Uptake of Calcium by the Endoplasmic Reticulum of the Frog Photoreceptor

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ABSTRACT   We studied retinal photoreceptors of Rana pipiens by using techniques designed to investigate calcium localization. Particularly useful were methods in which intracellular sites of calcium uptake were detected by incubation of saponin-treated isolated retinas in calcium-containing media, with oxalate present as a trapping agent. With these procedures, cell compartments accumulate deposits, which can be shown to contain calcium by x-ray microanalysis. Calcium accumulation was prominent in the rough endoplasmic reticulum in the myoid region. In addition, deposits were observed in agranular reticulum and in certain Golgi-associated compartments of the myoid region, in mitochondria, in axonal reticulum, and in agranular reticulum of presynaptic terminals. Calcium was also detected in the endoplasmic reticulum of retinas fixed directly upon isolation, by a freeze-substitution method. The factors influencing accumulation of calcium in the endoplasmic reticulum were evaluated by a semiquantitative approach based on determining the relative frequency of calcium oxalate crystals under varying conditions. Calcium accumulation was markedly enhanced by ATP. Studies with a nonhydrolyzable ATP analogue (adenylyl-imidodiphosphate) and with inhibitors of the sarcoplasmic reticulum $Ca^{2+}-Mg^{2+}$ ATPase (mersalyl and tetracaine) indicated that this ATP-dependent calcium uptake reflects an energy-dependent process roughly comparable to that in the sarcoplasmic reticulum.

Calcium ions play regulatory roles in a variety of cell types. Most extensively studied is skeletal muscle, in which Ca^{2+} regulates the contraction-relaxation cycle (21, 69). In skeletal muscle (30, 67, 69) and also in cardiac (17, 23, 25, 81, 98) and smooth muscle (18, 71), the sarcoplasmic reticulum, which is a specialized form of the smooth endoplasmic reticulum, is largely responsible for the control of concentrations of calcium ions free in the sarcoplasm. By means of an energy-dependent $Ca^{2+}-Mg^{2+}$ ATPase, the sarcoplasmic reticulum can transport calcium ions into its interior (38, 85) where the ions are sequestered by binding to calsequestrin and other binding proteins.

Much less is known about the regulation of calcium concentration in cells of the nervous system. There is reason to suspect that the situation may resemble that in muscle, at least to some extent. Though few details are understood, there is good evidence that Ca^{2+} plays a focal regulatory part in neurons, participating, for example, in regulation of transmitter release (20, 40, 47, 57). Neuronal mitochondria can accumulate calcium (2, 66), as can mitochondria in other cell types, and calcium deposits are observed in synaptic vesicles (11, 36, 70, 75, 83) and possibly in neuronal coated vesicles (10, 87), under a variety of conditions. It is thought more likely, however, that short-term physiological regulation of calcium in nerve axons and terminals depends on other organelles, chiefly elements of the system of agranular sacs and tubules generally referred to as the smooth endoplasmic reticulum or the axonal reticulum (9, 19, 37, 38, 52, 53, 83). It has been suggested that the smooth endoplasmic reticulum has a role in "depolarization-secretion" coupling, analogous to that of the sarcoplasmic reticulum in "excitation-contraction" coupling (52, 53).

Calcium ions are thought to play highly significant roles in photoreceptors of vertebrates (29, 41, 45, 58) and invertebrates (4, 14), both in the initial response of the photoreceptor to light (15, 27, 99) and in the subsequent events of neurotransmitter release (46, 77). There is, however, controversy as to the mechanisms of calcium involvement. For invertebrate

photoreceptors, an ATP-dependent uptake system, in what is thought to be smooth endoplasmic reticulum, seems to participate in regulating calcium levels (92–95). In vertebrate photoreceptors, which are a cell type quite different from those of invertebrates, calcium has been detected in the outer segment disks (84) and possibly in synaptic vesicles (76 and our own unpublished observations), but little is known about calcium regulation in these cells.

In the present study of frog retinal photoreceptors we demonstrate that the endoplasmic reticulum (ER),[1] including notably the rough ER, can take up calcium and that this uptake is ATP dependent. Calcium accumulation by the ER occurs by mechanisms similar to those of the sarcoplasmic reticulum at least in that they depend on the hydrolysis of the terminal phosphate group of ATP, and are inhibited by agents that also inhibit the Ca^{2+}–Mg^{2+} ATPase of the sarcoplasmic reticulum. For preliminary accounts of certain aspects of this work, see references 35 and 88.

MATERIALS AND METHODS

General: *Rana pipiens,* ~ 2.5–3 in in body length, were obtained from the Lake Champlain Frog Farm, Alberg, VT. Most of the experiments used frogs that had been maintained for a few days to several weeks on a 12-h light/ 12-h dark artificial lighting cycle and had been fed meal worms twice weekly. The ATP analog and inhibitor experiments, as well as the experiments performed for quantitation of ATP effects, involved frogs kept near a window in laboratory lighting conditions and fed meal worms daily from arrival until being killed, a period of at least 10 d (86).

Frogs were dark adapted before decapitation, and retinas were isolated under dim red light (Kodak safety filter No. 2, Eastman Kodak Co., Rochester, NY) into frog Ringer's solution (NaCl, 111 mM; NaHCO₃, 2.3 mM; KCl, 2 mM; CaCl₂, 1.2 mM; D-glucose, 5 mM; HEPES, 20 mM; pH 7.4).

All solutions were made up in distilled H₂O that was additionally passed through an ion-exchange demineralizer so as to produce a specific resistance of > 0.5–1 MΩ.

Unless otherwise indicated, our microscopy was done on material fixed as described below, then dehydrated in ethanol (with 15 mM oxalate in the first of the ethanol series [50%]) and embedded in Epon (see reference 88). Thin sections were cut at a thickness of either 70–90 nm (silver-gold interference color) or 224–240 nm (blue interference color), and viewed under the electron microscope unstained, or occasionally after staining with uranyl and/or lead (90, 96). Some blue sections were also cut using glycerol in place of water in the microtome boat (63).

Calcium Oxalate Method Based on That of McGraw and Blaustein: The principal procedure used to demonstrate accumulation of calcium is based on that of McGraw, Blaustein, and co-workers (7, 51–53; see reference 88). The method in essence: retinas were preincubated in a high-K⁺ Ringer's solution (NaCl, 5 mM; KCl, 145 mM; MgCl₂, 1.4 mM; KH₂PO₄, 2 mM; HEPES–Tris buffer, 20 mM; pH 7.5) containing 0.25 mg/ml saponin. Saponin renders the plasma membrane highly permeable to large, charged molecules—most likely owing to the complexing of saponin with sterols in the membrane; the permeability of compartments such as the ER or mitochondria should be less affected because the bounding membranes of these organelles have less cholesterol than does the plasma membrane (e.g., see reference 62 for additional discussion and references). After preincubation, the retinas were incubated with gentle agitation, in the light, in a similar high-K⁺ Ringer's solution lacking saponin but including 15 μM CaCl₂, 15 mM potassium oxalate (Sigma Chemical Co., St. Louis, MO), 2 mM ATP (Calbiochem-Behring Corp., La Jolla, CA), 5 mM phosphoenol pyruvate (Sigma Chemical Co.), and 1 U/ ml of pyruvate kinase (Sigma Chemical Co.). Retinas were then fixed in Karnovsky's fixative (39) containing 15 mM oxalate, and postfixed in a cacodylate-buffered solution of 1% OsO₄.

