The Influence of Calcium on the Rabbit Lens Sodium Pump

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Purpose. Experiments were conducted to examine how elevation of calcium in the lens impairs the lens sodium pump.

Methods. Rabbit lenses were incubated in the presence or absence of calcium ionophore A23187. ^{86}Rb uptake by the intact lens was measured as an index of sodium pump activity. Na,K-ATPase (ouabain-sensitive adenosine triphosphate [ATP] hydrolysis) activity was determined in membrane material isolated from the lens. Lens ion content and ATP content also were determined.

Results. Rabbit lenses gained calcium after exposure to calcium ionophore A23187 or ionomycin. Ionophore-treated lenses also gained sodium and lost potassium. A diminished rate of ^{86}Rb uptake observed in ionophore-treated lenses suggests that elevation of lens calcium leads to sodium pump inhibition. In contrast, the rate of ^{86}Rb efflux was not altered by A23187, indicating that elevated lens calcium causes little change in passive cation permeability. Membranes isolated from A23187-treated lenses were found to have normal Na,K-ATPase activity. However, calcium had a small direct inhibitory effect upon the Na,K-ATPase activity measured in freshly prepared lens membranes isolated from control (nonionophore-treated) lenses. Using a luciferase assay, A23187-treated lenses were found to have a normal ATP content.

Conclusions. Calcium may impair the ability of the lens Na,K-ATPase to pump ions in the intact lens, but appears to leave the ATP-hydrolyzing capability of the isolated enzyme unchanged. Invest Ophthalmol Vis Sci 1993;34:405–412.

In human senile cataracts, the concentration of sodium within the lens almost certainly will be elevated if there is opacification of the lens cortex.[1,2] In fact, the more severe the cortical opacity, the more pronounced is the increase in lens sodium. Opaque lenses with high sodium levels also will generally exhibit a diminished potassium content and an elevated calcium content.[3] These changes in electrolyte composition are deleterious to the lens and contribute to the loss of cortical transparency. For example, the osmotic disturbance resulting from the altered sodium and potassium levels causes the lens to accumulate water, leading to cell swelling and damage. Protein synthesis patterns also may be disturbed by altered lens sodium and potassium levels.[4] In addition, calcium may be linked directly to the mechanisms of opacification.[5,6]

Lens sodium is maintained at a low concentration compared to the concentration in aqueous humor. Sodium that leaks into the lens presumably is pumped out by the sodium pump, the Na,K-ATPase. The so-

dium pump transports potassium in the opposite direction, maintaining a high lens potassium concentration as it balances the continual passive outward potassium leak. In human cortical cataract, the lens sodium and potassium pump-leak balance fails; the Na,K-ATPase is no longer capable of keeping pace with the ion leaks. However, studies of Na,K-ATPase activity in human cataract have produced conflicting results, some suggesting an increased enzyme activity[7] and some a decreased activity.[8,9] Based on our own studies,[10] we believe the Na,K-ATPase activity may be unchanged in the cataractous lens.

Our working hypothesis is that although the cataractous lens may have adequate Na,K-ATPase enzyme, the lens is unable to pump sodium and potassium at a rate high enough to maintain ionic equilibrium. Perhaps conditions within the cataractous lens impair the sodium pump. One factor that may cause such altered sodium pump activity is calcium. This divalent cation is always elevated in cataractous lenses that have a high sodium content. A direct cytotoxic effect of calcium upon the lens cation pump was proposed by Hightower and Hind,[11] who found that pump activity could be reduced by elevating lens calcium 100-fold.

The present study examined how elevation of lens calcium impairs the lens sodium pump. We performed experiments using rabbit lenses exposed to calcium ionophores that cause a gradual increase in lens calcium as the ion leaks inward. ^{86}Rb flux studies were performed to examine the sodium pump in ionophore-treated lenses that had accumulated calcium.

MATERIALS AND METHODS

Lenses were obtained from 2–3 kg albino rabbits killed with an overdose of sodium pentobarbital administered via a marginal ear vein. The procedures used in these studies conformed to the ARVO Resolution on the Use of Animals in Research. After it was dissected from the eye, each lens was immersed in 20 ml of Tyrode's solution that contained 145 mmol/l NaCl, 6 mmol/l KCl, 2.5 mmol/l CaCl$_2$, 1.2 mmol/ MgCl$_2$, 5.5 mmol/l dextrose, and 5 mmol/l N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) at pH 7.4 and 37°C.

