Imaging of intracellular calcium stores in single permeabilized lens cells

GRANT C. CHURCHILL1 AND CHARLES F. LOUIS1,2
Departments of 1Biochemistry and 2Veterinary Pathobiology, University of Minnesota, St. Paul, Minnesota 55108

Churchill, Grant C., and Charles F. Louis. Imaging of intracellular calcium stores in single permeabilized lens cells. Am. J. Physiol. 276 (Cell Physiol. 45): C426–C434, 1999.—Intracellular Ca2+ stores in permeabilized sheep lens cells were imaged with mag-fura 2 to characterize their distribution and sensitivity to Ca2+-releasing agents. Inositol 1,4,5-trisphosphate (IP3) or cyclic ADP-ribose (cADPR) released Ca2+ from intracellular Ca2+ stores that were maintained by an ATP-dependent Ca2+ pump. The IP3 antagonist heparin inhibited IP3, but not cADPR-mediated Ca2+ release, whereas the cADPR antagonist 8-amino-cADPR inhibited cADPR, but not IP3-mediated Ca2+ release, indicating that IP3- and cADPR were operating through separate mechanisms. A Ca2+ store sensitive to IP3, cadPR, and thapsigargin appeared to be distributed throughout all intracellular regions. In some cells a Ca2+ store insensitive to IP3, cADPR, and thapsigargin, as well as a Ca2+ store that appears insensitive to all these agents.

Inositol 1,4,5-trisphosphate; cyclic ADP-ribose; calcium pools; mag-fura 2; epithelium

Many extracellular signals increase cytosolic Ca2+ by activating the release of Ca2+ from intracellular stores (2). In the best-characterized mechanism for Ca2+ release, the second messenger inositol 1,4,5-trisphosphate (IP3) activates intracellular Ca2+ channels (2). Although its mechanism is not as well characterized as that of IP3, cyclic ADP-ribose (cADPR) also activates Ca2+ release from intracellular stores in sea urchin eggs (6, 20) and certain mammalian cells (5, 9, 11, 13, 14, 18).

Intracellular Ca2+ stores have commonly been characterized by their sensitivity to Ca2+-releasing agents, either physiological or pharmacological. Most cells contain Ca2+ pools that are sensitive to IP3, thapsigargin, and ionomycin (2), and some cells contain a Ca2+ pool that is sensitive to cADPR (5, 6, 9, 11, 13, 14, 18, 20). These Ca2+ pools overlap to varying degrees, suggesting that the intracellular Ca2+ stores are functionally organized into distinct compartments sensitive to only certain agents (2, 6, 9, 11, 13, 14, 18, 20). The location of intracellular Ca2+ stores sensitive to IP3, inhibitors of the intracellular Ca2+ pump, activators of ryanodine receptors, or ionomycin has been revealed by direct imaging of permeabilized cells, the organelles of which contain a Ca2+-sensitive dye (11, 12, 15–17, 26, 27, 29).

In contrast, the location of cADPR-sensitive Ca2+ stores has not been defined, because the cells containing the Ca2+ stores that have been imaged were unresponsive to cADPR (17, 29). Nevertheless, it has been demonstrated that certain intracellular Ca2+ stores such as the envelope of isolated nuclei (10) and isolated zymogen granules (9) are sensitive to cADPR and IP3.

The ocular lens is a transparent tissue containing only two cell types: fiber cells, which lack organelles and make up the bulk of its mass, and epithelial cells, which contain organelles and form a single layer on its anterior surface (22). In the lens the loss of Ca2+ homeostasis is implicated in the loss of transparency and cataract formation (7), so it is important to better define the mechanisms by which Ca2+ is regulated in the lens. Duncan and co-workers (8) demonstrated that permeabilized human lens cells in suspension exhibit thapsigargin-sensitive ATP-dependent Ca2+ uptake and IP3-mediated Ca2+ release. However, the location of the intracellular Ca2+ stores, their sensitivity to other Ca2+-releasing agents, and the overlap among the various Ca2+ pools are unknown for lens cells of any species.

The objective of this study was to characterize the intracellular Ca2+ stores of mammalian lens cells in terms of their distribution and sensitivity to Ca2+-releasing agents. Intracellular Ca2+ stores in permeabilized cells were imaged with mag-fura 2 and fluorescence microscopy (15). A sheep lens cell culture system (28) was used in which the cells exhibit agonist-mediated Ca2+ signaling (4) as well as cell-to-cell Ca2+ waves (3). We conclude that lens cells contain IP3-, cADPR-, and thapsigargin-sensitive intracellular Ca2+ stores that are distributed throughout the cell, as well as Ca2+ stores that are insensitive to all these agents that are localized in a juxtanuclear region.

