^{2+} -selective with a small single channel conductance (Favre *et al.*, 1996). Together with the results of the conventional fura-2 experiments, these observations suggest that CGS 21680 up-regulates the activity of the PKA-activatable endo-membrane Ca^{2+} -ATPase (Schatzmann, 1989; Tao *et al.*, 1992), resulting in decreased efflux of Ca^{2+} as a consequence of competition between the up-regulated endo-membrane Ca^{2+} -ATPase and the plasma membrane Ca^{2+} -pump for cytosolic Ca^{2+} . Accelerated clearance of Ca^{2+} from the cytosol of CGS 21680-treated, FMLP-activated neutrophils is probably achieved through the action of these two Ca^{2+} -ATPases operating in unison.

Upregulation of the endo-membrane Ca^{2+} -ATPase would also explain the decreased store-operated influx of Ca^{2+} into CGS 21680-treated, FMLP-activated neutrophils. Accelerated activation and/or increased efficiency of this system would result in enhancement of re-sequestration of cytosolic Ca^{2+} . Utilisation of endogenous Ca^{2+} for re-filling of the stores would in turn decrease the requirement for exogenous cation, with a consequent reduction in the magnitude of the subsequent store-operated influx.

This mechanism of CGS 21680-mediated acceleration of clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils is supported by experiments in which thapsigargin, a highly selective inhibitor of the endo-membrane-ATPase (Lytton *et al.*, 1991), was used. Treatment of neutrophils with this agent abolished the CGS 21680-

mediated reduction in the efflux of Ca^{2+} from FMLP-activated neutrophils. The observed restoration of efflux by thapsigargin in CGS-treated neutrophils also demonstrates that the AR agonist does not affect the calmodulin-dependent plasma membrane Ca^{2+} -ATPase.

The influence of CGS 21680 on superoxide production by, and release of elastase from stimulated neutrophils was also investigated and compared with the effects of this agent on the handling of cytosolic Ca^{2+} by these cells. At the same concentrations which accelerated the clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils, CGS 21680 inhibited both superoxide generation and elastase release. As was the case with Ca^{2+} clearance, thapsigargin neutralised the inhibitory effects of CGS 21680 on superoxide production and elastase release, demonstrating a mechanistic link between these events.

The involvement of cAMP in mediating the effects of CGS 21680 on Ca^{2+} homeostasis and on the production/release of inflammatory mediators by activated neutrophils was strengthened by the following observations: i) intracellular concentrations of cAMP were increased following exposure of the cells to CGS 21680 and ii) the selective $A_{2A}R$ receptor antagonist ZM 241385 almost completely antagonised the effects of CGS 21680 on intracellular cAMP, Ca^{2+} clearance, superoxide production and elastase release. ZM 241385-mediated antagonism of the cAMP-elevating and anti-inflammatory interactions of CGS 21680 with neutrophils demonstrates that these effects are mediated via the adenylate cyclase-coupled $A_{2A}R$.

Interestingly, the effects of ZM 241385 *per se* on neutrophils were opposite to those of CGS 21680. At the single concentration tested, this agent attenuated the FMLP-activated transient increase in neutrophil cAMP in the setting of apparent interference with the sequestration of incoming Ca^{2+} during store-operated influx of the cation. This latter contention is based on the observation that during the late stages of the fura-2 fluorescence response of FMLP-activated neutrophils, when fluorescence has subsided to close to baseline values, there was a transient increase in fluorescence intensity and a subsequent delay in the return to basal fluorescence in ZM 241385-treated cells. These events coincided with store-operated influx of Ca^{2+} and are

compatible with interference with sequestration of incoming cation. In an additional series of experiments, which were not included in the current study, we observed that following the return to basal fluorescence, re-stimulation of fura-2-loaded, ZM 241385-treated neutrophils with FMLP resulted in immediate peak fura-2 fluorescence responses which were similar to those of control re-stimulated cells. These observations confirm that sequestration, albeit at a reduced level of efficiency, as opposed to an efflux mechanism, is responsible for the eventual return to baseline fluorescence in ZM 241385-treated, FMLP-activated neutrophils.

ZM 241385-mediated impairment of Ca^{2+} sequestration is the probable consequence of attenuated production of cAMP by FMLP-activated neutrophils, resulting in failure to up-regulate the cAMP-PKA-activatable, Ca^{2+} -sequestering endo-membrane Ca^{2+} -ATPase. Dysregulation of Ca^{2+} homeostasis may also explain the increased release of elastase from ZM 241385-treated FMLP/CB-activated neutrophils. These observations support the contention that endogenously-generated adenosine mediates the FMLP-induced increase in cAMP through autocrine interactions with $A_{2A}R$ (Iannone *et al.*, 1989), which may result in restoration of Ca^{2+} homeostasis and down-regulation of the pro-inflammatory activities of these cells.

In conclusion, the results presented here demonstrate that CGS 21680, as is the case with other cAMP-elevating agents such as rolipram (Anderson *et al.*, 1998), accelerates the re-sequestration of cytosolic Ca^{2+} in FMLP-activated neutrophils, probably by up-regulation of the endo-membrane Ca^{2+} -ATPase, leading to inhibition of Ca^{2+} -dependent neutrophil functions, while ZM 241385 has opposite effects on these. If they can be selectively targeted onto immune and inflammatory cells, AR agonists operative at the level of $A_{2A}R$ represent a novel group of anti-inflammatory agents which may be useful in the treatment of those disorders involving hyperactivity of neutrophils.

Chapter 5

Concluding Comments

5.1 Concluding Comments

The inability of corticosteroids to directly affect the immediately/early-activatable pro-inflammatory functions of human neutrophils *in vitro*, has been confirmed in the current study (Chapter 2). Neither dexamethasone nor fluticasone had any meaningful inhibitory effects on superoxide production by and release of elastase from chemoattractant-activated neutrophils, while in some cases potentiation of these activities was evident. Moreover, prolonged exposure (for up to 6 hours) of neutrophils also failed to affect the synthesis by, and release of IL-8 from the cells. Due to the high level of spontaneous apoptosis which was evident at 6 hours (R. Cockeran, Department of Immunology, University of Pretoria), I was unable to reliably extend the time-course of my experiments beyond this, which may explain the difference between my results and those reported by Cox (1995). The latter author reported that treatment of neutrophils with dexamethasone for 24 hours resulted in a significant decrease in the synthesis of IL-8 by these cells.

The apparent insensitivity of neutrophils to corticosteroids may be explained by several co-existent and possibly interactive mechanisms. Firstly, neutrophils are pre-programmed to respond instantly to receptor-mediated activation with chemoattractants, cytokines and opsonized particles, independently of *de novo* protein synthesis. Secondly, neutrophils are insensitive to the apoptosis-inducing actions of corticosteroids (Cox, 1995; Meagher *et al.*, 1996), due possibly, but not necessarily limited to, the paucity of mitochondria in these cells (Peachman *et al.*, 2000). Thirdly, the relatively high ratio of GR β to GR α in the neutrophil results in a high frequency of GR α /GR β heterodimers which have only 15-20% of the transactivating activity of GR α homodimers (Strickland *et al.*, 2001). Indeed, GR β lacks the steroid binding domain which makes it unable to bind glucocorticoids, reduces its affinity for DNA recognition sites, abolishes its ability to transactivate glucocorticoid-sensitive genes and makes it function as a dominant inhibitor of GR α through the formation of antagonistic GR α /GR β heterodimers (Strickland *et al.*, 2001).

Taken together, all of this seems to suggest that neutrophils are not particularly responsive to the anti-inflammatory actions of corticosteroids. Nevertheless, important indirect regulatory effects of corticosteroids on neutrophils mediated via down-regulation of the synthesis, release and/or expression of neutrophils-targeted pro-inflammatory polypeptides (adhesion molecules, cytokines) cannot be excluded.

A glucocorticoid-induced decrease in L-selectin expression has been demonstrated on neutrophils in the maturation pool of the bone marrow and may constitute an important mechanism in the reduction of PMN recruitment into inflammatory sites (Nakagawa *et al.*, 1999). This decrease was detectable 8-12 hours after GC treatment.

The ineffectiveness of corticosteroids in controlling the pro-inflammatory activities of neutrophils set the scene for the subsequent, and indeed the most important aspects of my laboratory research, which were to identify pharmacologic agents which would effectively suppress neutrophil NADPH oxidase activity and granule enzyme release. Given the well-recognised anti-inflammatory actions of cAMP-elevating agents, which include inhibition of the early-activatable pro-inflammatory activities of neutrophils (Moore & Willoughby, 1995), in the setting of the recent development of pharmacologic agents such as second generation type 4 PDE inhibitors (Torphy, 1998; Wang *et al.*, 1999) and subtype-selective agonists of adenosine receptors (Rieger *et al.*, 2001; Sullivan *et al.*, 2001), my research, much of which is original, was directed at identifying the anti-inflammatory potential and biochemical mechanisms of action of the latter group of agents.

