Calcium – An "All-round Player" in the Cornea

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The cornea is an essential light-transmitting component of the visual system. Corneal pathologies are therefore often associated with impaired vision due to loss of its transparency. The involvement of calcium ions in these processes was traditionally associated with their appearance as different mineral precipitates (e.g., band keratopathy), especially in the corneal stroma. During the last decade calcium has been the focus of tremendous interest and research efforts as the result of accumulated knowledge on its major role in several intracellular signaling pathways. These studies pointed out the importance of Ca^{2+} mediated signaling in a large number of pathophysiological processes. Recent studies report similar findings in the cornea, suggesting a potential role for Ca^{2+} signals in various corneal diseases and in their treatment. A better understanding of the mechanisms underlying these signals might therefore shed new light on intracellular processes influencing visual acuity.

The cornea

The cornea is a transparent tissue that comprises the anterior one-sixth of the external layer of the human eye and is continuous with the opaque sclera. It acts as a powerful converging lens through which light passes on its way to the retina [1].

The cornea is composed of three cellular layers, two epithelia and a thick stroma. The superficial stratified epithelium and the deep simple squamous endothelium are separated from the stroma by their basement membranes, termed Bowman's and Descement's respectively. The stroma comprises about 90% of the corneal thickness and lacks vascular supply. It contains multiple layers of parallel collagen bundles and glucosaminoglycans, which are synthesized by fibroblasts (keratocytes). Bundles in successive layers are organized at right angles to each other [1].

Calcium signals

Ca^{2+} ions play a central role in intracellular signal transduction pathways, mediating a wide range of cellular processes that may be tissue specific (e.g., synaptic transmitter release [2]) or generic (e.g., programmed cell death [3]). To mediate such diverse functions (sometimes in the same cell), Ca^{2+} signals have to be organized both spatially and temporally, creating a calcium-coding system.

Intracellular Ca^{2+} signals are defined as stimulus-dependent changes in the cytoplasmic Ca^{2+} concentration. Cells have two optional sources of Ca^{2+} for generating such signals: a practically infinite extracellular compartment, and a more limited intracellular store located in different organelles such as the endoplasmic reticulum [4] and the mitochondria [4,5].

Ca^{2+} signaling in excitable as well as non-excitable cells usually occurs due to stimulation by various factors. These stimuli trigger a cascade of events, resulting in increased cytosolic inositol 1,4,5-trisphosphate (IP_3) levels, which in turn induce Ca^{2+} release from intracellular pools. Two types of intracellular ligand-gated Ca^{2+} channels activated by ryanodine and inositol-phosphate mediate Ca^{2+}-release from these internal pools [6].

At the whole-cell level, Ca^{2+} fluxes present as a series of repetitive increases in $[Ca^{2+}]in$ (spikes) or Ca^{2+} oscillations. These oscillations are believed to be a central mechanism in Ca^{2+} signaling, supplying an effective means of "information coding." Oscillations may encode information by two modulation types [7]:

- Amplitude modulation – the translation of an analogue signal into changes in the amplitude of a reference signal. The frequency of the signal remains constant.
- Frequency modulation – the translation of analogue signals into changes in the frequency of a reference signal, while the signal amplitude remains constant.

The signaling system is organized in a hierarchial manner. The *elementary* event is a Ca^{2+} signal produced by a localized group of functionally linked channels [8,9]. Such an event results in a *spark*, a local $[Ca^{2+}]$ elevation, which may propagate across the cell in a regenerative manner producing a calcium *wave* [8].

$[Ca^{2+}]in$ = intracellular calcium concentrations

Ca^{2+} oscillations are believed to constitute a significant component of the cellular signaling system. Nevertheless, the full range of physiological effects associated with Ca^{2+} oscillations has yet to be revealed.

Calcium in the cornea

A large number of mediators influence the function of both corneal epithelium and endothelium. Many of these mediators exert their action by altering $[Ca^{2+}]in$. The intracellular dynamics of Ca^{2+} are therefore of major importance in the understanding of signaling processes within and between adjacent cells [10]. To monitor these signaling mechanisms one might use a laser scanning confocal microscope able to image changing Ca^{2+} concentrations as a function of time [Figure 1].

