The Influence of Calcium on Protein Synthesis in the Rabbit Lens

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Biosynthesis of lens proteins, as assessed by the incorporation of \(^{14}\)C-histidine, was investigated in young rabbit lenses cultured under conditions designed to specifically elevate lens calcium. While such lenses rarely were obtained without small degrees of Na/K imbalances, experiments with ouabain-treated lenses over comparable times indicated that changes solely in Na/K levels did not alter synthesis of lens crystallins. On the other hand, with far smaller changes in Na/K levels, excess calcium accumulation obtained by exposing lenses to A23187 or high levels of medium calcium invariably led to diminished synthesis of lens proteins. An increase in lens calcium from 0.2 mM to 0.6 mM led to a small but statistically insignificant decline in protein synthesis, while an increase to 1.4 mM or 1.9 mM resulted in a decline to values 50% and 11%, respectively, of the control. The results indicate that calcium may be important in influencing protein synthesis in the lens. Invest Ophthalmol Vis Sci 24:1422-1426, 1983

Since abnormally high concentrations of calcium often accompany cataract formation,\(^3\,^4\) it is important to consider the variety of physiologic processes that may be affected by alterations in lens calcium. For instance, inactivation of the Na/K-ATPase transport system recently was reported in opaque rabbit lenses in which membrane-bound calcium was elevated experimentally fourfold.\(^4\) Another potentially important role for calcium might be that of regulating protein synthesis, particularly in view of the recently reported inhibitory actions of sodium-potassium imbalances on crystallins synthesis.\(^5\,^7\) Since the role of calcium in biosynthesis of lens proteins has received little attention, the present study attempts to determine the effects of elevated calcium levels in rabbit lenses on the rate of incorporation of \(^{14}\)C-labelled histidine by lens proteins. Experiments are undertaken to measure protein synthesis in lenses cultured under conditions designed to increase the calcium content in the absence of poisons that might directly influence protein metabolism.

**Materials and Methods.** To assess the influence of calcium on protein synthesis, lenses were precultured for 15-20 hours in a variety of media (Table 1) at 21° C to facilitate calcium accumulation in the lens. Pre-culture media, buffered with HEPES (5 mM), pH 7.3, contained either 20 mM or 1 mM CaCl\(_2\) in addition to 5 mM glucose, 2 mM MgCl\(_2\), 5 mM KCl, and 100 to 120 mM NaCl to achieve an osmolality of 300 mOsm. Following preculture, all lenses were transferred to TC199 at 37° C. Except where indicated, TC199 was a complete culture medium containing bicarbonate (2.2 g/l) and pregassed with 5% CO\(_2\), balanced with oxygen and nitrogen. When the concentration of calcium normally present in TC199 (2 mM) was increased in 10 mM, bicarbonate and phosphate were replaced by HEPES (5 mM) to prevent calcium precipitation. The pH was maintained at 7.3. Lens culture at 37° C was limited to 5 hours, a period sufficient to measure amino acid incorporation.

To measure the extent of incorporation of \(^{14}\)C-histidine in 4- to 5-week-old rabbit lenses, lenses were incubated at 37° C for 5 hours in 2.5 ml TC199 media containing between 10 and 50 μCi labeled histidine at a specific activity of 343 Ci/mmol. After culture, lenses were blotted on water-moistened filter paper and homogenized in 1 ml of Tris/Ion buffer solution: KCl (120 mM), NaCl (20 mM), MgCl\(_2\) (2 mM), CaCl\(_2\) (0.2 mM), and Tris (50 mM) at pH = 7.3. Homogenates were centrifuged at 37,000 g for 20 min at 4° C on a Sorvall® RC-5B (Newtown, CT) centrifuge, and the supernatant (Sn) decanted for analysis. In the majority of experiments, radioactive histidine was assayed in this fraction following precipitation of 10% trichloroacetic acid (TCA) and washing (three times in TCA). A small sample (0.1 ml) of the TCA extract was assayed for radioactive histidine by adding to 5 ml of Dimilune-30\(\text{™}\) scintillation fluid and counting on a Hewlett-Packard® Liquid Scintillation System, Model 300C. The precipitate of a 0.1-ml Sn sample was dissolved in 0.5 ml of 2 N NaOH for several hours. Hyamine was not used because of variable degrees of quenching. A 0.1-ml sample was assayed for radioactivity in 5 ml scintillation fluid, and 0.1 ml was assayed for protein content by Bio-Rad assays, using standards of bovine serum albumins in NaOH or buffer. Typical protein
recoveries following extraction and washing were between 90 and 95%.

