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Review

The mechanisms of calcium homeostasis and signalling in the lens

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ABSTRACT

Excessive Ca^{2+} can be detrimental to cells and raised levels of Ca^{2+} in human lenses with cortical cataract have been found to play a major role in the opacification process. Ca^{2+} homeostasis is therefore, recognised as having fundamental importance in lens pathophysiology. Furthermore, Ca^{2+} plays a central role as a second messenger in cell signalling and mechanisms have evolved which give cells exquisite control over intracellular Ca^{2+} ($[Ca^{2+}]_i$) via an array of specialised regulatory and signalling proteins. In this review we discuss these mechanisms as they apply to the lens. Ca^{2+} levels in human aqueous humour are approximately 1 mM and there is a large, 10,000 fold, inwardly directed gradient across the plasma membrane. In the face of such a large gradient highly efficient mechanisms are needed to maintain low $[Ca^{2+}]_i$. The Na^+/Ca^{2+} exchanger (NCX) and plasma membrane Ca^{2+}-ATPase (PMCA) actively remove Ca^{2+} from the cells, whereas the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) sequesters Ca^{2+} in the endoplasmic reticulum (ER) Ca^{2+} store. In lens epithelial cells the dominant role is played by the ATPases, whilst in the fibre cells NCX activity appears to be more important. Usually, $[Ca^{2+}]_i$ can be increased in a number of ways. Ca^{2+} influx through the plasma membrane, for example, is mediated by an array of channels with evidence in the lens for the presence of voltage-operated Ca^{2+} channels (VOCCs), receptor-operated Ca^{2+} channels (ROCCs) and channels mediating store-operated Ca^{2+} entry (SOCE). Ca^{2+} signalling is initiated via activation of G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTK) of which the lens expresses a surprisingly diverse array responding to various neurotransmitters, hormones, growth factors, autocoids and proteases. Downstream of plasma membrane receptors are IP_3-gated channels (IP_3Rs) and ryanodine receptors (RYRs) located in the ER, which when activated cause a rapid increase in $[Ca^{2+}]_i$ and these have also been identified in the lens. Through an appreciation of the diversity and complexity of the mechanisms involved in Ca^{2+} homeostasis in normal lens cells we move closer to an understanding of the mechanisms which mediate pathological Ca^{2+} overload as occurs in the process of cataract formation.

Calcium (Ca^{2+}) has long been recognised to be of key importance to lens physiology and pathology. The earliest reports of disturbances to cellular Ca^{2+} in cataract came a century ago (Burge, 1909) when increased Ca^{2+} was measured in cataractous human lenses. It was, however, the seminal paper of Duncan and Bushell (1975) which brought the role of Ca^{2+} to the forefront of modern cataract research. In this paper they measured the ionic content of human lenses with cortical and nuclear cataract (Fig. 1) and showed that all lenses with cortical cataract also had raised intracellular Ca^{2+}, while those with pure nuclear cataract had Ca^{2+} concentrations similar to those of clear unaffected lenses. They also noted that associated with increased Ca^{2+} was a reduction in lens dry weight, indicating that protein loss in cortical cataract could be linked to Ca^{2+} overload. In subsequent experiments, the free Ca^{2+} concentration was shown to be highest in localised regions of cortical opacity (Duncan and Jacob, 1984a). The mechanisms underlying Ca^{2+}-induced opacification have since become a major focus of cataract research. Studies by Hightower and Farnum (1985) showed that incubating human lenses in medium containing high levels of Ca^{2+} (20 mM) resulted in a loss of transparency in the cortex of the lens. Further experiments confirmed the role of Ca^{2+}, demonstrating Ca^{2+} overload and consequent Ca^{2+}-induced proteolysis in *in vivo* and *in vitro* models of cataract (David and Shearer, 1984; Marcantonio et al., 1986) including the organ cultured human lens (Sanderson et al., 2000). The Ca^{2+}-activated protease calpain was implicated in this process (David and Shearer, 1984) and this family of proteases remains a major focus in cataract pathophysiology (Biswas et al., 2005). Clearly, an understanding of the regulation of Ca^{2+} is of prime importance in understanding the processes that lead to cortical opacification and this has led to a much wider appreciation of the role of Ca^{2+} in the lens, specifically in relation to Ca^{2+} signalling. This area of research was pioneered by George Duncan (Duncan et al., 1993) and was the

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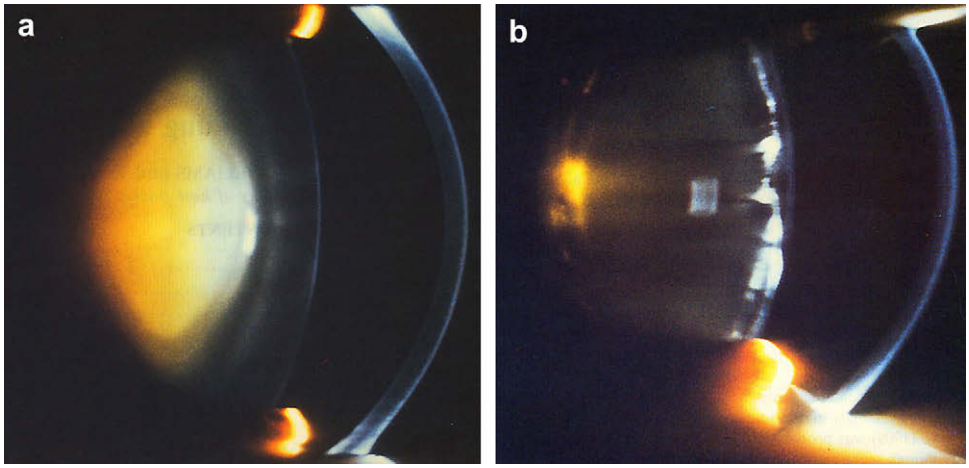

Fig. 1. *In vivo* slit lamp photographs of lenses with a) nuclear and b) cortical cataract. Lenses with cortical opacities have raised $[Ca^{2+}]_i$, whereas in those with pure nuclear cataract $[Ca^{2+}]_i$ is comparable to clear lenses (Duncan and Bushell, 1975). Note that 70% of cataracts have cortical involvement. Images are taken from Marcantonio et al. (1980).

dominant theme of his research career. Due in large part to his enthusiasm for the subject, Ca^{2+} is now recognised as a key mediator of signalling within lens cells, and a multitude of Ca^{2+} linked receptors has been identified in the lens.