Interestingly, CGS 21680 was found to be the most potent of the three adenosine receptor subtype agonists tested, implicating the adenylate cyclase/cAMP-coupled $A_{2A}R$ subtype as being primarily involved in regulating neutrophils pro-inflammatory activity. The involvement of this adenosine receptor subtype, as well as that of cAMP, was underscored by the action of ZM 241385, which attenuated the enhancing effects of CGS 21680 on neutrophil cAMP and the inhibitory actions of the $A_{2A}R$ agonist on FMLP-activated superoxide production and elastase release. The effects of CGS 21680 on neutrophil cAMP and pro-inflammatory activities were associated with definite alterations in Ca^{2+} handling by the cells following exposure to

FMLP. These were characterised by accelerated clearance of Ca^{2+} from the cytosol by FMLP-activated neutrophils, which was compatible with a cAMP-dependent mechanism involving up-regulation of the endo-membrane Ca^{2+} -ATPase. I am not aware of previous reports documenting these cAMP-dependent enhancing effects of CGS 21680 on restoration of Ca^{2+} homeostasis to chemoattractant-activated neutrophils.

The effects of ZM 241385 *per se* (in the absence of CGS 21680) on neutrophils are noteworthy. Treatment of the cells with this agent completely attenuated the FMLP-mediated transient increase in intracellular cAMP in the setting of prolongation of the elevation in the cytosolic Ca^{2+} concentration, increased release of elastase and a modest increase in superoxide production. These observations are compatible with the involvement of neutrophil-derived adenosine in the autocrine restoration of Ca^{2+} homeostasis and down-regulation of the pro-inflammatory activities of these cells. Complete attenuation of the FMLP-activated increase in cAMP by ZM 241385 further suggests that endogenous adenosine operating via subtype $A_{2A}R$ and cAMP, as opposed to other potential endogenous regulators such as prostaglandin E_2 operating via EP_2 and EP_4 receptors (Talpain *et al.*, 1995), is primarily involved in the physiological restoration of Ca^{2+} homeostasis to activated neutrophils. Although adenosine has been proposed by others to function as a physiological anti-inflammatory agent (Cronstein, 1994), the mechanistic data reported here are, to my knowledge, original.

With respect to ongoing and future research on the anti-inflammatory potential of subtype $A_{2A}R$ agonists, particularly in controlling neutrophils, there are several aspects which merit brief comment. Considerable effort is being devoted to the development of $A_{2A}R$ agonists with improved selectivity (Rieger *et al.*, 2001; Sullivan *et al.*, 2001), and at least one of these is currently undergoing phase 1 clinical trials (Dr M Johnson, Glaxo Smith Kline, UK). In the case of neutrophil-mediated inflammation, selectivity may be improved and side-effects reduced by combining an $A_{2A}R$ agonist with a selective inhibitor of PDE 4B2, the predominant type found in human neutrophils (Wang *et al.*, 1999). Such a combination strategy may increase efficacy in the setting of reduced toxicity. I have recently been reliably informed that exposure of certain types of inflammatory cells (unspecified) to corticosteroids is

accompanied by upregulation of $A_{2A}R$ (Dr M Johnson, Glaxo Smith Kline, UK), which, bearing in mind the apparent insensitivity of neutrophils to corticosteroids, may, or may not, justify the combined use of $A_{2A}R$ agonists and corticosteroids, as is currently the case with long-acting β_2 -agonists and corticosteroids. Corticosteroids appear to activate transcription of the gene encoding the $A_{2A}R$, in the setting of deactivation of the gene encoding the A_3R . Finally, potential side-effects may also be minimised through the development of inhaled $A_{2A}R$ agonists for the treatment of inflammatory airway disorders (Dr M Johnson, Glaxo Smith Kline, UK).

Future research is required to establish *in vivo* anti-inflammatory activity of A_{2A} agonists, optimal routes of administration of these agents, as well as assessment of anti-inflammatory potency in combination with other classes of anti-inflammatory agents especially type 4 phosphodiesterase inhibitors and development of selective and more potent A_{2A} agonists.

In conclusion, the results presented in this thesis demonstrate that the $A_{2A}R$ agonist, CGS 21680, possesses neutrophil-directed anti-inflammatory activities which are dependent on activation of adenylate cyclase and up-regulation of the Ca^{2+} sequestering/re-sequestering endo-membrane Ca^{2+} -ATPase. Although promising, no such agent is currently available for clinical application and further research is required to optimise selectivity and anti-inflammatory activity in the setting of minimal toxicity.

Chapter 6

References

References

- Ackerman N, Martinez S, Thieme T & Mirkovich A. Accumulation of rat polymorphonuclear leucocytes at an inflammatory site. *J Pharmacol Exp Ther* **221**: 701-707, 1982.
- Adams DH, Wang L, Burnett D, Stockley RA & Neuberger JM. Are neutrophils important in mediating the tissue damage of liver allograft rejection? *Gastroenterology* **96**: A570, 1989.
- Alonso A, Bayon Y, Mateos JJ, & Sanchez Crespo M. Signaling by leucocyte chemoattractant and Fc γ receptors in immune-complex tissue injury. *Lab Invest* **78**: 377-392, 1998.
- Anderson R, Glover A, Koornhof HJ & Rabson AR. *In vitro* stimulation of neutrophil motility by levamisole. Maintenance of cAMP levels in chemotactically stimulated human neutrophils. *J Immunol* **117**: 428-432, 1976.
- Anderson R. The activated neutrophil: formidable forces unleashed. *SAMJ* **85**: 1024-1028, 1995.
- Anderson R & Goolam Mahomed A. Calcium efflux and influx in FMLP-activated human neutrophils are chronologically distinct events. *Clin Exp Immunol* **110**: 132-138, 1997.
- Anderson R, Goolam Mahomed A, Theron AJ, Ramafi G & Feldman C. Effect of rolipram and dibutyryl cAMP on resequestration of cytosolic calcium in FMLP-activated human neutrophils. *Brit J Pharmacol* **124**: 547-555, 1998.

Bainton DF. Developmental biology of neutrophils and eosinophils. In: Gallin JI, Goldstein IM, Snyderman R, editors. *Inflammation: Basic Principles and Clinical Correlates* (2nd Edition). New York: Raven Press Ltd. p303-324, 1992.

Ballard PL, Carter JP, Graham BS & Baxter JD. A radioreceptor assay for evaluation of the plasma glucocorticoid activity of natural and synthetic steroids in man. *J Clin Endocrinol Metab* **41**: 290-304, 1975.

Bánfi B, Schrenzel J, Nusse O, Lew DP, Ligeti E, Krause KH & Demaurex N. A novel H (+) conductance in eosinophils: unique characteristics and absence in chronic granulomatous disease. *J Exp Med* **190**: 183-194, 1999.

Bánfi B, Maturana A, Jaconi S, Arnaudeau S, Laforge T, Sinha B;Ligeti E, Demaurex N & Krause KH. A mammalian H(+) channel generated through alternative splicing of the NADPH oxidase homolog NOH-1. *Science* **287**: 138-142, 2000.

Barnes PJ. Molecular mechanisms of glucocorticoid action in asthma. *Pulm Pharmacol Ther* **10**: 3-19,1997.

Barnes PJ & Adcock IM. NF- κ B: a pivotal role in asthma and a new target for therapy. *Trends Pharmacol Sci* **18**: 46-50, 1997.

Barnes PJ & Adcock IM. Transcription factors and asthma. *Eur Respir J* **12**: 221-234, 1998.

Barton AE, Bayley DL, Mikami M, Llewellyn-Jones CG & Stockley RA. Phenotypic changes in neutrophils related to anti-inflammatory therapy. *Biochim Biophys Acta* **1500**: 108-118, 2000.

Bassaris HP, Lianou PE, Mallioris KN, Skoutelis AT, Votta EG, Kyriazopoulou VE & Papavassiliou JT. Effects of methylprednisolone and dexamethasone on polymorphonuclear leucocyte adherence *in vivo*. *Drugs Exp & Clin Res*. **13**: 267-271, 1987.

Beatty K, Robertie P, Senior RM & Travis J. Determination of oxidized alpha-1-proteinase inhibitor in serum. *J Lab Clin Med* **100**: 186-192, 1982.

Berman ME & Muller WA. Ligation of platelet/endothelial cell adhesion molecule 1 (PECAM-1/CD31) on monocytes and neutrophils increases binding capacity of leucocyte CR3 (CD11b/CD18). *J Immunol* **154**: 299-307, 1995.