MATERIALS AND METHODS

Materials. cADPR and 8-amino-cADPR were generous gifts from Dr. Timothy Walseth (University of Minnesota). Sheep eyes were obtained from John Morrell (Sioux Falls, SD). Medium 199 and Hanks’ balanced salt solution (HBSS) were obtained from Gibco (Grand Island, NY). Fetal calf serum was obtained from Hyclone (Logan, UT). Mag-fura 2-AM was obtained from Molecular Probes (Eugene, OR). Ionomycin, thapsigargin, o-IP3, and L-IP3 were obtained from LC Laboratories (Woburn, MA). Saponin, digitonin, ADP-ribose, 8-B-DNP, ATP, creatine, creatine kinase (porcine), heparin (6-kDa average fragment size), and all other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Cell culture. Primary cultures of cells isolated from the equatorial region of fresh sheep lenses were prepared as described previously (28). The sheep lens epithelial cells used in this study were cultured for 4–34 days.

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Monitoring intracellular store Ca\(^{2+}\) concentration. Cells were maintained in HBSS supplemented with 10 mM HEPES (HBSS-H, pH 7.2) before they were loaded with mag-fura 2 by incubation in 6 µM mag-fura 2-AM for 1 h at 37°C, which promotes compartmentalization of the dye into intracellular organelles (15). After they were loaded, cells were rinsed three times with HBSS-H and incubated for 20 min at 37°C to promote complete hydrolysis of the AM ester. The glass coverslip with attached cells formed the bottom of a microincubation culture chamber (model MS 2000, Medical Systems, Greenvale, NY). The chamber was mounted on the stage of an inverted epifluorescence microscope (model IM 35, Zeiss) supported on a vibration-isolated table (Technical Manufacturing, Peabody, MA). Cells were viewed through a ×40, 1.3-NA, oil-immersion objective lens (Fluor 40, Nikon, Melville, NY).

Mag-fura 2 was excited with light from a 50-W mercury lamp alternately filtered to 340 or 380 nm. Fluorescence emission was filtered to >510 nm, focused with a ×20 lens, and monitored with a silicon intensified target camera (model VE-1000, Dage-MTI, Michigan City, IN). The camera's gain and kilovolts were set to manual and initially adjusted for the fluorescence intensity of intact cells but were increased to control the volume in the chamber (0.3–5 ml). Ca\(^{2+}\)-releasing agents were added to the cells by dilution of the stock solution (never >1% of the final volume bathing the cells) into ~100 µl of the intracellular buffer and addition of this to the chamber. Then 100 µl of the intracellular buffer were removed and readed five times from different regions of the microincubator to ensure complete mixing of the added compound.

Data analysis and presentation. Data were analyzed only from regions that exhibited a stable mag-fura 2 fluorescence ratio for at least 5 min in the absence of a Ca\(^{2+}\)-releasing agent. The results are from single experiments that are representative of the most frequently observed response for a given treatment. The number of independent experiments (n) is taken as the response of a field of 5–30 cells to a given treatment. All experiments were repeated a minimum of three but typically many more times.

Ca\(^{2+}\) concentrations are not presented as absolute values but, rather, as the ratio of mag-fura 2 fluorescence at 340-nm excitation to that at 380-nm excitation, because the amount of Mg\(^{2+}\) bound to mag-fura 2 is unknown, making the estimate of Ca\(^{2+}\) concentration potentially inaccurate (16, 27). Nevertheless, changes in Ca\(^{2+}\) concentration are reflected by the mag-fura 2 340 nm-to-380 nm fluorescence ratio, which is directly proportional to the concentration of free Ca\(^{2+}\) (dissociation constant = 53 µM) (15, 25).