Measurements of Lens Composition

To determine the lens sodium, potassium, calcium, and magnesium content, each lens was first blotted on moistened filter paper and then digested in 30% nitric acid. Ion concentrations in the acid digest were measured by atomic absorption spectroscopy as described previously.[12] Lens adenosine triphosphate (ATP) was measured using a luciferase bioluminescence assay kit (Sigma, St. Louis, MO). Lenses were homogenized in ice-cold buffer. The ATP concentration then was determined immediately in diluted aliquots of the homogenate.

^{86}Rb Uptake Experiments

^{86}Rb was used as a tracer for potassium. ^{86}Rb (1 μCi/ ml) was added to the Tyrode's solution for 60 min. At the end of this 1 hr uptake period, the lens was removed from the radioactive solution, gently blotted dry, weighed, and digested in 30% nitric acid. Radioactivity in the acid digest was determined by scintillation counting. Uptake data were computed as a ratio: [(counts per minute/mg lens water)/(cpm/ml medium)].

^{86}Rb Efflux Experiments

Lenses were first allowed to accumulate ^{86}Rb under control conditions for 20 hr. Radioactive lenses then were placed in a large volume of nonradioactive Tyrode's solution for 100 min to allow the complete washout of ^{86}Rb from extracellular space. Each lens then was transferred to an efflux chamber, described in detail earlier.[13] Every 10 min, the Tyrode's solution in the efflux chamber was replaced, forcing out the original solution that was collected. Radioactivity within the displaced solution was counted to determine the ^{86}Rb lost by the lens during that period. The ^{86}Rb efflux rate constant was computed as described by Delamere and Duncan.[14]

Measurements of Na,K-ATPase Activity

Na,K-ATPase activity was measured in a partially purified membrane-enriched preparation obtained from fresh (nonincubated) lenses or from lenses incubated in the presence of calcium ionophore for a specified time period. Lens material was homogenized in an ice-cold buffer that contained 0.3 mol/l sucrose, 10 mmol/l HEPES-HCl, and 2 mmol/l dithiothreitol at pH 7.4. As described earlier,[15] the membrane-enriched preparation was obtained using a three-stage differential centrifugation procedure. The protein content of the membrane material was determined using the Peterson[16] modification of the Lowry method.[17]

Na,K-ATPase activity was determined by measuring the rate of hydrolysis of (τ-^{32}P) ATP by lens membrane material in the presence and absence of 10^{-3} mol/l ouabain. Lens membrane material was incubated for 20 min at 37°C in a solution containing 100 mmol/l NaCl, 10 mmol/l KCl, 3 mmol/l MgCl$_2$, 40 mmol/l histidine-HCl, 11 μg/ml alamethicin, and 5 mmol/l ATP at a pH of 7.4, buffered with CaCl$_2$-EGTA to a specified free calcium concentration.[15] ATP hydrolysis was determined by extracting liberated ^{32}P as a phosphomolybdate complex that was extracted into benzene isobutyl alcohol (1:1, volume:volume) and quantified by scintillation counting.[15]

RESULTS

The Influence of A23187 On Lens Ion Composition

Rabbit lenses were incubated for periods up to 24 hr in the presence and absence of 5 μmol/l A23187, a calcium ionophore. Lenses exposed to A23187 gained calcium (Fig. 1A). Over a period of 24 hr, the lens calcium content was increased to 2.50 ± 0.13 mmol/ kg lens water from a control value of 0.35 ± 0.5 (mean ± SE, n = 4). Lenses incubated in the presence of 5 μmol/l A23187 also gained sodium and lost potassium (Fig. 1B). After 24 hr, the sodium content of A23187-treated lenses increased from 13.3 ± 0.3 to 93.5 ± 2.0 mmol/kg lens water, whereas the potassium content diminished from 132.6 ± 0.7 to 79.6 ± 1.4 (mean ± SE, n = 4).