1. Intracellular Ca^{2+} concentration in the lens

In order for Ca^{2+} to fulfil its role as a signalling molecule and to prevent the toxic effects of Ca^{2+} overload, intracellular Ca^{2+} is very tightly regulated and the concentration of Ca^{2+} in the cytoplasm ($[Ca^{2+}]_i$) is orders of magnitude less than the extracellular environment. The Ca^{2+} concentration in aqueous humour has been recorded at 1.34 mM in human (Ringvold et al., 1988) and 1.27 mM in primate (Cole, 1974) compared to a resting $[Ca^{2+}]_i$ in the order of 100 nM in lens epithelial cells. Reported values for $[Ca^{2+}]_i$ in lens epithelial cells are given in Table 1 and all are of a similar magnitude. $[Ca^{2+}]_i$ has also been measured in fibre cells using either Ca^{2+}-sensitive dyes or ion sensitive electrodes (Table 1) and the data show that $[Ca^{2+}]_i$ in fibre cells is greater than in epithelial cells.

The measurements described so far ($[Ca^{2+}]_i$) are of free Ca^{2+} and it is this fraction that regulates cellular processes and is actively controlled in Ca^{2+} homeostasis. However, it should be appreciated, that the majority of Ca^{2+} in the lens is in a bound or sequestered form. Therefore, values for total lens Ca^{2+}, measured by atomic absorption spectrophotometry, are greater than those measured for $[Ca^{2+}]_i$ since these include both the bound and the free fractions (Table 1). Included within the bound fraction is Ca^{2+} sequestered in organelles and that bound to protein (Duncan and van Heyningen, 1977) and membranes (Vrensen et al., 1995). There are also reports that there are intercellular structures that store Ca^{2+} between mature fibre cells (Vrensen et al., 1995; Dahn and Prescott, 2003).

2. Mechanisms of Ca^{2+} homeostasis

To maintain the *status quo* against such a large Ca^{2+} gradient, cells possess a variety of Ca^{2+} buffers, pumps and exchangers to keep $[Ca^{2+}]_i$ low. There are also, however, mechanisms that increase the concentration of free Ca^{2+} in the cytoplasm and at any

Table 1
Measurements of Ca^{2+} concentration in the lens.

Cell type	Technique	Species	$[Ca^{2+}]_i$	Reference
Cultured lens epithelial cells	Ca^{2+}-sensitive fluorimetric dye (FURA-2)	Human	78 ± 33 nM	Williams et al. (1993)
			96 ± 20 nM	Riach et al. (1995)
		Rabbit	49.5 ± 8.1 nM–101.8 ± 52 nM	Rafferty et al. (1994)
		Sheep	94 ± 16 nM	Churchill et al. (1996)
			132 ± 2 nM	Churchill and Louis (2002)
		Cow	70.8 ± 5.9 nM	Samadi et al. (2002)
			42.1 ± 2.5 nM	Samadi et al. (2005)
		Pig	65 ± 16 nM	Okafor et al. (2003)
Lens fibre cells in intact lens	Ca^{2+}-sensitive fluorimetric dye (FURA-2)	Mouse	0.3 μM (surface fibres)	Gao et al. (2004)
			0.7 μM (central fibres)	
	Ion sensitive electrodes	Frog	0.56 μM (posterior)	Jacob (1983)
			0.1 μM (anterior)	
		Rat	1.8 μM	Duncan and Jacob (1984b)
		Rabbit	33 ± 3 μM (young)	Hightower et al. (1985)
			51 ± 7 μM (old)	
		Human	9 μM (<40 yrs)	Duncan et al. (1989)
			15 μM (>40 yrs)	
Isolated lens fibre cells	Ca^{2+}-sensitive fluorimetric dye (Fluo-3)	Rat	0.11 ± 0.01 μM (outer fibres)	Srivastava et al. (1997)
			0.09 ± 0.01 μM (inner fibres)	
Lentoid bodies	Ca^{2+}-sensitive fluorimetric dye (FURA-2)	Sheep	0.37 ± 0.03 μM	Churchill and Louis (2002)
Whole lens	Atomic absorption spectrophotometry	Frog	250 μM	Baldwin and Bentley (1980)
		Rat	628 ± 117 μM	Duncan and Jacob (1984b)
		Rabbit	250 ± 50 μM (young)	Hightower et al. (1985)
			499 ± 50 μM (old)	
		Human	900 μM (nuclear cataract)	Duncan and Bushell (1975)

one time $[Ca^{2+}]_i$ is determined by the balance between these opposing processes. Cells utilise two main mechanisms to extrude Ca^{2+}, the Na^+/Ca^{2+} exchanger (NCX) and the plasma membrane Ca^{2+}-ATPase (PMCA). Both systems have been identified in the lens and have been shown to regulate $[Ca^{2+}]_i$. In addition to efflux to the extracellular fluid, Ca^{2+} is also sequestered in the endoplasmic reticulum (ER). This is achieved by the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) which also plays a critical role in maintaining low $[Ca^{2+}]_i$ by pumping Ca^{2+} into the intracellular stores. Mitochondria also sequester Ca^{2+}, expressing a uniporter that allows Ca^{2+} to move into the mitochondrial matrix driven by the mitochondrial membrane potential. Under normal circumstances the mechanisms that lead to $[Ca^{2+}]_i$ increase, be it via Ca^{2+} influx channels or release from intracellular stores, are very tightly regulated and are an integral part of Ca^{2+} signalling. The very large increases in Ca^{2+} recorded in cortical cataract, however, indicate that in some circumstances Ca^{2+} homeostasis breaks down and influx overwhelms the ability of lens cells to remove Ca^{2+} from the cytosol. What is not clear, however, is whether this is due to an unregulated increase in Ca^{2+} influx, an impaired ability to remove excess Ca^{2+} or a combination of both processes, and these questions remain fundamental to the current hypotheses to explain cataract development.

