The Influence of Calcium-Free EGTA Solution upon Membrane Permeability in the Crystalline Lens of the Frog

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Abstract Potential difference, resistance, cation content, and $^{86}\text{Rb}^{+}$ efflux were measured in frog lenses maintained in normal or calcium-free EGTA Ringer's solution. Exposure of the lens to calcium-free solution resulted in a rapid fall in potential and resistance, together with a twofold increase in $^{86}\text{Rb}^{+}$ efflux rate. These rapid changes were not due to an alteration in cation distribution between the lens and its environment. However, the alteration in $^{86}\text{Rb}^{+}$ efflux rate could be explained on the basis of the fall in potential. These findings suggested that removal of calcium from the bathing medium caused a rapid increase in sodium permeability alone. This suggestion was substantiated by the results of experiments where the response of the lens to low calcium solution was determined in a medium in which 90% of the sodium had been replaced by sucrose.

Introduction

The lens is a transparent, avascular organ held by suspensory ligaments between the aqueous and vitreous humors of the eye. It is comprised of tightly packed cells, referred to as lens fibers, with a single layer of epithelial cells at the anterior surface. The entire lens is bounded by an acellular elastic capsule. Lenticular metabolism is directed entirely toward growth and maintenance of transparency inasmuch as the lens plays an important role in visual accommodation.

Several early investigators have shown that lens opacities occur in hypocalcemic animals (Swan and Salit, 1941; Evans and Kern, 1931), and that similar opacities develop under low calcium conditions in vitro (von Bahr, 1940). Analysis of the cation content of mammalian lenses has demonstrated that a lack of calcium in the bathing medium results in a gain of sodium and loss of potassium (Harris and Gehrsitz, 1951; Merola et al., 1960). Also, the influence of calcium levels upon tracer fluxes in the lens has been reported (Thoft and Kinoshita, 1965; Becker and Cotlier, 1965; Becker, 1962). A similar dependence of intracellular sodium and potassium levels upon external calcium has been found in a wide range of cell types (Manery, 1969). These observations have been attributed to a general increase of cell membrane permeability in low calcium environments (Manery, 1969; Thoft and Kinoshita, 1965). However, recent investigations of the behavior of HeLa cells (Morrill and Robbins, 1967),
frog skin (Curran and Gill, 1962), and frog muscle (Kimizuka and Koketsu, 1963) suggest that low calcium effects on membranes can be attributed, for the most part, to an increase of sodium permeability alone.

Several approaches have been used to investigate permeability properties of the lens. Studies in which microelectrodes have been introduced into the lens have demonstrated a potential difference of approximately \(-70\) mV between the interior of the lens fibers and the bathing solution (Paterson and Rae, 1974). This potential, referred to as the lens potential, has been shown to be independent of the position of the electrode within the lens, implying the interior of the lens to be isopotential (Duncan, 1974). This phenomenon has been attributed to a high degree of coupling between lens cells, probably via permeable gap junctions (Duncan, 1969 b; Eisenberg and Rae, 1976). The lens potential is sensitive to changes of ionic composition in the bathing medium in a manner closely predicted by the Hodgkin-Katz-Goldman equation (Duncan, 1969 a; Delamere and Duncan, 1977). Duncan (1969 b), by measuring the time-course of the lens potential depolarization in response to an increase in the external potassium concentration, showed the site of the potential determining membranes to be at the periphery of the lens.

The lens fibers, which are several millimeters long, 8-10 \(\mu\)m wide, and 2-5 \(\mu\)m thick (Kronfeld, 1969), have been shown by electron microscopy to be very tightly packed with minimal extracellular space (Cohen, 1965). In fact, Yorio and Bentley (1976) found that the extracellular space of the amphibian lens, including the acellular capsule, was equivalent to between 7 and 12% of the tissue water, and that 80% of this space was in the lens capsule. It is therefore unlikely that a microelectrode, with a tip diameter of <1 \(\mu\)m, introduced into the fiber mass of the lens will enter an extracellular region. Furthermore, inasmuch as the lens capsule is freely permeable to ions and larger molecules, it cannot be considered to contribute to the electrical characteristics of the lens.