Variations, ATP Analogues, Inhibitors: The following variations on the above procedure were performed: (*a*) ATP was omitted from the incubation solution; (*b*) ATP was omitted from, and 1 mg/ml apyrase (Sigma Chemical Co.) added to, the incubation solution; (*c*) either oxalate or calcium was omitted from all solutions; (*d*) calcium was omitted from all solutions, and 10 mM EGTA (Sigma Chemical Co.) was added to the solutions, before postfixation; (*e*) incubations were performed with various concentrations of

either calcium (15, 30, 60, or 120 μM) or ATP (2, 4, 6, 8 mM); (*f*) either 1 mM of the SH reagent mersalyl (Sigma Chemical Co.) or 10 mM of the local anesthetic tetracaine (Sigma Chemical Co.) was added to the incubation solution; (*g*) ATP was omitted from, and 6 mM adenylyl-imidodiphosphate (AMP-PNP) (Boehringer Mannheim, Mannheim, Federal Republic of Germany) added to, the incubation solution.

Quantification of Crystals in the McGraw–Blaustein Preparations: The frequency of calcium oxalate deposits ("crystals") in ATP-treated preparations was compared to that in no-ATP preparations. Comparisons were made either between whole retinas or between the pair of half-retinas cut from a single retina. Thin sections of samples were viewed under the electron microscope, and the number of crystals per cell (myoid region) was counted directly. In addition, the number of crystals per unit area (myoid region) was determined from electron micrographs of these samples. Area was measured by overlaying a morphometric grid over the photographs. The counts in these experiments were generally done blind; i.e., the microscopist was unaware of the history of the preparations being examined.

Freeze Substitution: The freeze substitution method was adapted from procedures of Feder and Sidman (24), Somlyo et al. (79), and Ornberg and Reese (63), and Henkart et al. (31, 32). Retinas were dissected from the dark-adapted frog into frog Ringer's solution. The retinas were blotted on filter paper (in the light) and placed on a small piece of aluminum foil (receptor side up). The retina, on foil, was then plunged into isopentane which had been supercooled to below its freezing point (−130°C) by stirring in a styrofoam cup placed in liquid nitrogen. After 90 s, the retina was transferred into a solution of OsO₄–oxalate-acetone (4% OsO₄, 20 mM oxalic acid) on dry ice for 44 h; the vials were then brought to −20°C for 2 h. The retina was next transferred to fresh OsO₄–oxalate solution on ice for 1 h; the vials were then brought to room temperature for 1 additional h. Tissue then underwent three 5-min changes of acetone, followed by three 5-min changes of propylene oxide at room temperature. Tissue was embedded in Araldite. Thin sections were examined unstained in the electron microscope.

Since the electron-dense deposits formed with osmium–oxalate–acetone procedures contain considerable quantities of osmium compounds along with calcium (31, 32, 88), control experiments were carried out to evaluate the possibility that the calcium we observe accumulates as a result of the presence of osmium rather than by formation of calcium oxalate (for observations suggesting this possibility, see references 42 and 64). We generated calcium-containing oxalate–osmium–acetone deposits by preincubating the tissue in high-K⁺ Ringers containing 0.2 mM CaCl₂ and 10 mM potassium oxalate, then fixing in Karnovsky's fixative (39) with 10 mM oxalate and postfixing in 5% osmium–acetone (see also reference 88). Four types of controls were examined: (*a*) oxalate was omitted from all solutions; (*b*) calcium was omitted from all solutions, and 10 mM EGTA was added to all solutions before postfixation; (*c*) osmium was omitted from the postfixation solution (retinas were treated with acetone alone); (*d*) preincubation and incubation steps were omitted; retinas were immediately fixed in Karnovsky's fixative plus oxalate, then postfixed in 5% osmium in acetone.

X-ray Microanalysis: Samples were prepared for x-ray microanalysis as follows: blue (220–240 nm) sections like the one in Fig. 11 were placed on 200 mesh copper grids carrying a carbon-coated Formvar film and then coated with another light carbon coat.

For x-ray analysis, the instrument used was a Philips transmission electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ), equipped with a 400 twin lens system, an eccentric goniometer stage, a scanning transmission electron microscope attachment, and an energy dispersive x-ray analyzing system. When examining a specimen in either the transmission or the scanning transmission electron microscope system, we first selected an area of interest under normal condenser conditions. For energy dispersive x-ray analysis in the transmission electron microscope mode, the size and shape of the beam was adapted to conform to the area of interest by judiciously using the condenser stigmators. In the scanning transmission electron microscope mode, a circular (point) analysis was used. An area of the unstructured "ground" cytoplasm, not more than one or two beam diameters away, was analyzed under the same conditions and used as a control. A "net counts" spectrum was obtained by subtracting the reading in the control area from the reading in the area of interest; the spectra herein are net counts spectra as were virtually all on which we relied for evaluation of the deposits we observed. In general, we found very little calcium in the ground cytoplasm (see also legend to Fig. 11).

Most analyses were obtained in the TEM mode. For most of the studies, the analyzing conditions were: spot size = 400 Å; tilt angle = 0°; kV = 60; and emission current equaled 40 μA.

RESULTS

Fig. 1 schematizes the structure of the frog photoreceptor and indicates the locations of the regions to which we refer. Note