In the following sections we describe the function of each of the major components of Ca^{2+} homeostasis or Ca^{2+} signalling. Particular emphasis is given to those components that have been described in the lens.

2.1. Na^+/Ca^{2+} exchange

The Na^+/Ca^{2+} exchanger (NCX) plays a fundamental role in Ca^{2+} homeostasis and cell signalling (Blaustein and Lederer, 1999). It is a low affinity, high capacity system, which, in forward mode, uses the inwardly directed Na^+ electrochemical gradient to drive Ca^{2+} efflux. The stoichiometry is typically $3Na^+$ to $1Ca^{2+}$ and it is therefore electrogenic and voltage sensitive. The NCX can function in both directions and under conditions of raised intracellular Na^+ or cellular depolarization, it switches to reverse mode, bringing Ca^{2+} into the cell. There are 3 known mammalian isoforms (Blaustein and Lederer, 1999) and NCX1, the most widely expressed, has been identified in the lens (Okafor et al., 2003; Tamiya and Delamere, 2006). The presence of a Na^+/Ca^{2+} exchange mechanism in the lens was first indicated in experiments using membrane vesicles isolated from bovine lens fibre cells (Galvan and Louis, 1988). Na^+/Ca^{2+} exchange activity was investigated in greater detail by Tomlinson et al. (1991) who demonstrated that in the intact rat lens, 55% of Ca^{2+} efflux from the lens was dependent on extracellular Na^+. Activity has since been confirmed in human lens as well as in other species (Ye and Zadunaisky, 1992; Duncan et al., 1993; Okafor et al., 2003). In terms of the relative role of the NCX in the lens, differences exist between epithelial and fibre cells. In epithelial cells, resting Ca^{2+} was only minimally affected by either removing extracellular Na^+ (Duncan et al., 1993) or applying the NCX inhibitor bepridil (Okafor et al., 2003). This agrees with kinetic models since $[Ca^{2+}]_i$ in lens epithelial cells is below the affinity of the NCX (0.6–6 μM) (Blaustein and Lederer, 1999) and it would therefore not be predicted to make a major contribution to Ca^{2+} efflux. However, when $[Ca^{2+}]_i$ was raised above resting levels following activation of G-protein-coupled receptors (GPCR), inhibition of the exchanger resulted in an amplified Ca^{2+} response in lens epithelial cells (Okafor et al., 2003). This suggests that in epithelial cells, NCX driven Ca^{2+} efflux is only significant when intracellular Ca^{2+} is increased, defining a role in modulating Ca^{2+} cell signalling. In contrast, in the intact lens, where Ca^{2+} fluxes across fibre cells predominate, a large proportion of the resting efflux is via Na^+/Ca^{2+} exchange (Tomlinson et al., 1991) and both removal of extracellular Na^+ (Tomlinson et al., 1991) or exposure to bepridil (Tamiya and Delamere, 2006) caused an increase in lens Ca^{2+} content. In fibre cells, therefore, it appears that NCX is active in regulating resting Ca^{2+} levels. This would be predicted since the resting levels of Ca^{2+} in fibre cells are higher than in epithelial cells (Table 1) and are in the range where predicted NCX kinetics would support removal of Ca^{2+} (Duncan and Jacob, 1984a; Gao et al., 2004).

Fibre cell Ca^{2+} homeostasis is highly significant in terms of lens transparency and inhibition of NCX results in Ca^{2+} overload and lens opacification (Tomlinson et al., 1991; Tamiya and Delamere, 2006). Importantly, conditions where intracellular Na^+ is raised, as occurs in the human lens with aging (Duncan et al., 1989) and in cortical cataract (Duncan and Bushell, 1975), may not only result in reduced efficiency of Ca^{2+} efflux but could also cause NCX to function in reverse mode further worsening the situation by driving Ca^{2+} into the lens. This scenario can be modelled experimentally by inhibiting lens Na^+, K^+-ATPase with ouabain, causing increased Ca^{2+} influx and opacification (Tomlinson et al., 1991). NCX has also been shown to have a significant role in the selenite model of cataract (Wang et al., 1992) and in mediating the globulization of isolated fibres (Srivastava et al., 1997). In both these cases NCX functions in reverse mode providing a pathway for Ca^{2+} influx and cellular Ca^{2+} overload. Interestingly, compounds now exist (KRB7943 and SN-6) that inhibit NCX only in reverse mode and their effects on lens opacification can now be studied (Tamiya and Delamere, 2006).

2.2. The plasma membrane Ca^{2+}-ATPase

The plasma membrane Ca^{2+}-ATPase (PMCA) is a high affinity Ca^{2+} pump which uses the energy from ATP hydrolysis to drive Ca^{2+} out of the cell against its electrochemical gradient (Carafoli, 1994). The main regulator of PMCA activity is calmodulin, which on binding Ca^{2+} stimulates Ca^{2+} efflux both by increasing V_{max} and decreasing the K_m for Ca^{2+}. The lipid environment has also been shown to be an important regulator of PMCA activity which is stimulated by acidic phospholipids and long-chain polyunsaturated fatty acids (Niggli et al., 1981). The phosphorylation status of PMCA also appears to be important and activity is stimulated by protein kinases, including PKA and PKC. Four genes code for the different isoforms (PMCA1-4) and multiple splice variants have been identified (Carafoli, 1994). Different isoforms of PMCA have been shown to have distinct characteristics affecting, for example, their affinity for calmodulin (Carafoli, 1994) and sensitivity to proteolysis (Guerini et al., 2003).

