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CALCIUM BINDING TO RETINAL ROD DISK MEMBRANES

A. H. NEUFELD, W. H. MILLER AND M. W. BITENSKY

Department of Ophthalmology and Visual Science, Department of Pathology, Yale University School of Medicine, New Haven, Conn. 06510 (U.S.A.)

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SUMMARY

Ca^{2+}, ATP and reducible disulfide linkages interact on disk membranes prepared from bovine rod outer segments. Two classes of ATP binding sites are described. At one, ATP increases the number of Ca^{2+} binding sites. ATP present at a second site enables dithiothreitol to decrease Ca^{2+} binding. Cyclic AMP and illumination do not affect the amount of Ca^{2+} bound under any of the conditions tested. The possible role of these interactions in visual excitation is discussed.

INTRODUCTION

Ca^{2+} has a central but only partially understood role in excitatory and secretory processes[1-3]. Recently, Yoshikomi and Hagins[4] reported that elevated Ca^{2+} levels could mimic light in visual excitation and Bitensky *et al.*[5] demonstrated that rod outer segments contain a light sensitive adenyl cyclase. Also, Kakiuchi and Yamazaki[6] have provided new evidence that Ca^{2+} can regulate the levels of cyclic adenosine 3',5'-monophosphate (cyclic AMP) in nerve tissue. In addition, ATP-dependent Ca^{2+} binding to brain microsomes[7] and renal membranes[8] has been reported. Because of the interaction of Ca^{2+} and cyclic AMP in various systems, we have investigated Ca^{2+} binding in rod outer segments. This paper presents evidence for ATP increased, and dithiothreitol decreased, binding of Ca^{2+} to rod outer segment disk membranes.

MATERIALS AND METHODS

Dark adapted bovine retinas were obtained from Hormel (Minnesota), $^{45}CaCl_2$ from New England Nuclear, liquid-scintillation compounds from Beckman.

Preparation of rod disk membranes

All procedures were carried out under dim red light and at ice temperature. Bovine rod disk membranes were isolated by flotation in sucrose. Dark adapted bovine retinas were thawed, suspended in 50 % (w/v) sucrose, and sonicated at 120 W for 15 sec (Branson sonifier) producing a homogeneous suspension of intact disk membranes. This suspension was centrifuged (Beckman L65) 5 h at 20000 × *g* in a SW-27

Abbreviation: EGTA, ethyleneglycol-bis(aminoethyl)tetraacetic acid.

swinging-bucket rotor. A thick red paste (the rod disk membranes) was harvested from the sucrose–air interface at the top of the centrifuged tube. Electron microscopic observation indicated intact rod disks with little cellular debris. The paste was suspended in 5.0 ml of : 130 mM KCl, 2 mM $MgCl_2$, and 20 mM imidazole (pH 7.3). This suspension was centrifuged (Sorvall RC-2) at 20000 × *g* for 30 min and the pellet resuspended in 5.0 ml of the above solution. This constituted the protein suspension used to initiate the experiment. In half the experiments, the protein suspension was illuminated (room fluorescent light) from this time onward.

Assay for Ca^{2+} binding

A typical experiment was carried out as follows: 0.25 ml of the above protein suspension was added to 5.0 ml of a room temperature (23°) solution: 130 ml KCl, 2 mM $MgCl_2$, 2 mM ATP, 7 mM aminophylline, 5 μM $^{45}CaCl_2$ ($5 \cdot 10^5$ counts/min per nmole) and 20 mM Tris (pH 7.3). Within a given experiment, the ATP and $CaCl_2$ concentrations were varied and dithiothreitol or cyclic AMP were present initially where indicated. The reaction mixture was shaken vigorously and incubated at 23° for 5 min. At the end of this time, the reaction mixture was chilled on ice and centrifuged at 10000 × *g* for 30 min. The pellet was washed 2 times by resuspension in 20 ml of an ice-cold solution: 130 mM KCl, 2 mM $MgCl_2$, 0.1 mM ethyleneglycol-bis(aminoethyl)tetraacetic acid (EGTA) and 20 mM imidazole (pH 7.3), followed by centrifugation at 10000 × *g* for 15 min. At this point the rod disks were intact by electron microscopic observation. Washing in the light or dark, varying the time of centrifugation, or increasing the number of washes did not significantly alter the results. The final pellet was resuspended in 0.5 ml 1 M NaOH and stored overnight at 5°. The dissolved basic protein solution was acidified with 0.1 ml concentrated HCl.

For radioactivity determinations, 0.1 ml of the above acidic protein solution was mixed with 10 ml of scintillation cocktail: 4.0 g PPO, 0.05 g POPOP, 150 ml absolute ethanol, 100 ml Beckman Bio-Solv BBS-3 made up to 1.1 l with toluene and counted in a Beckman LS.