Factors that affect intracellular Ca^{2+} signaling may be "physiological" – involved in homeostasis, or "pathological" – which result from various insults. So far, research on different corneal models (especially bovine, rabbit and human) has supplied circumstantial evidence for the involvement of Ca^{2+} signals in two basic cellular functions:

● Wound healing – a complex process that comprises an initial inflammatory reaction followed by reorganization, cell growth and migration. In epithelial injuries, concomitant mitotic activity is an additional crucial component [10–13].

● Regulation of ion and fluid transport – the primary physiological activity of the cornea that is performed mainly by the endothelium. This regulation enables adjustment to changing external conditions. It may be demonstrated by exposure to a hypotonic environment, causing corneal swelling. Upon exposure, corneal cells perform a regulatory volume decrease intended to restore original cell volume. RVD has been characterized in the corneal epithelium and was shown to involve Ca^{2+}-induced Ca^{2+} release initiated by ryanodine receptors from intracellular stores [10,11, 14,15].

Calcium and the corneal physiology
Calcium in the corneal epithelium

The most superficial layer of the cornea is of ectodermal origin and is continuous with the epithelium of the bulbar conjunctiva. It is the major light-refracting component of the eye and serves as an anatomic barrier. The CEP consists of five to seven layers of extremely accurately arranged non-keratinized epithelial cells. The germinative stem cells in its periphery (the limbus) are responsible for its regenerative capabilities. The overall thickness of the epithelium is approximately 40–50 μm.

The superficial layers of the CEP, which are primarily involved in the tissue's protective function, contain large quantities of tight junctions. Metabolically, these cells are relatively quiescent. In deeper layers both metabolic activity and
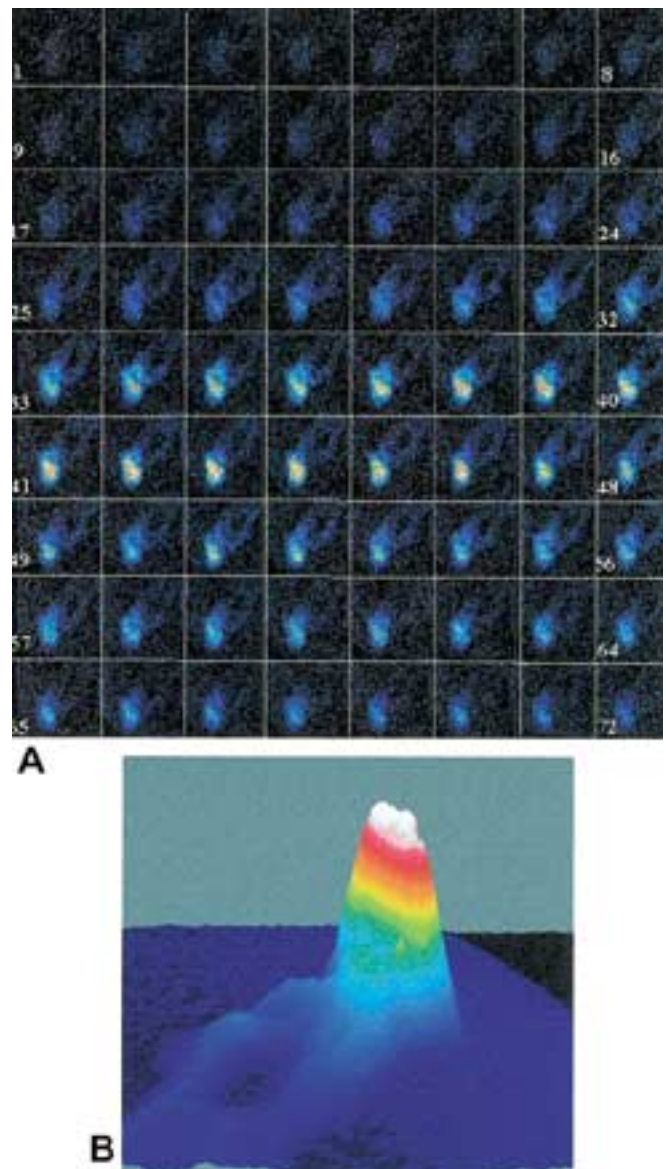
RVD = regulatory volume decrease
CEP = corneal epithelium