In some experiments, lenses were exposed to 5 μmol/l A23187 for 1 hr and incubated an additional 19 hr in control Tyrode's solution. These lenses also manifested an elevated sodium and calcium content together with a reduced potassium content. In these
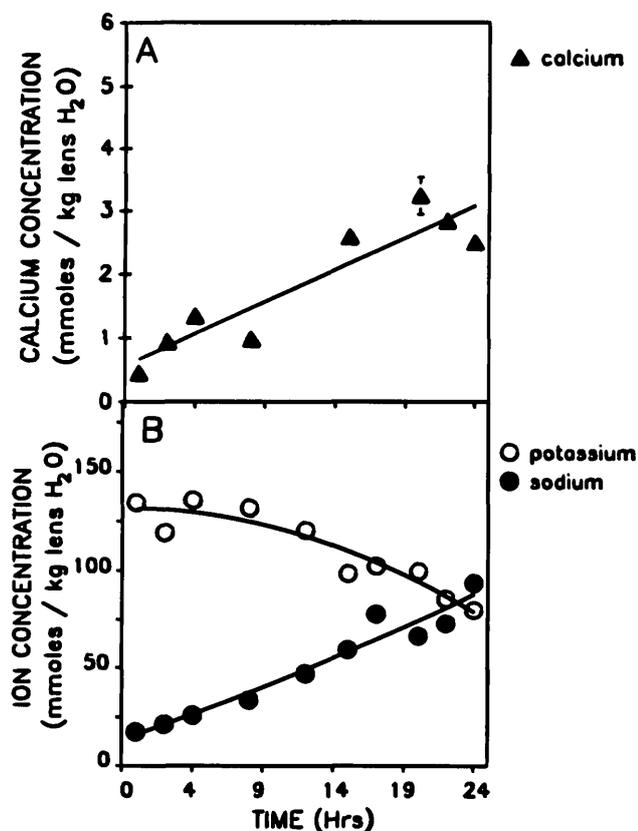

FIGURE 1. The calcium content (A) and sodium and potassium content (B) of rabbit lenses incubated in the presence of 5 μmol/l calcium ionophore A23187. The horizontal axis indicates the incubation time. Each point represents the means of data from four lenses. The standard error is indicated by a vertical bar. (For most points, the error bar is smaller than the symbol.)

lenses, the sodium, potassium, and calcium was 34.6 ± 2.6, 120.7 ± 0.3, and 0.71 ± 0.19 mmol/kg lens water, respectively (mean ± SE, n = 4).

To test whether the lens responds similarly to another calcium ionophore, we measured the electrolyte composition of lenses incubated in the presence of 5 μmol/l ionomycin. The calcium gain observed in lenses cultured 20 hr in the presence of ionomycin was greater than that measured in A23187-treated lenses (Fig. 2A). Five micromolar ionomycin also caused a greater elevation of lens sodium and loss of lens potassium than 5 μmol/l A23187 (Fig. 2B).

Distribution of Accumulated Calcium

Experiments were performed to examine where accumulated calcium is distributed within the cell mass of A23187-treated lenses. Lenses were incubated for 24 hr in Tyrode's solution that contained ^{45}CaCl$_2$ (0.5 μCi/ml) in the presence or absence of 5 μmol/l A23187. At the end of the incubation period, each lens was rinsed three times in nonradioactive Tyrode's solution and gently blotted on moistened filter paper. The capsule/epithelium then was removed from each lens. The lens fiber mass from each lens was frozen in liquid nitrogen, and a 6 mm anterior/posterior core was removed using a motor-driven trephine. The core then was sliced into six anterior/posterior sections. ^{45}Ca determined in the epithelium/capsule and the six anterior/posterior sections is shown in Figure 3. In lenses incubated for 24 hr with no A23187, ^{45}Ca was detected almost exclusively in the capsule/epithelium. Much of this radioactive calcium probably is located extracellularly. In lenses exposed to 5 μmol/l A23187, considerable amounts of ^{45}Ca were observed in the capsule/epithelium. Lesser amounts of ^{45}Ca were detected in the six anterior/posterior sections. As expected, material from the lens nucleus (sections 3 and 4) contained the least amount of accumulated calcium.