The presence of a Ca^{2+}-ATPase in the lens was first suggested by Hamilton et al. (1979) and further evidence was provided by Hightower et al. (1980). Ca^{2+}-ATPase activity has since been measured in lenses of all mammalian species investigated (Iwata et al., 1984; Borchman et al., 1988) including human (Borchman et al., 1989). Although, these early experiments did not distinguish between Ca^{2+}-ATPase activity in the plasma membrane and the ER (see below), they did demonstrate sensitivity to calmodulin showing that PMCA contributed to the activity measured (Iwata et al., 1984; Iwata, 1985). Both PMCA and SERCA pumps are now recognised to play an important role in Ca^{2+} homeostasis in lens epithelial cells (Duncan et al., 1993) and each has been shown to contribute approximately 50% of the total activity (Zeng et al., 1995). All 4 PMCA isoforms are expressed in both bovine (Bian et al., 2000) and human (Marian et al., 2007) lens epithelial cells and although multiple splice variants have been identified in other tissues there is, as yet, no evidence that this is the case in the lens (Bian et al., 2000). Early reports indicated Ca^{2+}-ATPase activity in both epithelial cells and cortical fibres (Hightower et al., 1980; Borchman et al., 1989), although, more recent studies suggest that

expression and activity are limited to the epithelium (Paterson et al., 1997; Marian et al., 2005).

Interestingly, although activity in the lens increases with age (Borchman et al., 1989), expression levels of the different Ca^{2+}-ATPase isoforms remain the same throughout life (Marian et al., 2008). Conversely, in human lenses with cataract, Ca^{2+}-ATPase activity was reduced by 50% compared to clear lenses (Paterson et al., 1997) although overall expression in cataractous lenses was unchanged. Recently, upregulation of PMCA2 was reported in the epithelia of cataractous lenses, presumably in compensation for increased intracellular Ca^{2+} levels (Marian et al., 2008). These studies highlight the importance of considering the control of PMCA activity at the functional as well as the transcriptional level. PMCA activity has been shown to be modulated by a range of different factors in the lens. Oxidation, for example, caused a reduction in Ca^{2+}-ATPase activity in lens cells (Borchman et al., 1989; Hightower and McCready, 1991) and oxidative stress might therefore be expected to impair the ability of the lens to maintain Ca^{2+} homeostasis. PMCA is also a substrate for Ca^{2+}-activated proteases (calpains), and increased calpain-mediated proteolysis was found to either increase or decrease activity depending on the PMCA isoform expressed (Guerini et al., 2003). Increasing lipid order, and hence membrane stiffness, has also been shown to increase PMCA activity (Tang et al., 2006). It is significant that lipid order has been shown to increase with age and cataract (Paterson et al., 1997) and PMCA activity might, therefore, also be expected to increase in these conditions. It remains to be elucidated, however, in exactly what capacity PMCA activity contributes to cataract: whether through upregulation, to mitigate increases in intracellular Ca^{2+}, or through loss of activity contributing to cataractogenic Ca^{2+} overload.

2.3. The sarco(endo)plasmic reticulum Ca^{2+}-ATPase

The sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) functions to pump Ca^{2+} from the cytosol into the ER Ca^{2+} store in most cell types. Like the PMCA it is a high affinity pump, powered by the hydrolysis of ATP, which drives Ca^{2+} against its concentration gradient into the ER. Its action, in combination with Ca^{2+} efflux mechanisms, is essential in maintaining a low resting $[Ca^{2+}]_i$. There are 3 isoforms (SERCA1-3) coded for by three genes and multiple splice variants, producing more than 10 different isoforms, have been described (Periasamy and Kalyanasundaram, 2007). The human lens expresses isoforms SERCA2b and SERCA3 (Liu et al., 1999). Functional differences between the isoforms, such as affinity for Ca^{2+} and pump velocity have been identified (Periasamy and Kalyanasundaram, 2007) and tissue-specific expression profiles presumably reflect the specific Ca^{2+} dynamics of particular cell types.

All SERCA isoforms are selectively and potently inhibited by the plant-derived sesquiterpene lactone, thapsigargin (Tg) (Thastrup et al., 1990) and it was application of this novel compound to lens epithelial cells (Duncan et al., 1993), that demonstrated the key role played by SERCA pumps in these cells. Inhibiting the pump led to an immediate increase in $[Ca^{2+}]_i$ clearly demonstrating the dynamic nature of the ER Ca^{2+} stores in lens epithelial cells. Although Tg inhibits all isoforms, it has been shown to increase expression of SERCA3 in cultured human lens epithelial cells, potentially indicating a greater role for this isoform in lens Ca^{2+} metabolism (Marian et al., 2007).

2.4. The mitochondrial uniporter

The mitochondrial uniporter is a Ca^{2+} transporter which allows movement of Ca^{2+} from the cytoplasm into the mitochondrial matrix (Spat et al., 2008). It is driven by the transmembrane potential across the inner mitochondrial membrane generated by the electron transport chain. It therefore serves to help limit increases in $[Ca^{2+}]_i$ (Ribeiro et al., 2003) and is recognised to play an important role in Ca^{2+} signalling (Spat et al., 2008). In the lens, mitochondrial Ca^{2+} signalling has not been specifically investigated. However, interestingly and contrary to what might be anticipated, the blocker of the Ca^{2+} uniporter, ruthenium red, inhibited lens opacification in organ cultured rat lenses exposed to the cataractogenic compound ciglitazone (Aleo et al., 2005). The role of mitochondria in modulating Ca^{2+} dynamics in lens cells, particularly in relation to cataractogenesis, certainly warrants further investigation.