Figure 1. Intracellular Ca^{2+} distribution in the corneal endothelium. **[A]** Confocal images of a cell, performed at 72 depth planes about 0.5 μm apart. **[B]** Pseudo-3D reconstruction of the 2D images shown above (height and color represent Ca^{2+} concentration)

intercellular communication gradually increase. Regulation of the CEP function involves a variety of substances, most of which mediate their effect by means of intracellular Ca^{2+} signals. The cellular responses generated by these materials are summarized in Table 1.

Muscarinic and adrenergic agonists

Cholinergic agonists are used for the treatment of glaucoma and for pupil contraction during ophthalmic surgery. Muscarinic agonists (e.g., carbachol) increase $[Ca^{2+}]in$ through both intracellular and extracellular sources. The influx from the membrane occurs through L-type Ca^{2+} channels. It was shown

that bovine CEP possess at least two types of muscarinic receptors – M1 and M2, which exert their function by stimulating the phosphatidyl-inositol pathway and by inhibiting cAMP production respectively [16]. α1- and β-adrenergic receptors influence [Ca^{2+}]in by stimulating cAMP production. In this case, Ca^{2+} influx occurs exclusively from the extracellular medium [17].

pH

The homeostasis of intracellular pH is a central factor in cellular function. Interactions of intracellular pH and intracellular Ca^{2+} are therefore of substantial importance. [Ca^{2+}]in and pH are known to influence CEP intercellular communication by facilitating gap junctions [18]. Recent studies on rabbit CEP revealed that elevation of pH leads to increased [Ca^{2+}]in, whereas acidification of the cell leads to only minor changes in [Ca^{2+}]in. This low pH tolerance may represent an adaptive mechanism designed to protect the CEP from conditions of local acidosis such as during prolonged eye closure [19].

Histamine

Histamine is a major inflammatory mediator responsible for a substantial part of the early cellular response to environmental irritations. Human CEP cells express H1 receptors, which increase phosphatidyl-inositol levels and [Ca^{2+}]in. Histamine stimulates various cell types to release inflammatory cytokines, especially interleukin-1, IL-6 and granulocyte macrophage colony-stimulating factor [12].

Bradykinin and endothelin

Bradykinin and endothelin are released during the later stages of the inflammatory reaction. Both stimulate CEP intracellular Ca^{2+} dynamics by activating specific receptors (human CEP express the B2-receptor subtype). Both substances appear to be primarily responsible for stimulating cell proliferation, thereby contributing to wound-healing processes. They appear not to be involved in the release of cytokines [13,20,21].

Platelet-activating factor and platelet-derived growth factor

PAF and PDGF are endogenous factors mediating inflammation and wound healing respectively. PAF receptors are known to affect intracellular Ca^{2+} in different tissues [22]. In the rabbit CEP, the PAF-induced Ca^{2+} signal seems to induce cyclo-oxygenase-2 gene expression [23]. Rabbit CEP expresses PDGF receptors of the β-subtype, apparently stimulating Ca^{2+} release from intracellular stores [24].

Local anesthetics

Local anesthetic agents like tetracaine, proparacaine and cocaine affect both Ca^{2+} regulating systems and mitochondrial function. After application of a local anesthetic agent, an

Table 1. Effects of endogenous mediators on the corneal epithelium and endothelium

Mediator	Effect on epithelial function
Histamine	Induces release of inflammatory cytokines
Bradykinin	Stimulates cell proliferation
Endothelin	Stimulates cell proliferation
PAF	Induces cyclooxygenase-2 gene expression

Mediator	Effect on endothelial calcium dynamics
Histamine	Biphasic response: transient [Ca^{2+}]in increase followed
Purine derivatives	by either a sustained elevation or by [Ca^{2+}]in oscillations
Bradykinin	Monophasic response:
Endothelin	Transient increase in [Ca^{2+}]in

increase in [Ca^{2+}]in is observed. This reduces the mitochondrial membrane potential, finally causing cytotoxic effects [25].

