Chapter 1

Theoretical background and aim of the study

The etiology and pathogenesis of renal diseases vary widely yet as chronic renal failure progresses, the manifestations in different patients are remarkably similar. These similarities make it possible to formulate common principles of pathophysiology that apply to the greater majority of patients, irrespective of the underlying pathogenic (infections, immunological, vascular or other) mechanism responsible for renal function destruction. (1) Intracellular calcium dyshomeostasis would appear to be one such common principle of the pathophysiology of chronic renal failure. This disturbance in intracellular calcium homeostasis involves many different cell types in the chronic renal failure patient. It is therefore possible that the abnormal intracellular calcium homeostasis, indicated in many different cell types in the chronic renal failure patient, may involve the body as a whole. Intracellular calcium homeostatic mechanisms are complex, and a large number of pathological factors are known to cause intracellular calcium dyshomeostasis. Many of these factors generally known to alter intracellular calcium status are indeed found in the chronic renal failure patient.

The present study comprises the investigation of intracellular calcium homeostasis and possible factors influencing intracellular calcium homeostasis in the chronic renal failure patient on maintenance haemodialysis treatment (MHT). The first introductory chapter deals with the theoretical background to the study and includes discussions on intracellular calcium signal transduction, intracellular calcium dyshomeostasis, possible manifestations of intracellular calcium

dyshomeostasis, status of intracellular calcium homeostasis in the chronic renal failure patient on maintenance haemodialysis treatment, and possible factors contributing to intracellular calcium dyshomeostasis in these patients. The aim of this study is presented in conclusion.

1) Intracellular calcium signal transduction

Ionised calcium (Ca^{2+}) is the most common signal transduction factor in cells ranging from bacteria to specialised neurons. (2) Unlike many of the other signal transduction elements, Ca^{2+} is required throughout the life of a typical cell since Ca^{2+} regulates many diverse cellular processes ranging from gametogenesis, fertilisation, cell proliferation, and specialised control functions including muscle contraction, exocytosis, energy metabolism, chemotaxis, and synaptic plasticity during memory and learning. (3) The vast quantity of the Ca^{2+} sensitive effectors responsible for the regulation of these processes are located in the cytosol. (4) It is therefore the free ionised Ca^{2+} fraction in the cytosol that is the most critical with regard to the regulation of these intracellular events. Unlike any other signal transduction element, Ca^{2+} is not synthesised or subsequently degraded during the signal transduction process. In contrast Ca^{2+} is moved and/or released and subsequently sequestered for every signal transduction cycle. In order for Ca^{2+} to be available for signal transduction functions, the cell is endowed with at least two mechanisms to increase Ca^{2+} in the cytosol, firstly by releasing Ca^{2+} from the intracellular storage sites and secondly by moving Ca^{2+} from the extracellular medium into the cytosolic space where the effectors are functionally distributed. (5) The intracellular storage sites include Ca^{2+} bound to the plasma membrane (anionic sites consisting of either phospholipids or membrane proteins), Ca^{2+} sequestered within intracellular organelles such as the endoplasmic reticulum, and Ca^{2+} both free and bound within the cytosol. (6) The various mechanisms capable of generating a rise in cytosolic free Ca^{2+} are

all passive processes since the cytosolic free Ca^{2+} is lower than the Ca^{2+} concentration in any of the calcium pools surrounding the cytosolic space. It is therefore not necessary to expend any energy to move calcium down its concentration gradient into the cytosol upon agonist activation. (3, 7) In contrast with these passive processes capable of generating a rise in the cytosolic free Ca^{2+}, the processes necessary to reduce the surplus Ca^{2+} at the end of the signal transduction cycle require the expenditure of energy. These energy expensive processes responsible for rapidly decreasing cytosolic free Ca^{2+} include the Ca^{2+}-ATPase in the plasma membrane and the Ca^{2+}-ATPase in the endo/sarcoplasmic reticulum or calciosome. The activation of these calcium pumps result in the transfer of Ca^{2+} from the cytosol to the extracellular medium and the sequestration of Ca^{2+} into the intracellular calcium storage organelles. The subsequent decrease in cytosolic free Ca^{2+} results in termination of the calcium response. (2, 8)

2) Intracellular calcium dyshomeostasis

It is imperative that the cytosolic free Ca^{2+} levels be maintained within normal physiological limits, since unlike any other signal transduction element, calcium dyshomeostasis is an intracellular trigger for cellular dysfunction and damage. (9) The derangements in intracellular calcium homeostasis, resulting in cellular dysfunction and damage, include the intracellular redistribution of calcium pools during the important early stages of cellular damage, followed by the influx of Ca^{2+} from extracellular to intracellular spaces. (6) Many pathogenic factors, resulting in cellular dysfunction and damage, affect cellular calcium regulation because their initial effect impair one or more of the following; energy metabolism, plasma membrane integrity, calcium translocation systems or calcium signalling. (10) The ensuing intracellular calcium dyshomeostasis aggravates the initial impairment of various cellular functions, due to the inappropriate activation of