2.5. Endoplasmic reticulum Ca^{2+} release channels

An important source of Ca^{2+} in cells is the ER Ca^{2+} store and release of Ca^{2+} during signalling is an essential process in most cells. The major second messenger responsible for the release of Ca^{2+} from ER stores is inositol-1,4,5-triphosphate (IP_3) which activates IP_3 receptor (IP_3R) channels, allowing release of Ca^{2+} into the cytoplasm. IP_3 synthesis in the lens was first demonstrated by Vivekanandan and Lou (1989) in rabbit lens epithelial cells, stimulated by various factors including serum and vitreous humour. In addition to IP_3R, the ER contains another group of Ca^{2+} release channels called ryanodine receptors (RYR), so called because of their affinity for the plant alkaloid of that name. RYRs are primarily activated by cytoplasmic $[Ca^{2+}]$ in the μm range, although, their activity can also be stimulated by cADP ribose (Berridge et al., 2003). In the lens there is a substantial body of research relating to the role of the ER in Ca^{2+} cell signalling, much of it originating from the Duncan laboratory. In the first experiments to investigate Ca^{2+} signalling in the lens, Duncan et al. (1993) demonstrated that IP_3 was able to directly stimulate Ca^{2+} release, presumably via IP_3R, in permeabilized human and bovine lens epithelial cells. This was later confirmed in cultured sheep lens cells where it was also shown that cADP ribose was also able to induce Ca^{2+} release from the Ca^{2+} stores (Churchill and Louis, 1998, 1999). There is further evidence that RYRs are involved in lens Ca^{2+} signalling from a study which showed expression of the non-muscle subtype, RYR3 in the human HLE-B3 cell line (Qu and Zhang, 2003).

2.6. Ca^{2+} influx channels

There are a variety of different types of channel which, when activated, allow Ca^{2+} entry into cells. Given the large electrochemical gradient that exists across the plasma membrane, this is a powerful way of rapidly increasing intracellular Ca^{2+} during cell signalling and the possibility of different combinations of channels enables a high degree of functional diversity between cell types. These channels include voltage-operated Ca^{2+} channels (VOCCs), receptor-operated Ca^{2+} channels (ROCCs) and second messenger operated Ca^{2+} channels (SMOCs). In addition, there are channels which mediate store-operated Ca^{2+} entry (SOCE) activated by depletion of the ER intracellular Ca^{2+} stores.

Although, VOCCs are primarily associated with excitable cells, there is also evidence for their expression in lens cells. The first indication of this came from Albrecht Fleckenstein, the "father of calcium antagonists", who found that the L-type Ca^{2+} channel blocker, verapamil, was effective in attenuating cataract formation in diabetic rats (Fleckenstein, 1983). Lenses from animals that had been treated with verapamil, also had a greatly reduced Ca^{2+} content. More recent studies of diabetic and radiation models of cataract also report decreased lens opacification following treatment with L-type Ca^{2+} channel blockers (Cengiz et al., 1999; Ettl et al., 2004; Kametaka et al., 2008). Direct evidence of functional expression of VOCCs in the intact lens has come from experiments

looking at either oscillations of membrane potential (Thomas et al., 1998) or trans-lens short-circuit current in the intact rabbit lens (Alvarez et al., 1995, 1997). In each case the L-type Ca^{2+} channel blocker, nifedipine, inhibited the oscillations, indicating that VOCCs are part of the signalling machinery in the whole rabbit lens. Verapamil has also been shown to inhibit Ca^{2+}-induced globulization in rat fibre cells (Srivastava et al., 1997), and a nifedipine sensitive inward current has recently been reported in the HLE-B3 human lens epithelial cell line (Meissner et al., 2007). Although indirect, these data provide convincing evidence, certainly in animal models, that expression of L-type voltage-gated Ca^{2+} channels in both lens epithelial and fibre cells is potentially an important route of Ca^{2+} influx which may contribute to pathological Ca^{2+} overload.

The presence of ROCCs in the lens has been found in the form of P2X purine receptors and the rat lens expresses all 7 isoforms (Suzuki-Kerr et al., 2008). Interestingly, different isoforms appear to be differentially expressed, for example, $P2X_2$ was found predominantly at the interface between epithelial and fibre cells, whilst $P2X_3$, $P2X_4$ and $P2X_6$ were present in the membranes between mature fibre cells. The physiological significance of such expression patterns, however, remains to be discovered.

Store-operated Ca^{2+} entry (SOCE) has been a focus of lens cell signalling research since the initial experiments that identified such a pathway in lens epithelial cells exposed to Tg (Duncan et al., 1993). Because Ca^{2+} is continually being released from the ER store, inhibition of SERCA by Tg causes the depletion of Ca^{2+} from the store and the activation of SOCE, also called capacitative Ca^{2+} entry. This phenomenon was also observed in intact rat lenses, where $^{45}[Ca^{2+}]$ influx was stimulated for up to 8 h after exposure to Tg (Thomas et al., 1999). In human lens epithelial cells, removal of extracellular Ca^{2+} eliminated the sustained Ca^{2+} increase that follows ATP or histamine induced store release (Riach et al., 1995) demonstrating that agonists as well as Tg can activate this pathway. In a detailed study, also in human lens epithelial cells, Williams et al. (2001) found that, SOCE was blocked by Zn^{2+} but was unaffected by nifedipine. In addition, SOCE was shown to have different kinetics when activated by agonists compared to when it was activated by Tg, suggesting that active SERCA pumps may play a role in shaping Ca^{2+} entry characteristics. The authors also showed that membrane potential depolarization, by increased extracellular K^+, caused a significant reduction in SOCE. Related to this is the finding that the hyperpolarising effect of activation of Ca^{2+}-activated K^+ (SK) channels, is an intimate part of Ca^{2+} signalling following GPCR agonists and RTK activation in the intact human lens (Rhodes et al., 2003). Also a role for SK channels in determining sensitivity to Ca^{2+} overload in cataract has been identified (Rhodes et al., 2006). Delineation of the mechanisms of Ca^{2+} influx remains a priority not least because pathways promoting Ca^{2+} influx may have important roles in the development of cortical cataract. It is only recently that questions regarding the molecular identity of this pathway have begun to be answered (Lewis, 2007), and a systematic characterisation of SOCE in lens has yet to be reported.

