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BIOCHEMICAL ASPECTS OF THE VISUAL PROCESS

XXV. LIGHT-INDUCED CALCIUM MOVEMENTS IN ISOLATED FROG ROD OUTER SEGMENTS

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Summary

Suspensions of isolated rod outer segments are shown to have a high calcium content of up to 11 moles calcium per mole rhodopsin. Osmotic lysis of the outer segments demonstrates the presence of two calcium fractions, a soluble one and a particulate one. The particulate fraction apparently coincides with the rod disks or with disk fragments. Illumination of intact rod outer segments in calcium-free ATP-containing Ringer solution has no measurable effect upon their total calcium content, but causes a significant shift of calcium from the particulate to the soluble fraction. This light effect is retained in lysed outer segments resuspended in calcium-free ATP-containing Ringer. These results support a function of calcium as a transmitter in light transduction in rod outer segments.

Recently Hagins [1] has proposed that signal transduction in the rod outer segment from the disk membranes, which contain the bulk of the visual pigment, to the outer membrane would involve Ca^{2+} as a transmitter. This calcium-coupling hypothesis of visual excitation is based on electrophysiological measurements in the intact retina, involving variations in the medium calcium concentration. Since hardly anything is known about the calcium content of rod outer segments and its intracellular distribution, it appeared to us that such information was needed. We report here experiments which show that intact frog rod outer segments have a high calcium content, distributed

TABLE I

Calcium content of frog rod outer segments isolated in various ways. The rod outer segment suspension is centrifuged after filtration, the sediment resuspended and centrifuged again. Calcium is determined in the resulting sediment and expressed as mol per mol rhodopsin with standard error of the mean. In parentheses the number of experiments (each in triplicate) is given. Statistical analysis by means of the Wilcoxon test gives the following *P*-values:

all values without sampling vs all values with sampling, $P = 0.003$
all values (+ATP) without EDTA vs all values with EDTA, $P = 0.46$
all values (± EDTA) with vs all values without ATP $P = 0.014$.

Medium	Without sampling	With sampling
Ringer, 3 mM ATP	11.0 ± 1.2 (21)	8.1 ± 1.7 (11)
Ringer, 3 mM ATP, 10 mM EDTA	12.8 ± 1.6 (4)	8.4 ± 1.6 (3)
Ringer, no ATP, no EDTA	7.8 ± 0.6 (10)	3.9 ± 0.6 (8)

TABLE II

Effect of illumination upon calcium content of intact frog rod outer segments. Isolated rod outer segments are washed twice with ATP-containing Ringer solution and the sediment is resuspended and divided into 4—6 aliquots. Two to three aliquots are then illuminated, while the others are kept in darkness. After centrifugation calcium is determined in sediments and supernatants. Results are expressed as percent of total calcium remaining in the sediment.

Medium	Outer segment sediment		Ratio	No. of experiments	Total No. of determinations	*P* value*
	Dark (%)	Light (%)				
Ringer	68.3	72.4	1.06	10	50	0.13
Ringer + 10 mM EDTA	67.9	67.7	1.00	2	12	0.23

*Determined by applying two-way analysis of variance to the experimental data.

TABLE III

Calcium distribution in intact and lysed frog rod outer segments. Isolated rod outer segments are washed twice with an ATP-containing Ringer solution, the sediment is resuspended and divided into different aliquots. After lysis the material is centrifuged and calcium is measured in sediment and supernatant. Results are expressed as percentage of total calcium in the sediment with standard errors of the mean and in parentheses the number of experiments (each in triplicate). *P* values calculated by means of the Wilcoxon test.

Preparation medium	Ringer	Ringer + 10 mM EDTA	*P* value
Intact outer segments	75 ± 5 (10)	68; 68	0.45
After lysis in 4 vol. H_2O	42 ± 3 (13)	0; 0*	0.03
P value	< 0.001	—	

*Lysis by addition of 4 vol. 10 mM EDTA.

over a soluble and a particulate fraction. In addition we show evidence that illumination, while not changing the total calcium content, causes a significant shift from the particulate to the soluble fraction. During the progress of these experiments two brief reports on light-induced calcium movements in rod outer segments have appeared [2, 3].

Rod outer segments are isolated from the excised retinas of dark-adapted frogs (*Rana esculenta*) by gentle shaking for 30 s in a modified Ringer solution, containing 112 mM NaCl, 3 mM KCl, 3 mM $MgCl_2$, 10 mM glucose, 3 mM ATP, sodium salt, and buffered to pH 7.4 with 10 mM Tris—HCl. After filtration over a stainless steel wire screen (60 mesh) the resulting suspension is

TABLE IV

Loss of particulate calcium from frog rod outer segments upon flash illumination. Isolated rod outer segments are washed twice with an ATP-containing Ringer solution, the sediment is resuspended and divided into different aliquots. The aliquots are either lysed after illumination (first row) or lysed immediately before illumination (third row) or lysed, centrifuged, resuspended in ATP-containing Ringer and illuminated (second row), After centrifugation calcium is determined in sediment and supernatant. Results are expressed both as percent of total calcium in the sediment and as the relative loss of calcium upon illumination.

