TAURINE ACTIVATION OF A BICARBONATE-DEPENDENT, ATP-SUPPORTED CALCIUM UPTAKE IN FROG ROD OUTER SEGMENTS

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Isolated frog rod outer segments (ROS) with a leaky plasma membrane showed a bicarbonate-dependent, ATP-activated ^{45}Ca accumulation. This calcium uptake requires magnesium and is specific for ATP; other nucleotides, ITP, GTP, UTP and the non-hydrolysable analogue of ATP β-γ-methylene ATP did not substitute for ATP. ^{45}Ca accumulation was inhibited by mersalyl, ethylmaleimide, ruthenium red, oligomycin and dicyclohexylcarbodiimide and was unaffected by ouabain. Addition of taurine to the incubation medium enhanced ^{45}Ca uptake in a concentration-dependent manner; increases of more than 100% being produced by 25 mM taurine. The taurine-induced stimulation of ^{45}Ca uptake was also sensitive to the tested inhibitors. The effect of taurine was only exerted on the bicarbonate-dependent, ATP-activated ^{45}Ca uptake. Calcium accumulation observed in the absence of ATP or in a tris-buffered medium was unaffected by taurine. Other amino acids, glycine, GABA, β-alanine, glutamic acid and the taurine analogue guanidinoethyl-sulfonate did not stimulate ^{45}Ca uptake. These results suggest that taurine is affecting a Mg-ATPase activity responsible for calcium accumulation in frog ROS.

INTRODUCTION

Taurine is the most abundant free amino acid in the retina of all the species studied so far (1, 2). Studies on the intraretinal distribution of

317

taurine have shown its prevalent localization in the photoreceptors (3, 4). Further work has shown that taurine is released from the retina, most likely from the photoreceptor cells, upon light stimulation (5–7). These observations have suggested an involvement of taurine in photoexcitation, but the steps or mechanisms of its action remain to be clarified. A possible involvement of taurine in the retinal processes regulating intracellular calcium concentration, particularly at the rod outer segments (ROS), has been suggested by our previous studies that showed a complex action of taurine on calcium accumulation by isolated retinal subcellular fractions (8, 9). At high external calcium concentrations, taurine reduces the accumulation of labeled calcium taken up by ROS through an ATP-independent process. At low calcium concentration, taurine markedly increases an ATP-dependent calcium uptake. This later effect was also observed by Kuo et al. in isolated of frog ROS disks (10). In the present work we describe some properties of the calcium process affected by taurine. This seems to concern an active calcium transport, linked to an ATP hydrolisis, and dependent on the presence of bicarbonate.

EXPERIMENTAL PROCEDURE

Dark adapted frogs were killed by decapitation and their eyes were enucleated. Retinas were teased away from the eyecups under Krebs-bicarbonate medium* without $CaCl_2$. The retinas were rinsed removing small amounts of pigment epithelium which occasionally adhered, and were transferred to test tubes containing 10 ml of Ca-free medium. Usually 20 retinas were pooled for each experiment. Rod outer segments were isolated by mildly vortexing the tubes for 1 min, then pelleted by centrifugation at 900 g for 10 min. The pellet was washed once with Ca-free Krebs-bicarbonate medium. In some experiments, ROS were purified in a Ficoll gradient, according to the procedure of Szutz and Cone (11). The ROS pellet was resuspended in 1 ml of 10% Ficoll (w/v) and was layered over a gradient formed by 5 ml 25% Ficoll and 5 ml 10% Ficoll. The gradient was centrifuged for 10 min at 58 000g (SW 40 rotor, 4°C). The floating ROS were collected at the interphase, then pelleted by diluting with Krebs-bicarbonate medium and centrifuging at 900g for 10 min. The pellet was washed twice with Krebs-medium.

^{45}Ca accumulation was assayed in a Krebs-bicarbonate medium (NaCl, 118 mM; KCl 4.7 mM; $MgSO_4$, 1.17 mM; KH_2PO_4, 1.2 mM, glucose, 5.6 mM and $NaHCO_3$, 25 mM) containing 0.2 μCi of $^{45}CaCl_2$ and the additions indicated in each experiment, in a final volume of 1 ml. pH of the incubation media was adjusted to different values by gassing with a 5% CO_2–95% O_2 mixtured or with pure O_2. The reaction was started by addition of the ROS suspension containing 50–70 μg of protein. After 10 min of incubation at 22°C, 0.3 ml aliquots of the suspension were withdrawn and centrifuged in a Beckman Microfuge. Pellets were