The final Ca^{2+} entry mechanism, for which there is evidence in the lens, is a non-selective cation conductance, which has been the subject of debate for many years. This was identified initially on the apical surface of frog lens epithelium (Jacob et al., 1985) and was subsequently found to be stretch-activated (Cooper et al., 1986). Similar, mechanosensitive conductances have since been reported (Churchill et al., 1996; Ohata et al., 1997) although the molecular identity has yet to be identified. A non-selective conductance has been shown to be activated as a result of oxidative stress to the lens (Duncan et al., 1988; Sanderson and Duncan, 1993) resulting in lens depolarization and increased Na^+ and Ca^{2+} content. These effects run parallel to the changes seen in the lens with ageing and cataract (Duncan and Bushell, 1975; Duncan et al., 1989) and it has been suggested that activation of such a channel could result in the pathophysiological changes in ion content observed in cataractous lenses. The transient receptor potential (TRP) group of ion channels contains members with similar properties to the non-specific cation conductance described, and it would be of future interest to investigate their role in the lens.

3. Ca^{2+} signalling in the lens

Since Ca^{2+} was identified as a second messenger, $[Ca^{2+}]_i$ has been shown to be involved in regulating numerous cellular processes, including gene expression, proliferation and cell death (Gomperts et al., 2003). There are two ways in which cells can increase the $[Ca^{2+}]_i$: firstly by the release of Ca^{2+} from internal Ca^{2+} containing stores and secondly by increasing permeability to extracellular Ca^{2+}, and it is the regulation of these processes, both temporally and spatially, in response to stimuli that is Ca^{2+} signalling. Each cell type is likely to possess a unique array of Ca^{2+} signalling components and for the lens these have been described in the previous section and are summarised in Fig. 2. We will now describe how the individual elements of the "Ca^{2+} tool kit" (Berridge et al., 2003) are coordinated to enable Ca^{2+} signalling and discuss the factors initiating Ca^{2+} signalling in the lens.

Ca^{2+} signalling is most widely initiated by stimulation of cell surface receptors. Two classes of plasma membrane receptor are responsible for detecting extracellular signals, relaying the message to the intracellular environment and causing the release of Ca^{2+} from intracellular stores. These are the G-protein-coupled receptors (GPCR), primarily of the $G_{q/11}$ subtype, and the receptor tyrosine kinases (RTK). On agonist binding, these receptors cause the activation of either phospholipase Cβ (PLCβ) or PLCγ, respectively. PLC is the enzyme responsible for hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) to IP_3 and diacylglycerol (DAG). IP_3 diffuses from the membrane to the endoplasmic reticulum Ca^{2+} store where it activates IP_3 receptor (IP_3R) channels which open to release Ca^{2+} into the cytoplasm. Following receptor stimulation, $[Ca^{2+}]_i$ can rapidly rise to approximately 1 μM and the rate of this increase is amplified by Ca^{2+}-induced Ca^{2+} release (CICR) whereby the local increase in $[Ca^{2+}]_i$ stimulates increased release from the ER both by sensitising the IP_3R and via the RYR. As previously described, depletion of ER Ca^{2+} stores following IP3R and RYR activation opens the SOCE pathway in the plasma membrane. SOCE has the dual function of enabling refilling of the store and prolonging the intracellular Ca^{2+} increase initiated by store release (Parekh and Putney, 2005). For many cell functions it is the long lasting increase in Ca^{2+} during this phase of the response that is vital and not the transient increased caused by the initial store release (Lewis, 2001). Following termination of the Ca^{2+} signal, homeostasis is restored by the action of the NCX, PMCA and SERCA pumps that restore $[Ca^{2+}]_i$ to resting levels.

3.1. Calcium mobilising receptors in the lens

The first Ca^{2+} signalling agonist to be identified in the lens was acetylcholine (Williams et al., 1993). Up to this point it was not widely anticipated that a non-excitable tissue, devoid of neural innervation, would respond to stimulation by a neurotransmitter. In these experiments, application of acetylcholine (ACh) caused a transient $[Ca^{2+}]_i$ increase and the response was blocked by the muscarinic receptor antagonist atropine, demonstrating the presence of muscarinic ACh receptors, classical GRPCRs, in the lens. In the same year, Knorr et al. (1993) used fluorescence spectroscopy to record Ca^{2+} increases in cultured bovine lens epithelial cells in suspension, after the application of the RTK ligand, platelet derived growth factor (PDGF). Since these pioneering studies, Ca^{2+}