RESULTS

Monitoring Ca\(^{2+}\) concentration in intracellular stores with mag-fura 2. The first step in this study was to determine whether, as reported previously in other cell types (15), the Ca\(^{2+}\) concentration in intracellular stores in lens cells could be directly monitored with mag-fura 2. After mag-fura 2 loading but before permeabilization, most cells exhibited a similar fluorescence ratio throughout the cells (Fig. 1Aa, ratio, cell 2); however, certain cells exhibited a higher fluorescence ratio (Fig. 1Ba, ratio, cell 1), likely because of the presence of mag-fura 2 in intracellular compartments. After the addition of the intracellular buffer with 300 nM Ca\(^{2+}\), the cells began to round up and pull apart from one another (Fig. 1Bb), and the mag-fura 2 ratio decreased in the cells that had an elevated fluorescence ratio (Fig. 1A, A and Bb, ratio). This decrease in the mag-fura 2 ratio was apparently due to the release of Ca\(^{2+}\) from intracellular stores and was likely due to the mechanical strain imposed on the cells during the change in morphology, since switching to low-Ca\(^{2+}\)-concentration medium can trigger a transient increase in cytosolic Ca\(^{2+}\) concentration (data not shown). After incubation for ~9 min in an intracellular buffer containing digitonin (10 µg/ml) without ATP, most cells were permeabilized on the basis of a loss of fluorescence intensity that was most evident over nuclear regions (Fig. 1Bc, 340 nm). The overall decrease in fluorescence intensity with permeabilization is not evident in Fig. 1Bc, because the camera's amplification (gain and kV settings) was increased to compensate for the loss of dye from the cytosol. The remaining fluorescence was concentrated in several regions that are likely organelles with trapped dye, which appeared as evenly fluorescent regions or punctate vesicular compartments (Fig. 1Bc, 340 nm). After the addition of 3 mM ATP, the mag-fura 2 fluorescence ratio increased rapidly for ~5 min and then more slowly over the next 20 min (Fig. 1Aa). These data demonstrate that mag-fura 2 can be used to monitor the Ca\(^{2+}\) concentration in the intracellular stores of sheep lens cells.

IP\(_3\) releases Ca\(^{2+}\) from intracellular stores. That the Ca\(^{2+}\) concentration in intracellular stores could be
monitored in permeabilized lens cells enabled the activity of Ca²⁺-releasing agents to be tested by directly adding them to the medium bathing the permeabilized cells. IP₃ mobilizes Ca²⁺ in many cell types (2), and 0.1–5 µM has been shown to mobilize Ca²⁺ from permeabilized human lens cells in suspension (8). Figure 2 shows the effect of various concentrations of IP₃ on the intracellular store Ca²⁺ concentration in a cytoplasmic region of a permeabilized sheep lens cell. The addition of submaximal concentrations (0.16–8 µM) of IP₃ resulted in a rapid release of only a fraction of the Ca²⁺ that could be released with maximal concentrations (≥16 µM) of IP₃ (Fig. 2). This phenomenon is termed quantal Ca²⁺ release and is commonly reported during IP₃-mediated Ca²⁺ release in other cell types (2, 21).

cADPR releases Ca²⁺ from intracellular stores. cADPR is known to mobilize Ca²⁺ from intracellular stores in sea urchin eggs (6, 20), as well as in certain mammalian cells (5, 9, 11, 13, 14, 18). To determine whether cADPR could release Ca²⁺ from permeabilized lens cells, cADPR was added to the buffer bathing permeabilized sheep lens cells. The effect of cADPR on the intracellular store Ca²⁺ concentration was variable; therefore, the results from three experiments are presented, which depict the different types of responses of permeabilized lens cells to added cADPR. In the experiment shown in Fig. 3A, 10 µM cADPR elicited a monophasic, nonquantal decrease in the intracellular store Ca²⁺ concentration; the rate of Ca²⁺ release was not accelerated by the addition of higher concentrations of cADPR. In contrast, in the experiment shown in Fig. 3C, 0.5 µM cADPR elicited a rapid, quantal decrease in the Ca²⁺ concentration in intracellular stores. The subsequent addition of 5 µM cADPR led to no further Ca²⁺ release, whereas the addition of 50 µM cADPR resulted in further quantal Ca²⁺ release (Fig. 3C). A similar quantal Ca²⁺ release is shown in Fig. 3D; however, in this experiment the cells were monitored for 10 min before the first addition of the cADPR, demonstrating that the Ca²⁺ concentration...
in the intracellular stores was stable until the addition of cADPR. These results indicate that although cADPR can mobilize Ca\(^{2+}\) from intracellular stores in sheep lens cells, the response to cADPR addition was more variable than that obtained with IP\(_3\) in terms of the rate of Ca\(^{2+}\) release and whether the Ca\(^{2+}\) release was quantal.