Preliminary experiments indicated that the amount of incorporation of $^{14}$C-labeled histidine in the water-soluble proteins of the mammalian lens depended on the amount of soluble radioactive in the pool and, therefore, was not used as a measure of protein synthesis in cultured lenses of 4- to 5-week-old New Zealand rabbits. Thus, synthesis is expressed as the percent of isotope measured in lens proteins relative to the total radioactive histidine measured in lens homogenates. Values so obtained represent a percent incorporation that is independent of the quantity of labeled histidine accumulated. That incorporation rather than indiscriminatory binding occurred was substantiated by the finding in preliminary experiments that radioactive histidine was not detected in the precipitated proteins of the nucleus, despite the presence of significant free histidine, probably since little, if any, protein synthesis occurs in the nucleus.

In several experiments, preparative separation of the water-soluble proteins present in the 37,000 g Sn was accomplished using a Beckman® High Performance Liquid Chromatography (HPLC) System, Model 362 (Berkeley, CA). Columns were of the silica type, BioRad TSK 400 in series with the TSK 250, both 300 mm × 7.5 mm. The eluent was a Tris-phosphate buffer (pH, 7.4) containing 100 mM KCl and 150 mM NaCl. Resolution was sacrificed for maximum collection of proteins by applying 250-μl samples, at a flow rate of 0.72 ml/min, monitored at 280 nm. A typical elution profile is illustrated in Figure 1, showing the four major peaks. Protein concentrations were measured in a total of 30 1-ml fractions over a total elution time of approximately 45 minutes; 95% of the protein applied to the columns was recovered in the eluate.

Proteins also were resolved by SDS polyacrylamide gel electrophoresis (PAGE) carried out for 3 to 5 hours at 20 mA/gel in a BioRad slab gel apparatus, model 220, using a 10% acrylamide gel and 5% acrylamide stacking gel. Preparation of samples in a 1% SDS buffer solution was similar to that reported by Garadi, Giblin, and Reddy.

Results. Table 1 summarizes data on $^{14}$C-histidine incorporation in cultured rabbit lenses containing different levels of calcium. Freshly excised lenses (group 1) precultured in control media TC199, incorporated 45.9% of the total radioactive histidine measured in the lens after 5 hours, as shown in the last column. The next four experiments attempted to elevate the content of lens calcium during preculture and maintain such levels during the culture period in which labeled histidine was taken up by the lens. Lenses exposed to high concentrations of calcium (group 2) contained as high as 1.4 mM are observed at the onset of culture at 37° C. The percent incorporation of labeled histidine was less than 4% of the mean in each case.

Table 1. Incorporation of $^{14}$C-histidine in rabbit lenses as a function of lens calcium