Combined use of corticosteroids and beta-blockers

Clinical reports suggest that combined treatment of corticosteroids (dexamethasone or prednisolone) and beta-blockers in patients with underlying epithelial defects might cause calcium phosphate deposits in the superficial stroma [26].

Sodium dodecyl sulfate

Sodium dodecyl sulfate is an anionic detergent used in different products (e.g., hair shampoo), which affects the integrity of the CEP. When applied to the corneal surface, it increases [Ca^{2+}]in inside the CEP by a factor of 2.5, subsequently causing intracellular acidification and cytotoxicity. The mobilization of Ca^{2+} originates primarily from intracellular stores [27,28]

Calcium in the corneal endothelium

The CEN consists of approximately 0.5 million hexagonal cells of neural crest origin. The cells have a uniform size (20 μm width, 5 μm thickness), and are cuboidal in shape. They have virtually no regenerative capability, leading to various deformations (regarding size and layer spacing) in old age. Resting [Ca^{2+}]in human CEN ranges from 30 to 100 nM [10].

The primary function of the corneal endothelium (together with the epithelium) is the regulation of the stromal tendency to absorb water. This is achieved by restricting the rapid movement of electrolytes (and subsequently water) from the neighboring anterior chamber to the cornea, thereby keeping the cornea both transparent and at a constant thickness. Two mechanisms are involved in carrying out this function:

- Tight junctions between adjacent cells form an anatomical barrier to the water's tendency to diffuse into the stroma. Perfusion with a Ca^{2+}-free medium leads to disassembly of these junctional complexes resulting in corneal swelling. Gap junctions, involved in intercellular communication rather than in barrier functions, seem to be insensitive to this manipulation [29]. While growing in a primary culture, this

IL = interleukin

PAF = platelet-activating factor
PDGF = platelet-derived growth factor
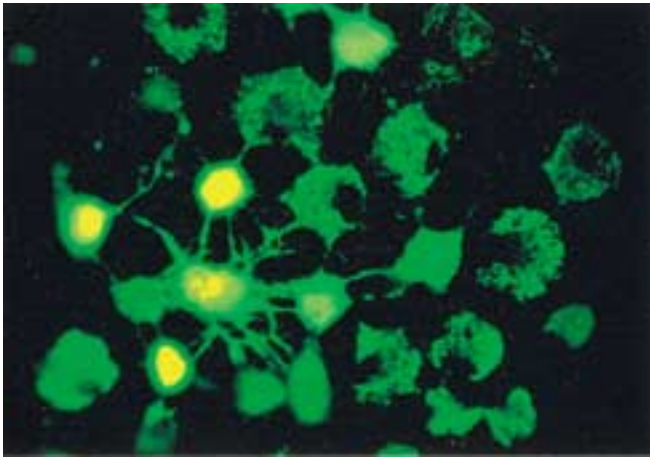CEN = corneal endothelium

Figure 2. Ca^{2+} in corneal endothelial cells in a primary culture (fluorescence microscope photography)

intercellular communication is established through intercellular connections [Figure 2].

- Endothelial pumps carry out active transport of ions and water from the stroma. The maximal pumping activity of an intact cornea was measured to be 6.7 $L/cm^2/hour$ against normal intraocular pressure. The molecular mechanism seems to involve bicarbonate pumps, probably in association with Na-K-ATPases found in large quantities in the cell membrane.

CEN cells regulate their activity by means of specific membrane receptors that usually resemble their counterparts on the epithelium [Table 1]. The presence of purinergic (type 1 and type 2), muscarinic and adrenergic receptors as well as receptors for histamine, prostaglandin E2, ANF, PAF and endothelin-1 on the CEN has been observed experimentally [11].

Histamine

Histamine modulates intracellular Ca^{2+} dynamics in a mechanism similar to that described in the epithelium. Cells exposed to histamine exhibit a biphasic response:

- A prompt and rapid increase in $[Ca^{2+}]$in.
- A sustained phase, presenting one of two distinct patterns:
  a) a plateau phase of $[Ca^{2+}]$in above baseline level, and
  b) Repeated oscillations in $[Ca^{2+}]$in.