The lens might therefore be considered as a giant cell, where lenticular cytoplasm is essentially in free communication, at least electrically, but is isolated from the extracellular bathing medium by the ion-restricting membranes of the peripheral lens cells.

This simplified model of the lens is also applicable to ion fluxes between the lens and bathing solution where ion flux rates and ion permeabilities quite accurately compare with those predicted using lenticular ion concentrations and the lens potential together with the theory of Hodgkin-Katz-Goldman (Delamere and Duncan, 1977).

The electrical resistance of the lens has been investigated by observing the change in lens potential resulting from the injection of a known current through another electrode placed within a different lens fiber. These investigations initially revealed data which agreed with the above model. The value of lens resistance, the lack of dependence of the resistance upon the position of the microelectrodes within the lens, and the dependence of resistance upon the ionic composition of the bathing solution all substantiated the hypothesis that the ion-restrictive membranes were located close to the surface of the lens fiber mass (Duncan, 1974). However, more recent studies have demonstrated the
likelihood of a finite resistance between cells in the interior of the lens, possibly the resistance of the intercellular junctions (Eisenberg and Rae, 1976; Duncan and Delamere, 1978).

In the present study, using the frog lens, we have examined changes in electrical parameters, cation content, and tracer fluxes in an attempt to better define the permeability dependence of the lens membranes upon external calcium.

**METHODS**

Northern frogs (*Rana pipiens*) were decapitated and the eyes were removed. The eyes were equatorially bisected and the suspensory ligaments of the lens were carefully cut away. The lens was gently removed from the globe and placed in a dish of Ringer's solution. All lenses were in the weight range of 10–15 mg.

The basic Ringer's solution used (Paterson et al., 1974) had a composition similar to the ionic composition of the aqueous humor of *Rana pipiens*. This solution was modified for particular experiments (Table I). The pH of all the solutions was adjusted to between 7.2 and 7.4. The calcium concentration in the normal Ringer's solution was 2 mM, which is close to that found in frog aqueous humor. Calcium-free solutions were checked by atomic absorption spectrophotometry and found to contain no detectable calcium. The calcium-free solutions also contained 1 mM EGTA to chelate calcium which might diffuse out of the lens.

**TABLE I**

| Solution       | NaCl | KCl | NaHCO₃ | Na₂HPO₄ | Na₂HPO₃ | MgSO₄ | CaCl₂ | Dextrose | EGTA | Sucrose |
|----------------|------|-----|--------|---------|---------|-------|-------|----------|------|---------|
| Normal         | 105  | 2.5 | 6.6    | 2.0     | 1.2     | 1.2   | 2.0   | 5.6      |      |         |
| Low calcium    | 105  | 2.5 | 6.6    | 2.0     | 1.2     | 1.2   | 2.0   | 5.6      | 1    | 210     |
| Low sodium (sucrose) | -   | 2.5 | 6.6    | 2.0     | 1.2     | 1.2   | 2.0   | 5.6      | 1    | 210     |
| Low calcium-low sodium | -   | 2.5 | 6.6    | 2.0     | 1.2     | 1.2   | 2.0   | 5.6      | 1    | 210     |

Electrophysiological Measurements

The lens was seated, anterior surface down, in a recess at the base of a Lucite chamber (E. I. Du Pont de Nemours & Co., Wilmington, Del.) The lens was submersed in bathing solution which was constantly flowing through the chamber. The flow rate was so adjusted that changes of ion concentrations in the chamber were completed within 60 s.