The Influence of A23187 On ^{86}Rb Uptake

^{86}Rb was used as a tracer to examine the inward movement of potassium into the lens. Lenses were incubated for specified times in the presence or absence of 5 μmol/l A23187. ^{86}Rb (1 μCi/ml) was added to the incubation solution during the final 60 min of the incubation period. Lenses exposed to A23187 had a significantly reduced ability to accumulate ^{86}Rb (Fig. 4). After only 2 hr in the presence of 5 μmol/l A23187, the lens ^{86}Rb uptake rate was diminished by 50%. The lens ^{86}Rb uptake capacity was reduced further during continued exposure to A23187.

Na,K-ATPase Activity in A23187-Treated Lenses

We performed experiments to test whether a loss of Na,K-ATPase caused the decreased ^{86}Rb uptake that
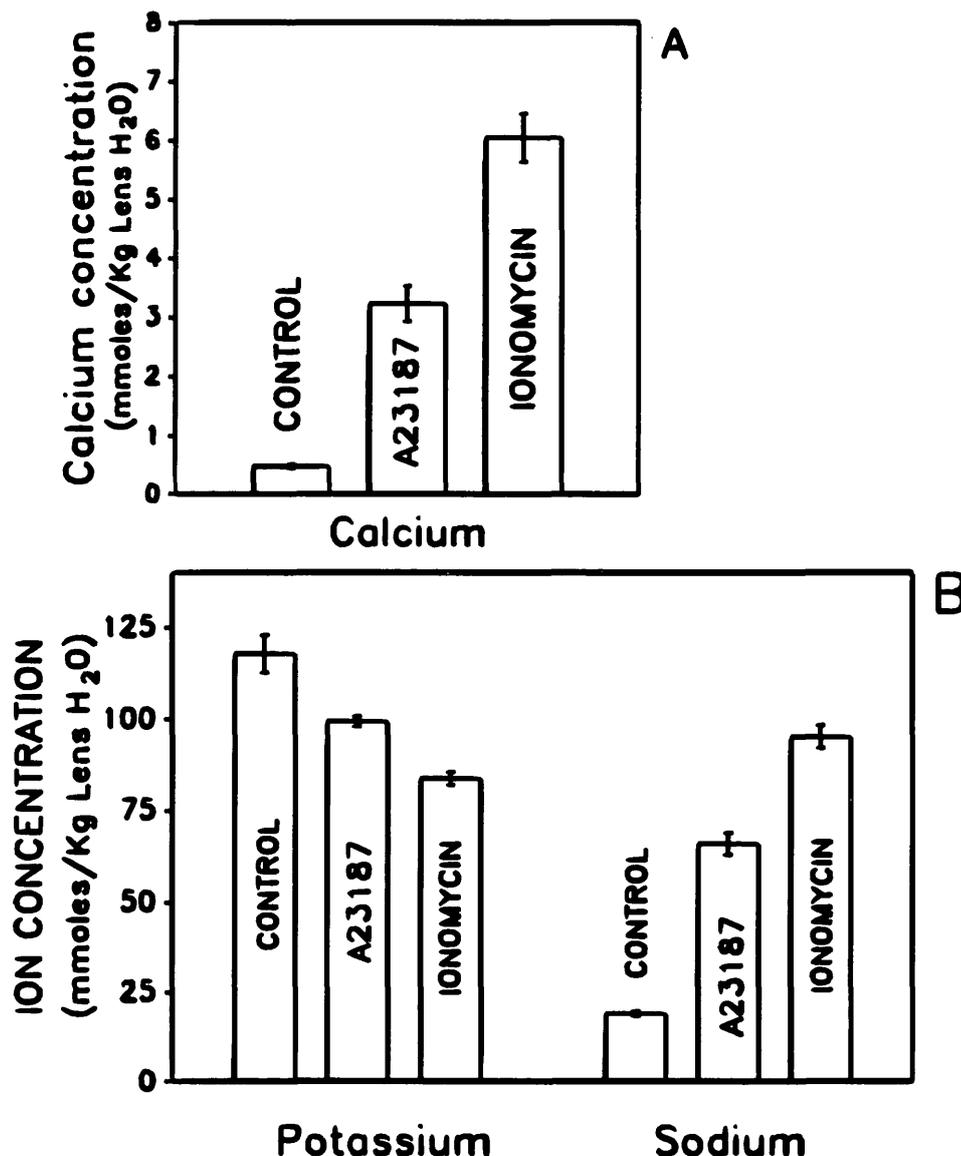
FIGURE 2. A comparison of the effects of A23187 and ionomycin showing the calcium content (A) and sodium and potassium content (B) of rabbit lenses incubated for 20 hr in the presence or absence (control) of 5 μmol/l A23187 or 5 μml/l ionomycin. Each bar represents the mean $\pm$ SE of results for four lenses.