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* In order to compare the present results with previous data, Krebs-bicarbonate rather than amphibia medium was used. The main difference between the two media is the presence of phosphate and high amount of bicarbonate in the mammalian, hence experiments with phosphate-free media and low bicarbonate media were also carried out (see Results).

washed with distilled water and solubilized with NCS (Tissue solubilizer, Amersham). Accumulated radioactivity was measured in the solubilized tissue after the addition of 10 ml of Tritosol (12). In some experiments, the pellet was rapidly washed with 2.5 mM unlabeled $CaCl_2$ and 1 mM EGTA containing medium to ensure the removal of the nonspecifically bound $^{45}CaCl_2$. After this treatment, the total radioactivity decreased by 10%–15%. All these procedures were carried out under a dim red light.

Cytochrome oxidase activity was measured as described by Schnaikman et al. (13). A didansyl-cysteine assay was carried out according to the procedure of Yoshikami et al. (14), by exposing ROS to the reagent (10 μM) for 1 min and then centrifuging the pellet before the light microscope observation. Proteins were measured by the procedure of Lowry et al. (15).

RESULTS

ROS preparations obtained by mild vortexing contain pigment epithelial cells, some erythrocytes and mitochondria from the inner segments. An estimate of the contribution of these contaminants to ^{45}Ca accumulation is necessary in order to be able to determine the part due to the isolated ROS. To this purpose, pure ROS were separated in a Ficoll gradient according to the procedure of Szutz and Cone (11), and the activity of cytochrome oxidase and the ATP-dependent calcium uptake were measured in the purified ROS floating in the Ficoll interphase and in the pellet containing unbroken cells and mitochondria. Table I shows that the cytochrome oxidase was concentrated in the pellet whereas the ROS fraction was devoid of enzyme activity. In contrast, the ATP-dependent calcium uptake was concentrated in the ROS layer and was practically absent

TABLE I

DISTRIBUTION OF CYTOCHROME OXIDASE ACTIVITY AND OF THE ATP-DEPENDENT CALCIUM UPTAKE ON A ROD OUTER SEGMENT PREPARATION FRACTIONATED ON A FICOLL GRADIENT

Fraction	Cytochrome oxidase (μatoms O_2/min/mg protein)	ATP-dependent Ca-uptake (nmol/mg protein)
10%–25%		
Ficoll interphase	N.D. (4)	2.5 ± 0.32 (6)
Pellet	2.01 + 0.16 (4)	0.1 ± 0.012 (6)
Crude preparation	2.67 ± 0.19 (4)	4.0 ± 0.01 (19)

ROS preparation was obtained as described in Experimental Procedure. Enzymatic activities and ^{45}Ca uptake were measured within 5 hr after excision of retinas. Results are the means ± SEM of the number of experiments indicated in parenthesis. N.D.: cytochrome oxidase activity not detectable.

from the pellet. The contamination of the ROS preparation with erythrocytes was estimated by directly counting the number of red cells per number of ROS under the microscope. This contamination was found to be less than 0.3%. In view of these results, all subsequent experiments were carried out in the crude ROS preparation. ROS in both the crude and the purified preparation, were found to have osmotically ruptured plasma membranes; the degree of leakiness was measured by the N-didansyl cysteine assay.

Figure 1 shows the time course of ^{45}Ca accumulation by frog ROS incubated in a Krebs-bicarbonate medium in the presence or the absence of ATP, and in media containing 25 mM taurine. ^{45}Ca uptake in a medium without ATP was very low, but was markedly increased by ATP and
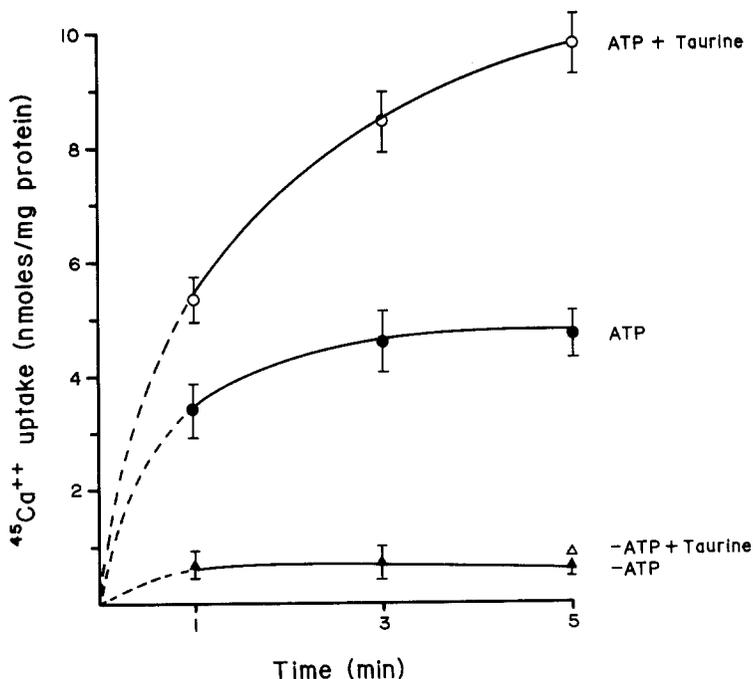