Bingle CD, Craig RW, Hanks BM, Eaton V, Zhou P & Whyte MKB. Mechanisms and consequences of apoptosis. *Am J Resp and Crit Care Med* **161**: A899 2000.

Blouin E, Halbwachs-Mecarelli L & Rieu P. Redox regulation of b2 integrin CD11b/CD18 activation. *Eur J Immunol* **29**: 3419-3431, 1999.

Borel JF. Effect of some drugs on the chemotaxis of rabbit neutrophils *in vitro*. *Experientia* **29**: 676-678, 1973.

Borregaard N & Cowland JB. Granules of the human neutrophilic polymorphonuclear leucocyte. *Blood* **89**: 3503-3521, 1997.

Bouma MG, Jeunhomme TMMA, Boyle DL, Dentener MA, Voitenok NN, Van Den Wildenberg FAJK & Burman WA. Adenosine inhibits neutrophil degranulation in activated human whole blood. Involvement of A₂ and A₃ receptors. *J Immunol* **158**: 5400-5408, 1997.

Bratt J & Heimburger M. Prednisolone interferes with neutrophil adhesion and neutrophil mediated endothelial injury. *Scand J Rheum* **28**: 308-313, 1999.

Brown SB & Savill J. Phagocytosis triggers macrophage release of Fas ligand and induces apoptosis of bystander leucocytes. *J Immunol* **162**: 480-485, 1999.

Burnett D, Chamba A, Hill SL & Stockley RA. Effects of plasma, tumour necrosis factor, endotoxin and dexamethasone on extracellular proteolysis by neutrophils from healthy subjects and patients with emphysema. *Clin Sci* **77**: 35-41, 1989.

Burns AR, Bowden RA, Abe Y, Walker DC, Simon SI, Entman ML & Smith CW. P-selectin mediates neutrophil adhesion to endothelial cell borders. *J Leucoc Biol* **65**: 229-306, 1999.

Burton JL & Kehrl ME Jr. Regulation of neutrophil adhesion molecules and shedding of Staphylococcus aureus in milk of cortisol- and dexamethasone-treated cows. *Am J Vet Res* **56**: 997-1006, 1995.

Capocidi C, Hanft S, Feoktistov M & Pillinger MH. Phosphatidylinositol 3-kinase mediates chemoattractant-stimulated, CD11b/CD18-dependent cell-cell adhesion of human neutrophils: Evidence for an ERK-independent pathway. *J Immunol* **160**: 1901-1909, 1998.

Cassatella MA. Neutrophil-derived proteins: Selling cytokines by the pound. *Adv Immunol* **73**: 369-509, 1999.

Cham BP, Gerrard JM & Bainton DF. Granulophysin is located in the membrane of azurophilic granules in human neutrophils and mobilizes to the plasma membrane following cell stimulation. *Am J Path* **144**: 1369-1380, 1994.

Church MK & Hughes PJ. Adenosine potentiates immunological histamine release from rat mast cells by a novel cyclic AMP- independent cell-surface action. *Br J Pharmacol* **85**: 3-5, 1985.

Clark RAF, Gallin JL & Fauci AS. Effects of *in vivo* prednisone on *in vitro* eosinophil and neutrophil adherence and chemotaxis. *Blood* **53**: 633-641, 1979.

Clark RA. Activation of the neutrophil respiratory burst oxidase. *Infect Dis* **179**: S309-S317, 1999.

Cox G. Glucocorticoid treatment inhibits apoptosis in human neutrophils. *J Immunol* **154**: 4719-4725, 1995.

Crockard AD, Boylan MT, Droogan AG, McMillan SA & Hawkins SA. Methylprednisolone-induced neutrophil leucocytosis—down-modulation of neutrophil L-selectin and Mac-1 expression and induction of granulocyte-colony stimulating factor. *Int J Clin Lab Res.* **28**: 110-115, 1998.

Cronstein BN, Kramer SB, Weissman G & Hirschhorn R. Adenosine: A physiological modulator of superoxide anion generation by human neutrophils. *J Exp Med* **158**: 1160-1177, 1983.

Cronstein BN, Rosenstein ED, Kramer SB, Weissmann G & Hirschhorn R, Adenosine: a physiologic modulator of superoxide anion generation by human neutrophils. Adenosine acts via an A₂ receptor on human neutrophils. *J Immunol* **135**: 1366-1371, 1985.

Cronstein BN, Levin RI, Belanoff J, Weissman G & Hirschhorn R. Adenosine: An endogenous inhibitor of neutrophil-mediated injury to endothelial cells. *J Clin Invest* **78**: 760-770, 1986.

Cronstein BN, Kramer SB, Rosenstein ED, Korchak HM, Weissman G & Hirschhorn R. Occupancy of adenosine receptors raises cyclic AMP alone and in

synergy with occupancy of chemoattractant receptors and inhibits membrane depolarization. *Biochem J* **252**: 709-715, 1988.

Cronstein BN & Haines KA. Stimulus-response uncoupling in the neutrophil: Adenosine A₂-receptor occupancy inhibits the sustained, but not the early, events of stimulus transduction in human neutrophils by a mechanism independent of actin-filament formation. *Biochem J* **281**: 631-635, 1992.

Cronstein BN, Levin RI, Phillips K, Hirschhorn R, Abramson SB & Weissmann G. Neutrophil adherence to endothelium is enhanced via adenosine A₁ receptors and inhibited via adenosine A₂ receptors. *J Immunol* **148**: 2201-2206, 1992.

Cronstein BN, Naime D & Firestein C. The antiinflammatory mechanism of methotrexate. Increased adenosine release at inflamed sites diminishes leucocyte accumulation in an *in vivo* model of inflammation. *J Clin Invest* **92**: 2675-2682, 1993.

Cronstein BN. Adenosine, an endogenous anti-inflammatory agent. *J Appl Physiol* **76**: 5-13, 1994.

Daffern PJ, Jagels MA & Hugli TE. Multiple epithelial cell-derived factors enhance neutrophil survival. Regulation by glucocorticoids and tumour necrosis factor-alpha. *Am J Respir Cell & Mol Biol* **21**: 259-267, 1999.

Dale DC, Liles WC, Llewellyn C, Rodger E & Price TH. Neutrophil transfusions: kinetics and function of neutrophils mobilized with granulocyte-colony-stimulating factor and dexamethasone. *Transfusion* **38**: 710-712, 1998.

Dandona P, Mohanty P, Hamouda W, Aljada A, Kumbkarni Y & Garg R. Effect of dexamethasone on reactive oxygen species generation by leucocytes and

plasma interleukin-10 concentrations: A pharmacodynamic study. *Clin Pharmacol Ther* **66**: 58-65, 1999.

Dandona P, Suri M, Hamouda W, Aljada A, Kumbkarni Y & Thusu K. Hydrocortisone-induced inhibition of reactive oxygen species by polymorphonuclear neutrophils. *Crit Care Med* **27**: 2442-2444, 1999.

Davenpeck KL, Zagorski J, Schleimer RP & Bochner BS. Lipopolysaccharide-induced leucocyte rolling and adhesion in the rat mesenteric microcirculation: regulation by glucocorticoids and role of cytokines. *J Immunol* **161**: 6861-6870, 1998.

Davis JM, Albert JD, Tracy KJ, Calvano SE, Lowry SF, Shires GT & Yurt RW. Increased neutrophil mobilization and decreased chemotaxis during cortisol and epinephrine infusions. *J Trauma-Injury Infect & Crit Care* **31**: 725-731, 1991.

Di Virgilio F, Lew PD, Anderson T & Pozzan T. Plasma membrane potential modulates chemotactic peptide-stimulated cytosolic free Ca^{++} changes in human neutrophils. *J Biol Chem* **262**: 4574-4579, 1987.

Dolmetsch RE, Lewis RS, Goodnow CC & Healy JI. Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. *Nature* **386**: 855-858, 1997.

Elsbach P & Weiss J. Oxygen-independent antimicrobial systems of phagocytes. In: Gallin JI, Goldstein IM, Snyderman R. editoris. *Inflammation: Basic Principles and Clinical Correlates* (2nd edition). New York: Raven Press Ltd. p663-700, 1992.

Erickson RW, Langel-Peveri P, Traynor-Kaplan AE, Heyworth PG & Carnutte JT. Activation of human neutrophil NADPH oxidase by phosphatidic acid or

diacylglycerol in a cell-free system. Activity of diacylglycerol is dependent on its conversion to phosphatidic acid. *J Biol Chem* **274**: 22243-22250, 1999.

Ezeamuzie CI & Philips E. Adenosine A₃ receptors on human eosinophils mediate inhibition of degranulation and superoxide anion release. *Br J Pharmacol* **127**:188-194, 1999.