we observed in lenses that gained calcium after A23187 treatment. Lenses were incubated for 24 hr in the presence or absence of 5 μmol/l A23187. At the end of the incubation period, membrane-enriched microsomal material was prepared from each lens. Each membrane-enriched microsomal preparation was assayed for Na,K-ATPase activity in the presence of a 10^{-8} mol/l free calcium concentration. The Na,K-ATPase activity determined in A23187-treated lens material was 643 $\pm$ 77 nM ATP hydrolyzed/hr/mg protein (mean $\pm$ SE, n = 7 lenses). This enzyme activity was not significantly different (P = 0.44) from the value of 580 $\pm$ 19 determined in lenses incubated without A23187.

The Direct Influence of Calcium and A23187 On Lens Na,K-ATPase Activity

Experiments were conducted to test whether the inhibition of Na,K-ATPase activity resulting from the pres-

ence of a high cytoplasmic calcium concentration could account for the diminished ^{86}Rb uptake we observed in A23187-treated lenses. Membrane-enriched microsomal material was prepared from fresh, nonincubated lenses and assayed for Na,K-ATPase activity at calcium concentrations of 10^{-8}, 10^{-6}, and 10^{-4} mol/l. Although the total lens calcium content is 2–3 $\times$ 10^{-4} mol/kg lens water, normal cytoplasmic free calcium concentrations of 10^{-7}–10^{-6} mol/l have been reported in the intact lens.[3] The free calcium concentration in A23187-treated lenses is not known. As a result of calcium buffering, it is likely to be considerably lower than the total calcium content, which increases to 2–3 $\times$ 10^{-3} mol/kg lens water after 24 hr. Na,K-ATPase activity was only slightly reduced in the presence of calcium (Fig. 5). Increasing the calcium concentration by four orders of magnitude (10^{-8} to 10^{-4}) reduced Na,K-ATPase activity by less than 20%.

In separate experiments, we also verified that Na,K-ATPase activity was not altered when the enzyme
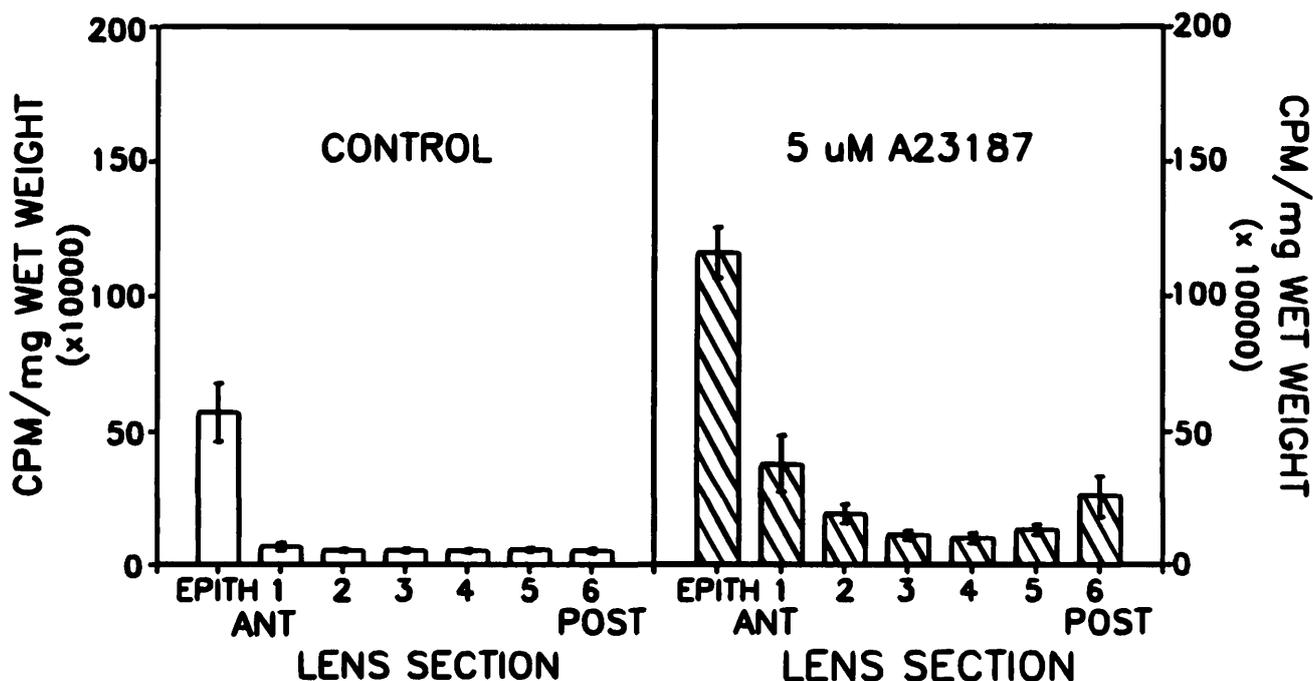
^{45}Ca in Lens Sections after 20 Hr