The primary response is not influenced by the presence of a Ca^{2+}-free medium, hence reflecting release from internal stores. The second phase in either case persists throughout the agonist application period but ceases immediately after its removal. The mechanism underlying these different patterns has not yet been elucidated.

As mentioned earlier, the Ca^{2+} oscillations are of particular interest as a mechanism for intracellular signaling. Further research on this phenomenon revealed that Ca^{2+} oscillations predominate during exposure to relatively low histamine concentrations (around 1 μM). Application of higher concentrations (within the above-mentioned "oscillatory range") increases the amplitude rather than the oscillation frequency,

implying amplitude modulation of the calcium signal. The extracellular Ca^{2+} probably serves as a reservoir for refilling intracellular stores. It is *not* directly required for generating oscillations.

It should be noted that histamine influences Ca^{2+} dynamics in several other epithelial cell types as well. It has been shown to affect human non-pigmented epithelium, bovine lens epithelium and human vascular endothelium, the latter performing cell signaling by means of frequency modulation of Ca^{2+} signals [10,30].

Bradykinin and endothelin-1

Application of bradykinin or endothelin-1 results in a transient increase in $[Ca^{2+}]$in that originates exclusively from intracellular pools. The CEN seems to be extremely sensitive to bradykinin; 1 nM elicits a nearly maximal response. Contrary to the response to histamine, these receptors undergo desensitization during prolonged agonist application.

Although the effects of bradykinin and endothelin-1 on $[Ca^{2+}]$ are of a transient nature, the cellular response might be long term. This may be explained by the fact that distal effectors of the signal transduction cascade (e.g., protein kinase C) may remain activated well beyond the transient Ca^{2+} elevation phase [10].

Purine derivatives

Different purine derivatives modulate CEN function through P2 receptors present on membranes of various fluid-transporting epithelia. Their main functions include modulation of K^+ and Cl^- channels, Na^+/K^+-ATPases and L-type Ca^{2+} channels. The P2 receptors expressed on CEN are of the P2Y-subtype, a metabotropic receptor family, which is further divided into P2y and P2u subtypes.

The most important P2 agonist is ATP, which acts on both receptor subtypes in a non-selective manner. Therefore, P2 receptors are likely to be activated during cell injury (which often causes ATP release into the extracellular medium). ATP, ADP, UTP and msATP elicit a biphasic response in which the initial transient peak is followed by a sustained steady state of elevated $[Ca^{2+}]$in or by Ca^{2+} oscillations. As described earlier, Ca^{2+} influx from extracellular sources becomes significant only during the second phase of the cellular response, while Ca^{2+} from internal pools dominates the first phase.

The cellular response mediated by P2 receptors resembles in many aspects the response observed during activation of histamine receptors [10]. Follow-up studies revealed that there is no mutual (heterologous) activation of these receptors. Nevertheless, both receptor types are indeed coupled to the same intracellular IP3-sensitive Ca^{2+} stores [11].

Cholinergic agonists

Clinical reports suggest that cholinergic agonists have variable effects on the CEN (their mechanisms of action resemble those described for the CEP):

- Acetylcholine seems to have a stimulating effect on CEN function by increasing fluid pumping activity.
- Carbachol might decrease ionic mobilization and cause corneal swelling [31].

Earlier research did not show any effect of carbachol on the intracellular Ca^{2+} dynamics [30].

Topical anesthetics

Preliminary reports link abuse of topical anesthetics like oxybuprocaine to decreased integrity of apical junctions between endothelial cells [32].

Calcium channel blockers

Ca^{2+} channel blockers are used for experimental purposes to elucidate the corneal function. Since some of these substances influence the endothelial Ca^{2+} levels, they may cause corneal edema and loss of transparency. While agents like verapamil, diltiazem, nifedipine and Cd^{2+} clearly diminish corneal function, other agents, e.g., Ni^{2+}, seem to have no effect on the cornea. These results suggest that CEN express predominantly L-type Ca^{2+} channels. In this case, the impaired endothelial function seems to reflect altered apical junction permeability [33].