Rod outer segment treatment	Dark pellet	Light pellet	% calcium lost upon illumination	No. of experiments	Total No. of determinations	*P* value*
Lysis in 4 vol. water after illumination	42.8	36.0	16	4	28	0.01
Lysis in 4 vol. water followed by centrifugation and illumination in Ringer	34.0	26.2	23	2	16	<0.001
Lysis in 4 vol. water before illumination	35.7	33.6	6	2	16	0.85

*Determined by applying two-way analysis of variance to the experimental data.

centrifuged for 10 min at 1200 × *g*, the sediment is suspended in Ringer solution and centrifuged again. The final sediment is suspended in the desired medium. The entire procedure takes less than 60 min.

This isolation method reproducibly yields a suspension, containing mainly intact rod outer segments, as examined by phase-contrast microscopy. Inclusion of ATP in the Ringer medium during isolation appears to give a higher yield of intact outer segments. The rhodopsin yield is 1.06 (S.E.: 0.08, 22 determinations) nmoles rhodopsin per retina, which is about half of the total rhodopsin present in the retina. Rhodopsin is determined as described elsewhere (Hendriks et al. [4]).

Calcium and magnesium are determined with a Pye Unicam SP 1950 double-beam atomic absorbance spectrophotometer. Supernatants are diluted with $LaCl_3$ (final concentration 0.5%) and used as such. Pellets are first digested in a mixture of H_2SO_4—$HClO_4$—HNO_3 (1:3:12, by vol.) and then measured quantitatively in 0.5% $LaCl_3$ solution against appropriate standard solutions.

The average calcium content of the rod outer segment preparations, after two centrifugations, is 11.0 (S.E.: 1.2, 21 determinations) moles calcium per mole rhodopsin. Further washings and centrifugations do not significantly decrease the calcium content. Upon isolation of intact as well as lysed rod outer segments in the absence of ATP the calcium content is significantly lower (Table I), which suggests the presence of an ATP-driven calcium pump. Hence, in all further experiments the Ringer solution contains 3 mM ATP. Addition of 10 mM EDTA to the Ringer solution has no effect on the calcium concentration in the sediment, indicating that virtually all calcium is sequestered. The suspension contains some mitochondrial contamination, because a minor part of the rods on microscopic examination are seen to

contain the myoid body. In addition, some melanin granules derived from the pigment epithelium are present. It is unlikely that a major part of the calcium in the frog rod outer segment suspension could be due to this contamination, since cattle rod outer segment preparations, free of mitochondrial and melanin contamination (Hendriks et al. [4]), also contain a high calcium level: 5.1 (S.E.: 1.5, 7 determinations) moles calcium per mole rhodopsin. Since the rhodopsin concentration in frog rod outer segments is 2.5 mM (Liebman [5]), the maximal calcium concentration must be about 28 mM, which is even slightly higher than that in squid axon mitochondria (20 mM, Baker [6]).

Table I also shows that sampling of an outer segment suspension by means of a constriction pipette clearly produces a significant ($P = 0.003$) calcium loss from the outer segments, noticeable also from higher calcium levels in the supernatant after centrifugation of the sample. While after a normal isolation procedure (without pipetting) only 1% of the remaining calcium appears in the supernatant during a third washing and centrifugation step, this percentage is increased to 25 after pipetting. Apparently pipetting damages a considerable fraction of the outer segments. This causes difficulties in obtaining reproducible aliquots, as far as the absolute amounts of calcium in the sediments are concerned. Therefore, we have always determined calcium in both sediment and supernatant after centrifugation and have expressed the results (Tables II, III and IV) as the percentage of total calcium remaining in the sediment for each aliquot.

Next, we have determined the effect of light on the total calcium content of the outer segments. Rod outer segments suspended in Ringer solution are illuminated immediately after sampling, either by a flash from a Rollei Strobofix E60 flash lamp through a Schott-Jena OG2 filter (about 50% bleaching of rhodopsin) or for 5 min by a 100 W tungsten lamp through 3 mm thick GG 3 and OG2 filters (nearly 85% bleaching). Illumination by either method, with or without EDTA present in the Ringer solution, causes no significant change in the percentage of total calcium remaining in the sediment, as compared to non-illuminated controls (Table II). Thus the calcium content of the rod outer segments does not change upon illumination. The absence of a measurable light effect on the calcium content of intact outer segments is not surprising, even if there would be a light-induced release of calcium from the disks, since the permeability of the outer membrane to calcium should be extremely low. This is generally the case for cell membranes (Hurlbut [7], Schatzmann [8]), and is also suggested by the fact that after two washings no further loss of calcium from intact outer segments is observed.