Fadok VA, Warner ML, Bratton DL & Henson PM. CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor (alpha v beta 3). *J Immunol* **161**: 6250-6257, 1998.

Favre CJ, Nüsse O, Lew DP & Krause K-H. Store-operated calcium influx: What is the message from the stores to the membrane? *J Lab Clin Med* **128**: 19-26, 1996.

Filep JG, Delalandre A, Payette Y & Foldes-Filep E. Glucocorticoid receptor regulates expression of L-selectin and CD11/CD18 on human neutrophils. *Circulation* **96**: 295-301, 1997.

Filep JG, Zouki C, Petasis NA, Hachicha M & Serhan CN. Anti-inflammatory actions of lipoxin A(4) stable analogs are demonstrable in human whole blood. modulation of leucocyte adhesion molecules and inhibition of neutrophil-endothelial interactions. *Blood* **94**: 4132-4142, 1999.

Flamand N, Boudreault S, Picard S, Austin M, Surette ME, Plante H, Krump E, Vallée M-J, Gilbert C, Naccache P, Laviolette M & Borgeat P. Adenosine, a potent natural suppressor of arachidonic acid release and leukotriene biosynthesis in human neutrophils. *Am J Respir Crit Care Med* **161**: 588-594, 2000.

Fleming SD, Edelman LS & Chapes SK. Effects of corticosterone and microgravity on inflammatory cell production of superoxide. *J Leucoc Biol* **50**: 69-76, 1991

Fonteh AN, Winkler JD, Torphy TJ, Heravi J, Udem BJ & Chilton FH. Influence of isoproterenol and phosphodiesterase inhibitors on platelet-activating factor biosynthesis in the human neutrophils. *J Immunol* **151**: 339-350, 1993.

Forbes LV, Truong O, Wientjes FB, Moss SJ & Segal AW. The major phosphorylation site of the NADPH oxidase component p67phox is Thr 233. *Biochem J* **338**: 99-105, 1999.

Forbes LV, Moss SJ & Segal AW. Phosphorylation of p67phox in the neutrophil occurs in the cytosol and is independent of p47phox. *Febbs Lett* **449**: 225-229, 1999.

Fozard JR, Pfannkuche H-J & Schuurman H-J. Mast cell degranulation following adenosine A₃ receptor activation in rats. *Eur J Pharmacol* **298**: 293-297, 1996.

Fredholm BB, Zhang Y & Van der Ploeg I. Adenosine A_{2A} receptors mediate the inhibitory effect of adenosine on formyl-Met-Leu-Phe-stimulated respiratory burst in neutrophil leucocytes. *Naunyn Schmiedebergs Arch Pharmacol* **354**: 262-267, 1996.

Fredholm BB. Purines and neutrophil leucocytes. *Gen Pharmacol* **28**: 345-350, 1997.

Fredholm BB. Adenosine and neuroprotection. *Int Rev Neurobiol* **40**: 259-280, 1997.

Freischlag JA, Colburn MD, Quinones-Baldrich WJ & Moore WS. Alteration of neutrophil function by heparin, dexamethasone, and enalapril. *J Surg Res* **52**: 523-529, 1992.

Fuenfer MM, Carr EA & HC Polk Jr. The effect of hydrocortisone on superoxide production by leucocytes. *J Surg Res* **27**: 29-35, 1979.

Gadaleta D, Verma M & Davis JM. Inhibition of neutrophil leukotriene generation by the 21-aminosteroid, U-74389F. *J Surg Res* **57**: 233-237, 1994.

Gainet J, Chollet-Martin S, Brion M, Hakim J, Gougerot-Pocidallo M & Elbim C. Interleukin -8 production by polymorphonuclear neutrophils in patients with rapidly progressive periodontitis: An amplifying loop of polymorphonuclear neutrophil activation. *Lab Invest* **78**: 755-762, 1998.

Geiszt M, Kapus A, Nemet K, Farkas L & Ligeti E. Regulation of capacitative Ca^{2+} influx in human neutrophil granulocytes. *J Biol Chem* **272**: 26471-26478, 1997.

Ghamberg CG, Valmu L, Fagerholm S, Kotovouri P, Ihanus E, Tian L & Pessamorikawa T. Leukocyte integrins and inflammation. *Cell Mol Life Sci* **54**: 549-555, 1998.

Goldstein DM, Roos D, Weissman G & Kaplan HB. Influence of corticosteroids on human polymorphonuclear function *in vitro*: reduction of lysosomal enzyme release and superoxide production. *Inflammation* **1**: 305-315, 1976.

Goldstein IM. Complement: Biologically active products. In: Gallin JI, Goldstein IM, Snyderman R, editors. *Inflammation: Basic Principles and Clinical Correlates* (2nd edition). New York: Raven Press Ltd. p33-62, 1992.

Goolam Mahomed A & Anderson R. Activation of human neutrophils with chemotactic peptide, opsonized zymosan and the calcium ionophore A23187, but not with a phorbol ester, is accompanied by efflux and store-operated influx of calcium. *Inflammation* **24**: 559-569, 2000.

Goulding NJ, Euzger HS, Butt SK & Perretti M. Novel pathways for glucocorticoid effects on neutrophils in chronic inflammation. *Inflammation Res* **47**: S158-165, 1998.

Greene WC. The interleukins. In: Gallin JI, Goldstein IM, Snyderman R, editors: *Inflammation: Basic Principles and Clinical Correlates* (2nd edition). New York: Raven Press Ltd. p 233-246, 1992.

Grynkiewicz G, Poenie M & Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* **260**: 3440-3450, 1985.

Guelfi JF, Courdouhji MK, Alvinerie M & Toutain PL. *In vivo* and *in vitro* effects of three glucocorticoids on blood leucocyte chemotaxis in the dog. *Vet Immunol Immunopathol* **10**: 245-252, 1985.

Gunther GR & Herring MB. Inhibition of neutrophil superoxide production by adenosine released from vascular endothelial cells. *Ann Vasc Surg* **5**: 325-330, 1991.

Ham EA, Soderman DD & Zanetti ME. Inhibition by prostaglandins of leukotriene B₄ release from activated neutrophils. *Proc Natl Acad Sci USA* **80**: 4349-4353, 1983.

Hannon JP, Bray-French KM, Phillips RM & Fozard JR. Further pharmacological characterization of the adenosine receptor subtype mediating inhibition of the oxidative burst in human isolated neutrophils. *Drug Dev Res* **43**: 214-224, 1998.

Harada N, Okajima K, Murakami K, Usune S, Sato C, Ohshima K & Katsuragi T. Adenosine and selective A(2A) receptor agonists reduce ischemia/reperfusion injury of rat liver mainly by inhibiting leucocyte activation. *J Pharmacol Exp Ther* **294**: 1034-1042, 2000.

Heimbürger M, Larfars G & Bratt J. Prednisolone inhibits cytokine-induced adhesive and cytotoxic interactions between endothelial cells and neutrophils *in vitro*. *Clin Exp Immunol* **119**: 441- 448, 2000.

Henderson LM, Chappell JB & Jones OT. Superoxide generation by electrogenic NADPH oxidase of human neutrophils is limited by the movement of a compensating change. *Biochem J* **255**: 285-290, 1988.

Hetherington SV & Quie PG. Human polymorphonuclear leucocytes of the bone marrow, circulation, and marginated pool: function and granule protein content. *Am J Hematology* **20**: 235-246, 1985.

Hill GE, Alonso A, Thiele GM & Robbins RA. Glucocorticoids blunt neutrophil CD/11b surface glycoprotein upregulation during cardiopulmonary bypass in humans. *Anaesthesia & Analgesia* **79**: 23-27, 1994.

Hirata F, Schiffman E, Venkatasubramanian K, Salomon D & Axelrod J. A phospholipase A₂ inhibitory protein in rabbit neutrophils induced by glucocorticoids. *Proc Natl Acad Sci USA* **77**: 2533-2536, 1980.

Hoeben D, Burvenich C & Massart-Leën A-M. Glucocorticoids and *in vitro* effects on chemiluminescence of isolated bovine blood granulocytes. *Eur J Pharmacol* **354**: 197-203, 1998.

Horan TD, English D & McPherson TA. Association of neutrophil chemiluminescence with microbicidal activity. *Clin Immunol Immunopathol* **22**: 259-269, 1982.

Humphreys JM, Stringer RE, Hart CA & Edwards SW. Effect of cytotoxic drugs on mature neutrophil function in the presence and absence of granulocyte-macrophage colony-stimulating factor. *Br J Haematol* **84**: 316-321, 1993.

Iannone MA, Wolberg G & Zimmerman TP. Chemotactic peptide induces cAMP elevation in human neutrophils by amplification of the adenylate cyclase response to endogenously produced adenosine. *J Biol Chem* **264**: 20177-20180, 1989.

