Calcium-Induced Opacification and Proteolysis in the Intact Rat Lens

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When intact rat lenses were incubated in artificial aqueous humor in the presence of 1 mM calcium and a sulfhydryl reagent p-chloromercuriphenyl sulfonate (pCMPS) a visible annular opacity developed within 4 hours. Combined photographic and ion-sensitive microelectrode investigations of the lenses demonstrated that the subsequent linear increase in opacification was accompanied by an increase in internal free calcium. Opacities were not observed in lenses incubated in the absence of either pCMPS or calcium. Gel electrophoresis of the soluble and urea-soluble fractions from lenses exposed to 1 mM calcium for periods of up to 14 hours showed no evidence for crystallin degradation and only minor proteolysis of cytoskeletal proteins. When lenses were incubated under identical conditions, but with 5 mM calcium, the degree of opacification increased up to approximately 8 hours and then remained constant. A progressive loss in cytoskeletal proteins was observed which correlated with a further increase in free calcium such that by 14 hours of incubation, when the internal calcium approached 1 mM, most of the spectrin and vimentin present in the cortex of the lens had disappeared. An unidentified 110-kilodalton protein also disappeared from lenses incubated in 5 mM calcium. These results indicate that proteolysis by calcium-dependent enzymes such as calpain may play a significant role in cytoskeletal regulation and metabolism in the lens. A role for cytoskeleton/membrane/crystallin interaction in calcium-induced opacification is discussed. Invest Ophthalmol Vis Sci 31:2405–2411, 1990

Recent investigations highlight the role played by calcium in the development of senile cortical cataract in humans.1,2 The importance of internal calcium concentration has also been established in several animal model systems. In organ-cultured bovine lenses, for example, a decrease in transparency was observed only in lenses with increased levels of calcium.3 In selenite cataract, calcium levels in the nuclear region of the rat lens, where the opacity is localized, are increased more than tenfold over control values.4 The development of selenite cataract is also associated with an increase in insoluble protein5 and extensive proteolysis.6

It is known that increased concentrations of calcium lead to the activation of proteases such as calpain7 and that calpain I, calpain II, and calpastatin, a specific endogenous inhibitor of calpain, are present in the lens.8 Calcium has also been found to produce aggregation of proteins when added to solutions of soluble lens proteins.9

There is currently much interest in the potential use of calpain inhibitors as agents for the prevention of cataract. Central to an understanding of calcium-dependent cataract formation, however, is the elucidation of the mechanism whereby calcium induces opacification and whether proteolysis is a necessary precondition for opacification in the intact lens. This study was undertaken to investigate these issues: (1) the relationship between intracellular free calcium and lens opacities and (2) the concomitant induction of lens proteolyses.

Materials and Methods

Lenses were dissected from Wistar rats killed by cervical dislocation. Each lens was washed in 4 ml of modified artificial aqueous humor (AAH) solution10 containing 1 or 5 mM CaCl₂ together with 40 µM of the sulfhydryl reagent p-chloromercuriphenyl sulfonate (pCMPS). The pCMPS was chosen since it has been shown to stimulate calcium influx into the rat lens without penetrating the cytoplasm.10 Lenses were then incubated in a capped vial at 36°C in 8 ml
of AAH containing 1 or 5 mM CaCl₂ and pCMPS for times up to 18 hr. Free-calcium levels in the lenses were measured using calcium-sensitive microelectrodes, inserted 150–200 μm into the posterior cortex, as described previously. Lenses were transferred from the incubation vials to small plastic petri dishes and photographed in AAH by transmitted light onto Ilford 100 ASA black-and-white film (Cheshire, U.K.). Negatives were scanned with 626-nm light in a densitometer (Joyce Loebl, Chromoscan 3, Joyce-Loebl, Gareshed, U.K.). This procedure was found to give more reproducible results than those obtained from lenses photographed against a black background (data not shown).

At the end of incubation the nucleus was removed from each lens using a trephine (2-mm internal diameter), and the cortex was frozen in liquid nitrogen and stored at −20°C. The cortex was homogenized in 400 μl of 40 mM Tris, pH 7.5, containing 5 mM Ethylene glycol-bis (Baminoethyl ether)N,N,N',N'-tetraetic acid (EGTA), 5 mM ethylenediaminetetraetic acid (EDTA), 1 mM N-ethyl maleimide, and 0.5 mM Phenylmethyl sulfonyl fluoride (PMSF) and centrifuged (10,000 × g for 15 min). The pellet was reextracted as described and then dissolved in 60 μl of 8 M urea containing 1 mM EDTA by shaking for 2 hr. A sample (20 μl) was mixed with an equal volume of sample buffer (6.25 mM Tris pH 6.8, 1.0% sodium dodecyl sulfate [SDS], 15% glycerol, and 5% v/v 2-mercaptoethanol), and 20 μl was used for electrophoresis. Discontinuous one-dimensional gel electrophoresis was done as described by Laemmli. Proteins were stained with colloidal Coomassie blue and the gels scanned at 626 nm, like the photographic negatives.