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Preculture medium (15 hr)</th>
<th>Culture medium (6 hr)</th>
<th>Lens Ca (mM)</th>
<th>Lens Na (mM)</th>
<th>Lens K (mM)</th>
<th>Incorp. cpm per lens $\times 10^2$</th>
<th>Total cpm per lens $\times 10^3$</th>
<th>% Incorp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TC199</td>
<td>TC199</td>
<td>0.20</td>
<td>11 ± 2</td>
<td>123 ± 5</td>
<td>407 ± 18</td>
<td>885 ± 49</td>
<td>45 ± 4</td>
</tr>
<tr>
<td>2</td>
<td>20 mM Ca</td>
<td>TC199</td>
<td>0.63</td>
<td>12 ± 2</td>
<td>120 ± 4</td>
<td>334 ± 14</td>
<td>831 ± 61</td>
<td>40 ± 4</td>
</tr>
<tr>
<td>3</td>
<td>20 mM Ca</td>
<td>TC199 + A23187</td>
<td>1.90</td>
<td>13 ± 3</td>
<td>118 ± 6</td>
<td>54 ± 8</td>
<td>472 ± 20</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>4</td>
<td>20 mM Ca</td>
<td>TC199 + 10 mM Ca</td>
<td>1.45</td>
<td>13 ± 3</td>
<td>115 ± 6</td>
<td>117 ± 12</td>
<td>511 ± 26</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>5</td>
<td>1 mM Ca</td>
<td>TC199 + 10 mM Ca</td>
<td>0.45</td>
<td>11 ± 2</td>
<td>125 ± 4</td>
<td>209 ± 11</td>
<td>498 ± 19</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>6</td>
<td>None</td>
<td>TC199 + ouabain</td>
<td>0.30</td>
<td>36 ± 5</td>
<td>94 ± 6</td>
<td>76 ± 5</td>
<td>169 ± 13</td>
<td>45 ± 4</td>
</tr>
<tr>
<td>7</td>
<td>TC199 + ouabain</td>
<td>TC199 + ouabain</td>
<td>0.75</td>
<td>68 ± 7</td>
<td>65 ± 8</td>
<td>81 ± 7</td>
<td>290 ± 20</td>
<td>28 ± 3</td>
</tr>
</tbody>
</table>

* Values shown are means ± standard error (SE) for n = 4–6 experiments; in columns 4, SE is less than 4% of the mean in each case.

% Incorp. is a normalization: incorporated cpm/total cpm.

Fig. 1. HPLC elution profile of water-soluble proteins from rabbit lens homogenates centrifuged at 37,000 g. The positions of the 1-ml fraction analyzed for labeled histidine, BioRad analysis, and subsequent PAGE analysis.
the ionophore might directly influence protein synthesis, an alternative method of raising lens calcium was employed. Thus, lenses (group 4) were precultured in medium A and transferred to TC199 containing a high concentration of calcium 10 mM. This technique led to a calcium concentration of 1.4 mM in the lens and a 50% decrease in the fraction of histidine incorporated.

This experiment was repeated except that lenses were precultured in a medium containing 1 mM calcium (experiment 5) prior to incubation in TC199 containing 10 mM calcium. Following the 5-hour culture period, the calcium concentration was 0.45 mM and the percent of histidine incorporation remained nearly normal at 42%. Thus, the only experimental conditions that produced a significant decline in the degree of incorporation were those that also produced significantly elevated levels of lens calcium.

Since experimental lenses containing excessive amounts of calcium also were characterized by a decline in the uptake of labeled histidine, as evident from reduced values of total cpm per lens, it was necessary to determine if diminished transport of histidine was sufficient to retard the rate of protein synthesis. Therefore, lenses were cultured in the presence of 0.1 mM ouabain for 5 hours to inhibit cation pump-dependent transport of amino acids. The data (Table 1, row 6) reveal that the percent of incorporation was still 44.9%, indistinguishable from control values, despite the decrease in the quantity of histidine incorporated and decrease in the ratio of lens to medium labeled histidine from 2.8 to 1.3 (data not shown). This demonstrates that a decrease in the amount of soluble radioactivity in the pool, as a result of reduced uptake of histidine, does not influence the percent of histidine incorporation.

To determine whether alterations of Na or K levels might have occurred and contributed to inhibition of protein synthesis, the concentration of these cations was measured in both the calcium-loaded lenses and the ouabain-treated lenses, as shown in Table 1. As evident, the changes in sodium and potassium concentrations were minimal in all experiments except those involving ouabain. Only in calcium-loaded lenses of experiment 5 did the ratio of K to Na decrease significantly from 11 to 9, compared to a decrease to 2.5 ± 0.4 (n = 4) for ouabain-treated lenses (group 6). The important finding here is that lenses exposed to ouabain (10^-4 M) for 5 hours showed evidence of a significant change in sodium and potassium levels while maintaining near-normal levels of calcium and normal rates of histidine incorporation (45%).