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[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; AMP-PNP, adenylyl-imidodiphosphate.
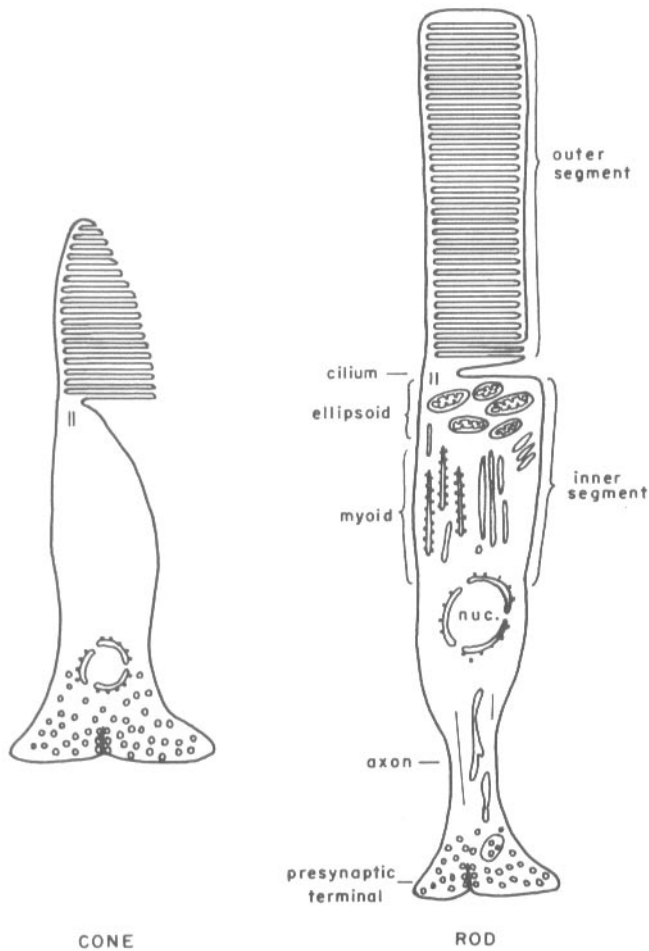
FIGURE 1 Schematic diagram, illustrating the major organelles and regions of the frog photoreceptor. Rod outer segments differ from those of cones in that rod disks, except for the most recently formed ones, are discrete, whereas cone disks are continuous with the plasma membrane. In addition, the rod synaptic terminal is separated from the nucleus by an axon, whereas the cone terminal abuts the nucleus.

that virtually all the cell's mitochondria are clustered in the "ellipsoid" region, and that the myoid contains the Golgi apparatus and most of the ER. For a detailed report on our prior studies of the morphology of the photoreceptor, see reference 55, in which we outline criteria for organelle recognition.

In this report, we omit discussion of mitochondrial localization of calcium, because mitochondrial uptake of calcium is a long-established phenomena. The methods that we have used do reveal calcium-containing deposits within mitochondria. With some of the methods we have used, calcium-containing deposits also are produced in the disks of rod outer segments and in synaptic vesicles; these localizations are expected in accordance with the literature cited at the beginning of this article, but we have not yet studied them in detail, partly because, as noted below, the methods used in the present report generate too much disruption of the outer segments for confident interpretation.

Calcium Oxalate Method

The procedures derived from those of McGraw et al. (52, 53), result in deposits ("crystals") of calcium oxalate with a

distinctive microscopic appearance (Figs. 2–7; the crystals exhibit a characteristic "bubbling" when subjected to the electron beam). Virtually all the crystals were located within membrane delimited compartments. The crystals show substantial levels of calcium detectable by x-ray microanalysis (Fig. 8). The formation of the crystals depends on uptake of calcium from the incubation medium (it is not observed when either Ca^{2+} or oxalate is omitted from the medium), and the crystals can generally be readily recognized as such in the electron microscope. Thus this approach offers appreciable advantages for studying the calcium accumulation system, when compared with more traditional methods, such as the ones based on precipitation with pyroantimonate; the latter methods produce deposits of complex, and apparently variable composition, whose interpretation has been very much in dispute. However, the McGraw–Blaustein method does result in considerable morphological distortion of the photoreceptors. Outer segments are often severely disrupted or absent, thus preventing adequate observation on these regions of the cells. Although sometimes, as in Fig. 7, the distortion of the myoid is relatively slight, it is more often that the intracellular organelles, especially the rough ER, are vesiculated, so as almost to resemble an in situ microsome preparation. In other work, we have noted comparable disruption in saponin-exposed retinas incubated in a variety of circumstances quite different from those in the present report and therefore have concluded, tentatively, that it is the saponin exposure that renders the photoreceptors subject to the observed effects. It may be, for example, that the rough ER is particularly sensitive because its permeability is minimally altered by saponin (see Materials and Methods), leaving it more subject to osmotic effects than are other compartments whose permeability barriers are compromised to a larger extent.

After treatment with the McGraw–Blaustein method, most of the structures containing crystals in the myoid region are derived from the ER. Most correspond to rough ER, reflecting the fact that rough ER is more abundant in the myoid than is smooth ER (55). Even in the unstained thin sections that we routinely used, ribosomes could be seen faintly, lining many of the crystal-containing vesicles (Fig. 2). When the thin sections were stained with uranyl acetate (which extracts the calcium oxalate crystals), virtually all profiles of the type that we thought to be rough ER in the unstained sections were readily seen to be ribosome-studded (Fig. 3). Preliminary studies of serial sections, in which alternating sections were viewed unstained and stained with uranyl acetate, confirmed that crystal-containing vesicles were studded with ribosomes.

Crystals in the myoid region were also observed in elements of the Golgi apparatus (Fig. 7). In addition, in all regions of the myoids, axons, and presynaptic terminals, crystals were present in the agranular reticulum (Figs. 4–6), including structures of the sort identifiable as smooth ER (cf. reference 55). In the terminals, crystals were found also in vacuoles (not shown), which other work in our laboratory indicates may either be derived from the endoplasmic reticulum, or may be involved in membrane recycling (22, 46).