Microelectrodes, made on a horizontal puller (Industrial Science Associates, Inc., Ridgewood, N. Y.), were filled with 3 M potassium chloride (KCl) by capillary action. They had tip resistances in the range 15–40 MΩ, and a tip diameter of <1 μm. The microelectrodes were mounted in Lucite holders filled with 3 M KCl, with sintered silver-silver chloride plugs serving as reversible junctions. The microelectrode holders fitted directly into the probes of a high impedance differential preamplifier (model M 750, W-P Instruments, Inc., New Haven, Conn.). The lens potential was measured by inserting one of a pair of microelectrodes into the superficial lens cortex using a hydraulic microdrive (Trent-Wells, Inc., South Gate, Calif.). The potential difference between the interior of the lens and the reference electrode in the bath was displayed on an oscilloscope (model 5113, Tektronix, Inc., Beaverton, Ore.), and recorded on a chart recorder.
For resistance measurements, a Ringer-agar bridge and reversible electrode were placed in the chamber to serve as a ground and a current sink. A second microelectrode was introduced into the superficial lens cortex using a manual micromanipulator. The angular separation of the electrodes within the lens, as defined by Eisenberg and Rae (1976), was always >60°. Lens resistance was measured by passing a square pulse of current between the second electrode and the agar bridge bath electrode, and the resulting change in the lens potential was recorded. The currents, normally 3 s in duration, were provided by a specialized amplifier (model M701, W-P Instruments, Inc.), controlled by a pulse generator (Series 800, W-P Instruments, Inc.), and were in the range 0.1-1 × 10⁻⁶ A. The amplitude and wave-form of the potentials and currents were monitored on the storage screen of the oscilloscope.

Applying Ohm's Law by dividing the magnitude of the voltage response by the current has been accepted (Duncan, 1969 b; Delamere and Duncan, 1977), in the frog and toad lens, to be a measure of lens resistance. Recent studies have revealed that a finite resistance might exist between lens fibers (Eisenberg and Rae, 1976; Duncan and Delamere, 1978). Additionally, voltage changes due to a high current density around the current passing electrode (Eisenberg and Rae, 1976) might contribute to part of the voltage response, although the large angular separation of the microelectrodes within the lens in our experiments would minimize this problem. Resistance measurements are therefore presented as ohms per lens, and not converted to specific values by considering lens membrane area.

**Ion Analysis**

The lens was completely freed of adhering tissues and then quickly blotted on clean filter paper before being placed in a preweighed Pyrex vessel. The vessel was then weighed to obtain the fresh weight of the lens and placed in a drying oven for 4 days, or until no further weight loss could be noted.

After water determinations, a known volume of 25% nitric acid was added to each vessel containing the dried lens, and the vessel was sealed and maintained at 65°C to achieve complete digestion of the lens. The resulting digest was diluted as necessary with double glass-distilled water, and sodium and potassium were determined by flame photometry using standards in the range of 10⁻⁵-10⁻⁴ M.

**Rubidium-86 (⁸⁶Rb) Fluxes**

Immediately after removal from the eye, lenses were incubated at ambient temperature (20°C) for 4 h in normal Ringer's solution containing 10 μCi/ml of ⁸⁶Rb. At the end of the loading period, lenses were briefly washed in nonradioactive solution and placed in a Lucite chamber containing 0.5 ml of normal medium. Bathing solution (5 ml) was then introduced through one port in the chamber, washing out the original solution through a second port where it was collected in a scintillation vial. This procedure was repeated at 10-min intervals, and the radioactivity in the displaced medium was assayed in a scintillation counter. After 2 h of using normal medium, the modified bathing solutions were used for a given period of time to wash the lens. At the end of the experiment, the lens was removed from the chamber, digested in 1 ml of 25% nitric acid, and a sample of the digest was counted as described above to determine the radioactivity remaining in the lens. The efflux rate constant (k) was calculated for each 10-min period as

\[
\frac{\ln R_{t_1} - \ln R_{t_2}}{10}
\]

where \( R_{t_1} \) represents the relative or percentage radioactivity remaining in the lens at time \( t_1 \), and \( R_{t_2} \) represents that remaining after an additional 10-min wash out.
RESULTS

Electrical Measurements

POTENTIAL DIFFERENCE The potential difference of frog lenses bathed in normal medium containing calcium was in the range -67 to -72 mV. Only those lenses which maintained a steady potential within that range for >20 min were used for further experimentation. In a number of experiments, stable but much less negative potentials, as low as -35 mV, could be measured in different regions of lenses which also exhibited potentials in the normal range. The significance of these lower potentials and their response to reduction of the calcium level in the bathing solution will be discussed later.