Specificity of IP\(_3\) - and cADPR-mediated Ca\(^{2+}\) release. To determine whether IP\(_3\) and cADPR released Ca\(^{2+}\) from intracellular stores through specific and separate mechanisms, the effect of specific antagonists and analogs of these Ca\(^{2+}\)-releasing messengers was evaluated. In the first approach the responses to IP\(_3\) and cADPR were assessed in the presence of the IP\(_3\) antagonist heparin (2) or the cADPR antagonist 8-amino-cADPR (31). Heparin (100 µg/ml) blocked the Ca\(^{2+}\) release mediated by 16 µM IP\(_3\), but not that mediated by 10 µM cADPR (Fig. 4A). Conversely, 8-amino-cADPR (40 µM) blocked the Ca\(^{2+}\) release mediated by 10 µM cADPR, but not that mediated by 16 µM IP\(_3\) (Fig. 4B). These results indicate that IP\(_3\) and cADPR are acting through separate mechanisms.

In the second approach the Ca\(^{2+}\)-releasing activity of IP\(_3\) or cADPR analogs was evaluated. IP\(_3\) is an enantiomer, and the naturally occurring o-form has a 100- to 1,000-fold greater affinity than the l-form for the IP\(_3\) receptor (24). In sea urchin eggs, cADPR is formed from \(\beta\)-NAD\(^+\) and is metabolized to ADPR (20). Ca\(^{2+}\) was not released from intracellular Ca\(^{2+}\) stores in permeabilized lens cells after the addition of 32 µM l-IP\(_3\), 50 µM ADPR, or 200 µM \(\beta\)-NAD\(^+\), but Ca\(^{2+}\) was released after the addition of 50 µM cADPR (Fig. 4C). Collectively, these results indicate that IP\(_3\) and cADPR release Ca\(^{2+}\) through specific mechanisms.

Spatial distribution and functional overlap of the intracellular Ca\(^{2+}\) stores sensitive to IP\(_3\), cADPR, and thapsigargin. The next set of experiments was designed to reveal whether lens cells contained a single intracellular Ca\(^{2+}\) store sensitive to all Ca\(^{2+}\)-releasing agents or multiple intracellular Ca\(^{2+}\) stores sensitive to only certain agents. Also, to determine whether functionally distinct Ca\(^{2+}\) stores were also spatially distinct, as demonstrated recently in astrocytes and myocytes (12), three regions of interest were defined: one over the nucleus, a second over a juxtanuclear region, and a third over a cytoplasmic region. An experiment in which the IP\(_3\)-sensitive Ca\(^{2+}\) stores were depleted before the addition of cADPR is shown in Fig. 5, A and C. The addition of 16 µM IP\(_3\) released a portion of the stored Ca\(^{2+}\) from all three regions monitored (Fig. 5, A and C), and the subsequent addition of 32 µM IP\(_3\) led to the release of additional Ca\(^{2+}\) (Fig. 5, A and C). The subsequent addition of 30 µM cADPR failed to release...
any additional Ca\(^{2+}\), indicating that IP\(_3\) had completely depleted the Ca\(^{2+}\) stores sensitive to cADPR. The addition of thapsigargin released a small amount of additional Ca\(^{2+}\) from all three intracellular regions. The addition of 10 \(\mu\)M ionomycin released the remaining Ca\(^{2+}\) from all the intracellular Ca\(^{2+}\) stores in all regions of the cell. The nuclear region appears to have ionomycin-insensitive Ca\(^{2+}\) stores, but this is an artifact arising from the low fluorescence intensity of mag-fura 2 in this region after permeabilization of the cells (Fig. 1B). The weaker the mag-fura 2 fluorescence, the more the ratio will be influenced by noise. The pixel intensities due to noise above the background will be approximately equal at 340- and 380-nm excitation, yielding a ratio of \(1\). This "noise" ratio is averaged with the ratio reporting Ca\(^{2+}\), presumably 0.6, on the basis of the other regions of the cell that have sufficient dye to accurately report on Ca\(^{2+}\), resulting in an apparent ratio of 0.8.