For protein determinations, 0.1 ml of the above acidic protein solution was diluted 10-fold and the method of Lowry *et al.*[9] was used with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Factors affecting Ca^{2+} binding

Bovine rod disk membranes bind Ca^{2+}. This binding of Ca^{2+} is not light-sensitive but can be affected markedly by physiological variables (Table I). ATP can affect the amount of Ca^{2+} bound to these membranes in an apparently complex way. The conditions for obtaining the data in Table I are described under MATERIALS AND METHODS. Freshly prepared membranes (control) bind some Ca^{2+} and ATP more than doubles the amount bound. In the presence of dithiothreitol alone, the amount of Ca^{2+} bound is equal to that bound under the control conditions. However, rod disk membranes introduced into a solution containing both ATP and dithiothreitol show little ability to bind Ca^{2+}. Addition of dithiothreitol a few minutes after introducing the membranes into the ATP enriched solution depletes the membranes of almost all bound Ca^{2+}. Cyclic AMP is without effect on the amount of Ca^{2+} bound.

TABLE I

Ca^{2+} BINDING TO ROD DISK MEMBRANES

Results are expressed in the form of mean ± S.E. followed by the number of observations in parentheses. The conditions are described under MATERIALS AND METHODS. The Ca^{2+} concentration was 5 μM.

Conditions	*nmoles Ca^{2+}/mg protein*	
	Dark	*Light*
(A) Control	0.30 ± 0.02 (10)	0.30 ± 0.02 (10)
(B) 2 mM ATP	0.70 ± 0.04 (12)	0.70 ± 0.04 (16)
(C) 3 mM dithiothreitol	0.30 ± 0.02 (3)	0.30 ± 0.02 (3)
(D) 2 mM ATP + 3 mM dithiothreitol	0.07 ± 0.01 (7)	0.05 ± 0.01 (9)
(E) 2 mM ATP + 3 mM dithiothreitol*	0.05 ± 0.01 (2)	0.05 ± 0.01 (2)
(F) 10 μM cyclic AMP	0.30 ± 0.02 (3)	0.30 ± 0.02 (3)
(G) 2 mM ATP + 10 μM cyclic AMP	0.80 ± 0.04 (7)	0.80 ± 0.04 (7)
(H) 2 mM ATP + 10 μM cyclic AMP + 3 mM dithiothreitol	0.05 ± 0.01 (3)	0.05 ± 0.01 (3)

* Dithiothreitol was added after a 4-min initial incubation with ATP alone.

In addition, we have obtained the same types of data with frog rod disk membranes prepared in a similar manner.

By the end of the 5-min incubation, the binding of Ca^{2+} appears to be independent of time. Longer incubation times or various washing times (*e.g.* 15, 30, 60 min initial clearing centrifugation) do not alter the amount of Ca^{2+} bound. Incubation in ice for varying times indicated that Ca^{2+} binding was stable at ice temperature. Thus, the system has probably reached equilibrium within 5 min and studies to determine binding constants have been carried out.

Determination of binding constants

The Ca^{2+} binding system exhibits saturation phenomena for ATP and Ca^{2+}. Fig. 1 shows the dependence of Ca^{2+} bound per mg of protein on the Ca^{2+} concentration in the presence and absence of ATP. A reciprocal plot (1/(nmoles Ca^{2+}/mg protein) *vs.* 1/Ca^{2+} concn.) indicates that ATP does not change the half maximal concentration for Ca^{2+} (5 μM Ca^{2+}) but does change the maximum amount of Ca^{2+} bound from 0.7 to 2.0 nmoles Ca^{2+}/mg protein at saturating Ca^{2+} concentrations. ATP, thus, increases the amount of Ca^{2+} bound to cow rod disk membranes by increasing the number of Ca^{2+} binding sites and not by increasing the affinity of the existing sites. As seen in Table I, the effect of ATP is different depending on the presence or absence of the reducing agent, dithiothreitol. As shown in Fig. 2, in the absence of dithiothreitol, ATP increases the amount of Ca^{2+} bound with a half maximal concentration of 60 μM ATP (obtained from a reciprocal plot of Ca^{2+} concentration/(nmoles Ca^{2+}/mg protein) *vs.* ATP concentration). In the presence of dithiothreitol, ATP decreases the amount of Ca^{2+} bound with a half maximal concentration of 0.2 mM ATP (obtained from a reciprocal plot of 1/(nmoles Ca^{2+}/mg protein) *vs.* ATP concentration).