Jarvis MF, Schultz R, Hutchison AJ, Hoi Du U, Sills MA & Williams M. A selective A₂ adenosine receptor agonist directly labels A₂ receptors in rat brain. *J Pharmacol Exp Ther* **251**: 888-893, 1989.

Jankowski A & Grinstein S. A noninvasive fluorimetric procedure for measurement of membrane potential. Quantification of the NADPH oxidase-induced depolarization in activated neutrophils. *J Biol Chem* **274**: 26098-26104, 1999.

Jayappa HG & Loken KI. Effect of antimicrobial agents and corticosteroids on bovine polymorphonuclear leucocyte chemotaxis. *Am J Vet Res* **44**: 2155-2159, 1983.

Jones CJ, Morris KJ & Jayson MIV. Prednisolone inhibits phagocytosis by polymorphonuclear leucocytes via steroid receptor mediated events. *Ann Rheum Dis* **42**: 56-62, 1983.

Jones SL, Knaus UG, Bokoch GM & Brown EJ. Two signalling mechanisms for activation of α M β 2 avidity in polymorphonuclear neutrophils. *J Biol Chem* **273**: 10556-10566, 1998.

Jordan JE, Zhao Z-Q, Sako H, Taft S & Vinten-Johansen J. Adenosine A₂ Receptor activation attenuates reperfusion injury by inhibiting neutrophil accumulation, superoxide generation and coronary endothelial adherence. *J Pharmacol Exp Ther* **280**: 301-309, 1997.

Jordan JE, Thourani VH, Auchampach JA, Robinson JA, Wang N-P & Vinten-Johansen J. A₃ adenosine receptor activation attenuates neutrophil function and neutrophil-mediated reperfusion injury. *Am J Physiol* **277**: H1895-H1905, 1999.

Kamei Y, Xu L, Heinzl T, Torchia J, Kurokawa R, Gloss B, Lin S-C, Heyman RA, Rose DW, Glass CK & Rosenfeld MG. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85**: 403-414, 1996.

Kolanus W, Nagel W, Schiller B, Zeitlmann L, Godar S, Stockinger H & Seed B. α L β 2 integrin LFA-1 binding to ICAM-1 induced by Cytohesin-1, a cytoplasmic regulatory molecule. *Cell* **86**: 233-242, 1996.

Kondo E & Kanai K. Mechanism of bactericidal activity of lysolecithin and its biological implication. *Jap J Med Sci & Biol* **38**: 181-194, 1985.

Koppenol WH. The basic chemistry of nitrogen monoxide and peroxynitrite. *Free Radic Biol Med* **25**: 385-391, 1998.

Krause K-H, Pittet D, Volpe P, Pozzan T, Meldolesi J & Lew DP. Calciosome, a sarcoplasmic reticulum-like organelle involved in intracellular Ca²⁺ -handling by

non-muscle cells: studies in human neutrophils and HL60 cells. *Cell Calcium* **10**: 351-361, 1989.

Krump E, Lemay G & Borgeat P. Adenosine A₂ receptor-induced inhibition of leukotriene B₄ synthesis in whole blood *ex vivo*. *Br J Pharmacol* **117**: 1639-1644, 1996.

Krump E, Picard S, Mancini J & Borgeat P. Suppression of leukotriene B₄ biosynthesis by endogenous adenosine in ligand-activated human neutrophils. *J Exp Med* **186**: 1401-1406, 1997.

Kurihara A, Ojima F & Tsurufuji A. Analysis of the effect of an antiinflammatory steroid, dexamethasone, on neutrophil chemotaxis in the Boyden chamber with a modified ⁵¹Cr-labeling method. *J Pharm Dyn* **7**: 742-754, 1984b.

Kuzniar J, Sajewicz W, Kopec W & Hruby Z. Chemiluminescence of neutrophils in patients with glomerulonephritis treated with methylprednisolone. *International Urology and Nephrology* **23**: 527-534, 1991.

Lagast H, Lew DP & Waldvogel FA. Adenosine triphosphatase-dependent calcium pump in the plasma membrane of guinea pig and human neutrophils. *J Clin Invest* **73**: 107-115, 1984.

Lam BK & Austen KF. Leukotrienes: Biosynthesis, Release and Actions. In: Gallin JI, Goldstein IM, Snydermann R, editors. *Inflammation, Basic Principles and Clinical Correlates* (2nd edition) New York, Raven Press Ltd, p139-147, 1992.

Lane SJ, Adcock IM, Richards D, Hawrylowicz C, Barnes PJ & Lee TH. Corticosteroid-resistant bronchial asthma is associated with increased c-fos expression in monocytes and T lymphocytes. *J Clin Invest* **102**: 2156-2164, 1998.

Lappin D & Whaley K. Adenosine A₂ receptors on human monocytes modulate C₂ production. *Clin Exp Immunol* **57**: 454-460, 1984.

Lauffenburger DA & Horwitz AF. Cell migration: A physically integrated molecular process. *Cell* **84**: 359-369, 1996.

Le Cabec V, Cowland JB, Calafat J & Borregaard N. Targeting of proteins to granule subsets is determined by timing and not by sorting: The specific granule protein NGAL is localized to azurophilic granules when expressed in HL-60 cells. *Proc Natl Acad Sci USA* **93**: 6454-6457, 1996.

Leslie CC. Properties and regulation of cytosolic phospholipase A₂. *J Biol Chem* **272**: 16709-16712, 1997.

Leusen JH, Verhoeven AJ & Roos D. Interactions between the components of the human NADPH oxidase : intrigues in the phox family. *J Lab Clin Med* **128**: 461-476, 1996.

Lew DP, Monod A, Waldvogel FA, Dewald B & Baggiolini M. Quantitative analysis of the cytosolic free calcium dependency of exocytosis from three subcellular compartments in intact human neutrophils. *J Cell Biol* **102**: 2197-2204, 1986.

Levine PH, Hardin JC, Scoon KL & Krinsky NI. Effect of corticosteroids on the production of superoxide and hydrogen peroxide and the appearance of chemiluminescence by phagocytosing polymorphonuclear leucocytes. *Inflammation* **5**: 19-27, 1981.

- Li X, Steeber DA, Tang MLK, Farrar MA, Perlmutter RM & Tedder TF. Regulation of L-selectin-mediated rolling through receptor dimerization. *J Exp Med* **188**: 1385-1390, 1998.
- Lim LH, Solito E, Russo-Marie F, Flower RJ & Perretti M. Promoting detachment of neutrophils adherent to murine postcapillary venules to control inflammation: effect of lipocortin 1. *Proc Natl Acad Sci USA* **95**: 14535-14539, 1998.
- Linden J. Cloned adenosine A₃ receptors: pharmacological properties, species differences and receptor functions. *Trends pharmacol Sci* **15**: 289-306, 1994.
- Liu Q, Wang Y & Thorlacius H. Dexamethasone inhibits tumour necrosis factor-alpha-induced expression of macrophage inflammatory protein-2 and adhesion of neutrophils to endothelial cells. *Biochem Biophys Res Comm* **271**: 364-367, 2000.
- Llewellyn-Jones CG, Hill SL & Stockley RA. Effect of fluticasone propionate on neutrophil chemotaxis, superoxide generation, and extracellular proteolytic activity *in vitro*. *Thorax* **49**: 207-212, 1994.
- Llewellyn-Jones CG, Harris TAJ & Stockley RA. Effect of fluticasone propionate on sputum of patients with chronic bronchitis and emphysema. *Am J Respir Crit Care Med* **153**: 616-621, 1996.
- Lomas DA, Ip M, Chamba A & Stockley RA. The effect of *in vitro* and *in vivo* dexamethasone on human neutrophil function. *Agents and Actions* **33**: 279-285, 1991.
- Lytton J, Westlin M & Hanley MR. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. *J Biol Chem* **266**: 17067-17071, 1991.

Macconi D, Zanoli AF, Orisio S, Longaretti L, Magrini L, Rota S, Radice A, Pozzi C & Remuzzi G. Methylprednisolone normalizes superoxide anion production by polymorphs from patients with ANCA-positive vasculitides. *Kidney Int* **44**:215-220, 1993.

MacGregor RR, Spagnuolo PJ & Lentnek AL. Inhibition of granulocyte adherence by ethanol, prednisone and aspirin measured with an assay system. *N Engl J Med* **291**: 642-646, 1974.

Mak JCW, Nishikawa M, Shirasaki H, Miyayasu K & Barnes PJ. Protective effects of a glucocorticoid on down-regulation of pulmonary β_2 -adrenergic receptors *in vivo*. *J Clin Invest* **96**: 99-106, 1995.