When lenses with initial potentials in the range -67 to -72 mV were exposed to calcium-free solution, a significant depolarization was always observed. Fig. 1 shows the time-course of depolarization in several typical experiments. Although there was some variability in the response of the potential to low calcium, in general the rate of depolarization was most rapid during the first 20 min, followed by a very slow fall until a steady potential was reached at about 1 h. The mean magnitude of the depolarization in 24 lenses was 37.1 ± 2.3 mV (mean ± SEM).

The depolarization rapidly reversed when the medium bathing the lens was changed to contain calcium. The degree of recovery of the potential appeared to be dependent upon how long the lens had been exposed to low calcium. As can be seen in Fig. 1, the potential of lenses exposed to low calcium for 20 min tended to return to closer its initial level than did the potential of lenses exposed to low calcium for longer periods. The rate of recovery showed a fast phase, followed by a more gradual phase, as was observed with depolarization.

For both depolarization and the recovery process, the lens potential began to change within the one minute required to change the composition of the solution bathing the lens.

RESISTANCE MEASUREMENTS The response of the lens potential to a square wave pulse of current passed through a microelectrode within the lens was measured every 2 min while the lens potential was being continually recorded under the conditions described above. The lenses that, during the establishment of an initial steady potential in normal calcium-containing bathing medium, showed significant variation in resistance measurements during a 20-min period were not used for further experimentation. Calcium-free bathing solution produced a fall in lens resistance. It is clear from Fig. 2 that the time-course of the resistance change closely followed that of the depolarization in low calcium. In 19 lenses, the resistance was reduced by 62.5 ± 3.2% after exposure to calcium-free medium for 1 h. The resistance rapidly increased when calcium was returned to the medium bathing the lens. The extent of the resistance recovery, as with the reversal of the depolarization, was dependent upon the time of previous exposure to low calcium medium.

In addition to alteration in the magnitude of the voltage response in calcium-free medium, the wave-form of the response also differed. Fig. 2 clearly demonstrates that, in normal calcium, the voltage response to a step current pulse is not a step voltage change, but an exponential-like rise (or fall), which
takes the order of seconds to reach a steady level. As the amplitude of the voltage response decreases in calcium-free solution, the rise time also decreases to the order of milliseconds. On return to normal calcium environment, the response returned to its original wave-form.

As mentioned above, in a number of cases, potentials as low as -35 mV were recognized in lenses which also showed -67 to -72 mV potentials. Although a systematic study of such low potentials was not undertaken, it was demonstrated
that the lower potentials responded to low calcium medium in a fashion similar to the response of potentials in the higher range. Resistance measurements on "low potential" lenses also gave similar results in low calcium medium as the "high potential" lenses.

Figure 2. Typical changes in potential and resistance during exposure to calcium-free solution for (A) 20 min and (B) 60 min. Voltage changes in response to current pulses (as described in Methods) are seen as "spikes" superimposed upon continual potential traces A and B. The resistance values (filled circles) were calculated from each "spike." The actual wave forms of selected "spikes" are shown and indicated by arabic numerals. It can be seen that the wave form changes significantly during exposure to calcium-free solution (described in Results).
Lens Cation Content

Levels of sodium, potassium, and water content in fresh and incubated lenses are given in Table II. Clearly, lenses incubated in a low calcium environment lose potassium and gain sodium relative to the control lenses incubated in normal calcium-containing medium. It is particularly important to note lens cation levels have changed very little during the first 20 min of exposure to low calcium medium. During that same time period, as described above, both the lens potential and resistance have fallen rapidly to close to their new steady values. In that membrane potentials and resistance are both functions of free ion concentrations, and permeabilities (Goldman, 1943; Hodgkin and Katz, 1949; Kimizuka and Koketsu, 1963), we applied the Hodgkin-Katz-Goldman equation to the cation data:

\[
E = \frac{RT}{ZF} \ln \left( \frac{P_{Na}/P_{K}}{K_{b}} + \frac{P_{Na}/P_{K}}{N_{a}} + \frac{P_{Cl}/P_{K}}{C_{b}} \right)
\]