various calcium sensitive enzymes. These calcium sensitive enzymes include various enzymes regulating the intracellular calcium signalling process and a whole array of degradative enzymes: such as phospholipases, proteases and endonucleases. Intracellular targets for these degradative enzymes include the plasma membrane, the mitochondria, the endoplasmic reticulum and the cytoskeleton. The calcium activated degradative processes result in plasma membrane bleb formation, nuclear membrane bleb formation, deterioration of mitochondrial structure and function, as well as chromatin condensation. (11) Since an inappropriate elevation in intracellular free calcium results in the activation of so many degradative processes, the cell is furnished with several calcium translocation systems to carefully regulate intracellular calcium activity. The function of the Ca^{2+}-ATPase pump, as explained above, is to rapidly obviate the surplus calcium at the end of a transduction cycle. (8) In addition, these calcium pumps also play an important role in decreasing intracellular free Ca^{2+} under conditions of relative intracellular Ca^{2+} overload. These pumps are well suited for their roles since they display dynamic, low capacity enzyme kinetics. A rise in cytosolic free calcium results in immediate activation of these pumps and the rapid extrusion out of the cell, and sequestration into intracellular organelles of calcium. In the face of a prolonged elevation in intracellular free calcium, a second high capacity, slow to become activated, calcium extrusion pump plays a more important role namely the Na^+/Ca^{2+}-exchanger. This pump extrudes Ca^{2+} ions to the outside of the cell in exchange for Na^+ ions. The sodium concentration gradient – high sodium concentration on the outside of the cell and low sodium concentration on the inside – provides the energy needed for Na^+/Ca^{2+}-exchanger activities. This sodium gradient is maintained by the Na^+/K^+-ATPase. (7) During prolonged intracellular free calcium elevation the roles of the Na^+/Ca^{2+}-exchanger and in combination the buffering of calcium by the mitochondria become more important. (11)

In fact, the mitochondrium becomes the single most important membrane system in the cell to act as a long-term calcium buffer and to handle large amounts of calcium whenever the need arises. (11) All the pumps mentioned play a significant role in maintaining calcium homeostasis. Plasma membrane integrity, on the other hand, fulfills a dual function in the maintenance of intracellular calcium homeostasis. An intact plasma membrane, impermeable to calcium ions forms a barrier to unwanted calcium inflow into the cell. Plasma membrane phospholipid and fatty acid composition are furthermore important in supporting the structure and function of the calcium homeostasis pumps. (12) Impairment of any of the mechanisms mentioned may lead to intracellular calcium dyshomeostasis.

3) Manifestations of intracellular calcium dyshomeostasis

A rise in intracellular free calcium can potentially activate a number of calcium sensitive enzymes that could culminate in cell dysfunction and damage. This cellular dysfunction and damage, resulting from the activation of calcium sensitive enzymes, could subsequently result in further aggravation of the initial rise in intracellular free calcium.

- As mentioned mitochondria become the single most important membrane system in the cell to act as long-term calcium buffers, but with the subsequent increase in mitochondrial calcium content functional and structural changes are triggered. The prolonged uptake of calcium by the mitochondria results in a reduction of cellular ATP content, since mitochondrial calcium transport takes precedence over oxidative phosphorylation. Calcium uptake occurs by an electrophoretic uniport that involves transport of calcium ions across the mitochondrial membrane without direct coupling to the movement of another ion. This net inward movement of positive charges, associated with due calcium flux, causes depolarisation of the mitochondrial membrane, which is immediately compensated for by an increase in the respiratory

activity followed by an increase in H^+ extrusion. Thus mitochondrial H^+ efflux coupled to electron transfer along the respiratory chain is the active energy-linked process driving the electrophoretic calcium uptake. This utilisation of the electrochemical gradient across the mitochondrial membrane for calcium uptake makes the electrochemical gradient unavailable for ATP production. (6) Furthermore, calcium uptake is followed by the activation of a mitochondrial associated phospholipase A_2. Phospholipase A_2 hydrolyses ester bonds of membrane phospholipids, specifically phosphatidylcholines containing the fatty acid arachidonic acid in the sn_2 position. Activation of this enzyme may therefore be responsible for the dramatic ultrastructural changes in the crystal membranes and mitochondrial swelling with a prolonged elevation in intracellular calcium. (9 - 11) The structural and functional changes of mitochondria upon prolonged elevation in intracellular calcium, most notably result in the further decrease of ATP production. Many of the enzymes responsible for decreasing intracellular free calcium are energy-dependent, therefore decreased ATP production could possibly result in decreased calcium extrusion. Therefore, mitochondrial dysfunction following the initial elevation in intracellular free calcium could result in the further progressive aggravation of intracellular calcium dyshomeostasis.

- An elevation in intracellular free calcium can possibly result in the activation of calpain, a neutral protease. Calpain activation results in degradation of cytoskeletal proteins such as actin and tubulin, and cytoskeletal and plasma membrane organisational proteins that are responsible for anchoring cytoskeletal elements to the plasma membrane. This results in alterations in the microfilament network and subsequently plasma membrane blebbing. Many of these organisational proteins restrict different membrane proteins to specific regions of the plasma membrane. Degradation of these organisational proteins

necessary for the restriction of membrane proteins results in the subsequent loss of cell polarisation and cell dysfunction. (9 - 11, 13)

- An inappropriate elevation in intracellular free calcium results in the unwanted production of oxygen radicals by phagocytic cells. Phagocytic cells such as neutrophils possess a H_2O_2/O_2^--generating NADPH oxidase. NADPH oxidase activation is triggered by the influx of calcium resulting in the production of oxygen radicals. (9, 14) Therefore, an inappropriate elevation in intracellular free calcium can result in the unwanted activation of the NADPH oxidase and the excessive production of oxygen radicals. In addition, oxidative stress, a condition in which the pro-oxidant: anti-oxidant ratio favours the former, is known to affect intracellular calcium homeostasis mechanisms. Disruption of many of the intracellular calcium homeostasis mechanisms due to an increased production of oxygen radicals, can potentially result in the elevation of basal intracellular free calcium. (15, 16) This disruption of intracellular calcium homeostasis mechanisms is the result of the reaction of oxygen radicals with cellular constituents. The superoxide anion is one of the oxygen radicals produced by the NADPH oxidase. The superoxide anion is not particularly reactive but is capable of diffusing over relatively large distances in the cell where, in the presence of iron or copper, a metal-catalysed Haber-Weiss reaction can occur with the formation of the highly reactive hydroxyl radical. (9) This hydroxyl free radical rapidly reacts with cellular constituents in its immediate vicinity and is the key radical species in causing cellular damage. With regard to intracellular calcium homeostasis the reaction with membrane lipids (to cause non-enzymatic lipid peroxidation), and the reaction with the sulphydryl groups contained in cellular proteins appear to be of particular importance. Lipid peroxidation can result in the loss of membrane integrity and a further influx of calcium into the cytosol due to the breakdown of the membrane barrier between the cytosol and the high