FIG. 1. Time course of the ATP-dependent ^{45}Ca accumulation by frog rod outer segments in the presence or the absence of 25 mM taurine. A ROS suspension isolated as described in Experimental Procedure, containing 50–100 μg protein, was incubated at 25°C, in a Krebs-bicarbonate medium, pH 7.4 containing 20 μM $^{45}CaCl_2$ during 1–5 min. Aliquots of the suspension (0.3 ml) were withdrawn at the indicated times, centrifuged and solubilized. The accumulated radioactivity was measured by scintillation spectrometry. Each point is the mean ± SEM for 4 separate experiments for 1 and 3 min respectively and of 19 experiments for 5 min.

TABLE II

THE EFFECT OF TAURINE AND OTHER AMINO ACIDS ON THE ATP-DEPENDENT [45]CA-
UPTAKE

Amino acids (10 mM)	[45]Ca uptake (nmol/mg protein)	
	− ATP	+ ATP
Control	0.41 ± 0.03	3.9 ± 0.16
Taurine (25 mM)	0.43 ± 0.04	8.2 ± 0.52
(10 mM)		6.7 ± 0.66
(5 mM)		5.5 ± 0.54
Glycine		4.6 ± 0.38
β-alanine		5.4 ± 0.67
Glutamic acid		4.2 ± 0.71
GABA		3.9 ± 0.42
Guanidinoethyl sulfonate		3.8 ± 0.45

ROS were incubated in a Krebs-bicarbonate medium containing taurine, amino acids and guanidinoethyl sulfonate at the concentrations indicated. Results are the means ± SEM of the number of experiments indicated in parenthesis.

further enhanced in the presence of taurine. The increase produced by ATP expressed as percent of controls was the same at all the times examined. Taurine-induced increase was seen to increase with time, and was maximal at 5 min. At this time, [45]Ca accumulation in the presence of 25 mM taurine was more than doubled while it was increased by 80% and 40% in the presence of 10 mM and 5 mM taurine respectively (Table II). Taurine did not affect the ATP-independent accumulation of [45]Ca. Other amino acids like GABA, glycine, glutamic acid, β-alanine and the taurine analogue, guanidinoethyl sulfonate did not modify the ATP-activated Ca accumulation either (Table II).

In order to test the specificity of ATP in promoting [45]Ca uptake, the effect of other nucleotides was examined. UTP, ITP, and CTP at concentrations ranging from 0.5 to 5 mM were ineffective in increasing [45]Ca accumulation. The stimulatory effect of taurine was also observed only in the presence of ATP. The activating effect of ATP might be due to a calcium binding, probably as CaATP to ROS membranes or to a process associated with the hydrolysis of the nucleotide. In order to discriminate among these possibilities, the ability of the non-hydrolizable analogue of ATP, β-γ-methylene ATP, to increase [45]Ca accumulation was tested. Figure 2 shows that β-γ-methylene ATP was ineffective as a substitute for ATP. The presence or absence of inorganic phosphate did not modify [45]Ca uptake.
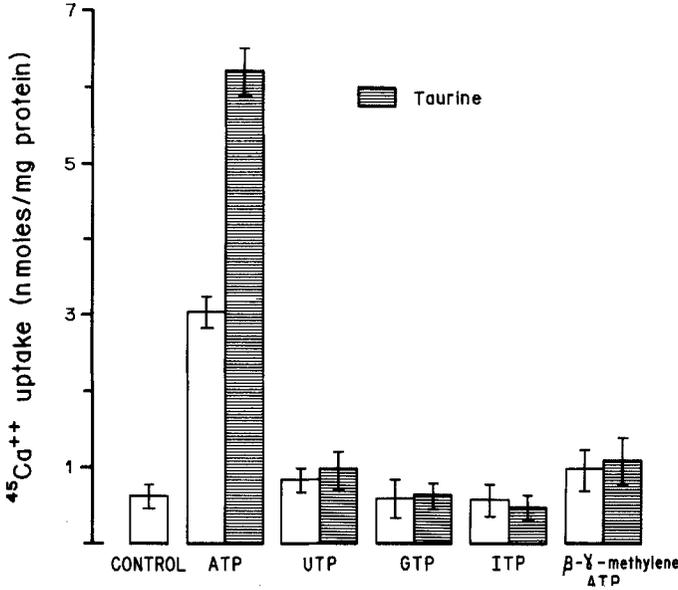
FIG. 2. The effect of nucleotides on ^{45}Ca uptake by frog rod outer segments, in the presence or absence of 25 mM taurine. The experimental conditions were as described in Figure 1. The nucleotides at a 1 mM concentration were present from the beginning of the incubation. Each bar represents the mean of 4 separate experiments ± SEM.