Proteins were transferred to nitrocellulose membranes for immunoblot analysis, essentially as described by Towbin et al. After blocking the membranes with 3% bovine serum albumin, the blots were probed as follows: (1) for spectrin, we used a monoclonal antibody to sheep erythrocyte spectrin, diluted 1:400, overnight at 4°C, (2) for vimentin, we used anti-IFA, a panspecific monoclonal antibody which recognizes a determinant common to all intermediate filament proteins, undiluted, overnight at 4°C, (3) for actin, we used monoclonal antiactin (Amersham, Aylesbury, Bucks, U.K.), diluted 1:1000, overnight at 4°C, and (4) for protein 115, we used a monoclonal antibody to the bovine lens fiber cell M₁₁5-kilodalton (kD) protein, diluted 1:1000, overnight at 4°C, and (4) for protein 115, we used a monoclonal antibody to the bovine lens fiber cell M₁₁5-kilodalton (kD) protein, diluted 1:1000, overnight at 4°C, and (4) for protein 115, we used a monoclonal antibody to the bovine lens fiber cell M₁₁5-kilodalton (kD) protein, diluted 1:1000, overnight at 4°C, and (4) for protein 115, we used a monoclonal antibody to the bovine lens fiber cell M₁₁5-kilodalton (kD) protein, diluted 1:1000, overnight at 4°C, and (4) for protein 115, we used a monoclonal antibody to the bovine lens fiber cell M₁₁5-kilodalton (kD) protein, diluted 1:1000, overnight at 4°C, and (4) for protein 115, we used a monoclonal antibody to the bovine lens fiber cell M₁₁5-kilodalton (kD) protein, diluted 1:1000, overnight at 4°C, and (4) for protein 115, we used a monoclonal antibody to the bovine lens fiber cell M₁₁5-kilodalton (kD) protein, diluted 1:1000, overnight at 4°C. Peroxidase-conjugated second antibodies (Sigma, St. Louis, MO) were diluted 1:2000 and applied for 1 hr at room temperature, with 3-amino-9-ethylcarbazole as the chromophoric agent.

All animals were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

### Results

Since pCMPS was being used under two conditions (1 mM and 5 mM Ca²⁺) to induce opacification, the effect of the sulfhydryl complexing agent on lens membrane potentials was monitored. The data (Table 1) confirm that a very large depolarization was induced, which appeared to be independent of the level of external calcium. This indicates that pCMPS induces a large change in membrane permeability, as has previously been suggested, with a consequential change in internal sodium and calcium. Since Ca²⁺ rather than Na⁺ is responsible for the major change in transparency during cataract formation, we investigated, in detail, the relationship between internal Ca²⁺ changes and opacification.

In the intact rat lens a visible white annular opacity could be observed after approximately a 4 hr incubation in AAH containing 40 μM pCMPS and 1 mM calcium. The degree of opacification appeared to increase with time. Lenses incubated in the absence of either calcium or pCMPS remained transparent for over 18 hr. This is illustrated in Figure 1 where a lens incubated in AAH containing 1 mM calcium and pCMPS for 14 hr (Fig. 1a) is shown together with a lens incubated for the same time in the absence of pCMPS (Fig. 1b).

To quantify the extent of the cataract as a function of time, lenses were photographed and analyzed by densitometric scanning as described. The data obtained for the lenses shown in Figure 1 are depicted in Figure 2. The degree of opacification of each lens was measured using calcium-sensitive microelectrodes (Fig. 4).

### Table 1. Membrane voltage measurements corresponding to pCa values given in Figure 4.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Control (mV)</th>
<th>40 μM pCMPS + 1 mM Ca²⁺ (mV)</th>
<th>40 μM pCMPS + 5 mM Ca²⁺ (mV)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>−73.9 ± 3.3 (4)</td>
<td>−18.1 ± 4.4 (4)</td>
<td>−20.2 ± 4.95 (2)</td>
</tr>
<tr>
<td>4</td>
<td>−70.3 ± 1.3 (6)</td>
<td>−14.1 ± 1.8 (3)</td>
<td>−21.7 ± 3.5 (4)</td>
</tr>
<tr>
<td>8</td>
<td>−64.1 ± 4.4 (3)</td>
<td>−6.3 ± 1.1 (2)</td>
<td>−5.5 ± 0.9 (3)</td>
</tr>
</tbody>
</table>

The voltage values are expressed as mean ± SEM (n).
Since the rat lens is known to contain calpains, lenses exposed to increased calcium for various times were extracted and both the soluble and urea-soluble fractions examined for evidence of protein degradation. Minor proteolysis only was observed in either fraction up to 14 hr. This is illustrated by the densitometer profiles of SDS gels shown in Figure 5 for the urea-soluble proteins from lenses incubated for 14 hr in the presence (Fig. 5a) or absence (Fig. 5b) of 1 mM calcium.