calcium containing pools. (9, 15, 17) Additionally, a change in membrane phospholipid and fatty acid composition as a result of lipid peroxidation may cause an alteration in the lipid microdomain of the Ca^{2+}-ATPase and the other enzymes responsible for lowering of intracellular free Ca^{2+}. The lipid microdomain surrounding an integral membrane protein determines the activity of the protein. This is mainly due to the support of the three-dimensional structure of the protein. Therefore, an optimal lipid environment is an important factor that can influence membrane protein function such that a change in the microdomain of the calcium pumps can result in the suppression of calcium extrusion. (12, 18-23) Furthermore, the calcium pumps responsible for decreasing calcium with the unwanted accumulation of calcium in the cytosol are highly sensitive to oxygen radicals. The unchecked production of oxygen radicals can possibly react with protein sulphydryl groups. The sulphydryl groups form part of the active center of the calcium pump, oxidation of these groups result in the suppression of calcium extrusion. (16, 24) Oxidative stress can also result in disruption of microfilament organisation. Thiol oxidation of actin molecules perturbs microfilament organisation. Microfilament organisation determines the size and distribution of the intracellular calcium stores, through the interaction with intracellular calcium store membranes. A change in the distribution of intracellular calcium stores can affect intracellular calcium signalling processes. During conditions of oxidative stress, the loss of both reduced pyridine nucleotides and protein thiols inhibits mitochondrial calcium sequestration resulting in the loss of this vital defence mechanism against elevated calcium while at the same time releasing calcium into the cytosol. (9) Lastly, one of the important properties of the calcium extrusion pump is its stimulation by calmodulin, which is due to a direct interaction of calmodulin with the pump. (25) Calmodulin binds four calcium ions, and this binding of calcium is accompanied by a substantial

conformational change whereupon the protein assumes an active form. The active form of calmodulin is now capable of interaction with the calcium pump. These high-affinity calcium-binding sites on the surface of calmodulin include methionine residues. Oxidation of these residues, due to the reaction of oxygen radicals with the sulphydryl group results in the alteration of calcium binding properties with the apparent loss of high-affinity calcium binding sites. The loss of the high-affinity calcium binding sites results in decreased activation of calmodulin and diminished capability of the up-regulation of calcium-pump activity. (26) This can result in decreased calcium-pump activity and the subsequent rise in intracellular free Ca^{2+}.

- An elevation in intracellular free calcium results in the activation of a cytosolic phospholipase A_2 (PLA_2). PLA_2 hydrolyses specific ester bonds of a specific membrane phospholipid; phosphatidylcholine with arachidonic acid in the sn_2 position. The activation of PLA_2 as a result of an elevation in intracellular free calcium upon agonist stimulation is a normal physiological response. It is when an unwanted elevation in intracellular free calcium occurs that PLA_2 activation can result in substantial membrane damage. PLA_2 activation consequently has been considered a candidate for causing cellular damage, since the loss of membrane integrity could result in an unwanted influx of calcium from the extracellular medium. (9, 27) Membrane integrity play an important role in the maintaining of cell viability, therefore an increase in membrane breakdown will result in an increase in membrane repair mechanisms. Acyltransferase is one of the enzymes responsible for membrane phospholipid and fatty acid resynthesis. For the adequate functioning of this enzyme ATP is required. One of the possible results of an elevation in intracellular free calcium is the reduced production of ATP. An unwanted elevation in intracellular free Ca^{2+} could therefore result in the perturbation of membrane repair due to the inadequate ATP production for acyltransferase activity.

- To ensure adequate signalling responses cells have evolved an ingenious mechanism of signalling based on presenting calcium as brief spikes organised as oscillating regenerative waves. (28) It is generally accepted today that calcium spiking is an important signalling mechanism and that various patterns of intracellular calcium oscillations are of physiological significance. (5) These kinds of responses are both spatially and temporally regulated and are obtained when cells are stimulated with concentrations of agonists representing the physiological situation. (5) This is in contrast with the calcium response obtained when cells are stimulated with high concentrations of agonist, i.e., a sustained elevation in intracellular free calcium and the subsequent return to baseline levels. Calcium appears to be the transduction element in these responses since receptors and enzymes displaying various degrees of sensitivity to the surrounding calcium concentration regulates the generation of these calcium patterns. Therefore, perturbation in intracellular free Ca^{2+} could result in the unwanted activation of these receptors and enzymes resulting in inappropriate calcium signalling responses. Following, is an account of these receptors and enzymes sensitive to calcium and the possible influence of an unwanted rise in intracellular free Ca^{2+} on calcium signalling processes. Agonist-receptor interaction stimulates these intracellular calcium oscillations. Initially, agonist-receptor interaction stimulates the activity of a phosphatidyl inositol specific phospholipase C (PI-PLC). Activation of this enzyme leads to the hydrolysis of phosphatidyl inositol 4,5-bisphosphate and formation of downstream effectors, water soluble 1,4,5-trisphosphate ($InsP_3$) and lipid soluble diacygliserol (DAG). The initial PI-PLC isoform activated as a result of receptor stimulation involves the activation of a specific PI-PLC isoform that is coupled to the receptor via a G-protein. This PI-PLC isoform appears to be largely insensitive to calcium. However, during the later phase of the response a second PI-PLC isoform, a calcium-dependent