An experiment in which the cADPR-sensitive Ca\(^{2+}\) stores were depleted before the addition of IP\(_3\) is shown in Fig. 5, D and F. The addition of 1 \(\mu\)M cADPR resulted in a slow release of Ca\(^{2+}\) from all three intracellular regions (Fig. 5, D and F). The subsequent addition of 30 \(\mu\)M cADPR did not release appreciably more Ca\(^{2+}\), and all three regions reached a new steady state soon thereafter. The addition of IP\(_3\) released additional Ca\(^{2+}\) from all three regions, and this release was accelerated by the addition of thapsigargin. The addition of ionomycin rapidly released all the remaining Ca\(^{2+}\) from all three regions.

Thapsigargin-, IP\(_3\)-, and cADPR-insensitive intracellular Ca\(^{2+}\) stores. In some cell types such as sea urchin eggs, the thapsigargin-sensitive Ca\(^{2+}\) pools completely overlap the IP\(_3\)- and/or cADPR-sensitive Ca\(^{2+}\) pools (20); however, in other cell types, these Ca\(^{2+}\) pools are separable. For example, cADPR releases Ca\(^{2+}\) from a thapsigargin-insensitive Ca\(^{2+}\) pool in T lymphocytes (14), and IP\(_3\) and cADPR, but not thapsigargin, release Ca\(^{2+}\) from secretory granules isolated from pancreatic acinar cells (9). Therefore, it was of interest to determine whether cADPR or IP\(_3\) could release Ca\(^{2+}\) from a thapsigargin-insensitive store in lens cells. To address this issue, mag-fura 2-loaded permeabilized lens cells were treated sequentially with thapsigargin, IP\(_3\), and cADPR (Fig. 6). Thapsigargin almost fully depleted all the intracellular Ca\(^{2+}\) stores in the cytoplasmic regions but only partially depleted the intracellular Ca\(^{2+}\) stores in a juxtanuclear region (Fig. 6, A and Bb). Neither the thapsigargin-sensitive nor the thapsigargin-insensitive Ca\(^{2+}\) stores released any additional Ca\(^{2+}\) in response to the subsequent additions of 16 \(\mu\)M IP\(_3\) or 30 \(\mu\)M cADPR (Fig. 6A). These data demonstrate that the thapsigargin-sensitive Ca\(^{2+}\) stores appear to completely overlap the IP\(_3\)- and cADPR-sensitive Ca\(^{2+}\) stores and that the thapsigargin-insensitive Ca\(^{2+}\) stores are also IP\(_3\) and cADPR insensitive. In some cell types, certain Ca\(^{2+}\) stores have been reported to contain a thapsigargin-resistant Ca\(^{2+}\) pump with an IC\(_{50}\) for thapsigargin of 5 \(\mu\)M compared with the more typical IC\(_{50}\) of \(0.2 \mu\)M (30); therefore, a higher concentration of thapsigargin was applied (10 \(\mu\)M) but was also without effect (Fig. 6A).

Mag-fura 2 fluorescence has been shown to respond to mitochondrial Ca\(^{2+}\) concentration (16); therefore, it was considered that the thapsigargin-, IP\(_3\)-, and cADPR-insensitive Ca\(^{2+}\) stores identified in sheep lens cells might be mitochondria. Mitochondrial Ca\(^{2+}\) can be released by agents that dissipate their electrochemical gradient (17). After the addition of thapsigargin to deplete all the thapsigargin-insensitive Ca\(^{2+}\) stores,
the addition of the proton ionophore 2,4-dinitrophenol did not release Ca\textsuperscript{2+} from the thapsigargin-insensitive Ca\textsuperscript{2+} stores, whereas ionomycin rapidly released this Ca\textsuperscript{2+} (Fig. 7). These results indicate that the thapsigargin-, IP\textsubscript{3}-, and cADPR-insensitive Ca\textsuperscript{2+} stores were not mitochondria.