TABLE III

THE EFFECT OF DIFFERENT DRUGS ON THE ATP-DEPENDENT, TAURINE STIMULATED CALCIUM UPTAKE BY FROG ROS

Condition		^{45}Ca uptake (nmol/mg protein)		
		− ATP	+ ATP	+ ATP + Taurine
No addition		0.41 ± 0.03	3.9 ± 0.16	8.2 ± 0.5
Ethylmaleimide	(500 μM)		0.48 ± 0.05	0.46 ± 0.06
Mersalyl	(16 μM)		0.66 ± 0.07	0.68 ± 0.07
Ruthenium red	(100 μM)		0.40 ± 0.03	0.45 ± 0.05
Olygomycin	(1 μg/ml)		1.69 ± 0.08	1.75 ± 0.09
DCCD	(100 μM)		1.89 ± 0.09	1.94 ± 0.12
Ouabain	(1 mM)		4.02 ± 0.04	—

ROS were obtained as described in Experimental Procedure and incubated 5 min at 22°C in the medium containing 20 μM ^{45}Ca Cl_2. Drugs were present from the beginning of the incubation. Results are the means ± SEM of 3–19 separate experiments.

The requirement for non-hydrolysed ATP raises the possibility that calcium uptake is mediated by a Mg-dependent ATPase. Indirect support for this idea comes from the observation of a stringent requirement for Mg ion in ^{45}Ca uptake (Figure 3). With the same idea the effect of dicyclohexylcarbodiimide (DCCD), an agent known to inhibit ATPases was tested on ^{45}Ca uptake. Table III shows that a 50% decrease of calcium accumulation was observed in the presence of the inhibitor. Another Mg-ATPase inhibitor in ROS, oligomycin, also produced a marked decrease in the ^{45}Ca accumulation. Ruthenium red, mersalyl and ethylmaleimide at the concentrations known to inhibit ATPase activity in mitochondria and plasma membranes of other cell types, practically abolished the ATP-dependent ^{45}Ca uptake by ROS. Ouabain did not affect ^{45}Ca accumulation. The taurine-induced increase was also markedly affected by all the inhibitors tested (Table III).

In previous work we have shown that the effect of taurine on ^{45}Ca accumulation is observed only in the presence of bicarbonate; therefore, the influence of this ion was analyzed in the present study. Figure 4 shows that the ATP-dependent calcium accumulation is detected only in the
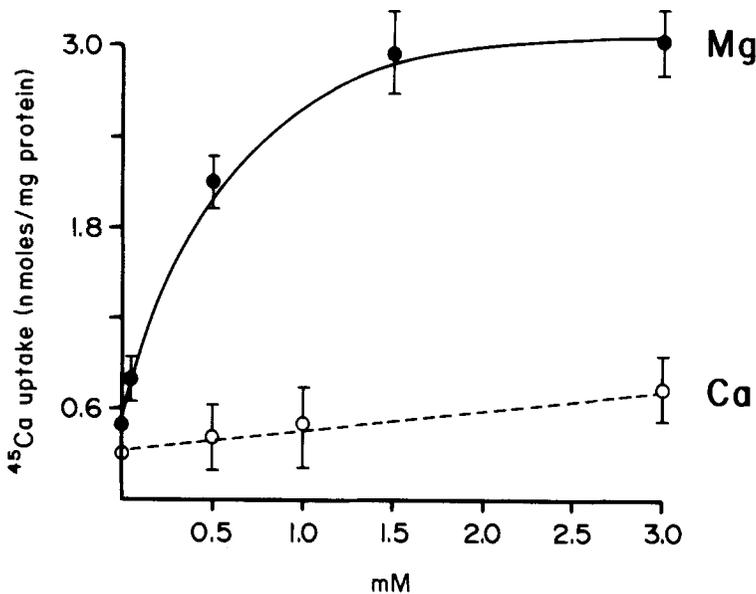