isoform is activated. It is hypothesised that the activation of this calcium dependent PI-PLC isoform by an increase in intracellular free calcium, plays a role in the generation of calcium oscillations. An inappropriate elevation in intracellular free calcium, therefore can result in perturbation of these intracellular calcium oscillations due to the unwanted activation of the calcium-dependent PI-PLC. It is the primary action of $InsP_3$ to liberate calcium ions from intracellular organelles, mostly calciosomes (the equivalent of the calcium pool in the sarcoplasmic reticulum of skeletal muscle) into the cytosol. (29, 30) $InsP_3$ releases calcium from these stores by binding to the $InsP_3$-sensitive calcium channels in the membranes of these organelles. The $InsP_3$-activated channel is composed of four non-covalently bound identical subunits. The $InsP_3$ binding domain resides at the extreme N-terminal region, whilst the C-terminal portion contains six putative membrane-spanning domains that make up, or at least contain the calcium channel. The region between the $InsP_3$-binding domain and the putative calcium-channel constitutes the so-called 'coupling' domain, some 1400-1500 amino acids in length, which lies in the cytoplasm. This so-called 'coupling' domain constitutes the site for modulatory actions. (5) This domain contains a site for calcium binding. There are at least four different types of $InsP_3$ receptors derived from three or four distinct genes. Between these different types of $InsP_3$ receptors there exist a strong homology in the membrane-spanning domains but large divergencies are indicated in the cytoplasmic modulatory domains. It is hypothesised that since the $InsP_3$-receptor contains a site for calcium binding in the modulatory domain and in the light of the fact that there exists receptor subtypes containing large divergencies in these modulatory domains, that the functional distribution of these $InsP_3$-receptor subtypes play a significant role in the regulation of these spatially and temporally organised calcium patterns. Indeed there are many indications that the $InsP_3$-receptor plays the central role as

possible regulator of these complex intracellular calcium signalling patterns. There are indications that an inappropriate elevation in intracellular free calcium may affect these $InsP_3$-receptor functions. This can be the result of one of two factors. An elevation in intracellular free calcium results in cytoskeletal disorganisation followed by a change in the structure of the calcium stores. The structure of these calcium stores can be controlled by the integrity of the cytoskeleton since the internal membranes are associated with the elements of the cytoskeleton and it has been shown that cytoskeletal disruption leads to fragmentation of the $InsP_3$-sensitive calcium stores. The intracellular calcium signalling patterns are spatially organised and these spatially organised calcium patterns are possibly determined by the functional distribution of $InsP_3$-receptor subtypes. Therefore the structure of the $InsP_3$-sensitive calcium store is an important determinant for the generation of these spatially organised calcium signalling patterns. The following calcium signalling processes are determined by the distribution of $InsP_3$-receptor subtypes: Upon agonist activation the intracellular calcium signal is initiated at a discrete subcellular locus adjacent to a small region of the plasma membrane. This intracellular calcium wave initiation domain does not simply result from a polarised distribution of agonist receptors but indications are that there may be specialised subcellular regions for intracellular calcium release containing $InsP_3$-receptor subtypes that are more sensitive to $InsP_3$. (31) Various intracellular calcium responses do not involve the entire cell and are therefore localised within a specific region. To avoid the undesirable activation of calcium-dependent processes elsewhere in the cell, calcium signalling patterns are produced locally which are confined to the relevant region. The production of such local calcium signalling patterns are largely determined by the spatial arrangement of the $InsP_3$-sensitive stores. (31) In cells displaying chemotaxis the polarisation of the cell changes with every new movement. There are indications of a calcium gradient within these

cells that underlies the activities of calcium-dependent processes that favour the polarisation and directed locomotion of these cells. The gradient changes continually as the polarisation of the cell changes. The direction of the new calcium gradient within cells is established by the reorganisation of the $InsP_3$-sensitive stores. (31) An elevation in intracellular free calcium not only results in the disorganisation of the calcium stores and the subsequent altered distribution of the $InsP_3$-receptors, but the free calcium concentration can also modulate the degree of $InsP_3$–evoked channel opening. The free calcium concentration is an important factor in determining the degree of $InsP_3$-gated channel opening. These $InsP_3$–gated calcium channels display a bell-shaped calcium response curve. In the physiological calcium concentration range (0.1 – 1 μM) small changes in the calcium concentration can have dramatic effects on the probability of channel opening. When the intracellular free calcium concentration is increased from 0.1 to about 0.3 μM there is a marked increase in the open-state probability, but when the calcium concentration is further increased to 1 μM a marked fall in the degree of $InsP_3$–evoked channel opening is observed. Therefore an increase in intracellular free calcium concentration to above 0.3 μM can result in an inhibition of $InsP_3$–evoked calcium release. (30) $InsP_3$ liberation not only results in releasing of calcium from intracellular calcium stores as explained in the previous section, but also activates calcium entry pathways in the plasma membrane. It is most probably not $InsP_3$ per se that activates the calcium channels in the plasma membrane, but a product of the enzymatic modification of $InsP_3$ namely inositol-1,3,4,5 tetrakisphosphate ($InsP_4$). The plasma membrane $InsP_3$-receptor has a lower affinity for $InsP_3$ but a higher affinity for $InsP_4$ when compared to the well-known endoplasmic reticulum $InsP_3$-receptor, therefore providing for such a role for $InsP_4$. $InsP_4$ is formed as a result of the