To determine whether Na/K imbalances over greater periods of time could influence protein synthesis, lenses (group 7) were precultured in ouabain (0.1 mM) for 15 hours. Under these conditions, the percent of labeled histidine incorporated in 5 hours was only 28% at a time when the Na/K ratio was 1.1 ± 0.1. However, the calcium level also was elevated by a factor of nearly 4.

To support the premise that the labeled histidine measured in the 37,000 Svedberg fraction was in fact that incorporated by the lens crystallins, preparative liquid chromatography was carried out as described in the Methods section. Protein samples of 25 μg were analyzed for labeled histidine in each fraction and subsequently resolved by SDS-PAGE. The activity measured in each peak, indicated for peak 1 as the cross-hatched area in Figure 1, from control lenses (n = 3) varied between 500 and 700 cpm/protein sample, an amount consistent with that observed for whole lens homogenates centrifuged at 37,000 g. Uniform incorporation of labeled histidine by the crystallins in normal lenses also was reported by Spector and Ki-noshita.11

Figure 2 shows the gel pattern of crystallins corresponding to peaks I-IV in Figure 1. Comparison of these bands, columns 4-7, to those of Liem-The and Hoenders12 suggests that these polypeptides are in fact from alpha, beta, and gamma crystallins. The alpha band splits into two separate bands when smaller amounts are applied.12 Protein samples from normal and calcium-loaded lenses also were applied to gels to determine if subtle changes may have occurred in the relative synthesis of the subunits of the crystallins. It is evident from Figure 2 that the number and position of the bands are quite similar in the control (column 2) and calcium-loaded lens (column 3), indicating the absence of any significant degradation or transformation of crystallins.

Discussion. Under incubation conditions which favor selective accumulation of calcium in young rabbit lenses, biosynthesis of lens crystallins appears to be impaired. On the basis of previous reports,5,6 it might be argued that either an imbalance in the Na/K concentration or a reduction in histidine influx is directly responsible for the observed decrease in protein synthesis. However, it is unlikely that a change in the concentration of sodium and potassium alone is suf-
**Fig. 2. SDS-PAGE bands of control rabbit lens (column 2) and calcium loaded lens (column 3) supernatants after centrifugation at 37,000 g. Columns 4-7 represent 25-μg samples of normal lens proteins separated by HPLC (peaks I-IV).**

Sufficient to retard protein synthesis in these lenses in view of the small changes in K/Na values in calcium-loaded lenses and the data obtained from ouabain-treated lenses. Thus, in lenses exposed to 0.1 mM ouabain for 5 hours, protein synthesis remained normal, despite a 25% reduction in the concentration of potassium. On the other hand, in lenses with far less extensive changes in the K/Na ratio but containing excessive calcium (1.4 mM for example), protein synthesis was reduced significantly. Experiments to maintain normal levels of calcium and abnormal levels of sodium and potassium during extended culture periods were unsuccessful. The results of 20-hour culture in the presence of ouabain merely demonstrated that a marked decrease (70%) in protein synthesis was accompanied by significant changes in the content of calcium, sodium, and potassium, and raise the possibility that inhibition of protein synthesis might be facilitated by alterations in all three cations.

Inhibition of protein synthesis by calcium-dependent alterations in histidine transport is possible but not likely in view of the evidence. In calcium-loaded lenses (group 4) with a 50% reduction in protein synthesis, the 14C-histidine uptake ratio (data not shown) decreased only to a value of 2.1, whereas following ouabain treatment, the uptake ratio fell to 1.3, yet protein synthesis was unaffected. This is also evident from the decrease in the amount of soluble radioactivity in the pool, or the total cpm/lens. Thus, a decrease in histidine influx is not sufficient to retard protein synthesis over the observed period of time.