FIGURE 3. The distribution of ^{45}Ca^{2+} in rabbit lenses incubated for 20 hr in the presence or absence (control) of 5 μmol/l A23187. All incubation solutions contained 0.5 μCi/ml ^{45}Ca^{2+}. The capsule/epithelium (EPITH) was examined separately from six anterior-posterior sections of a 6 mm core removed from the optical axis of the lens. Each bar represents the mean ± SEM of results from eight lenses.

assay was performed in the presence of 5 μmol/l A23187. Higher concentrations of A23187 have been reported to selectively inhibit Na,K-ATPase activity.[18]
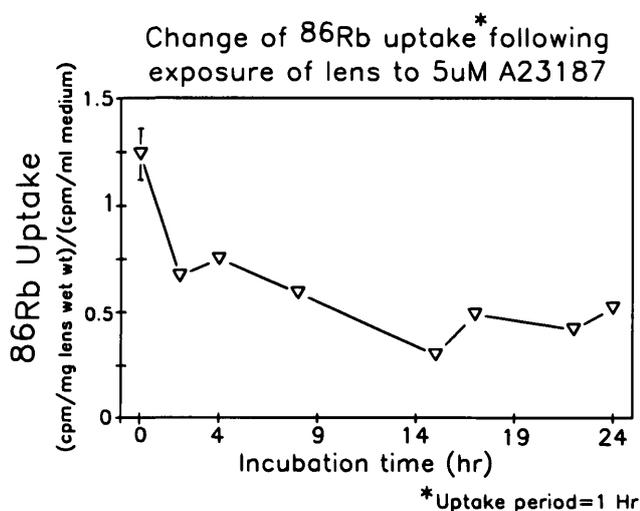

FIGURE 4. The influence of A23187 on ^{86}Rb uptake by the rabbit lens. ^{86}Rb uptake was measured over period of 1 hr. Lenses were exposed to A23187 (5 μmol/l) for the time indicated on the horizontal axis. Each point represents data from four lenses. The standard error is indicated by a vertical bar. (For most points, the error bar is smaller than the symbol.)

Measurement of ATP Level in A23187-Treated Lenses

Obviously, the Na,K-ATPase requires an adequate supply of ATP. To test whether ATP is available in the A23187-treated lens, we used a simple luciferase assay. In lenses incubated for 20 hr in the presence of 5 μmol/l A23187, the ATP content was 0.9 ± 0.2 mmol/
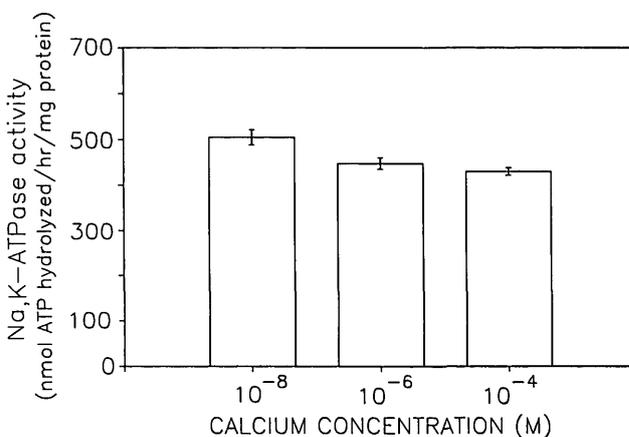