Fig. 3. Effect of MgCl$_2$ concentration on ^{45}Ca uptake by frog rod outer segments. The ROS preparation was incubated as described in Figure 1, in a Krebs-bicarbonate medium containing 20 μM ^{45}CaCl$_2$ and varying concentrations of MgCl$_2$. The dashed curve represents ^{45}Ca accumulation in a Mg-free medium at different Ca concentrations.

KREBS – BICARBONATE   KREBS-TRIS

$^{45}Ca^{++}$ uptake (nmoles/mg protein)

ATP + Taurine

ATP

−ATP

7.2   7.4   7.7   8.0   7.2   7.4   7.7   8.6

pH   pH

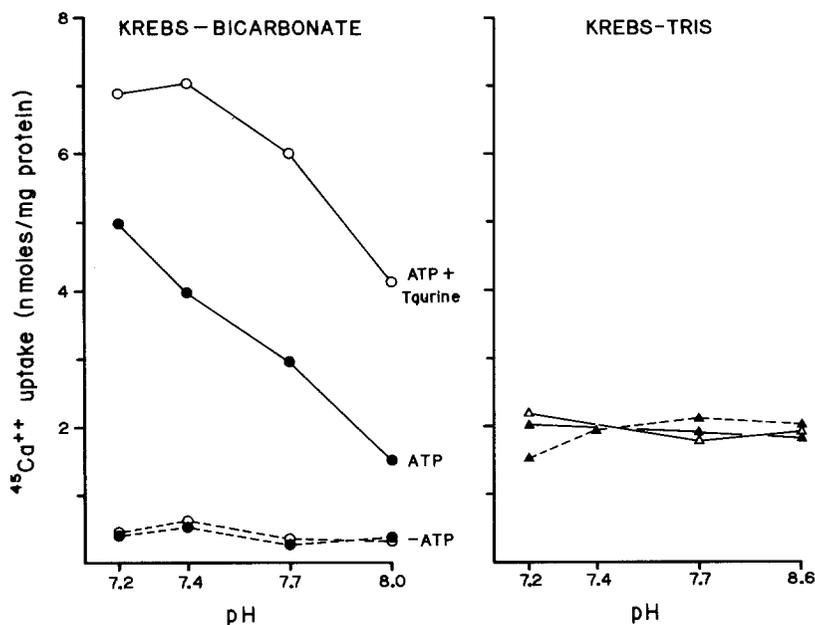FIG. 4. The effect of taurine on ^{45}Ca accumulation in a Krebs-bicarbonate medium or in a medium in which TRIS replaced bicarbonate, at different pH. The pH of the incubation media was adjusted by gassing with a 5% CO_2–95% O_2 mixture. The solid and dotted lines connect the means of determinations made in the presence or the absence of ATP, respectively, (O) Taurine, (●) without taurine, in experiments made in Krebs-bicarbonate medium; (△) Taurine, (▲) without taurine, in experiments made in Krebs-Tris medium. Each point is the mean of 4–7 separate experiments, SEM were lower than 12% in all cases.

presence of bicarbonate. This effect was observed at a range of bicarbonate concentration from 2.5 to 25 mM. Replacement of bicarbonate by TRIS lead to the suppression of the ATP-stimulatory effect; an ATP-independent calcium binding was observed under these conditions. The effect of taurine was exerted on the bicarbonate-stimulated Ca accumulation (Figure 4).