**DISCUSSION**

Intracellular Ca\textsuperscript{2+} stores in permeabilized sheep lens cells were imaged with mag-fura 2 to characterize their distribution and sensitivity to Ca\textsuperscript{2+}-releasing agents. The intracellular Ca\textsuperscript{2+} stores exhibited ATP-dependent Ca\textsuperscript{2+} uptake and IP\textsubscript{3}- and cADPR-mediated Ca\textsuperscript{2+} release. These results are consistent with the previous report of IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release in permeabilized human lens cells (8) and with the ability of an inhibitor of phospholipase C to prevent agonist-mediated cytosolic Ca\textsuperscript{2+} increases in sheep lens cells (4). In permeabilized lens cells the response to cADPR was less consistent than the response to IP\textsubscript{3} in regard to
IP$_3$- and cADPR-sensitive Ca$^{2+}$ stores in lens cells appeared to be functionally distinct in terms of the relative overlap of the two Ca$^{2+}$ pools. In some experiments the IP$_3$- and cADPR-sensitive Ca$^{2+}$ pools completely overlapped, whereas in other experiments cADPR released only a portion of the IP$_3$-releasable Ca$^{2+}$ pool. Similarly, the portion of the thapsigargin-sensitive Ca$^{2+}$ pool released by IP$_3$ was variable. Taken together, these results indicate heterogeneity in the Ca$^{2+}$ stores in lens cells. This heterogeneity was not manifested as spatially distinct Ca$^{2+}$ stores sensitive to IP$_3$ or cADPR, however, indicating that the Ca$^{2+}$ stores might be organized into distinct compartments on a scale that is below the spatial resolution of conventional microscopy or organized along the axis through which the cells are viewed. Inasmuch as conventional microscopy was used in this study, only the average Ca$^{2+}$ in all stores through the thickness of the cell was detected.

Regardless of the intracellular organization of the Ca$^{2+}$ stores, our data indicate the presence of at least three types of functionally distinct Ca$^{2+}$ stores in lens cells. These Ca$^{2+}$ pools may represent physically distinct Ca$^{2+}$ stores with different complements of Ca$^{2+}$ channels and pumps. In one type of Ca$^{2+}$ store there would be channels activated by IP$_3$ and cADPR as well as a thapsigargin-sensitive Ca$^{2+}$ pump. In the second type of Ca$^{2+}$ store there would be Ca$^{2+}$ channels activated by IP$_3$ but not cADPR as well as a thapsigargin-sensitive Ca$^{2+}$ pump. In the third type of Ca$^{2+}$ store there would be no Ca$^{2+}$ channels activated by either IP$_3$ or cADPR and no thapsigargin-sensitive Ca$^{2+}$ pump. The relative abundance of each of these Ca$^{2+}$ stores in any intracellular region would determine the degree of functional overlap among the various Ca$^{2+}$ pools in that region.

This third type of Ca$^{2+}$ store that was insensitive to IP$_3$, cADPR, and thapsigargin was functionally and spatially distinct from the Ca$^{2+}$ stores sensitive to these...
agents. This store's juxtanuclear location indicates that it may be the Golgi apparatus, although this could not be proven with organelle-selective dyes, because they failed to resolve the organelle distribution in lens cells (data not shown). IP$_3$- and thapsigargin-insensitive Ca$^{2+}$ pools have been reported previously in other cell types (16, 23, 30). Similar to lens cells, a Ca$^{2+}$ store in a juxtanuclear region exhibited reduced responsiveness to IP$_3$ in BHK-21 cells; however, unlike lens cells, this store was sensitive to thapsigargin (17). Hofer et al. (17) proposed that the partial Ca$^{2+}$ release attained in the intracellular regions containing the Golgi apparatus arose from superimposition of the Golgi apparatus and the intracellular regions containing the Golgi apparatus. Additionally, Ca$^{2+}$, cADPR, and thapsigargin and that these stores are also present in some lens cells that are insensitive to Ca$^{2+}$-releasing agents and changes in the relative sizes of the Ca$^{2+}$ pools sensitive to a given agent.

In conclusion, we have demonstrated that sheep lens cells contain Ca$^{2+}$ stores that are sensitive to IP$_3$, cADPR, and thapsigargin and that these stores are distributed throughout the cell. Additionally, Ca$^{2+}$ stores are also present in some lens cells that are insensitive to IP$_3$, cADPR, thapsigargin, and 2,4-dinitrophenol and are localized in a juxtanuclear region. It will be important to examine whether depletion of any of these Ca$^{2+}$ stores is responsible for Ca$^{2+}$ regulation defects that result in cataract formation.

We thank Tim Walseth (University of Minnesota) for generously providing cyclic ADP-ribose and 8-amino-cyclic ADP-ribose and for helpful discussions.

G. C. Churchill was partially supported by a doctoral dissertation fellowship awarded by the Graduate School, University of Minnesota. This research was supported by National Eye Institute Grant EY-05684.

Address for reprint requests: C. F. Louis, Dept. of Veterinary Pathobiology, University of Minnesota, 1988 Fitch Ave., Rm. 295, St. Paul, MN 55108.

Received 15 April 1998; accepted in final form 12 October 1998.

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