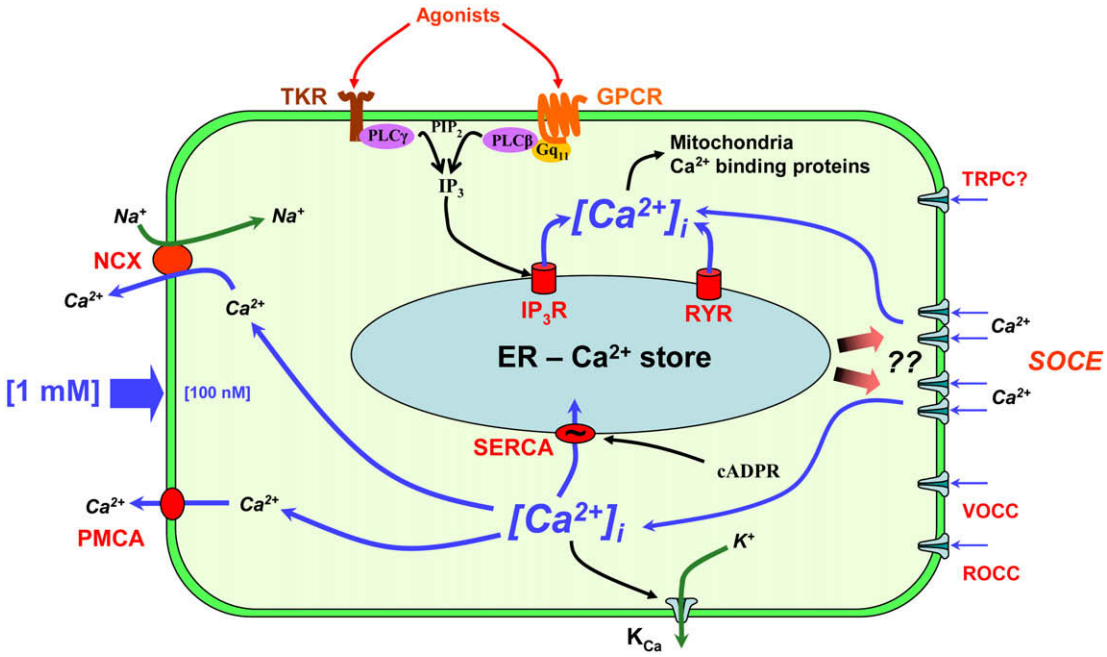

Fig. 2. Summary of mechanisms of Ca^{2+} homeostasis and signalling in lens. See text for details.

responses to a range of different GPCR and RTK agonists have been seen in lens cells from human and several animal species (Table 2). The most recently discovered receptors were activated by thrombin which increased $[Ca^{2+}]_i$ via protease activated receptor type 1 (PAR1) in both equatorial and central anterior epithelial cells of the intact human lens (James et al., 2005). Thrombin receptors are unusual in that they are activated when thrombin, a protease most commonly associated with blood coagulation, cleaves a peptide bond which releases an integral, tethered ligand allowing it to interact with the receptor binding site. Their expression exemplifies the surprising diversity of Ca^{2+}-coupled receptors in the lense.

3.2. Spatial differences in Ca^{2+} signalling in the intact lens

The single most widely applied technique to investigate cellular Ca^{2+} dynamics is the use of Ca^{2+} indicator dyes to track changes in $[Ca^{2+}]_i$ in real time. The incorporation of Ca^{2+} indicator dyes into cells is often achieved by the use of cell permeable ester derivatives (e.g. FURA-2 AM) where in many, but not all cells, endogenous esterases cleave the ester side chain, rendering the dye impermeable and thus trapped inside the cell. Importantly, lens fibre cells appear to lack the necessary esterase activity to activate and trap ion sensitive dyes and cannot be loaded by this method (Bassnett et al., 1994; Collison and Duncan, 2001). This property of the lens, however, has enabled researchers, using ester derivatives of Ca^{2+} indicator dyes, to record pure epithelial cell Ca^{2+} responses from intact lense, a technique that was developed and first employed in the Duncan lab. In a detailed study of the intact human lens (Collison and Duncan, 2001) it was shown that there are distinct differences in the relative amplitude of responses to ACh and EGF across the epithelium. The response to ACh was greater than both ATP and histamine in the central anterior region of the epithelium, but only a relatively small response occurred at the equator. The reverse was the case for EGF which induced a large response at the equator but no response in the central anterior epithelium. Related to this is the finding that, although EGF receptor (EGFR) is evenly distributed across the human lens epithelium, the level of PLCγ was found to be greater in equatorial cells compared to central anterior cells (Maidment et al., 2004) thus accounting for the gradient of signalling activity observed. Similar spatial signalling differences have been observed in animal models. In the intact rat lens epithelium a regional pattern of functional receptor expression has also been found such that at the equator adrenaline dominated the Ca^{2+} responses with only a relatively small response to ACh, while in the central anterior cells ACh produced the largest Ca^{2+} increase with no response from adrenaline (Hu et al., 2008). Additionally, in chicken lens, activated EGFR was detected at the equator (annular pad) and superficial fibres but was absent in central anterior epithelial cells (Ireland, 2005). These differences presumably reflect the requirement for spatial control of downstream function such as volume regulation or cell proliferation.

Table 2
Ca^{2+} mobilising receptors in the lens.

Receptor	Subtype	Species	Reference
Acetylcholine	Muscarinic	Human	Williams et al. (1993)
Acetylcholine	Muscarinic	Rat	Duncan et al. (1994)
Acetylcholine	Muscarinic	Rabbit	Thomas et al. (1998)
Acetylcholine	Muscarinic	Chicken	Oppitz et al. (2003)
ATP	P2U ($P2Y_2$)	Human	Riach et al. (1995)
ATP	–	Rabbit	Duncan et al. (1996)
ATP	P2U ($P2Y_2$)	Sheep	Churchill and Louis (1997)
ATP	–	Rat	Hu et al. (2008)
Bombesin	–	Rabbit	Rafferty et al. (1994)
Calcium	–	Human	Chattopadhyay et al. (1997)
EGF	–	Rabbit	Vivekanandan and Lou (1989)
EGF	–	Human	Collison and Duncan (2001)
Endothelin	–	Pig	Okafor and Delamere (2001)
Epinephrine	α1A	Sheep	Churchill and Louis (1997)
Epinephrine	–	Rat	Hu et al. (2008)
Histamine	H1	Human	Riach et al. (1995)
Histamine	–	Rabbit	Duncan et al. (1996)
PDGF	–	Cow	Knorr et al. (1993)
PDGF	–	Human	Riach et al. (1995)
PDGF	–	Rabbit	Duncan et al. (1996)
Prostaglandin	FP	Human	Mukhopadhyay et al. (1999)
Serotonin (5-HT)	–	Rabbit	Vivekanandan and Lou (1989)
Thrombin	PAR1	Human	James et al. (2005)