Mandell GL, Rubin W & Hook EW. The effect of an NADH oxidase inhibitor (hydrocortisone) on polymorphonuclear leucocyte bactericidal activity. *J Clin Invest* **49**:1381-1388, 1970.

Marone G, Thomas L & Lichtenstein TL. The role of agonists that activate adenylate cyclase in the control of cAMP metabolism and enzyme release by human polymorphonuclear leucocytes. *J Immunol* **125**: 2277-2283, 1980.

Martin-Martin B, Nabokina SM, Blasi J, Lazo PA & Mollinedo F. Involvement of SNAP-23 and syntaxin 6 in human neutrophil exocytosis. *Blood* **96**: 2574-2583, 2000.

Martin-Padura I, Lostaglio S, Schneemann M, Williams L, Romano M, Fruscella P, Panzeri C, Stoppacciaro A, Ruco L, Villa A, Simmons D & Dejana E. Junctional Adhesion Molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol* **142**: 117-127, 1998.

McFadden ER. Inhaled glucocorticoids and acute asthma: therapeutic breakthrough or non-specific effect?. *Am J Respir Crit Care Med* **157**: 677-678, 1998.

McGarrity ST, Stephenson AH & Webster RO. Regulation of human neutrophil functions by adenine nucleotides. *J Immunol* **142**: 1986-1994, 1989.

Meagher LC, Cousin JC, Seckl JR & Haslett C. Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. *J Immunol* **156**: 4422-4428, 1996.

Minkenberg I & Ferber E. Lucigenin-dependent chemiluminescence as a new assay for NADPH-oxidase activity in particulate fractions of human polymorphonuclear leucocytes. *J Immunol Meth* **71**: 61-67, 1984.

Mohr FC & Fewtrell C. Depolarization of rat basophilic leukemia cells inhibits calcium uptake and exocytosis. *J Cell Biol* **104**: 783-792, 1987.

Montero M, Alvarez J, Garcia-Sanchez J. Agonist-induced Ca^{2+} influx in human neutrophils is secondary to the emptying of intracellular calcium stores. *Biochem J* **277**: 73-79, 1991.

Moore AR & Willoughby DA. The role of cAMP regulation in controlling inflammation. *Clin Exp Immunol* **101**: 387-389, 1995.

Moroz C & Stevens RH. Suppression of immunoglobulin production in normal human B lymphocytes by two T-cell subsets distinguished following *in vitro* treatment with adenosine. *Clin Immunol Immunopathol* **15**: 44-51, 1980.

Morris DD, Strzemienski PJ, Gaulin G & Spencer P. The effect of corticosteroid administration on the migration, phagocytosis and bactericidal capacity of equine neutrophils. *Cornell Vet* **78**: 243-252, 1988.

Mueller H, Motulsky HJ & Sklar LA. The potency and kinetics of the beta-adrenergic receptors on human neutrophils. *Mol Pharmacol* **34**: 347-353, 1988.

Nagel W, Schilcher P, Zeitlmann L & Kolanus W. The PH domain and the polybasic c domain of cytohesin-1 cooperate specifically in plasma membrane association and cellular function. *Mol Biol Cell* **9**: 1981-1994, 1998.

Nakagawa M, Bondy GP, Waisman D, Minshall D, Hogg JC & van Eeden SF. The effect of glucocorticoids on the expression of L-selectin on polymorphonuclear leucocytes. *Blood* **93**: 2730-2737, 1999.

Niwa Y, Somiya K, Miyachi Y, Kanoh T & Sakane T. Luminol-independent chemiluminescence by phagocytes is markedly enhanced by dexamethasone, not by other glucocorticoids. *Inflammation* **11**: 163-174, 1987.

Nolte D, Lorenzen A, Lehr HA, Bokoch GK & Sklar LA. Reduction of post-ischemic leucocyte-endothelium interaction by adenosine via A₂ receptor. *Naunyn Schmiedebergs Arch Pharmacol* **346**: 234-237, 1992.

Oda T & Katori M. Inhibition site of dexamethasone on extravasation of polymorphonuclear leucocytes in the hamster cheek pouch microcirculation. *J Leucoc Biol* **52**: 337-342, 1992.

Ogryzko W, Schiltz RL, Russanova V, Howard BH & Nakatani Y. The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**: 953-959, 1996.

Ohta N, Shimaoka M, Imanaka H, Nishimura M, Taenaka N, Kiyono H & Yoshiya I. Glucocorticoid suppresses neutrophil activation in ventilator-induced lung injury. *Crit Care Med* **29**: 1012-1016, 2001.

Oishi K, Raynor RL, Charp PA, & Kuo JF. Regulation of protein kinase C by lysophospholipids. *J Biol Chem* **263**: 6865-6871, 1988.

Okusa MD, Linden J, Huang L, Rieger JM, Macdonald TL & Huynh LP.A(2A) adenosine receptor-mediated inhibition of renal injury and neutrophil adhesion. *Am J Physiol Renal Physiol* **279**: F809-F818, 2000.

Olah ME & Stiles GL. Adenosine Receptor Subtypes: Characterization and therapeutic Regulation. *Annu Rev Pharmacol Toxicol* **35**: 581-606, 1995.

Olds JW, Reed WP, Eberle B & Kisch AL. Corticosteroids, serum, and phagocytosis: *in vitro* and *in vivo* studies. *Infect Immun* **9**: 524-529, 1974.

Ongini E & Fredholm BB. Pharmacology of adenosine A_{2A} receptors. *Trends Pharmacol Sci* **17**: 364-372, 1996.

Parmely MJ, Zhou WW, Edwards ICK, Borcharding DR, Silverstein R & Morrison DC. Adenosine and a related carbocyclic nucleoside analogue selectively inhibit tumour necrosis factor- α production and protect mice against endotoxin challenge. *J Immunol* **151**: 389-396, 1993.

Peachman KK, Lyles DS & Bass DA. Mitochondrial differences in glucocorticoid treated eosinophils and neutrophils. *Am J Resp and Crit Care Med* **161**: A899, 2000.

Penner R, Matthews G & Neher E. Regulation of calcium influx by second messengers in rat mast cells. *Nature* **334**: 499-504, 1988.

Perretti M, Croxtall JD, Wheller SK, Goulding NJ, Hannon R & Flower RJ. Mobilizing lipocortin 1 in adherent human leucocytes downregulates their transmigration. *Nature Med* **2**: 1259-1262, 1996.

Persellin RH & Ku LC. Effects of steroid hormones on human polymorphonuclear leucocyte lysosomes. *J Clin Invest* **54**: 919-925, 1974.

Pettit EJ & Hallett MB. Localized and global cytosolic calcium changes in neutrophils during engagement of CD11b/CD18 integrin visualised using confocal laser scanning reconstruction. *J Cell Sci* **109**: 1689-1694, 1996.

Phillips TR, Yang WC & Schultz RD. The effects of glucocorticoids on the chemiluminescence response of bovine phagocytic cells. *Vet Immunol Immunopathol* **14**: 245-256, 1987.

Phillis JW. The selective adenosine A₂ receptor agonist, CGS 21680, is a potent depressant of cortical neuronal activity. *Brain Res* **509**: 328-330, 1990.

Pichyangkul S, Schick D, Schober W, Dixon G & Khan A. Increased expression of adhesive proteins on leucocytes by TNF alpha. *Exp Hematology* **16**: 588-593, 1988.

Poucher SM, Keddie JR, Sing P, Stoggall SM, Caulkett PWR, Jones G & Collis MG. The *in vitro* pharmacology of ZM 241385, a potent non-xanthine A_{2A} selective adenosine receptor antagonist. *Br J Pharmacol* **115**: 1096-1102, 1995.

Prentki M, Wollheim CB & Lew DP. Ca²⁺ homeostasis in permeabilized human neutrophils: characterisation of Ca²⁺ sequestering pools and the action of inositol 1,4,5-triphosphate. *J Biol Chem* **259**: 13777-13782, 1984.

Prescott SM. A thematic series on kinases and phosphatases that regulate lipid signaling. *J Biol Chem* **274**: 8345-8346, 1999.

Ramkumar V, Stiles GL, Beaven MA & Ali H. The A₃ adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells. *J Biol Chem* **268**: 16887-16890, 1993.

Ramos CL, Pou S, Britigan BE, Cohen MS & Rosen GM. Spin trapping evidence for myeloperoxidase-dependent hydroxyl radical formation by human neutrophils and monocytes. *J Biol Chem* **267**: 8307-8312, 1992.

Rhinehart JJ, Balcerzak SP, Sagone AL & LoBuglio AF. Effects of corticosteroids on human monocyte function. *J Clin Invest* **54**: 1337-1343, 1974.

Rieger JM, Brown ML, Sullivan GW, Linden J & Macdonald TL. Design, synthesis and evaluation of novel A_{2A} adenosine receptor agonists. *J Med Chem* **44**: 531-539, 2001.