ATP Dependence of Calcium Uptake

As already noted, the calcium oxalate crystals produced in the McGraw–Blaustein procedure are directly identifiable in the electron microscope. Only when there are just a very few small crystals present in the cell, as in some of our control preparations, is it sometimes difficult to distinguish occasional
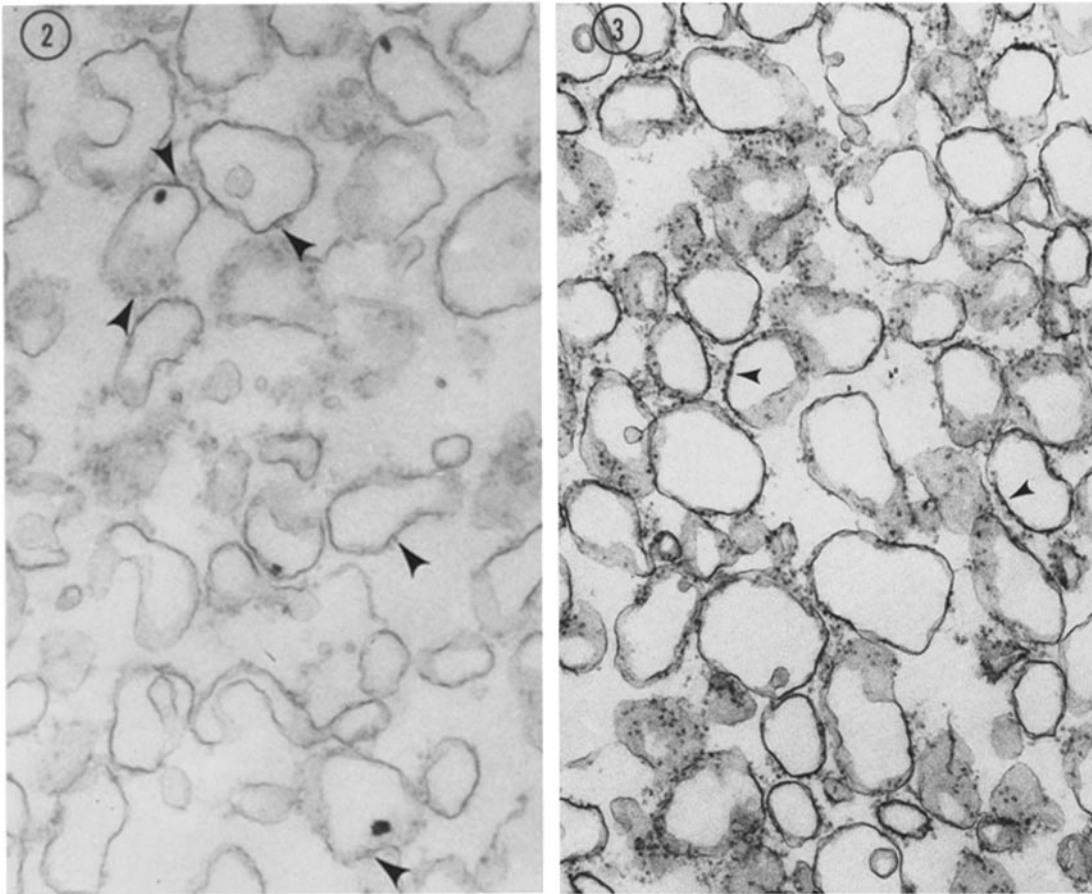
FIGURES 2 and 3 Vesicles in the myoid regions of rod cells prepared with the calcium oxalate procedures based on those of McGraw and Blaustein.. Fig. 2: The thin section used for this micrograph was not stained with uranyl or lead. Ribosomes (arrowheads) are faintly visible along the surfaces of many of the cisternae, including ones containing calcium-oxalate crystals. × 53,000. Fig. 3: The thin section used for this micrograph was stained with uranyl acetate. Ribosomes are clearly observed on the membranes of most of the vesicles (arrowheads). Crystals are no longer seen in any of the vesicles, apparently having been dissolved by the stain. × 39,000.
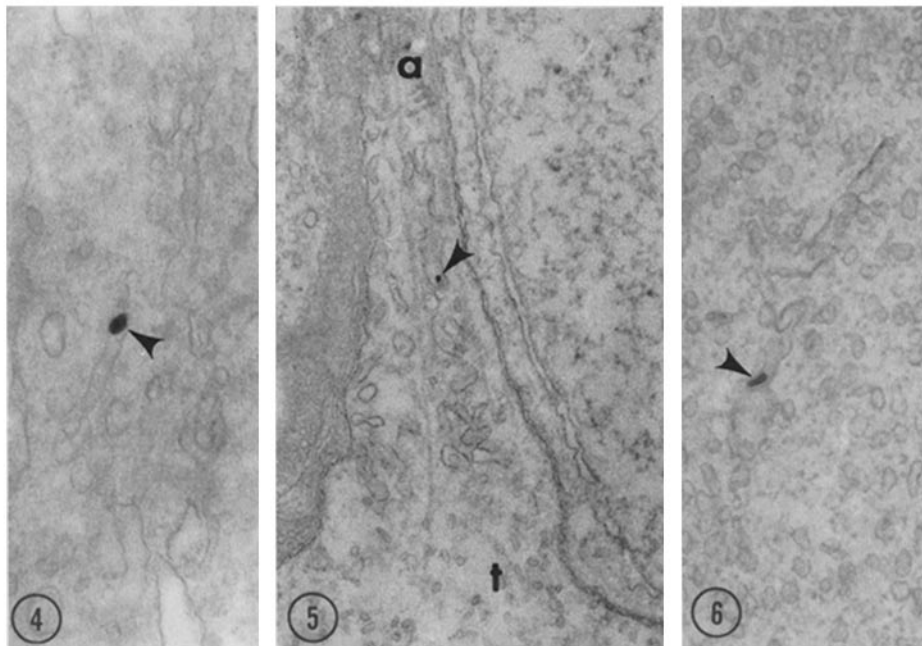
FIGURES 4–6 The thin sections used for these micrographs were not stained with uranyl or lead. They are from McGraw–Blaustein preparations. Fig. 4: From the myoid region of a rod cell. The arrowhead points to agranular reticulum containing a calcium oxalate crystal. × 54,000. Fig. 5: Axonal (a) and terminal (t) regions of a rod cell. A calcium oxalate crystal is present in the axonal reticulum (arrowhead). × 24,000. Fig. 6: Portion of a rod terminal showing a calcium oxalate crystal in the agranular reticulum (arrowhead). × 63,000.
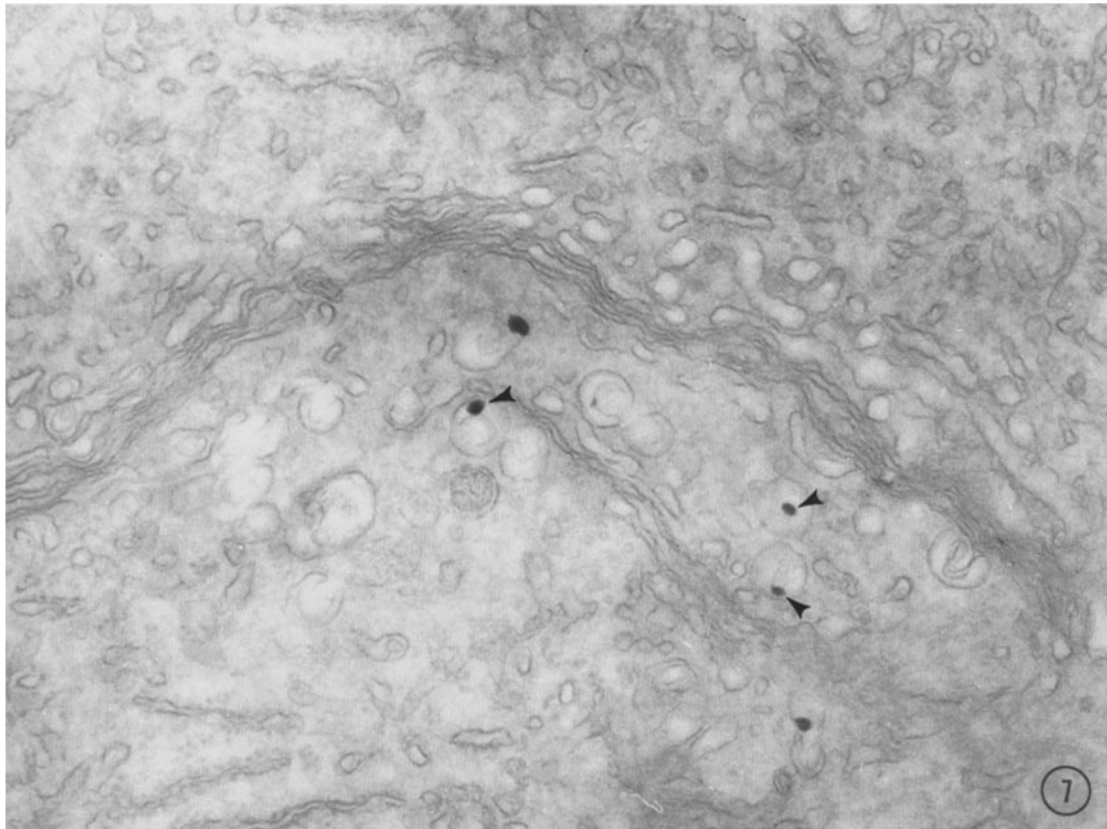
FIGURE 7 Golgi apparatus in the myoid region of a rod cell (McGraw–Blaustein preparation; thin section not stained with uranyl or lead). Crystals are seen in vacuoles at the trans aspect of the Golgi (arrowheads). Given the degree of disruption engendered by the methods, we cannot be sure whether these represent distorted sacs or structures that actually are vacuoles. We occasionally see crystals within expansions at the margins of sacs in the Golgi stack, which is consistent with either possibility since these expansions are one likely source of vesicles or vacuoles budding from the apparatus. × 48,000.
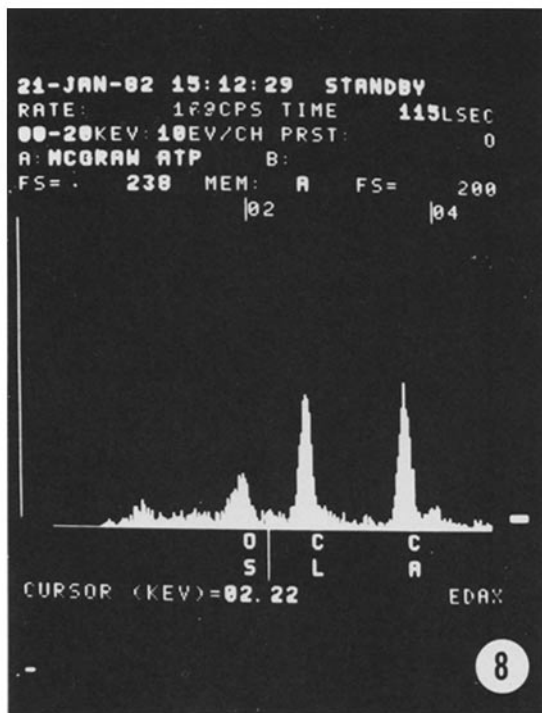