The time course of opacification and protein analysis was repeated using lenses incubated in AAH containing 5 mM calcium in place of 1 mM calcium to stimulate the net influx of calcium. The dependence of lens opacification on the time of incubation in 5 mM calcium is shown in Figure 6. The degree of opacification in the first 4 hr was very similar to that observed in the 1 mM calcium experiment. However, opacification then increased more slowly until approximately 8 hr at which point it reached a plateau. Gel electrophoretic analysis of the soluble proteins from these lenses revealed no detectable changes in the profiles, although at high gel loadings faint lower-molecular-weight bands were observed which may correspond with crystallin degradation products (data not shown). The SDS gel patterns of the urea-soluble fractions revealed major decreases in several protein bands (Fig. 5c) in marked contrast to the situation in control and 1 mM Ca\(^{2+}\) lenses (Figs. 5a–b). Gel electrophoresis of the urea-insoluble pellet revealed only trace levels of these polypeptides (data not shown).

The time course of loss of three of these components from the urea-soluble fraction as determined by densitometric scans of Coomassie blue-stained gels is shown in Figure 7. For lenses incubated in AAH containing 5 mM calcium it can be seen that the profiles of protein degradation closely mirror that observed for the influx of calcium into the lens (Fig. 4).

The first component shown in Figure 7a underwent electrophoresis as a doublet and was calculated to have a molecular weight of approximately 230–240 kD. The provisional identification of this protein as spectrin was confirmed using immunoblotting with a monoclonal antibody raised to sheep erythrocyte spectrin (Fig. 8b). Examination of the blots confirmed the marked reduction in spectrin observed with the Coomassie-blue staining. In addition these blots clearly showed spectrin immunoreactivity associated with 162- and 155-kD polypeptides, prominent in gels from lenses exposed for 8 and 11 hr to 5 mM calcium. These bands were barely visible in lenses incubated for 14 hr indicating that the lens spectrin was being degraded via the formation of transient lower-molecular-weight intermediates.

The second component of the urea-soluble fraction
analyzed was found to have a molecular weight of approximately 57 kD; it was identical with vimentin by immunoblotting experiments using antibodies directed both against vimentin (data not shown) and intermediate filaments (Fig. 7b). As was the case with spectrin, vimentin degraded almost totally after 14 hr of incubation as judged both by densitometry (Fig. 7b) and by analysis of the blots (Fig. 8c).

The third component whose loss is shown in Figure 7c was found to have a molecular weight of approximately 110 kD. It was thought initially that this protein might be identical with the 115-kD lens fiber cell-specific extrinsic membrane protein described by FitzGerald. However on immunoblots this protein did not cross-react with a monoclonal antibody raised against the lens cell-specific cytoskeletal component. The protein displayed a similar degradation time course to that of spectrin.

The urea-soluble fractions were also examined with an antibody raised against actin. The result shown in Figure 8d indicates that, unlike the other cytoskeletal components probed with specific antibodies, there is appreciable actin immunoreactivity present after a 14-hr incubation in 5 mM calcium. This antibody, while reacting with both lens actin and bovine muscle actin, also cross-reacted with vimentin. The staining reaction with this antibody (Fig. 8d) confirmed the result shown in Figure 8b for the degradation of vimentin.

To test whether inclusion of a calpain inhibitor could inhibit either the opacification or protein degradation, lenses were incubated in AAH containing 5 mm calcium in the presence or absence of 50 μM E-64 (trans-epoxy succinyl-L-leucylamido 4-guanidino butane) for up to 14 hr. This thiol protease inhibitor had no significant effect on either the protein degradation or the opacification. It is not known however whether E-64 is able to penetrate the lens under these study conditions.

**Discussion**

In this study we attempted to correlate the degree of opacification in intact rat lenses with both protein degradation and intracellular free calcium in the lens.