Rivkin I, Foschi GV & Rosen CH. Inhibition of *in vitro* neutrophil chemotaxis and spontaneous motility by anti-inflammatory agents. *Proc Soc Exp Biol Med* **153** : 236-240, 1976.

Rose FR, Hirschhorn R, Weissmann G & Cronstein BN. Adenosine promotes neutrophil chemotaxis. *J Exp Med* **167**: 1186-1194, 1988.

Røshol H, Skrede KK, Aëro CE & Wijk P. Dexamethasone and methylprednisolone affect rat peritoneal phagocyte chemiluminescence after administration *in vivo*. *Eur J Pharmacol* **286**: 9-17, 1995.

Rossi AG, Cousin JM, Dransfield I, Lawson MF, Chilvers ER & Haslett C. Agents that elevate cAMP inhibit human neutrophil apoptosis. *Biochim Biophys Res Comm* **217**: 892-899, 1995.

Roth JA & Kaeberle ML. Effects of *in vivo* dexamethasone administration on *in vitro* bovine polymorphonuclear leucocyte function. *Infect Immun* **33**: 434-441, 1981.

Roussel E & Gingras MC. Transendothelial migration induces rapid expression on neutrophils of granule-release VLA6 used for tissue infiltration. *J Leucoc Biol* **62**:356-362, 1997.

Ruchaud-Sparagano MH, Walker TR, Rossi AG, Haslett C & Dransfield I. Soluble E-selectin acts in synergy with platelet-activating factor to activate neutrophil beta 2- integrins. Role of tyrosine kinases and Ca²⁺ mobilization. *J Biol Chem* **275**: 15758-15764, 2000.

Saito N, Pulford KA, Breton-Gorius J, Masse JM, Mason DY & Cramer EM. Ultrastructural localization of the CD68 macrophage-associated antigen in human blood neutrophils and monocytes. *Am J Pathol* **139**: 1053-1059, 1991.

Sajjadi FG, Takabayashi K, Foster AC, Domingo RC & Firestein GS. Inhibition of TNF- α expression by adenosine. Role of A₃ adenosine receptors. *J Immunol* **156**: 3435-3442, 1996.

Sakamoto S, Okanoué T, Itoh Y, Sakamoto K, Nishioji K, Nakagawa Y, Yoshida N, Yoshikawa T & Kashima K. Intercellular adhesion molecule-1 and CD18 are involved in neutrophil adhesion and its cytotoxicity to cultured sinusoidal endothelial cells in rats. *Hepatology* **26**: 658-663, 1997.

Salmon JE & Cronstein BN. Fc γ -receptor-mediated functions in neutrophils are modulated by adenosine receptor occupancy. *J Immunol* **145**: 2235-2240, 1990.

Schatzmann HJ. The calcium pump of the surface membrane and of the sarcoplasmic reticulum. *Annu Rev Physiol* **51**:473-485, 1989.

Scheinman RI, Cogswell PC, Lofquist AK & Baldwin AS Jr. Role of transcriptional activation of I κ B α in mediation of immunosuppression by glucocorticoids. *Science* **270**: 283-286, 1995.

Scheinmann RI, Gualberto A, Jewell CM, Cidlowski JA & Baldwin AS Jr. Characterization of mechanisms involved in transrepression of NF- κ B by activated glucocorticoid receptors. *Mol Cell Biol* **15**: 943-953, 1995.

Schleimer RP, Freeland HS, Peters SP, Brown KE & Derse CP. An assessment of the effects of glucocorticoids on degranulation, chemotaxis, binding to vascular endothelium and formation of Leukotriene B₄ by purified human neutrophils. *J Pharmacol Exp Ther* **250**: 598-605, 1989.

Schrenzel J, Serrander L, Bánfi B, Nusse O, Fouyouzi R, Lew DP, Demaurex N & Krause KH. Electron currents generated by the human phagocyte NADPH oxidase. *Nature* **392**: 734-737, 1998.

Seeds MC, Nixon AB, Wykle RL & Bass DA. Differential activation of human neutrophil cytosolic phospholipase A2 and secretory phospholipase A2 during priming by 1,2-diacyl- and 1-0-alkyl-2-acylglycerols. *Biochim Biophys Acta* **1394**: 224-234, 1998.

Segal BH, Leto TL, Gallin JI, Malech HI & Holland SM. Genetic, biochemical, and clinical features of chronic granulomatous disease. *Medicine* **79**: 170-200, 2000.

Sengelov H, Kjeldsen L & Borregaard N. Control of exocytosis in early neutrophil activation. *J Immunol* **150**: 1535-1543, 1993.

Shang T, Yednock T & Issekutz AC. Alpha9 beta1 integrin is expressed on human neutrophils and contributes to neutrophil migration through human lung and synovial fibroblast barriers. *J Leucoc Biol* **66**: 809-816, 1999.

Shimoyama M, Shimmura S, Tsubota K & Oguchi Y. Suppression of nuclear factor kappa B and CD18-mediated leucocyte adhesion to the corneal endothelium by dexamethasone. *Invest Ophthalm Vis Sci* **38**: 2427-2431, 1997.

Shiose A & Sumimoto H. Arachidonic acid and phosphorylation synergistically induce a conformational change of p47phox to activate the phagocyte NADPH oxidase. *J Biol Chem* **275**: 13793-13801, 2000.

Simchowicz L & Cragoe EJ Jr. Na⁺-Ca²⁺ exchange in human neutrophils. *Am J Physiol* **254**: C150-164, 1988.

Simchowicz L, Foy MA & Cragoe EJ. A role for Na/Ca exchange in the generation of superoxide radicals by human neutrophils. *J Biol Chem* **265**:13449-13456, 1990.

Smolen JE, Petersen TK, Koch C, O'Keefe SJ, Hanlon WA, Seo S, Pearson D, Fossett MC & Simon SI. L-selectin signaling of neutrophil adhesion and degranulation involves p38 mitogen-activated protein kinase. *J Biol Chem* **275**: 15876-15884, 2000.

Snyderman R & Uhing RJ. Chemoattractant stimulus-response coupling. In: Gallin, JI, Goldstein IM & Snyderman R, editors. *Inflammation: Basic principles and Clinical Correlates*. (2nd edition) New York: Raven Press Ltd. p 421-439, 1992.

Springer TA. Traffic signals for lymphocyte recirculation and leucocyte emigration: The multistep paradigm. *Cell* **76**: 301-314, 1994.

Steel HC, Matlola NM & Anderson R. Inhibition of potassium transport and growth of mycobacteria exposed to clofazimine and B669 is associated with a calcium-independent increase in microbial phospholipase A2 activity. *J Antimicrob Chemother* **44**: 209-216, 1999.

Steinbeck MJ, Khan AU & Karnovsky MJ. Intracellular singlet oxygen generation by phagocytosing neutrophils in response to particles coated with a chemical trap. *J Biol Chem* **267**: 13425-13433, 1992.

Steward AG & Harris T. Adenosine inhibits platelet-activating factor but not tumour necrosis factor- α -induced priming of human neutrophils. *Immuol* **78**: 152-158, 1993.

Stewart M & Hogg N. Regulation of leucocyte integrin function: Affinity vs. avidity. *J Cell Biochem* **61**: 554-561, 1996.

Stiles GL. Adenosine receptors. *J Biol Chem* **267**: 6451-6454, 1992.

Strickland I, Kisich H, Hauk PJ, Vottero A, Chrousos GP, Klemm DJ & Leung DYM. High constitutive glucocorticoid receptor β in human neutrophils enables them to reduce their spontaneous rate of cell death in response to corticosteroids. *J Exp Med* **193**: 585-593, 2001.

Sullivan GW, Carper HT & Mandell GL. The specific type IV phosphodiesterase inhibitor rolipram combined with adenosine reduces tumor necrosis factor- α -primed neutrophil oxidative activity. *Int J Immunopharmacol* **17**: 793-803, 1995.

Sullivan GW & Linden J. Role of A_{2A} adenosine receptors in inflammation. *Drug Dev Res* **45**: 103-112, 1998.

Sullivan GW, Rieger JM, Scheld WM, Macdonald TL & Linden J. Cyclic AMP-dependent inhibition of human neutrophil oxidative activity by substituted 2-propynylcyclohexyl adenosine A_{2A} receptor agonists. *Br J Pharmacol* **132**: 1017-1026, 2001.

Suzuki H, Yokomizo S, Wakamoto S, Watanabe K, Hirose K, Akamatsu N & Tanoue K. Translocation of sLe(x) on the azurophilic granule membrane to the plasma membrane in activated human neutrophils. *J Electron Microscopy* **49**: 359-370, 2000.