FIGURE 8 Energy dispersive x-ray net (EDAX) counts spectrum of a crystal in an ER vesicle in the myoid region of a rod cell from a McGraw–Blaustein preparation. Strong calcium and chlorine peaks, as well as an osmium peak, are observed. Calcium: $K\alpha = 3.69$ keV; $K\beta = 4.01$ keV; chlorine: $k\alpha = 2.62$ keV; osmium: $M\alpha = 1.91$ keV.

crystals from glycogen or other particles. In our experience, each crystal in control or experimental preparations that we thought from microscopic observation to be calcium oxalate, proved to contain calcium when analyzed with x-ray microanalysis. This enabled us to utilize a semiquantitative approach, which is based on counting the number of crystals formed, to investigate the ATP dependence of calcium accumulation in the myoid region.

Variation in the concentration of calcium (from 15 to 120 μM) or of ATP (from 2 to 8 mM) leads to no significant differences in the frequency of crystals. This suggests that the concentrations of calcium and of ATP present in the medium saturate the calcium accumulation system insofar as the formation of visible calcium oxalate crystals is concerned. (Note that inasmuch as we did not utilize a buffer system such as Ca^{2+}–EGTA, the absolute effective concentration of Ca^{2+} in the medium is likely to be higher than that which we added, owing to effects such as efflux from the retina itself. Note also that the medium contains an ATP-regenerating system: pyruvate kinase plus phosphenol pyruvate.)

Preparations incubated in the presence of ATP exhibit a marked enhancement of calcium accumulation in the ER, when compared with preparations incubated without added ATP. Fig. 9 illustrates a representative experiment and its legend summarizes the results of our 19 experiments to evaluate the effects of ATP. Though the magnitude of the ATP effect varies appreciably from cell to cell and from retina to retina, the retinas incubated without added ATP never contain the large numbers of crystals characteristic of many cells in retinas incubated with added ATP.
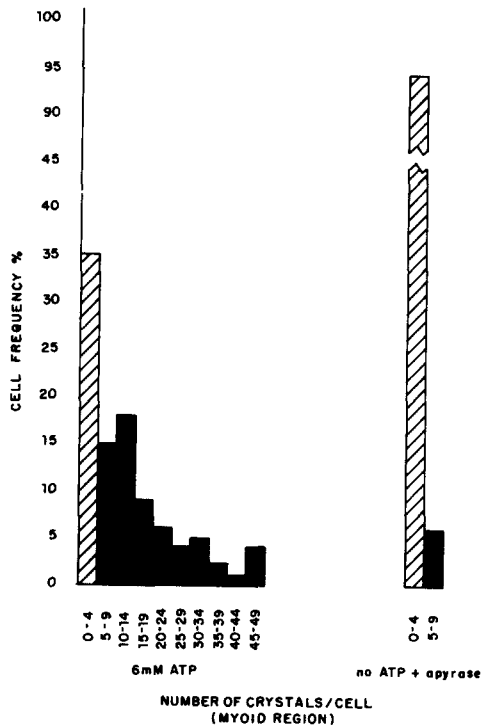
FIGURE 9 Comparison of the frequency of cells containing the indicated range of calcium oxalate crystals per cell in two half-retinas, cut from a single retina. One half-retina was incubated with 6 mM ATP, the other with no ATP + 1 mg/ml apyrase. For each sample the number of crystals in the myoid region was counted for 40 cells. Crystals were observed only in vesicles, which were chiefly derived from ER. In the presence of ATP, not only did the maximum number of crystals per cell increase, but the percentage of cells containing a large number of crystals also increased (solid bars). We regard the 0–4 crystals category as background, since occasionally the identity of a structure (crystal vs. glycogen or other particle) was difficult to ascertain without analytical microscopy. Nineteen such ATP vs no-ATP experiments were evaluated statistically in a pairwise fashion. The presence of ATP significantly ($P < 0.05$) increased the percentage of cells containing greater than four crystals per cell. For the ATP preparations, the mean percentage of cells with greater than four crystals per cell was 40 ± 9 (SEM); for the no ATP preparations, 10 ± 1.

Our quantitation is based on counts of numbers of crystals per thin section of a cell. Therefore, systematic differences in cellular volume between ATP and no-ATP preparations could lead to error in interpretation of the data. However, when we compare paired half-retinas on the basis of numbers of crystals per unit area (Table I), the same effect of ATP is noted as when we base the comparison on numbers of crystals per cell profile.