phosphorylation of $InsP_3$ by $InsP_3$-kinase. These two processes, i.e., the release of calcium from the intracellular calcium storage organelles and the influx of calcium from the extracellular medium, are highly coordinated and possibly regulated by calcium. The $InsP_3$–kinase responsible for the formation of $InsP_4$ is calcium activated, therefore $InsP_3$ liberation results in the releasing of intracellular calcium and subsequently the activation of $InsP_3$-kinase. The subsequent formed $InsP_4$ triggers the influx of calcium from the extracellular medium. An unwanted increase in intracellular free Ca^{2+} could result in the perturbation of the interaction between these processes. (30) It is not only the calcium release and calcium influx pathways that are regulated by intracellular free calcium, but the pathways resulting in the termination of intracellular calcium signalling responses are also influenced by intracellular free calcium. These processes; calcium release, calcium influx and calcium efflux are highly coordinated. It was indicated that pulsatile calcium extrusion occurred synchronously with the cytosolic calcium spikes. The activity of the Ca^{2+}-ATPase mostly regulates the calcium extrusion and calcium sequestration at the end of the response. In the calcium range 0.1 – 0.3 μM, where $InsP_3$-gated channel opening is increasing very steeply with a rise in intracellular free calcium, calcium extrusion is moderately activated. In the calcium range 0.3 – 1 μM where $InsP_3$–gated calcium channel opening decreases with increasing free intracellular calcium, calcium extrusion rises sharply as intracellular free calcium increases. (30) Intracellular calcium per se plays an important role in the coordination of these processes. Three processes activated by the increase in intracellular free calcium results in the extrusion of calcium by the Ca^{2+}-ATPase. An increase in intracellular free calcium results in calmodulin activation. In the absence of calmodulin only a small increase in the calcium transport rate is indicated with the increase in intracellular free calcium. But in the presence of calmodulin, a small increase in intracellular free calcium

results in a dramatic increase in calcium pumping activity. The calcium-calmodulin-dependent activation of the Ca^{2+}-ATPase calcium pump increases the capacity or Vmax of the pump 15-20 fold and the efficiency two-fold, i.e., lowers the Km for calcium. The calcium-calmodulin complex binds to the Ca^{2+}-ATPase resulting in an increase in pump activity and the more rapid extrusion of calcium. The enzyme protein kinase C (PKC) also plays a significant role in the activation of the Ca^{2+}-ATPase. PKC is activated as a consequence of both the initial rise in the diacylglyserol (DAG) content of the plasma membrane and the transient rise in intracellular free calcium. Upon activation PKC moves from the cytosol to the plasma membrane. When PKC associates with the plasma membrane, it undergoes a conformational change to become a calcium-sensitive, plasma membrane associated protein kinase. The activity of this form of the kinase is regulated by the rate of calcium-cycling across the plasma membrane. (29) PKC activated by calcium leads to phosphorylation of the Ca^{2+}-ATPase pump, resulting in a 4 - 6 fold increase in pump activity. A third process possibly contributing to the coordination of calcium oscillations is the calcium-mediated activation of PLA_2. PLA_2 activation leads to the release of arachidonic acid. Arachidonic acid possibly performs a dual function in the coordination of intracellular calcium signalling patterns, i.e., arachidonic acid can inhibit $InsP_3$-evoked calcium mobilisation and can at the same time activate calcium extrusion. (31) In conclusion, many calcium sensitive receptors and enzymes are responsible for the generation of the complex calcium signalling patterns. In order to assure adequate signalling responses, these various processes are highly coordinated. Since the receptors and enzymes responsible for the generation of the calcium signalling patterns are calcium sensitive, calcium seems to be the modulator of its own signalling cycle. Therefore, perturbation in intracellular calcium could culminate in disturbed calcium signalling responses.

4) Intracellular calcium status in chronic renal failure

It was Massry who suggested that chronic renal failure is a state of calcium toxicity. (32) Soft tissue calcification is indeed a common finding in chronic renal failure (CRF), with an increase in calcium content known to occur in the cornea, skin, blood vessels, brain, peripheral nerves, heart, lungs, pancreas, liver, epididymal fat and testis of patients as well as animals with chronic renal failure. (33) Intracellular free calcium would appear to reflect this increased tissue content of calcium. An elevation in basal intracellular free calcium in various cell types in CRF is documented, indicating a potential association between this increase in the calcium content of tissues with an elevation in basal intracellular free calcium. A summary of published results of basal intracellular free calcium in humans and rats with chronic renal failure can be seen in Table 1. The cell types in which the levels have already been determined include brain synaptosomes, pancreatic islets, cardiac myocytes, thymocytes, B cells, T cells, neutrophils, adipocytes, and platelets. (33-37, 40-42, 44-46, 48, 50-51)

4.1) Pathological factors associated with CRF possibly contributing to the derangements in intracellular calcium homeostasis

There are a multitude of possible factors contributing to the elevation in basal intracellular free calcium in the CRF patient including secondary hyperparathyroidism, oxidative stress, deranged phospholipid and fatty acid metabolism and reduced ATP concentrations.