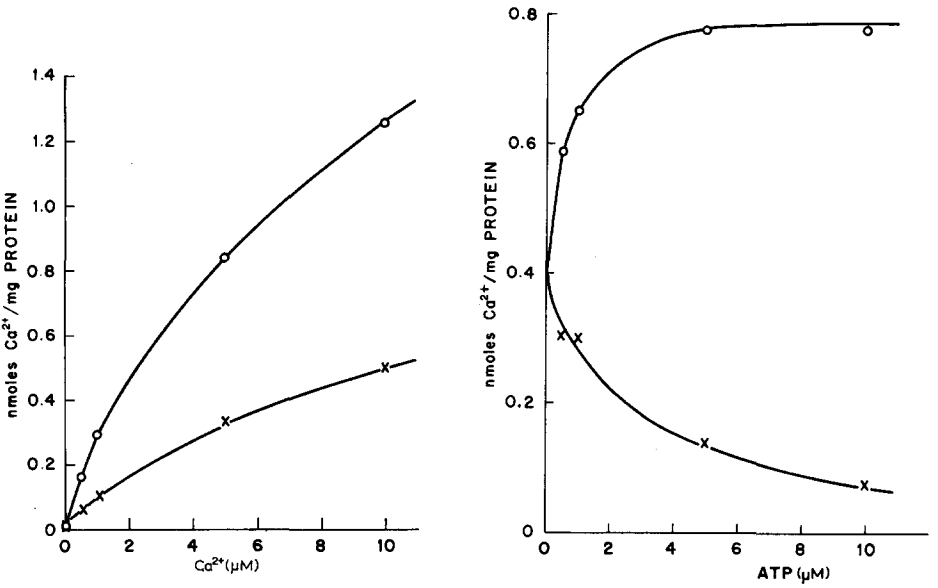

Fig. 1. The dependence of Ca^{2+} binding on the Ca^{2+} concentration: O—O, with 1.0 mM ATP; ×—×, without ATP. These data were obtained in the light and the conditions are described under MATERIALS AND METHODS.

Fig. 2. The dependence of Ca^{2+} binding on the ATP concentration: O—O, without dithiothreitol; ×—×, with 3.0 mM dithiothreitol. These data were obtained in the light and the conditions are described under MATERIALS AND METHODS. The Ca^{2+} concentration was 5 μM.

ATP hydrolysis and specificity of Ca^{2+} binding

Although ATP is needed for maximal Ca^{2+} binding, the latter does not appear to depend on the hydrolysis of ATP to ADP + P_i. Preliminary measurements of the rate of [γ-^{32}P]ATP hydrolysis by the cow rod disk membrane preparation show no effect of adding 0.1 mM Ca^{2+} to the ATPase assay medium.

The ability of the membranes to bind low concentrations of Ca^{2+} in the presence of high concentrations of Mg^{2+} demonstrates the specificity of the binding. Washing the membranes in EGTA does not remove this bound Ca^{2+}; the Ca^{2+} may be inside the disk and, therefore, not available to EGTA, or tightly complexed with the outside of the membrane.

General conclusions

The simplest explanation of our data is that two ATP sites exist that can affect Ca^{2+} binding to rod disk membranes. In the absence of ATP a certain amount of Ca^{2+} can bind to these membranes and no reducible disulfide linkages are exposed. At saturating concentrations, all ATP sites are occupied resulting in an increase in the amount of Ca^{2+} bound, presumably through an increase in the number of Ca^{2+} sites and the exposure of a reducible disulfide linkage. This disulfide linkage may be intra- or intermolecular. If dithiothreitol is present, or added after the ATP, the disulfide linkage is reduced and Ca^{2+} is liberated from the membranes. Other possibilities exist, for example two distinct Ca^{2+} sites with different sensitivities to ATP and dithiothreitol.

No light-dark or cyclic AMP difference is apparent, however, under any of the conditions tested. We do not feel that this rules out the involvement of this system in visual excitation. Light–dark and/or cyclic AMP sensitivity may have been lost due to the method of preparation or may not be favored under the conditions tested. In a number of systems, especially those involving permeability changes, and particularly brain and kidney, an interaction between Ca^{2+} and cyclic AMP has been postulated[3]. Possible roles for Ca^{2+} and cyclic AMP in visual excitation have recently been discussed[4,5] and the Na^+ permeability change that occurs in rod outer segments[10] may be somewhat analogous to that which occurs in brain and kidney, although they lead to different physiological responses. The Ca^{2+} binding system, like the cyclic AMP system, may very well be a generalized phenomenon. Our observations of specific Ca^{2+} binding in a membrane preparation from this highly specialized tissue further imply a role for Ca^{2+} in visual excitation. The relationship of the interaction of Ca^{2+}, ATP and dithiothreitol on the cow rod disk membranes to visual excitation remains elusive. ATP and the redox state of disk sulfhydryl groups could regulate the amount of free and bound Ca^{2+} *in vivo* and Ca^{2+} could regulate the phosphodiesterase activity and, therefore, cyclic AMP concentration[6], or play a role in the membrane events involved in the depolarization and hyperpolarization leading to electrical activity in the rod outer segment.

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