DISCUSSION

Light stimulation of rhodopsin triggers a set of biochemical reactions that lead to a reduced sodium current through the photoreceptor plasma membrane, consequently changing the electrical potential across the membrane (16, 17). Since the disk membranes in which rhodopsin is located are physically separated from the plasma membrane, a signal

must be transmitted from the disks to the plasma in order to trigger hyperpolarization. Haggins and Yoshikami (18) have proposed that this signal is carried by calcium ions, which after being released from within the disks when rhodopsin is bleached, subsequently diffuse to the plasma membrane to block sodium channels. Although some inconsistencies still exist about the hypothesis proposing calcium as the intracellular messenger for visual transduction, it has been demonstrated that the light regulated sodium channels of the cell membranes are highly sensitive to calcium (23). Therefore, cytoplasmic calcium levels should be kept low in order to maintain the resting state of the photoreceptors. Sequestration of calcium into the disks has been suggested as a mechanism of calcium removal from the cytoplasm. However, studies on the accumulation of calcium by the disks are scarce and controversial Ca-Ca exchange has been detected in cattle and frog ROS disks (24, 25). An active calcium uptake has been observed under certain conditions, in cattle ROS (26) but not in frog purified disks (25). Some reports on Mg-dependent nucleotide triphosphatase activities have been published (27, 28), but they have not been related to calcium transport. The results of the present study appear to confirm the existence of an active ATP-dependent Ca uptake in frog ROS, which markedly differs, by its pharmacological properties, from the mechanism of calcium exchange previously described (24). Since we are dealing with a leaky preparation, this process is most likely occurring at the ROS disks.

The effect of Mg on ^{45}Ca uptake suggests that the observed process relates to an active mechanism and that it is not simply a manifestation of possible calcium binding in the form of Ca-ATP, to superficial sites of the membrane. Lowering the Mg concentration in the incubation medium markedly reduced the ATP-dependent ^{45}Ca uptake. Since Ca and Mg bind to ATP with nearly equal affinity (29), omission of Mg should have increased the concentration of CaATP, and consequently, more and not less calcium should have been bound in the absence of Mg. The mechanism of this ATP-dependent Ca uptake remains to be clarified. It could represent a similar activity to that of a Mg ATPase responsible for the maintenance of low calcium levels in erythrocytes or for the extrusion of calcium across the sarcolemma (30, 31); however, direct evidence, namely the demonstration of coupling between calcium transport and ATP hydrolisis is still lacking. An alternate possibility is that phosphate, coming from the hydrolysis of ATP, could bind in some way to ROS membranes and that the phosporylated membrane complex would in turn increase the efficiency of calcium uptake. In bovine erythrocytes, an ATP-dependent calcium binding has been related to the formation of membrane-bound phosphoinositides, formed in the presence of ATP and Mg (32). These possibilities are now being investigated in our laboratory.

The process described in the present work requires the presence of bicarbonate. The effect of bicarbonate might be exerted at several sites on the Ca uptake mechanism. A stimulatory effect of this anion has been observed on Mg ATPases in different nerve preparations, including the retina (33); bicarbonate could also contribute to stabilize a Ca-phosphate binding, thus increasing calcium accumulation, or alternately, it might modify the membrane permeability thus making substrates or cofactors available to disk membranes. The importance of bicarbonate in retinal function has been emphasized by the observations on the effect of bi-carbonate omission on biochemical and physiological aspects of retinal activity. Replacement of bicarbonate by TRIS or phosphate in order to maintain a constant pH leads to the selective loss of the b-wave and a decline in the amplitude of the a-wave of the electroretinogram (34). Also, omission of bicarbonate leads to deleterious effects on metabolic retinal function (35). To our knowledge, the influence of bicarbonate on calcium transport in retina has not previously been described, although an effect of bicarbonate on mobilizing the calcium available for contraction has been observed in nuscle of both vertebrates and invertebrates (36, 37).

Taurine produced a marked stimulatory effect on the ATP-dependent ^{45}Ca-uptake. The concentration at which taurine is exerting this action, 5–25 mM, is within the range of physiological concentrations of taurine in ROS (4). Taurine-induced increase of ^{45}Ca accumulation was only observed in the presence of ATP, and not of other triphosphate nucleo-tides, inorganic phosphate or of the non-hydrolizable ATP analogue β-γ-methylene ATP, thus suggesting that its effects are linked to an ATPase activity. Also, the taurine stimulatory effect was blocked by ATPase inhibitors. More work is necessary to elucidate the mechanism of taurine action on ^{45}Ca uptake. The effects of taurine previously observed on calcium accumulation processes in retina had shown a strict requirement of bicarbonate, which has not been explained until now. The results of the present work, showing a bicarbonate-dependent calcium uptake proc-ess, offer a possible explanation for the taurine effect by relating it to a bicarbonate-requiring reaction. Although the mechanism of taurine action is still unclear, the observations reported here provide further support for its involvement in processes responsible for calcium regulation in the rod outer segments.

ACKNOWLEDGMENTS

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