3.3. Heterogeneity in receptor expression

Clear differences between the species are apparent in these studies and no single animal species can be regarded as a complete

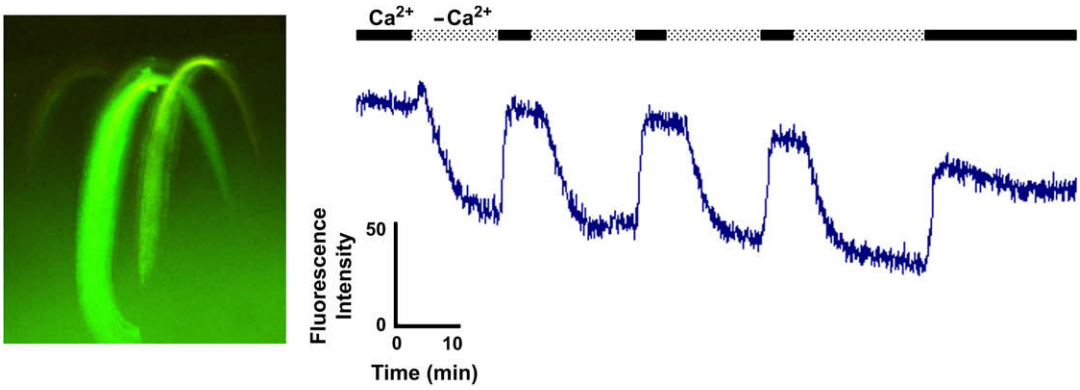

Fig. 3. Measurement of Ca^{2+} dynamics in human lens cortical fibre cells. a) Individual fibre cells loaded by dye injection with Fluo4. b) Changes in $[Ca^{2+}]_i$ in human lens fibre cells as a result of removing extracellular Ca^{2+} ($-Ca^{2+}$). For details of procedures refer to Hu et al. (2008).

model of the human lens. For example, despite robust $[Ca^{2+}]_i$ increases in rat and sheep lens epithelial cells, no response to adrenaline occurs in the human lens (Churchill and Louis, 1997; Collison and Duncan, 2001; Hu et al., 2008). Also, even when the same agonist induces responses in different species, characterisation studies show that there are differences in receptor subtype expression. In the case of muscarinic receptors, native human lens epithelial cells express the M_1 subtype (Collison et al., 2000) while in rat and rabbit, M_3 is the dominant subtype (Rhodes et al., 2002).

Differences in receptor subtype expression have also been found between native and cultured cells and in the human lens cell line HLE-B3, it is the M_3 subtype that predominates and not M_1 as in the native epithelium (Collison et al., 2000). In the rabbit cell line NN1003A, no responses to either ACh or carbachol were recorded (Duncan et al., 1996), in contrast to the robust responses recorded in whole rabbit lenses (Rhodes et al., 2002). Changes in receptor expression have also been found to occur in cultured capsular bags, where cells on the anterior capsule, although retaining their original hexagonal appearance, lose their response to ACh after 6 weeks in culture. This is in contrast to *ex vivo* capsular bags, obtained from donors who had undergone cataract surgery more than one year prior to death, where the ACh response was retained (Collison et al., 2004).

3.4. Ca^{2+} signalling in lens fibre cells

Up to this point almost all studies on lens Ca^{2+} signalling have been done in either lens epithelial cells in culture or the *in situ* epithelium. The only attempts to study fibre cell Ca^{2+} signalling have been carried out using either isolated fibre preparations (Srivastava et al., 1997) or lentoid bodies (Churchill and Louis, 2002). However, recent research carried out in the Duncan lab has been able to study dynamic changes in $[Ca^{2+}]_i$ in fibre cells in the intact lens (Hu et al., 2008). By confocal microscopy of fibre cells injected with the cell impermeant Ca^{2+} indicator Fluo4, it was found that cortical fibre cells in intact rat lenses respond to both adrenergic and purinergic GPCR agonists with biphasic Ca^{2+} responses. These findings show that far from being a region of quiescent cells, passively under the control and regulation of the overlying epithelium, the outermost layers of cortical fibres have an independent and active Ca^{2+} tool kit of their own. The intracellular Ca^{2+} dynamics of human cortical fibre cells is also being studied using these techniques. Fig. 3 shows the relatively large changes in $[Ca^{2+}]$i that were observed when the extracellular Ca^{2+} concentration was changed from 1 mM to zero. In contrast to this, in epithelial cells no change in baseline $[Ca^{2+}]$ was seen when changing the extracellular Ca^{2+} concentration (Williams et al., 2001). This is interesting as it suggests that human fibre cells have a substantial inward "leak" current which may be of significance when considering Ca^{2+} increases in ageing and cataract.

4. Conclusions and future directions

The foundations laid down by George Duncan and others have firmly established Ca^{2+} at the heart of our ideas about cataract formation, and Ca^{2+} homeostasis and signalling are now key disciplines in lens research, due in large part to the research of the Duncan lab. In addition, excellent studies from other labs on PMCA and NCX in the lens have shown that these processes are not only fundamental to lens homeostasis, but may also be important in cataract development and remain an important area for future research. Although, numerous Ca^{2+} mobilising receptors have been identified, much remains to be discovered about how Ca^{2+} enters lens cells or if alterations in this process are factors in cataract formation. There have recently been major advances in the study of SOCE and a comprehensive analysis, in both normal and cataractous lenses, of the novel pathways that mediate this route for Ca^{2+} entry is needed. Since the development of new techniques that make it possible to investigate Ca^{2+} dynamics in all cell types of the intact lens, research in this area of lens physiology has entered a new and exciting phase. A detailed knowledge of Ca^{2+} metabolism in normal lens and how this changes in cataract is a future goal which will not only be instructive in determining the role of Ca^{2+} in cataract but also in the development of future strategies for treatment and prevention of this disease.

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