FIGURE 5. The influence of calcium on lens Na,K-ATPase activity. In the presence of 10^{-8}, 10^{-6}, or 10^{-4} mol/l calcium, Na,K-ATPase activity was determined in membrane material isolated from fresh, nonincubated rabbit lenses. Each bar represents the mean ± SE of data from four lenses.

kg lens water (mean ± SE, n = 4 lenses). This value was not significantly different from the ATP content of 1.1 ± 0.2 mmol/kg lens water determined in lenses incubated for 20 hr under control conditions. An ATP content of 1.28 ± 0.6 mmol/kg lens water was determined in fresh lenses. This value is equivalent to 312 nM of ATP per lens, which is similar to the ATP values reported recently by Winkler and Riley.[19]

The Influence of A23187 On ^{86}Rb Efflux

If monovalent cation permeability of the lens membranes were to increase, the result would be a pattern of lens sodium gain and potassium loss similar to that we observed in A23187-treated lenses. ^{86}Rb efflux experiments were performed to test whether lens potassium permeability increases as lenses gain calcium in the presence of A23187. Using lenses pre-loaded with ^{86}Rb and washed to clear extracellular label, the pattern of ^{86}Rb efflux was first established in control Tyrode's solution. Adding 5 μmol/l A23187 to the incubation solution caused no detectable change in the ^{86}Rb efflux rate (Fig. 6). Exposing the lens to 5 μmol/l A23187 for a longer time also did not significantly alter the ^{86}Rb efflux rate. The ^{86}Rb efflux constant measured in lenses incubated with 5 μmol/l A23187 for 8 hr was $4.2 ± 0.2$ min^{-1} × 10^{-4} (mean ± SE, n = 4) compared to a control value of $4.9 ± 0.8$ × 10^{-4}.

These ^{86}Rb efflux experiments suggest that 5 μmol/l A23187 may not significantly alter lens membrane potassium permeability. It also can be inferred that A23187 does not increase lens membrane sodium permeability, because such a permeability change would depolarize the lens and this too would alter the
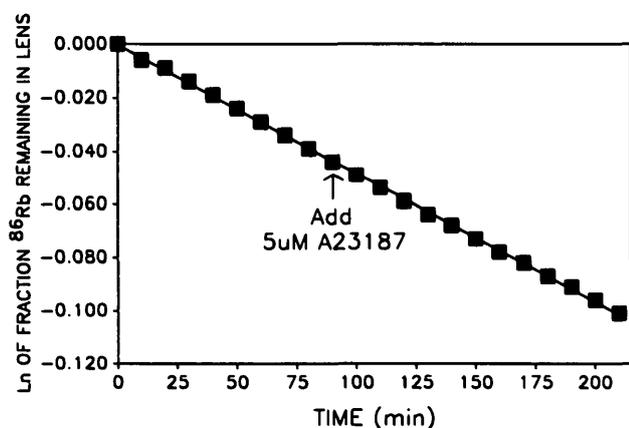

FIGURE 6. The influence of A23187 upon the rate of ^{86}Rb efflux from the rabbit lens. Before the start of the experiment, lenses were loaded with ^{86}Rb for 20 hr in control Tyrode's solution and washed for 100 min to clear label from extracellular spaces. The loss of ^{86}Rb was monitored for 90 min in control conditions. A23187 (5 μmol/l) then was added (as indicated by the arrow) for the remainder of the experiment. Each point represents a mean of data from eight lenses. (The SEM is smaller than the symbol size).