A simple photographic procedure involving densitometric scanning of negatives generated data which could be used to provide a quantitative measure of the extent of lens opacification with time. Using this technique it was shown that, after a lag period of approximately 1 hr, the degree of opacification in lenses incubated in AAH containing 1 mM calcium and pCMPS increased linearly with time. At the end of the 14 hr incubation period, when there was a six- to sevenfold increase in lens opacification, the free calcium had increased fivefold from 2 μM (pCa = 5.8 ± 0.07) to 11 μM (pCa = 4.95 ± 0.23). It should be noted that free calcium levels were measured using calcium-sensitive microelectrodes inserted into the posterior face of the lens, whereas the annular opacity was localized predominantly in the equatorial region of the lens. The observed increase in free calcium, which resulted in marked opacification, was not accompanied by a major degradation of lens crystallins.
Fig. 5. Densitometer traces of urea-soluble fractions separated in 12.5% SDS gels and stained with Coomassie blue. (a) 1 mM Ca\(^{2+}\) and 40 \(\mu\)M pCMPS; (b) no Ca\(^{2+}\) and 40 \(\mu\)M pCMPS; (c) 5 mM Ca\(^{2+}\) and 40 \(\mu\)M pCMPS. Arrows in Figure 5b show the positions of MW markers (kD): from the left hand side, myosin (205), \(\beta\) galactosidase (116), phosphorylase b (97.4), bovine serum albumin (66), egg albumin (45), carbonic anhydrase (29), and trypsinogen (24).

Markedly different results were obtained when lenses were incubated in AAH containing 5 mM calcium and pCMPS. In these lenses the intracellular free calcium rose rapidly after 4 hr to approach millimolar concentrations after approximately 8 hr (Fig. 4). This period was found to correspond with that of major lens cytoskeletal degradation.

After a 14 hr incubation in 5 mM calcium, the lenticular levels of spectrin, vimentin, and an unidentified 110-kD component had dropped almost to zero (Fig. 7). The disappearance of spectrin and vimentin was confirmed by immunoblot analysis. As described by us previously, the degradation of spectrin appears to proceed via the generation of fragments of approximately 155 kD.

These results are consistent with the hydrolysis of the cytoskeletal components by an endogenous calcium-activated protease. Calpain II purified from rat lenses has been found to be activated above 50 \(\mu\)M calcium ion and to require 400 \(\mu\)M calcium for maximal activity. Thus calpain II may be implicated in the differential cytoskeletal proteolysis we observed.

In the intact lens, the lenticular calcium-activated protease displays high activity toward cytoskeletal proteins such as vimentin and spectrin but lower apparent activity toward actin and lens crystallins. Since crystallins are present at high concentrations in
the lens, proteolysis may be masked when samples are analyzed by gel electrophoresis. Some evidence for minor crystallin degradation to smaller-molecular-weight components was obtained by overloading the gel lanes. Previous studies with purified rat lens calpain II have shown that α- and β- but not γ-crystallin can be degraded to lower-molecular-weight species by incubation with the enzymes. In agreement with our results in the intact lens, the proteolytic activity of purified calpain II toward insoluble lens proteins appeared to be higher.

It is interesting to compare the time course for lens opacification in 1 mM with that in 5 mM calcium in the light of our results. Lenses exposed to 1 mM calcium show an almost linear increase in opacification after a lag period of 1 hr; this trend continues at least up to 16 hr (16-hr integral values, 9960 and 10,600). The degree of opacification in lenses exposed to AAH containing 5 mM calcium, on the other hand, increased little after 8 hr of incubation. Thus if one lens from an animal is incubated for 14 hr in 1 mM calcium and the contralateral lens in 5 mM calcium, the 1 mM calcium-treated lens appears more opaque.

The major difference between these two lenses is that the 5 mM calcium-treated lens contains little cytoskeletal framework after 14 hr. These data indicate that the cytoskeleton may play an important role in calcium-induced transparency loss, which has at least two phases. At moderately increased calcium levels, opacification occurs without major degradation of intracellular proteins and may be the result of calcium-stimulated interactions between the membrane-cytoskeletal network and crystallins, as described by Clark et al. Such a calcium-induced interaction between the structural elements of the lens could be reversible. In lenses subjected to greater calcium increases, the process of opacification appeared to slow down at the time that major degradation of the cytoskeleton became apparent. This may be due to the loss of cytoskeletal elements as centers of aggregation. This phase of calcium-induced trauma, which does appear to involve massive degradation by endogenous proteases, would be expected to be largely irreversible.

**Key words:** calcium, cytoskeleton, lens, spectrin, cataract

**Fig. 7.** Relative content of cytoskeletal proteins by densitometry of 12.5% SDS gels stained with Coomassie blue. Points represent the integrated areas of the protein peaks, relative to control values taken to be 100%. (a) Relative content of spectrin. (b) Relative content of vimentin. (c) Relative content of a 110 kDa urea-soluble polypeptide.
Fig. 8. Identification of poly-peptides. In all figures, lane 1, no pCMPS and 1 mM CaCl₂; lane 2, 40 μM pCMPS and 5 mM CaCl₂, after 14 hr of incubation. (a) Urea-soluble proteins separated in 12.5% SDS gels and stained with Coomassie blue. (b) Western blot from a 6% SDS gel, probed with an antibody to sheep erythrocyte spectrin. (c) Western blot from 12% SDS gel, probed with an antibody to intermediate filaments. (d) As for c, probed with anti-actin.

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