Although calcium-dependent biosynthesis of proteins has not been reported previously in the lens, the concept is not new and has been observed in the rat parotid gland, where perhaps calcium acts indirectly on the intracellular messengers cyclic AMP or GMP. Whether or not calcium exerts an inhibitory influence on crystallin synthesis directly or indirectly by altering the ATP content in the lens, for example, is not known, and further studies are necessary. While a number of physiologic changes might occur in lenses precultured at 21°C in the modified media employed in these experiments, it is important to remember that control and experimental incubation conditions generally differed only with respect to the calcium content or the presence of an agent like A23187 to specifically alter calcium levels. Regardless of the mechanism, the results of this study involving normal rabbit lenses suggest that when considering the influence of cations on biosynthesis of lens proteins, elevated levels of calcium also may be critically important, as are possible synergistic interactions with sodium or potassium. At present, conditions are being tested to permit extended culture of lenses in which Na/K levels can be altered without also affecting a change in lens calcium. In addition, cell-free systems of proteins will be investigated in order to better distinguish between the effects of calcium, sodium, and other ions on the synthesis of the individual crystallins.

**Key words:** calcium, lens, protein synthesis, cataract

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Sorbitol, resulting from glucose metabolism through aldose reductase, may play a role in diabetic complications such as cataracts, neuropathy, and vasculopathy. Sulindac (Clinoril®) and sorbinil, two inhibitors of aldose reductase, decreased sorbitol formation in cataract or nerve tissue incubated in high glucose TC-199 media. Sulindac, a widely used anti-rheumatic drug, may have clinical applications in preventing diabetic complications. Invest Ophthalmol Vis Sci 24:1426-1429, 1983

The presence of the polyol pathway in animal lens and nerve and the role of sorbitol in formation of diabetic cataracts and neuropathy is well established. In the diabetic rat lens, the accumulation of non-diffusible sorbitol creates a hyperosmotic state leading to lens swelling and increased permeability to cations, sodium, and potassium. Compensatory pump activity occurs, until the pump is overwhelmed and intracellular cations and water increase. Eventually, the clarity of the lens is lost and a cataract is formed. In the rat lens, aldose reductase (AR) inhibition prevented sorbitol production with its subsequent pathologic steps. However, in human diabetic cataracts, sorbitol increases are not uniform and its role in cataract formation requires further investigation. Similarly, the polyol pathway in other tissues has obvious implications for the etiology of diabetic vasculopathy or neuropathy.

Fatty acids, flavonoids, 3,3-tetramethylene glutaric acid, and Alrestatin, have been shown, in vitro, to effectively inhibit AR of animal and human lenses. Lens incubation with high glucose allowed evaluation of AR inhibition in vitro. Both in vitro and in animal experiments, Alrestatin inhibited AR and decreased sorbitol formation. However, drug toxicity, including hepatotoxicity, in human clinical trials obviated its usefulness. More potent, less toxic AR inhibitors are being sought for therapy of diabetic neuropathy, cataracts, and retinopathy. Recently, we found that the antirheumatic drugs, salicylate, indomethacin, oxyphenbutazone, and sulindac, inhibit AR of animal and human lenses. Now we report that sulindac [cis-5-fluoro-2-methyl-1-p-(methylsulfonyl) benzylindene-3 acetic acid] (Fig. 1), is an inhibitor of sorbitol formation in human cataracts, rat lens, and sciatic nerve. The inhibitory effect of sulindac was compared with that of sorbinil (d-6-fluoro-spirochroman-4'-imidazolidine-2'-5' dione), another potentially clinically useful AR inhibitor.

Materials and Methods. From 250-g albino rats, the lenses were removed after enucleation with ether anesthesia and then weighted. Rat lenses were placed in 1 ml of TC 199 bicarbonate media containing 5.5 mM glucose (low) or 35.5 mM glucose (high glucose) and incubated at 37°C for 24 hours in round-bottom, Kjeldahl flasks. Sulindac or sorbinil were both tested in lenses and nerve using the contralateral tissue as control. Sciatic nerves were obtained from 2-kg albino rabbits immediately after death. The nerves were dissected longitudinally into pieces of equal size (ap-