4.1.1) Secondary hyperparathyroidism

It is hypothesised that an elevation in PTH levels in these patients could be the main cause for the increase in basal intracellular free calcium in the various cell types. (33, 44)

Table 1. Intracellular free Ca^{2+} in various cell types in humans and rats with CRF

Cell type	Model	CRF $[Ca^{2+}]i$	Normal $[Ca^{2+}]i$	Fluorescent calcium indicator	Reference
Neutrophils	HD patients	73±3.6nM	42±0.09nM	Fura-2	34
Neutrophils	HD patients	92nM	60nM	Fura-2	35
Neutrophils	HD patients	60±1.2nM	46±1.2nM	Fura-2	36
Neutrophils	HD patients	79±5.1nM	52±3.4nM	Fura-2	37
Neutrophils	HD patients	68±1.1nM	57±1.1nM	Fura-2	38
	CRF rats	133±3.1nM	104±1.3nM		
Neutrophils	CRF rats	149±2.7nM	108±2.4nM	Fura-2	39
Platelets	CRF patients	117±33nM	47±14nM	Fura-2	40
Platelets	HD patients	137±8.86nM		Fura-2	41

Table 1. (continue) Intracellular free Ca^{2+} in various cell types in humans and rats with CRF

Cell type	Model	CRF $[Ca^{2+}]i$	Normal $[Ca^{2+}]i$	Fluorescent calcium indicator	Reference
Platelets	CRF patients	138±16nM	83±7nM	Fura-2	42
Platelets	CRF patients	173±44nM	150±30nM	Indo-1	43
T-cells	HD patients	87nM	55nM	Fura-2	44
B-cells	HD patients	115±5.2nM	86±4.4nM	Fura-2	45
Brain synaptosomes	CRF rats	437±18nM	345±9nM	Fura-2	46
Brain synaptosomes	CRF rats	445±10nM	353±7.5nM	Fura-2	47
Islets of Langerhans	CRF rats	252±7.4nM	137±4.5nM	Fura-2	48
Heart cells	CRF rats	101±3.2nM	56±1.6nM	Fura-2	49

Indeed, this hypothesis finds much credit, since prevention of the elevation in PTH levels by parathyroidectomy or vitamin D therapy, or by the prevention of the action of PTH by calcium channel blocker administration, resulted in the normalisation of basal intracellular free calcium levels. (35, 37, 48, 51-53) It is generally accepted that patients with CRF have secondary hyperparathyroidism and elevated blood levels of PTH. (45) PTH tightly regulates plasma calcium and phosphate concentrations by modulating the activity of specific cells in the bone and kidney. (54, 55) In the normal functional kidney, PTH is known to increase calcium reabsorption and to stimulate phosphate excretion. PTH also activates the renal synthesis of the active vitamin D metabolite, which, in turn is able to stimulate or inhibit intestinal absorption of calcium and phosphate and is able to increase or decrease osteoclast mediated bone resorption depending on the concentration of vitamin D. (56) These actions of PTH are classical hormonal functions. It is with an abnormal elevation in PTH levels that PTH targets many other cell types. It has been shown that PTH can cause a sharp increase in the basal intracellular free calcium content of pancreatic islets cells, thymocytes (57), cardiac myocytes, hepatic cells, adipocytes, kidney cells, and osteoblasts. This effect of PTH is receptor-mediated. PTH activates the voltage-dependent calcium channels. This PTH-mediated calcium influx can be blunted or prevented by calcium channel blockers such as verapamil or nifedipine. The influence of PTH on non-conventional targets is supported by recent data which demonstrated that the mRNA for the PTH/PTHrP receptor is present in non-classical targets for PTH such as heart, brain, spleen, aorta, ileum, skeletal muscle, lung and testis. (33, 54) In addition to the experimental evidence for the central role of PTH in the elevation of intracellular free calcium in different cell types, indications suggest deranged cellular functions in association with an elevation in PTH. These include studies indicating deranged cellular functions in combination with

either an elevation in PTH or an elevation in PTH accompanied by an elevation in intracellular free calcium. Intracellular free calcium plays a paramount role in the regulation of a multitude of cellular functions. Intracellular calcium dyshomeostasis in CRF may thus be a major contributor to abnormal cellular function in uraemia.

4.1.2) Oxidative stress

Chronic renal failure patients receiving maintenance haemodialysis treatment (MHT) are continuously exposed to oxidative stress. Oxidative stress is a state of increased pro-oxidant levels and/or a deficit in the anti-oxidant capacity. In the MHT patient both an increase in pro-oxidant production and a decrease in anti-oxidant mechanisms are indicated. The increase in pro-oxidant production is mainly due to the relentless activation of polymorphonuclear leucocytes (PMNL) during haemodialysis. During extracorporeal circulation activation of PMNL's results in increased free oxygen radical production. (58) This activation of PMNL's is the result of the exposure of the blood constituents to the dialyser membrane. Various pathways are activated resulting in PMNL activation. These pathways include both complement-dependent and complement-independent PMNL activation. (59) Complement-dependent activation leads to the production and binding of C5a and C5a des Arg to specific PMNL receptors inducing aggregation, adherence to endothelial cells, releasing of oxygen radicals, degranulation, arachidonic acid metabolism, and enhanced phagocytotic capacity. (60) Complement-independent activation of PMNL's is the result of either direct stimulation of PMNL's due to interaction with the dialyser membrane or binding of monocyte-derived cytokines to their respective PMNL receptors. Cytokine exposure primes the respiratory burst to generate elevated levels of oxidants. (9, 61) There are indications that the activation of PMNL's during extracorporeal circulation involves an increase in intracellular free calcium. (59, 60, 62, 63) The process