Cells incubated without added ATP exhibit a few crystals in the ER. This is true even with the addition of apyrase to the incubation medium to hydrolyze residual endogenous ATP. The average size of these crystals is similar to that of crystals formed in the presence of added ATP, and we have established by x-ray microanalysis that some of the crystals contain calcium.

Our observations on locations of the crystals were based predominantly on thin sections with silver-gold interference color that were cut and collected on a water surface. To determine whether possible extraction of crystals during sectioning would affect our findings, we also cut thicker (purple-blue) sections, some of which were cut directly onto glycerol in the microtome knife-boat (63). The average diameter of the crystals is increased somewhat by these procedures, which might conceivably indicate that some extraction does occur in our routine procedures, but may be due simply to inclusion of a greater amount of crystal in a thicker section. There was no systematic difference in location, diameter, or frequency of crystals, which would affect, for example, our ATP vs. no-ATP results. Except for rare instances, none of the preparations we used showed the small holes associated with loss of calcium oxalate crystals during sectioning or subsequent steps.

AMP-PNP, Mersalyl, Tetracaine

To evaluate whether the terminal "high-energy" phosphate bond of ATP is required for calcium accumulation in our system, as it is in others (8, 61, 78), we substituted the nonhydrolyzable ATP analogue, AMP-PNP, for ATP in the incubation medium. As seen in Fig. 10, when this substitution is made, formation of calcium oxalate crystals is at control levels.

Tetracaine and mersalyl have been shown to inhibit ATP hydrolysis and calcium uptake by the sarcoplasmic reticulum (30, 37, 49), and to block uptake of calcium by other smooth ER (7, 94). When these agents are added to our incubation medium, ATP-dependent accumulation of calcium oxalate is inhibited (Fig. 10). In fact, the mersalyl and tetracaine preparations contained the fewest crystals of all our material, and

TABLE I

Oxalate Crystal Formation in Myoid Region

		Dependence on ATP		
	ATP	Number of oxalate crystals per unit area	Total number of oxalate crystals	Area
Exp. 1	+	0.13	415	3,185
	−	0.03	150	4,307
		$\dfrac{ATP}{no\ ATP} = 4.3$		
Exp. 2	+	0.07	245	3,330
	−	0.03	92	3,011
		$\dfrac{ATP}{no\ ATP} = 2.3$		
Exp. 3	+	0.12	348	2,840
	−	0.02	49	3,013
		$\dfrac{ATP}{no\ ATP} = 6.0$		
Exp. 4	+	0.06	146	2,644
	−	0.01	24	2,109
		$\dfrac{ATP}{no\ ATP} = 6.0$		

Number of crystals per unit area in ATP vs. no-ATP experiments was determined for four pairs of corresponding half-retinas. Incubations were performed with 15 mM oxalate, and 15 μM calcium or 30 μM calcium, with or without 6 mM ATP. 40 cells for each experimental and paired control were photographed under the electron microscope. For each cell, the number of crystals in the myoid region was counted from the photograph. The area of the myoid region was determined by overlaying a morphometric grid on the photograph. Area is expressed in arbitrary units based on the morphometric grid.
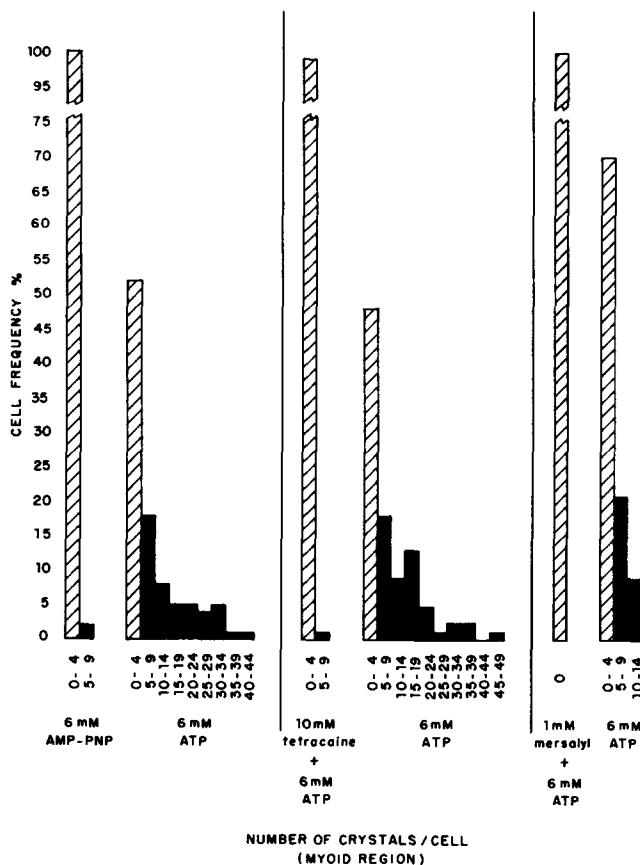
FIGURE 10 The effect of ATP, ATP analogue, or ATP inhibitor upon ATP-dependent calcium uptake. Corresponding half-retinas were incubated with 15 mM oxalate, 15 μM calcium, and ATP and/or other agent, as specified in the illustration. The ATP preparation served as control. For each experimental and paired control ($n \geq$ 3), we counted the number of crystals per myoid region of 40 cells. The range of percentage of cells with greater than four crystals per cell was 0–14 in the presence of AMP-PNP, 0–1 in the presence of tetracaine, and 0–1 in the presence of mersalyl.

this effect was reproducibly the case in all three of the experiments we did with the inhibitors. (We have not carried out sufficient numbers of direct comparisons on paired half-retinas for a thoroughgoing comparison of the small numbers of crystals in our no ATP controls with the apparently smaller numbers in the inhibitor-exposed preparations, but the inhibitors have consistently given numbers of crystals at the lowest end of the range for these controls.)

None of the conditions we observe to reduce crystal abundance in the McGraw–Blaustein preparations have a differential effect on particular compartments—all the calcium-accumulating structures are affected.

Freeze Substitution

Freeze substitution offers considerable theoretical advantage for the localization of calcium, because it minimizes redistribution of ions during the crucial early stages of tissue preparation (24, 32, 73, 80). The principal problem with freeze substitution for most cell types and tissues is that freezing conditions adequate for ion localization are usually realized only in the outermost 10–20 μm of the tissue (31, 32, 73, 89). In our freeze substitution experiments, the outer segments of many of the photoreceptors detached during the procedure.

Good preservation of the inner segments extended only down to the level of the nuclei of the rod cells; more interior zones of the retina showed extensive ice crystal damage. We were, therefore, only able to study calcium-containing deposits in a limited portion of the cell; fortunately, this included the myoid region of the cell, which contains the bulk of the cell's ER. (See also the legend for Fig. 11).