Suzuki Y, Nishio K, Takeshita K, Takeuchi O, Watanabe K, Sato N, Naoki K, Kudo H, Aoki T & Yamaguchi K. Effect of steroid on hyperoxia-induced ICAM-1 expression in pulmonary endothelial cells. *Am J Physiol-Lung Cell Mol Physiol* **278**: L245-252, 2000.

Taylor A, Das AM, Getting SJ, Flower RJ & Perretti M. Subacute treatment of rats with dexamethasone reduces ICAM-1 levels on circulating monocytes. *Biochem Biophys Res Comm* **231**: 675-678, 1997.

Takenawa T, Ishitoya J & Nagai Y. Inhibitory effect of prostaglandin E₂, forskolin, and dibutyryl cAMP on arachidonic acid release and inositol phospholipid metabolism in guinea pig neutrophils. *J Biol Chem* **261**: 1092-1098, 1986.

Talpain E, Armstrong RA, Coleman RA & Vardey CJ. Characterisation of the PGE receptor subtype mediating inhibition of superoxide production in human neutrophils. *Br J Pharmacol* **114**: 1459-1465, 1995.

Tao J, Johansson JS & Haynes DH. Stimulation of dense tubular Ca^{2+} uptake in human platelets by cAMP. *Biochim Biophys Acta* **110**: 29-39, 1992.

Thelen M, Dewald B & Baggiolini M. Neutrophil signal transduction and activation of the respiratory burst. *Physiol Rev* **73**: 797-822, 1993.

Tintinger GR, Theron AJ, Steel HC & Anderson R. Accelerated calcium influx and hyperactivation of neutrophils in chronic granulomatous disease. *Clin Exp Immunol* **123**: 254-263, 2001.

Torphy TJ. Phosphodiesterase enzymes: Molecular targets for novel antiasthma agents. *Am J Resp Crit Care Med* **157**: 351-370, 1998.

Trowald-Wigh G, Håkansson L, Johannisson A & Edqvist LE. The effect of prednisolone on canine neutrophil function: *In vivo* and *In vitro* studies. *Acta Vet Scand* **39**: 201-213, 1998.

Umeki S & Soejima R. Hydrocortisone inhibits the respiratory burst oxidase from human neutrophils in whole cell and cell free systems. *Biochim Biophys Acta* **1052**: 211-215, 1990.

Underwood DC, Bochnowicz S, Osborn RS, Kotzer CJ, Luttmann MA, Hay DWP, Gorycki P, Christensen SB & Torphy TJ. Antiasthmatic activity of the second generation phosphodiesterase 4 (PDE4) inhibitor SB207495 (Ariflo) in the guinea pig. *J Pharmacol Exp Ther* **287**: 988-995, 1998.

Van Dyke K, Van Dyke C, Udeinya J, Brister C & Wilson M. A new screening system for non-steroidal anti-inflammatory drugs based upon inhibition of chemiluminescence produced from human cells (granulocytes). *Clin Chem* **25**: 1655-1661, 1979.

Van Kooyk Y, Van Vliet SJ & Figdor CG. The actin cytoskeleton regulates LFA-1 ligand binding through avidity rather than affinity changes. *J Biol Chem* **274**: 26869-26877, 1999.

Van Overveld FJ, Vermeire PA & DeBacker WA. Induced sputum of patients with chronic obstructive pulmonary disease (COPD) contains adhesion-promoting, therapy-sensitive factors. *Inflam Res* **49**: 8-13, 2000.

Varani K, Gessi S, Dionisotti S, Ongini E & Borea PA. [³H]-SCH58261 labelling of functional A_{2A} adenosine receptors on human neutrophils. *Br J Pharmacol* **123**: 1723-1731, 1998.

Villagrasa V, Navarette C, Sanz C, Berto L, Perpina M, Cortijo J & Morcillo EJ. Inhibition of phosphodiesterase IV and intracellular calcium levels in human polymorphonuclear leucocytes. *Methods Find Exp Clin Pharmacol* **18**: 239-245, 1996.

Walker BAM, Hagenlocker BE, Douglas VK & Ward PA. Effects of adenosine on inositol 1,4,5-triphosphate formation and intracellular calcium changes in formyl-met-leu-phe-stimulated human neutrophils. *J Leucoc Biol* **48**: 281-283, 1990.

Walker BAM, Jacobson MA, Knight DA, Salvatore CA, Weir T, Zhou D & Bai TR. Adenosine A₃ receptor expression and function in eosinophils. *Am J Respir Cell Mol Biol* **16**: 532-537, 1997.

Wan W, Sutherland GR & Geiger JD. Binding of the adenosine A₂ receptor ligand [³H] CGS 21680 to human and rat brain: Evidence for multiple affinity sites. *J Neurochem* **55**: 1763-1771, 1990.

Wang P, Wu P, Ohleth KM, Egan RW & Billah MM. Phosphodiesterase 4B2 is the predominant phosphodiesterase species and undergoes differential

regulation of gene expression in human monocytes and neutrophils. *Mol Pharmacol* **56**: 170-174, 1999.

Ward PA. The chemosuppression of chemotaxis. *J Exp Med* **124**: 209-225, 1966.

Watson RW, O'Neill A, Brannigen AE, Coffey R, Marshall JC, Brady HR & Fitzpatrick JM. Regulation of Fas antibody induced neutrophil apoptosis is both caspase and mitochondrial dependent. *FEBS Letters* **453**: 67-71, 1999.

Webb JL, Polak JM & Evans TJ. Effect of adhesion on inducible nitric oxide synthase (iNOS) production in purified human neutrophils. *Clin Exp Immunol* **123**: 42-48, 2001.

Weinrauch Y, Elsbach P, Madsen L, Foreman A & Weiss J. The potent anti-*Staphylococcus aureus* activity of a sterile rabbit inflammatory fluid is due to a 14-kD phospholipase A2. *J Clin Invest* **97**: 250-257, 1996.

Werr J, Eriksson EE, Hedqvist P & Lindbom L. Engagement of beta2 integrins induces surface expression of beta1 integrin receptors in human neutrophils. *J Leucoc Biol* **68**: 553-560, 2000a.

Werr J, Johansson J, Eriksson EE, Hedqvist P, Ruoslahti E & Lindbom L. Integrin alpha(2)beta (1) (VLA-2) is a principal receptor used by neutrophils for locomotion in extravascular tissue. *Blood* **95**: 1804-1809, 2000b.

Wheller SK & Perretti M. Dexamethasone inhibits cytokine-induced intercellular adhesion molecule-1 upregulation on endothelial cell lines. *Eur J Pharmacol* **331**: 65-71, 1997.

Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P & Halbwachs-Mecarelli L. Biology of Disease. Neutrophils: Molecules, functions and pathophysiological aspects. *Lab Invest* . **80**: 617-653, 2000.

Wolberg G, Zimmerman TP, Hienstra K, Winston M & Chu LC. Adenosine inhibition of lymphocyte-mediated cytotoxicity: possible role of cyclic adenosine monophosphate. *Science* **187**: 957-959, 1975.

Wolfe AP. Sinful repression. *Nature* **387**: 16-17, 1997.

Wright DG & Malawista SE. Mobilization and extracellular release of granular enzymes from human leucocytes during phagocytosis: inhibition by colchicine and cortisol but not by salicylate. *Arthritis Rheum* **16**: 749-758, 1973.

Yoshida N, Yoshikawa T, Nakamura Y, Takenaka S, Sakamoto K, Manabe H, Nakagawa S & Kondo M. Methylprednisolone inhibits neutrophil-endothelial cell interactions induced by interleukin-1beta under flow conditions. *Life Sci* **60**: 2341-2347, 1997.

Youssef PP, Triantafillou S, Parker A, Coleman M, Roberts-Thompson PJ, Ahern MJ & Smith MD. Effects of pulse methylprednisolone on cell adhesion molecules in the synovial membrane in rheumatoid arthritis. Reduced E-selectin and intercellular adhesion molecule 1 expression. *Arthritis Rheum* **39**: 1970-1979, 1996.

Zak-Nejmark T, Jankowska R, Malolepszy J, Jutel M, Kraus-Filarska M & Nadobna G. Spontaneous motility and chemotaxis of neutrophils is influenced by glyocorticosteroid therapy. *Arch Immunol Ther Exp (Warsz)* **44**: 77-80, 1996.

Zimmerman GA, Prescott SM & McIntyre TM. Endothelial cell interactions with granulocytes: tethering and signalling molecules. *Immunol Today* **13**: 93-99, 1992.

Zouki C, Ouellet S & Filep JG. The anti-inflammatory peptides, antinflammins, regulate the expression of adhesion molecules on human leucocytes and prevent neutrophil adhesion to endothelial cells. *FASEB J* **14**: 572-580, 2000.