^{86}Rb efflux pattern.[14] In separate experiments, we measured the lens membrane potential and verified that there is no detectable change of membrane voltage during the first 60 min of exposure to 5 μmol/l A23187.[20]

DISCUSSION

Calcium ionophore A23187 caused the rabbit lens to progressively gain calcium. Ionomycin, another calcium ionophore, caused a slightly greater increase of lens calcium content. This may be explained on the basis that a pair of A23187 molecules, but only a single ionomycin molecule, is needed to shift one calcium ion across the plasma membrane.[21]

In the presence of calcium ionophores, lenses gained sodium and lost potassium. Both A23187 and ionomycin are relatively specific for calcium,[21] and it is unlikely that the presence of either ionophore directly brings about the sodium and potassium changes. Instead, it seems reasonable to hypothesize that calcium gained by the ionophore-treated lens makes the tissue unable to regulate its sodium and potassium composition. In cataractous human lenses and cataractous lenses from animal models, a high lens calcium level is almost always associated with a high sodium and low potassium content.[3] The same is true for rabbit lenses exposed to insults such as hydrogen peroxide, iodoacetate, or chlorpromazine.[12,22,23] Thus, a considerable amount of evidence supports the hypothesis that elevated lens calcium levels impair the sodium/potassium regulation mechanism in this tissue. The reverse is not true, at least in the rabbit lens, because sodium and potassium levels can be changed markedly by ouabain without detectable effect upon the lens calcium content.[22]

We observed a very clear reduction of ^{86}Rb uptake in A23187-treated lenses. A similar effect of A23187 upon ^{86}Rb uptake has been reported in rat brain synaptoneurosomes.[24] The reduced ^{86}Rb uptake rate indicates that in the ionophore-treated lens, the ability of the sodium pump to shift ions is impaired, perhaps because of the high lens calcium level. Such impaired sodium pump activity would produce the pattern of progressive sodium gain and potassium loss observed in A23187-treated lenses in the present study (Fig. 1). Based on our ^{86}Rb efflux experiments, it seems unlikely that increased potassium or sodium permeability contributes to the lens sodium and potassium redistribution seen in the ionophore-treated lens.

Our findings support Hightower and Hind's hypothesis that impairment of the sodium pump may be the principal cause of sodium and potassium changes in the high-calcium lens.[11] However, partially purified membranes isolated from A23187-treated lenses were found to have the same amount of Na,K-ATPase activity as membranes isolated from control lenses. In this

respect, the ionophore-treated lens resembles human cataractous lenses, which can have markedly disturbed sodium and potassium levels (indicating that the sodium pump cannot maintain ion balance), but contain normal quantities of Na,K-ATPase.[10,25]

Under conditions of elevated lens calcium, the calcium ATPase probably works at close to maximal velocity, consuming a substantial fraction of the available ATP within the lens. For this reason, and also because of the possibility that lens ATP generation may be impaired by calcium, we tested whether the ATP concentration is depleted in the A23187-treated lens. However, A23187-treated lenses were found to contain a normal amount of ATP, suggesting that active sodium pump impairment in these lenses was not likely caused by a lack of fuel.

Our experiments suggest that ionophore-treated lenses may contain normal amounts of Na,K-ATPase but that the activity of this enzyme is inhibited when lens calcium increases. Calcium can have a direct inhibitory effect upon Na,K-ATPase activity,[26] and calcium ions compete with sodium ions at the cytoplasmic side of the Na,K-ATPase.[27] However, some cells respond to A23187-induced calcium challenge with an increase of Na,K-ATPase activity.[28,29] In isolated, partially purified lens membranes, we observed that increasing the free calcium concentration by four orders of magnitude caused only a modest (15%) reduction of Na,K-ATPase activity. It was not possible for us to judge whether this degree of calcium inhibition of Na,K-ATPase in isolated membranes could fully account for the degree of sodium pump inhibition seen in lenses exposed to A23187. More or less Na,K-ATPase inhibition by calcium may take place in situ. Furthermore, we have no value for the free calcium concentration in A23187-treated lenses, where calcium buffering by cytoplasmic constituents is likely to result in a calcium activity significantly lower than the total lens calcium content detected by atomic absorption spectroscopy. It also should be recognized that other factors, such as calmodulin, might modify the calcium sensitivity of the enzyme in the intact lens. It is quite likely that the interaction of calcium ions with Na,K-ATPase in an isolated membrane preparation is different from the interaction that takes place in an intact cell.[30]

Another possibility is that sodium transport mechanisms could be impaired through the action of proteases that are known to be activated by calcium in the intact lens.[31] In addition, Garner and coworkers[32] showed there are circumstances in which ion transport and ATP hydrolysis by the Na,K-ATPase may become uncoupled.

Key Words

ATP, calcium, lens, Na,K-ATPase, ^{86}Rb uptake.

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