where \(E\) represents the potential; \(P_{Na}, P_{K}, \) and \(P_{Cl}\) represent lens membrane permeability to sodium, potassium, and chloride respectively; \(K_{b}, N_{a}, \) and \(C_{b}\) represent the bathing medium ion concentrations as provided in Methods; \(K_{b}, N_{a}, \) and \(C_{b}\) represent lens ion concentrations, and \(R, T, Z\) and \(F\) have their usual meanings. We compared the predicted lens potential calculated using cation concentrations from fresh lenses with that using cation data from lenses incubated for 20 min in low calcium medium. The values, 12.8 mM/kg lens water for lens chloride content, \(P_{Na}/P_{K} = 0.02,\) and \(P_{Cl}/P_{K} = 1.15,\) were taken from Delamere and Duncan (1977).

The fresh lens values predict a membrane potential of \(-61\) mV. The validity of such quantitative calculations for a preparation which elicits more than one steady potential will be discussed later. However, on the assumption that no change of ion permeabilities or lens chloride occurs, the computed depolarization due to the cation redistribution after 20 min in calcium-free solution would be \(<1.0\) mV. This, therefore, indicates that cation changes alone cannot account for the observed large depolarization. It is more likely explained by a change of ion permeability. In fact, the depolarization toward the equilibrium
potential for sodium (+52.3 mV) suggests that the relative permeability of sodium to potassium \((P_{Na}/P_{K})\) has increased. Certainly, an increase of lens membrane permeability toward at least one charged species would lead to an explanation of the fall of lens resistance during the first 20 min of exposure to low calcium solutions.

**86Rb Efflux**

The passive flux of potassium or rubidium out of the lens can readily be related to potassium permeability across the lens membranes. Observation of the 86Rb efflux rate constant for the lens revealed a significant increase to a new steady-state efflux rate upon exposure to calcium-free solution (Fig. 3). The efflux rate returned to control levels upon exposing the lens to a bathing solution containing calcium. The ratio of the efflux rate constant in normal calcium environment to that in low calcium \((k/k')\) in eight lenses was 0.52 ± 0.03.

However, it is known that potassium efflux in the lens closely follows the theoretical behavior (Kimizuka and Koketsu, 1963) which predicts that potassium efflux increases in response to depolarization of the lens potential (Duncan, 1974). The theoretical rate constant ratio \((k/k')\) resulting from depolarization can be calculated from the equation of Kimizuka and Koketsu (1963):

\[
k/k' = P_{R}/P_{R}^{'}\cdot \exp\left[\frac{zR}{2RT} (E - E')\right]
\]

where \(P_{R}\) and \(P_{R}^{'}\) are the permeabilities of the ionic species \(R\) in the control and test solution, and \(E\) and \(E^{'}\) are the potentials in those solutions. Using this equation and assuming that the change in efflux rate in low calcium is due solely to the depolarization (i.e., no change in potassium permeability occurs), the theoretical rate constant ratio \((k/k')\) for the observed 37.1 mV depolarization was 0.48. The close agreement with the measured ratio (0.52) implies little alteration
in potassium permeability. This finding would seem to substantiate the suggestion that the response of the lens to low calcium environment was principally due to an increase in sodium permeability rather than a change in potassium permeability.

**Observation of Low Calcium Effects in Low Sodium Bathing Solutions**

If the large potential and resistance change in the lens induced by low calcium bathing solutions were principally due to an increase in sodium permeability, then the observed electrical changes should be reduced in solutions where the sodium has been replaced by an impermeable molecule. Similarly, the increase in $^{86}$Rb efflux rate in low calcium medium would be reduced if the lens depolarization were reduced.

As substitutes for 90% of the sodium in the bathing solution, we separately examined choline, Tris, and sucrose. With sucrose as a substitute, it was shown that changing the solution bathing the lens from one containing normal calcium to one containing no calcium caused a depolarization of $<10$ mV without any demonstrable effect on resistance (Fig. 4 A). Similarly, there was a minimal

![Graph A](image1.png)

**NORMAL Na**

**LOW Na**

![Graph B](image2.png)

**Figure 4.** The effect of calcium-free solution upon (A) lens potential and resistance and (B) $^{86}$Rb efflux rate constants in normal and low sodium environments. The potential trace and resistance measurements (filled circles in A) demonstrate that the effects of calcium-free solution are greatly diminished when 90% of the sodium has been replaced by sucrose (see text for details). Similarly, changes in the $^{86}$Rb efflux rate constants (B) seen in calcium-free solutions are greatly reduced in a low sodium environment.
change in $\text{^{86}Rb}$ efflux rate upon changing the bathing solution to low calcium-low sodium (sucrose) solution (Fig. 4 B).