responsible for the reduction of molecular oxygen to superoxide anion is catalysed by a multicomponent NADPH-oxidase and this process is activated and regulated by intracellular free calcium. It is therefore anticipated that an increase in intracellular free calcium will result in an increase in oxygen radical production. (64) Anti-oxidant mechanisms include various enzymes responsible for the reduction of the oxygen radicals to water. These anti-oxidant enzymes include superoxide dismutase, glutathione peroxidase and catalase. Superoxide dismutase results in the dismutation of the superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2). In the following reactions the H_2O_2 formed is reduced to H_2O by either glutathione peroxidase or catalase. This subsequent reduction of H_2O_2 prevents the potential formation of hydroxyl radicals. If H_2O_2 accumulates due to insufficient glutathione peroxidase and catalase activity, hydroxyl radicals may be formed by the heavy metal catalysed Fenton reaction. During the glutathione peroxidase catalysed reduction of hydrogen peroxides, glutathione (GSH) serves as an electron donor, and the glutathione disulphide (GSSG) formed in the reaction is subsequently reduced back to GSH by glutathione reductase at the expense of nicotinamide adenine dinucleotide phosphate (NADPH). (9) NADPH is produced by normal activity of the hexose monophosphate shunt. (65) In MHT patients, a metabolic block of the pentose phosphate shunt has been described that impairs the reduction of oxidised glutathione due to the reduced NADPH formation. (65, 66) Most of the enzymes responsible for the reduction of oxygen radicals to H_2O contain certain trace elements at their active sites. Trace element levels are altered in MHT patients. The dialysis procedure further could appreciably alter the body's supply of trace elements by removing some, whereas contaminating trace elements in the dialysis fluid could be transferred to the patient. A reduction in the trace elements necessary for anti-oxidant enzyme activity can result in reduced anti-oxidant activity and therefore an increase in oxidative stress. The concentrations

of Zn and Se for instance are reduced in the MHT patient. These trace elements are necessary for normal activity of the anti-oxidant enzymes; superoxide dismutase and glutathione peroxidase. (67-69) Other trace elements catalyse the production of hydroxyl radicals from hydrogen peroxide, via the heavy metal catalysed Fenton reaction. An increase in these trace elements would therefore result in an elevation in oxidative stress. (68) Under conditions of oxidative stress, when the cell must cope with large amounts of H_2O_2, the role of glutathione oxidation exceeds the slower rate of GSSG reduction by glutathione reductase, and GSSG accumulates. To avoid the detrimental effects of increased intracellular levels of GSSG (e.g. formation of mixed disulphides with protein thiols) the cell actively secretes GSSG, which can lead to depletion of the intracellular glutathione pool. (9) In addition vitamins A, E and C are powerful anti-oxidants. Vitamin E and †-carotene are lipid-soluble anti-oxidants preventing radical mediated breakdown of poly-unsaturated fatty acids and vitamin C acts as a reductant in the cell, both regenerating oxidised vitamin E and preventing oxidation of cytosolic thiols. (9, 65, 66) In the CRF/MHT patient an increase in oxidative stress can result in the overconsumption of available anti-oxidant vitamins.

The increase in the oxidative stress in the CRF patient receiving maintenance haemodialysis due to both an increase in pro-oxidant production and a decrease in anti-oxidant activity, can potentially result in intracellular calcium dyshomeostasis. Unwanted oxygen radical production can result in the oxidation of membrane polyunsaturated fatty acids. Oxidation of membrane polyunsaturated fatty acids can subsequently result in an increase in the permeability for calcium ions causing an unwanted influx of calcium ions. (58, 65, 70) Furthermore, an unwanted oxidation of membrane polyunsaturated fatty acids can result in the derangement of membrane fatty acid composition possibly

due to an increase in the saturated fatty acid/unsaturated fatty acid ratio. The membrane fatty acid composition is an important factor regulating membrane-associated protein function. It is well known that various membrane-associated enzymes are highly sensitive to the membrane micro-environment. The Ca^{2+}-ATPase calcium pump responsible for lowering intracellular free calcium is one such membrane-associated enzyme that is very sensitive to membrane fatty acid composition. (71-73) An increase in oxidative stress will furthermore result in the oxidation of cellular proteins. Many of the membrane-associated proteins are rich in SH-groups which are targets for oxygen radical action. The Ca^{2+}-ATPase is a thiol-rich protein at the cytosol-membrane surface. (74) Therefore, an increase in the oxidative stress may result in oxidation of the SH-groups of the pump and suppression of the pump's activity. (75)

4.1.3) Inhibition of the calcium pump

A circulating inhibitor of the calcium pump is indicated in the plasma of CRF patients. This inhibitor is dialysable, smaller than 3000 molecular weight, heat-stable, and said to be protease resistant. (76, 77) It is hypothesised that this inhibitor of the calcium pump may be one of the factors contributing to the elevation in intracellular free calcium in various cells in the CRF patient.