In the freeze-substituted material, electron dense deposits are localized in elongate cisternae in the myoid region of the photoreceptors (Fig. 11). The most prominent of this cisternae are identifiable as rough ER by their appearance and distribution; for comparison, see Fig. 12 which shows the electron dense deposits in the rough ER generated by the oxalate-osmium–acetone method we previously have utilized, without freezing (88). Like the latter deposits, the deposits produced in the freeze-substituted material proved to contain calcium (Fig. 13).

Our control experiments to evaluate the possibility that the osmium in the deposits augments the calcium levels beyond
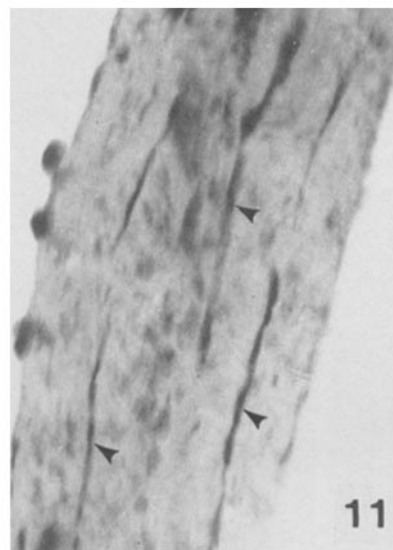

FIGURE 11 Myoid region of a rod cell in a freeze-substitute preparation. As in our own work on other material, and the experience of other laboratories, freeze-substituted material often looks "muddier" than comparable material prepared by conventional fixation, owing in part to the retention of more material in the background cytoplasm. In the present case this effect is exacerbated by our use of relatively thick sections and of section support and coating procedures, as is required for the analytical techniques. Therefore, we focused our attention on the electron-dense deposits observed in the readily identifiable cisternae of rough ER illustrated in this micrograph (arrowheads). We had hoped that the methods would permit us to detect calcium in such sites as the ground cytoplasm, where small amounts—either free, or bound to proteins—might be expected. We sampled the material in which we had obtained appreciable calcium peaks in the ER (as in Fig. 13) and in three of the half-dozen samples examined thus far, we could locate points from which we obtained at least marginal calcium signals. Unfortunately, such signals have turned out to be uninterpretable since, as can be seen in this micrograph, some ER cisternae are sectioned tangentially or in such a plane as to extend only part way through the section, generating relatively pale-looking deposits that are very difficult to see under the electron-optical conditions used for our analytical microscopy; in at least some cases careful examination indicated that we had included a portion of such a deposit in what seemed to be a region of ground cytoplasm. × 30,000.
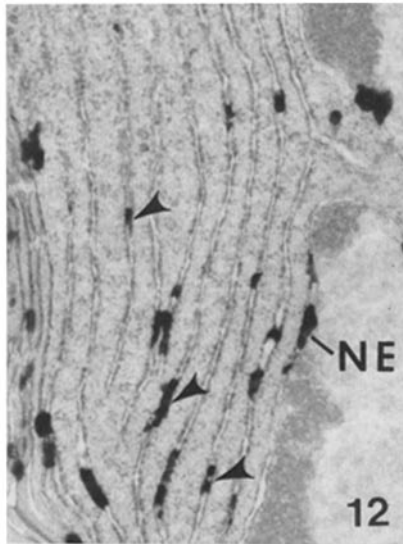
FIGURE 12 Part of the myoid region of a rod cell treated with the oxalate–osmium–acetone procedure. Electron-dense deposits are observed in cisternae of rough ER (arrowheads) and in the nuclear envelope (NE). × 31,000.
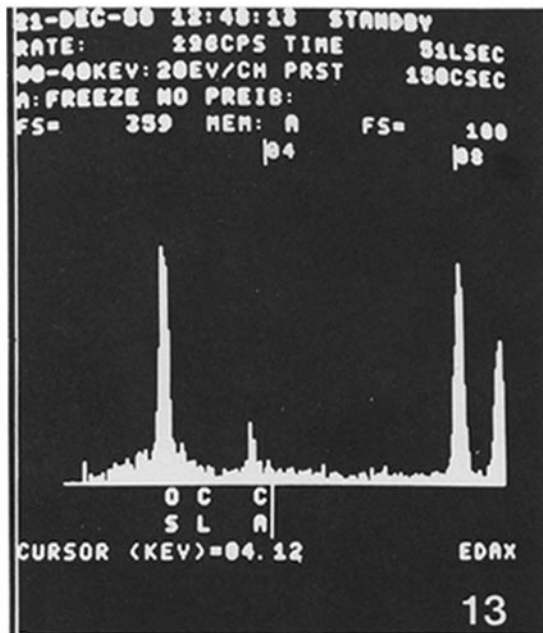

FIGURE 13 Energy dispersive x-ray net (EDAX) counts spectrum of electron-dense deposits in cisternae of rough ER, from the myoid region of a rod cell. The retina was prepared by the freeze substitution procedure. The x-ray spectrum shows clear osmium (Os) and calcium (Ca) peaks. (The absence of the strong Cl peak of Fig. 8 reflects the use of Araldite rather than Epon as the embedding medium.) Calcium: $K\alpha = 3.69$ keV; osmium: $M\alpha = 1.91$ keV; $L\alpha = 8.91$ keV; $L\beta = 10.35$ keV. (Beam width = 1,000 × 600 Å).

those present before exposure to osmium indicate that such an effect is very unlikely to be significant in our tissues: if either calcium or oxalate, or both, are omitted from the medium, we obtain the same sort of electron-dense precipitates as seen in the full media, but the most any of these contain are trace amounts of calcium, at the limit of sensitivity of our x-ray analysis. If we omit incubation altogether, and simply fix in glutaraldehyde–oxalate, followed by osmium–acetone, the precipitates again form but no calcium is detect-

able in them. If preparations are preincubated in calcium plus oxalate, but osmium is omitted from the acetone postfixation, tiny crystals are observed in the myoid, and these contain detectable levels of calcium.

DISCUSSION

It is often presumed that calcium accumulation is a widespread role of the ER, especially of the smooth ER, but this has in fact been demonstrated only for a handful of cell types (for nervous tissue see 7–9, 19, 31, 32, 53, 68, 83, 92–95). Our results indicate that the endoplasmic reticulum in the myoid region of the frog retinal photoreceptor can accumulate calcium in an ATP-dependent fashion. Our studies with a nonhydrolyzable ATP analogue, AMP–PNP, and with inhibitors of the sarcoplasmic reticulum ATPase, mersalyl and tetracaine, strongly suggest that the ATP-dependent calcium uptake reflects an energy-dependent process, perhaps a calcium pump comparable to that of the sarcoplasmic reticulum. The rough ER of the myoid region seems to represent a major site of calcium accumulation, along with the agranular reticulum of the myoid, axon, and terminal.