The results obtained with substitution of sodium by choline or Tris were equivocal because both choline and Tris appeared to depolarize directly the lens potential and subsequently to increase the $\text{^{86}Rb}$ efflux. Thus, the influence of low calcium was superimposed upon the effects of choline and Tris. It was apparent, however, that under those conditions, reduced calcium levels did not cause a large depolarization, and there was no significant change in the resistance.

DISCUSSION

In common with most cellular tissues (Manery, 1969), and the mammalian lens (Harris and Gehrsitz, 1951), the frog lens gains sodium and loses potassium in a calcium-free environment. The most striking observation, however, is that whereas the time-course of the cation changes is in the order of hours, the major alteration of the lens potential, resistance, and $\text{^{86}Rb}$ efflux occurs within 20 min.

The cation redistribution toward the levels in the bathing medium has been attributed in the lens and other tissues to a general increase in cell membrane permeability (Harris and Gehrsitz, 1951; Thoft and Kinoshita, 1965; Manery, 1969). The 62.5% reduction of the frog lens resistance supports that concept to the extent that the permeability to at least one mobile charged species has increased. The large depolarization of the lens potential during the first 20 minutes of exposure to calcium-free solutions cannot be fully explained by the cation changes alone, but, on the basis of the Hodgkin-Katz-Goldman equation, it could feasibly be the result of a relatively greater increase of sodium rather than potassium permeability. Although the $\text{^{86}Rb}$ efflux increases in calcium-free solutions, the increase can be largely explained by the change of lens potential, and so offers little evidence of a potassium permeability increase. Kimizuka and Koketsu (1963) have even suggested that calcium-free media might result in a decrease in potassium permeability in frog sartorius muscle fiber membranes.

Under low sodium conditions, the influence of calcium removal had a much reduced effect upon the lens potential and resistance, and $\text{^{86}Rb}$ efflux from the lens. Explaining this in terms of the Hodgkin-Katz-Goldman equation, the depolarizing effect of an increased sodium permeability will be less when the term $\text{Na}_0$ (external sodium concentration) is reduced. Similarly, the low $\text{Na}_0$ explains the absence of a large resistance decrease due to an elevation of sodium permeability in calcium-free solutions. The smaller depolarization also accounts for the smaller $\text{^{86}Rb}$ efflux rate change.

The evidence suggests, therefore, that the early effects of calcium-free media upon the lens are mainly due to increased sodium permeability. Changes in cation distribution or electrical parameters in low calcium media in HeLa cells (Morrill and Robbins, 1967), frog muscle (Kimizuka and Koketsu, 1963), and frog skin (Curran and Gill, 1962) have also been attributed principally to elevations of sodium permeability.

The low calcium-induced modification of the voltage response to a current pulse during the resistance measurement is of considerable interest. The long
time-course of the voltage change in normal calcium solution has been attributed to lens membrane surface area (Eisenberg and Rae, 1976) or to complex current pathways between fibers with the bulk of the lens (Duncan and Delamere, 1978). We are presently investigating the low calcium-induced reduction of the time-course of the voltage change in terms of both hypotheses.

The response of "low potential" lenses to calcium-free solutions is somewhat puzzling. Rae (1974) also reported lower potentials in frog lenses, which at the same time exhibited potentials in the order of $-70 \text{ mV}$. He attributed the low potentials to measurements obtained when the microelectrode tip was in the extracellular space rather than within a cell. However, the present observed depolarization of these low potentials in calcium-free solutions is difficult to explain on the basis of that hypothesis. The results, in fact, suggest that some of the cells within the lens might have different permeability characteristics and perhaps different ion content from the majority of cells and hence are not adequately described by the Hodgkin-Katz-Goldman equation as applied to the whole lens.

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