Calcium and the corneal pathophysiology

Some pathophysiological processes reflect impaired Ca^{2+} homeostasis at either the extracellular or intracellular levels:

Pathologies associated with extracellular calcium

Elevated extracellular Ca^{2+} concentrations, occurring in various physiological and metabolic disorders, may result in depositions of Ca^{2+} in the cornea. These depositions are found primarily in and around Bowman's membrane. They usually display a distinct band-like pattern, though diffuse calcifications might occur as well. This condition is sometimes accompanied by conjunctival calcifications.

Band keratopathy

This is the most common corneal disorder associated with deposition of minerals. It is characterized by a band-form deposition of Ca^{2+} salts, primarily hydroxyapatite, in Bowman's membrane and the superficial stroma. The depositions develop initially in the peripheral part of the interpalpebral fissure and are limited to the cornea, leaving the neighboring limbus unaffected.

Band keratopathy is the outcome of a variety of pathological conditions that elevate Ca^{2+} and phosphate levels in the blood or interstitial fluids beyond their precipitation threshold. This is caused either by directly increasing $[Ca^{2+}]/[P^{4+}]$ or by an indirect increase triggered by pH elevation or evaporation. The most common conditions associated with band keratopathy include:

- Ocular degenerative diseases – chronic uveitis, phthisis bulbi, absolute glaucoma, juvenile rheumatoid arthritis, interstitial keratitis.
- Trauma – exposure to different chemicals.
- Hypercalcemia – hyperparathyroidism, vitamin D intoxication, renal failure, etc. [1].

Topical application of steroid-phosphate preparations or of multiple eye drops was reported as another possible factor for the development of band keratopthy [34].

During early phases, the peripheral depositions do not impair vision. In later phases, if untreated, the keratopathy might expand and interfere with the visual axis. The treatment of band keratopathy consists of surgical removal of the epithelium and subsequent application of EDTA (a Ca^{2+} chelator) on the underlying Bowman's membrane [1].

Calcareous degeneration

This is a rare form of corneal calcification characterized by depositions involving the full thickness of the cornea. This type of degeneration is usually associated with severely injured eyes or with necrotic neoplasms [1,35].

Alkali injury

Lime ($Ca(OH)_2$) and its derivatives are a common cause of ocular burns and might cause corneal Ca^{2+} precipitates. These depositions are the result of a defensive reaction of the CEP intended to minimize the penetration of such substances into the eye. This reaction produces Ca^{2+} soaps that precipitate in the cornea and cause corneal opacities. Immediate treatment is irrigation of the burn with liquid to prevent further damage. Thereafter starts a long-term treatment intended to stimulate epithelial regeneration and prevent inflammation and other complications [1,36].

Pathophysiologic processes associated with intracellular calcium

Pathologies associated with intracellular Ca^{2+} dynamics, whether injury or disease, affect primarily the metabolically active layers, i.e., the epithelium and the endothelium, the latter being particularly prone to insults. The most prominent clinical manifestation of these pathologies is corneal edema. This state evolves due to loss of the cells' barrier function and interference with their active pumping mechanisms, resulting in loss of transparency and impaired vision. Corneal edemas may be divided into two distinct forms:

- Stromal edema – usually the first evidence of endothelial or epithelial injury. The swelling of the stroma is directed inward, leaving no evidence outside the eye. Clinically, stromal edema usually precedes epithelial edema.
- Epithelial edema – caused by increased permeability of the epithelial cells that results in intracellular swelling. This type of edema usually leads to impaired vision and patient discomfort. It should be mentioned that this condition is also dependent on the intraocular pressure, as fluid will accumulate only when this pressure exceeds the endothelial pumping pressure [1].

Conclusions

Ca^{2+} ions seem to play a major role in various aspects of the corneal pathophysiology. While extracellular Ca^{2+} depositions are well associated with certain pathological states and are reasonably well understood, intracellular Ca^{2+} dynamics and its relation to the pathophysiology of the cornea are still in early phases of research. The exploration of these mechanisms might contribute substantially to our understanding of the corneal function in health and disease.

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