Methodological Issues

Using methods derived from those of McGraw et al. (52, 53), we precipitated calcium with oxalate, which enabled us to detect intracellular sites where calcium is accumulated from the incubation medium, and to determine the extent of this uptake, semiquantitatively, under varying conditions. Our success in demonstrating calcium uptake in the frog photoreceptor very likely is related to the extensive disruption of cell structure resulting from the exposure to saponin and the subsequent incubation. We regard our preparation as falling somewhere between intact cells and the microsome preparations that have been used to advantage in the study of Ca^{2+} accumulation in muscle (17, 26, 30, 82, 97) and other cell types (6, 59, 60). Calcium oxalate procedures, fundamentally similar to those we used, have been extensively employed to study calcium uptake in sarcoplasmic reticulum vesicles (1, 5, 17, 49, 54, 73, 97) and to some extent, also, in isolated subcellular fractions of other cell types. These methods have not, however, been widely applied to tissue as such. Our findings suggest that calcium oxalate methods may prove broadly useful for studying tissues, such as the retina, where isolation of individual cell types and of corresponding cell fractions is still not feasible.

The McGraw–Blaustein approach demonstrated calcium in the ER of our in vitro incubation system. That the ER of photoreceptors contains calcium in vivo is supported by our freeze substitution results, indicating that the ER of rapidly frozen photoreceptors does contain appreciable concentrations of calcium. Together with our prior results utilizing oxalate–osmium–acetone methods, which provide reasonable morphological preservation of the tissue (88), these observations make it unlikely that the results of our calcium oxalate in vitro incubation procedures are due to some unanticipated redistribution of enzymes resulting from the disruption of cell structure; nor do we reveal some sort of unexpected oxalate accumulation system, with calcium accumulation being an incidental accompaniment. The observation that calcium uptake is via an ATP-dependent mechanism which parallels, in some respects, the well-studied system of the sarcoplasmic reticulum, also suggests that, in our in vitro system, we are exploring phenomena relevant to the situation in vivo.

A limited amount of calcium apparently does accumulate in the ER of the frog photoreceptor, even in the absence of added ATP. If our impressions that mersalyl and tetracaine reduce this accumulation prove accurate, they may imply that such uptake is enzymatic or carrier mediated. Alternatively, the uptake of calcium in the absence of added ATP might depend on the passive binding of calcium to divalent cation-binding sites like the ones we have detected in our prior work (55); one obvious possibility is that such sites normally serve in the sequestration of calcium within the reticulum, once the ions have been transported across the membrane. However, we cannot yet definitively rule out the possibility that some ATP remains, or is generated, in our no-ATP preparations, but is inaccessible to added apyrase.

Calcium Uptake by the ER

That the rough ER is importantly involved in uptake of calcium is an unusual observation. One of the possible precedents is the pancreatic acinar cell, where the rough ER also seems to be centrally involved (91). Most prior studies on neurons and the work on invertebrate photoreceptors cited at the beginning of this article, have focused on smooth surfaced compartments such as those in axons and terminals; only passing mention has been made of the rough ER (31). As considered further below, vertebrate photoreceptors differ markedly from most other neurons both morphologically (the axon is unusually short, and the cell body is attached to a very large outer segment, where active calcium release or uptake may occur), and physiologically (the potential changes that spread from the outer segment to the terminal do so without triggering an action potential). Thus it probably is not surprising that the photoreceptors differ from "typical" neurons in the pattern of calcium storage.

In neurons, identification of smooth-surfaced sacs and tubules as bona fide smooth ER is complicated by the fact that, in these cells, smooth ER lies adjacent to similar looking smooth-surfaced structures of a very different nature (33, 55). This makes the identification of the structures that accumulate calcium in neurons as smooth ER somewhat tentative. (This matter is far more than a semantic issue; see references 33, and 55.) Our findings indicate that unambiguously identifiable ER—that with ribosomes attached—is involved in calcium uptake in the photoreceptor. Furthermore, in the photoreceptor, there is extensive direct continuity between rough ER and smooth ER in the myoid and also in the axon and terminal (55). (This situation is comparable to the sarcoplasmic reticulum of muscle which, at least at certain developmental stages, is also directly continuous with the rough endoplasmic reticulum [48].) Therefore, the generally accepted conclusion that at least some of the smooth compartments that participate in calcium accumulation in other neurons are truly ER is strengthened by our observations that the ER takes up calcium in the photoreceptor.

(Note also that, strictly speaking, our methods lack the resolution to rule out that the Ca^{2+} in the rough ER actually accumulates in very short smooth zones of the reticulum interspersed with, and immediately continuous with, ribosome-studded portions rather than directly where ribosomes are present.)

Implications

Two working hypotheses seem plausible to account for the pattern of calcium-accumulating compartments that we observe. On the one hand, it has been widely believed that the concentration of calcium ions in the cell's cytoplasm is maintained at physiologically regulated levels in part through the intervention of mitochondria (3, 13, 16). In the photoreceptor cell, the mitochondria are concentrated at the ellipsoid pole of the inner segment (Fig. 1). Hence, most of the cell body and the axon and terminal lack what is potentially an important system for storage of calcium ions. Conceivably the ER of the myoid has been pressed into service to compensate, in part, for this.

On the other hand, it is also possible that the involvement of the myoid ER in accumulating calcium reflects participation of the ER in production and/or transport of calcium-accumulating enzyme systems for use elsewhere in the cell. The photoreceptor almost certainly does require calcium-accumulating capacities in membrane systems at both poles of the cell: the outer segments and the presynaptic terminals. Our observations may relate to the role of the ER as the site of synthesis of membrane components and enzyme systems destined for one or both ends of the photoreceptor. The endoplasmic reticulum of the myoid is involved in synthesis of outer segment membrane components (12, 33, 50, 51, 56, 65, 100) and probably in formation of materials destined for synaptic vesicles (55, 83). The myoid ER has been demonstrated to be continuous with portions of the axonal agranular reticulum (55), which could permit direct movement of those enzymes of the Ca^{2+} system not requiring Golgi processing (48), from the myoid ER into the axonal system. The Golgi apparatus seems also to take part in forming membrane systems slated for each end of the cell (33, 34, 44, 72, 74, 83); our observations of calcium-accumulating capacities in Golgi associated systems might relate to this as well as to functioning of the Golgi apparatus itself (28, 43). However, to ascertain whether our observation of calcium uptake by the ER, and especially by Golgi-associated structures, is related to the destiny of transported ER-derived components will require improved methods, adequate for use in comparing the calcium-accumulating capacities of the myoid region in more detail with those found in the outer segments or axons and terminals.

A vast literature has documented ATP-dependent calcium oxalate precipitation in sarcoplasmic reticulum vesicles (1, 5, 17, 30, 49, 73, 97) and in microsomes (often of mixed origins) from a number of tissues (6, 59, 60, 91). In light of the above studies, and considering the findings reported here, it may well turn out that calcium accumulation is a widespread, if not ubiquitous, function of the endoplasmic reticulum.

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