4.1.4) Inhibition of the Na^+-K^+-ATPase

There are indications of an elevation in plasma levels of natriuretic factor in CRF. Natriuretic factor is a potent inhibitor of the Na^+-K^+-ATPase. Na^+-K^+-ATPase plays an important role in the maintenance of intracellular calcium homeostasis. Na^+-K^+-ATPase maintains the sodium gradient across cellular membranes, a high sodium concentration extracellularly and a low sodium concentration on the inside. This sodium gradient provides the energy necessary for the

extrusion of calcium via the Na^+-Ca^{2+}-exchanger. A decrease in the activity of the Na^+-K^+-ATPase may ultimately result in an elevation in intracellular free calcium. (78)

4.2) Results of an elevation in basal intracellular free calcium in chronic renal failure

4.2.1) Deranged membrane phospholipid metabolism

It is indicated that the content of phospholipids in brain synaptosomes and specifically phosphatidyl inositol (PI), phosphatidyl serine (PS), and phosphatidyl ethanolamine (PE) are significantly reduced in CRF. This reduction in the phospholipid content is attributed to the increase in basal intracellular free calcium caused by the chronic exposure to PTH. (79) Phospholipids are important components of the cell membrane and play an important role in maintaining membrane fluidity and membrane protein function. (80) Changes in phospholipid composition of cell membranes may therefore affect the activity of proteins and enzymes present in the membranes. (81, 82) The various enzymes responsible for lowering of intracellular free calcium, Ca^{2+}-ATPase, Na^+/Ca^{2+}-exchanger and the Na^+/K^+-ATPase are extremely sensitive to membrane phospholipid composition. (83, 84) Indeed, the changes in synaptosomal phospholipid composition are associated with changes in Na^+/K^+-ATPase activity. This change in the activity of the enzymes responsible for lowering of intracellular free calcium may thus possibly aggravate the initial increase in intracellular free calcium. Chronic exposure to PTH has been shown to inhibit the activity of many cellular enzymes. It is therefore possible that chronic exposure to excess PTH may also inhibit the activity of enzymes involved in the synthesis of phospholipids. (79, 85)

4.2.2) Deranged membrane fatty acid synthesis

It is indicated in the CRF patient that intracellular free calcium accumulation results in inhibition of various cellular enzymes. One of the enzymes possibly inhibited by an elevation in intracellular free calcium is carnitine palmitoyl transferase (CPT). (79) Adequate CPT activity is important in the maintaining of a favourable acyl CoA/free CoA ratio. CPT maintains a favourable acyl CoA/free CoA ratio by forming acylcarnitine from carnitine and acyl CoA. Membrane fatty acid turnover depends on both long chain acyl CoA synthetase and lysophospholipid acyl CoA transferase (LAT). The former generates acyl CoA from free fatty acids and CoA, while the latter reacylates lysophospholipids by using acyl CoA as substrate. When the rate of formation of acyl CoA becomes different from that of its utilisation for reacylation, acyl CoA and the acyl CoA/free CoA ratio become altered, leading to disturbances in membrane fatty acid turnover. In the CRF patient reduced activity of CPT is indicated. (85) Under such circumstances, the lack of buffering activity of CPT alters the acyl CoA/free CoA ratio, and eventually the availability of fatty acid moieties for reacylation are impeded. Additionally, the relatively high concentrations of free CoA may further act as an inhibitor of the enzyme LAT. This possible reduced activities of CPT and LAT may result in the inadequate repairing of membrane fatty acids damaged by free radicals and the loss of membrane integrity. (85)

4.2.3) Decreased ATP production

An elevation in basal intracellular free calcium inhibits mitochondrial oxidation, resulting in the decrease of basal and stimulated ATP production. (53, 78)

5) Aim of this study

Over the past decade a significant amount of work has been done on intracellular calcium in chronic renal failure. It should however be

remembered that the term intracellular calcium is used relatively non-specifically, as can be seen from the various publications on the subject where the expression *intracellular calcium* is used to denote anything from total cellular calcium to diffusible calcium content, depending on the methods available in specific laboratories. The complete picture of the intracellular calcium status, including a comparison between the intracellular content of the various calcium compartments, the characteristics of the transmembrane calcium movement, the possible causes which may contribute to the assumed intracellular calcium disturbances in CRF, as well as the contribution of such disturbances to the pathogenesis of chronic renal failure still need to be clarified. At this point in time it can, with a relative amount of certainty, be assumed that both the total intracellular calcium content and the intracellular free Ca^{2+} are increased in end stage chronic renal failure patients. This despite a small number of contradictory reports. Very little is, however, known about the movement across the cell membranes as well as about the intercompartmental shifts. The parathyroid hormone disturbance in chronic renal failure is at present seen as the major contributor to the deranged calcium status, but the evidence for such an assumption is, however tempting, still far from conclusive.

The aim of this study was to contribute to the knowledge on intracellular calcium in chronic renal failure by

a) Examining the intraneutrophilic free cytosolic Ca^{2+} content in end stage chronic renal failure patients on maintenance haemodialysis treatment. The aim is not to evaluate the influence of renal failure *per se* on calcium metabolism, but rather to determine the status of intracellular calcium in the maintenance haemodialysis patient as it presents as a result of renal failure, dialysis treatment and medication. The rationale is that firstly the disturbed cellular functions and other

effects of the disturbed calcium content are functions of the existing calcium status and secondly that the application of stringent exclusion criteria would eliminate too many patients from the already small available group.

b) Examining the characteristics of the transmembrane movement of calcium, i.e., the calcium fluxes.

c) Investigating the intracellular distribution of calcium in the neutrophils of the patients by means of electron microscopy.

d) Exploring the possibility of a link between the alterations in intracellular calcium status and factors known to influence the calcium status. The factors investigated in this study include the lipid composition of the membrane, the oxidative status as reflected by anti-oxidant vitamin levels, the levels of parathyroid hormone, and ionised serum calcium.

In order to establish conditions inducive to accurate research, the techniques for calcium determinations, which did not previously exist in the current laboratory, had to be developed and evaluated. This constituted a rather large part of the project.

The rest of the chapters of this dissertation are presented in the following sequence

Chapter 2: Evaluation of the techniques for the determination of intracellular free calcium, transmembrane calcium fluxes and intracellular distribution of calcium

Chapter 3: Materials and methods

Chapter 4: Results

Chapter 5: Discussion and conclusions

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