In other cell types where calcium has an important (transmitter) function (nerve, muscle), the calcium is largely sequestered in subcellular structures (mitochondria, sarcoplasmic reticulum). Therefore we have tried to establish the existence of separate calcium pools in outer segments by determining calcium content before and after osmotic lysis, Lysis is induced by adding 4 vol. water (or 10 mM EDTA) to the suspension, followed by violent shaking on a Vortex mixer for 10 s. Light microscopy indicates that this procedure

causes complete destruction of the typical rod outer segment structure. Only small fragments (maximal dimension about 5 μm) remain visible. Calcium determinations after centrifugation show that calcium is indeed solubilized after this procedure (Table III, lower row). Hence, this lysis method enables us to distinguish between a soluble and a particulate calcium fraction, the latter coinciding with the rhodopsin containing sediment.

The lysed rod outer segment material, in contrast to intact rod outer segments, loses all calcium upon treatment with 10 mM EDTA-containing Ringer. Since in the outer segments no other intracellular compartments than the rod disks are present, it is reasonable to assume that the particulate fraction represents calcium present either inside remaining intact disks or bound to disk membranes.

Next, we have determined the effect of illumination on the distribution of the calcium between the two fractions. When the intact rod outer segments are flash illuminated and then lysed, a significant loss of calcium into the supernatant occurs, as compared to the non-illuminated controls (Table IV, upper row). This means that illumination causes a shift of calcium from the particulate to the soluble fraction in intact rod outer segments.

Thus having established that a particulate fraction of the outer segment suspension loses calcium upon illumination, the question arises whether the same fraction after destruction of the rod outer segments is still sensitive to light. Rod outer segments are lysed in darkness, the particulate material is sedimented and resuspended in Ringer solution. Illumination again causes a significant release of calcium from the particulate material (Table IV, second row), about equal to that in the experiments with lysis following illumination. Thus the mechanism by which calcium is released by illumination remains intact upon lysis. However, when the rod outer segments are illuminated immediately after lysis, i.e. suspended in a 5-fold diluted Ringer solution, no effect of light is seen (Table IV, third row). This suggests either that the low ion concentrations (Na^+, K^+, Mg^{2+}) in the diluted Ringer solution or the relatively high calcium concentration in the medium after lysis (about 10^{-5} M) inhibits the light sensitive calcium release mechanism.

Magnesium is also present in rod outer segments in rather high concentration (1.4 moles magnesium per mol calcium). Upon lysis it also turns out to be divided into a particulate and a soluble fraction (particulate fraction: 18.1%, S.E. 0.6, 4 determinations). Illumination of the lysed rod outer segments in a magnesium-free Ringer solution (required for accuracy of the magnesium determination) causes no change in the magnesium distribution (particulate fraction: 18.5%, S.E. 0.6, 4 determinations), whereas 9.2% (S.E. 0.2, 4 paired determinations) of the particulate calcium goes into solution. Although this calcium loss is only about half of the calcium loss in a magnesium-containing Ringer solution, the effect is still significant. This finding indicates that the light-induced calcium release is specific for this bivalent cation.

The question arises whether the calcium released by light had been present within the disks or had been bound to the outside of the disk

membrane. It is remarkable that after prior lysis and removal of the soluble calcium, the light-induced calcium release is completely retained. This means that the calcium involved in the light effect belongs to the particulate fraction, which consists of calcium either bound to the disk membrane or present within the disks. Lysis in the presence of EDTA or with EDTA added afterwards removes all calcium from the outer segments, which finding might seem to favor binding to the membrane. However, the ongoing discussion on the mechanism of calcium release in stimulated nerve (Baker [6]) and muscle (Ebashi and Endo [9]) — release by downhill efflux vs release from calcium binding sites on membrane proteins — suggests that cautious interpretation is required.

Thus we have demonstrated that isolated rod outer segments have a high calcium content. An appreciable fraction of this calcium is particle-bound and appears to be either sequestered inside the disks or bound to the disk membranes. Illumination releases a significant portion of the particulate calcium. This finding would seem to support Hagins' hypothesis that the light-induced decrease of the sodium permeability of the outer membrane of the rod outer segment is caused by calcium ions released from the disks upon photolysis of rhodopsin. Further experiments will be required to determine the relationship between the amount of calcium released and the percentage of rhodopsin bleached, as well as to settle the question whether the released Ca^{2+} were bound on the outside of the disk membrane or were present inside the disks, either free or membrane-associated.

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