J. Membrane Biol. 122, 203–213 (1991)
0022263191000944

The Journal of
Membrane Biology

Diltiazem at High Concentration Increases the Ionic Permeability of Biological Membranes

Antonio Caretta, Robert T. Sorbi, Peter J. Stein†, and Roberto Tirindelli
Istituto di Fisiologia Umana, Università di Parma, 43100 Parma, Italy, and †Department of Ophthalmology and Visual Sciences, School of Medicine, Yale University, New Haven, Connecticut 06540

Summary. The effects of diltiazem, a drug which inhibits the calcium channels in cardiac muscle as well as the light-sensitive channels in photoreceptor cells, were studied on ionic fluxes in both membrane and intact cell preparations. Diltiazem nonselectively increased the ionic permeability to both anions and cations in photoreceptor rod outer segment and synaptic membrane vesicles as well as in intact erythrocytes. Under our conditions, the estimated threshold for the diltiazem effect varied between 12.5 and 200 μM. In each case the concentration dependence exhibited the sigmoidal shape characteristic of positive cooperativity. The effect of diltiazem on ionic fluxes from phospholipid vesicles were strongly influenced by phospholipid composition and membrane charge. By contrast, diltiazem inhibited the efflux of ^{86}Rb from photoreceptor cells of intact aspartate-isolated retina, an effect opposite to that of diltiazem on ionic permeabilities in photoreceptor membrane vesicle preparations.

These data raise serious doubts on the specificity of diltiazem as a calcium channel blocker or as a cGMP channel blocker when used at concentrations higher than 10 μM.

Key Words diltiazem · photoreceptors · membrane permeability · phospholipid vesicles

Introduction

Diltiazem is used as an inhibitor of the calcium slow channels of plasma membranes and is commonly employed in clinical practice. Both the L- and D-*cis*-isomers of this drug have been recently reported to affect the cGMP-activated channel in the photoreceptor membranes [9, 11, 15, 18, 20], which plays a crucial role in phototransduction [5, 12].

In attempting to duplicate the ion flux experiments which identified diltiazem as a specific agent for blocking cGMP channel in photoreceptor membranes, we made the surprising observation that this drug increased membrane permeability to a variety of anions and cations (Ca^{2+} included) when tested on photoreceptor membrane vesicles, at a concentration substantially higher than the clinically useful concentration (0.3–1 μM). We extended these observations to synaptic membrane vesicles and intact erythrocytes. However, we found that diltiazem blocked rubidium efflux from photoreceptor cells of the intact aspartate-isolated retina, mimicking the effect of light. Experiments with phospholipid vesicles indicated that net membrane charge played an important role in the expression of diltiazem effect. They indicate that the drug can increase membrane permeability in reconstituted vesicles devoid of protein. Thus, a component of diltiazem response may be the result of an interaction directly with membrane phospholipids, not only with channel proteins.

Materials and Methods

Photoreceptor membranes were prepared from bovine or toad retinas as previously described [6] and usually washed in distilled water (three times with 2 ml/mg rhodopsin) to remove soluble proteins. The A_{280}/A_{500} ratio of emulphogene-solubilized membranes was 2.01 ± 0.08 (n = 7) for bovine membranes and 2.32 and 2.38 for two toad preparations, thus ruling out significant contamination. Photoreceptor membranes were chromatographed through a concanavalin A-Sepharose column (Pharmacia) (i.d. 1 cm, height 3 cm, flux rate 200 μl/min). No binding of photoreceptor membranes was observed, suggesting that the vesicles were inside out (with carbohydrate groups on the intravesicular surface).

Synaptosomes were prepared from rat brain according to [1].

Bovine erythrocytes were collected at a local slaughterhouse, centrifuged three times in 130 mM NaCl, 20 mM TRIS-HCl, at pH 7.8, 6 mM glucose, resuspended in the same solution at a hemoglobin concentration of 50 mg/ml (as measured from absorbance at 540 nm) with 20 μCi/ml ^{86}RbCl and, after 30 min incubation, the membrane permeability was tested as above described, using erythrocytes equivalent to 5 mg hemoglobin.

To perform permeability studies, separate membrane samples (0.6 mg) were centrifuged at 15,000 × g for 15 min in 150 mM KCl containing 20 mM Tris-HCl, at pH 7.7. The supernatant

was carefully removed and 1 μCi of the tested radiotracer ion was added to the pellet. After 1 hr incubation at 0°C, the pellet was resuspended in 1 ml test solution, injected into a small celite column (80 μl volume), perfused with a solution (130 mM KCl, or 150 mM NaCl in case of bovine erythrocytes, 1 mM EDTA, 25 mM Tris-HCl, at pH 7.7) driven by a peristaltic pump at flux rate of 3 ml/min. After having discarded the first 5 ml, samples of 0.5 ml were collected every 10 sec into scintillation vials by means of an automatic drop collector. Tests were made by modifying the perfusing solution from the 4th sample onward. Efflux rates were calculated as the fraction of remaining radioactivity being lost per minute [8]. The effect of diltiazem on the efflux rate of ions is expressed as

$$(K_{diltiazem} - K_o)/K_o$$

where $K_{diltiazem}$ is the efflux rate in the presence of diltiazem and K_o is the efflux rate in control conditions. The threshold for diltiazem effect was defined as the lowest diltiazem concentration that increased the efflux rate of both the 5th and 6th samples above that of the 4th sample in all the three membrane preparations on which we measured the concentration-effect relationship (Fig. 5 and Table 2; 6 preparations). Since the efflux rates are declining in time (*see* control traces, Figs. 1–4), the minimum effective diltiazem concentration is likely to be lower than our estimates. Results are given as mean ±SD of three experiments. It was checked that either L- or D-diltiazem (up to 2 mM) did not affect radioactive counting efficiency.

For retina efflux experiments, toads (*Bufo bufo*) were dark adapted over night, killed by decapitation; the eye was hemidissected under dim red light and 0.5 μCi of the radioactive isotope diluted in 6 μl Ringer were injected between the retina and the pigment epithelium; the retina was incubated in the presence of the radioactive isotope for 2 min. The retina was then placed in a perfusion chamber as described in [8]. The perfusion fluid was driven by a peristaltic pump at 2 ml/min. After 10 min perfusion in darkness with physiological solution, the fluid was collected by means of an automatic drop collector in vials. The solutions were either Ringer (101 mM NaCl, 2.68 mM KCl, 1.90 mM $CaCl_2$, 2.1 mM $MgCl_2$, 10 mM aspartate-Tris, 15 mM Tris-HCl, pH 7.8) or choline (110 mM choline chloride, 1 mM EDTA, 10 mM aspartate-Tris, 15 mM Tris-HCl, pH 7.8) or KCl-Ringer (105 mM KCl, 1.9 mM $CaCl_2$, 2.1 mM $MgCl_2$, 10 mM aspartate-Tris, 15 mM Tris-HCl, pH 7.8).

Thioacetamido-fluorescein guanosine cyclic 3′-5′ phosphate (SAF-cGMP) binding measurements were performed at 18°C as previously described [6].

Arsenazo III (AIII) was used to measure the effects of diltiazem on calcium efflux from phospholipid vesicles. To prepare the vesicles, a chloroform-methanol solution of phospholipids was dried in a rotavapor and the phospholipids (60 mg) were resuspended in 1 ml of a solution containing 50 mM arsenazo III, 100 mM $CaCl_2$ (or $BaCl_2$, in case of centrifugation experiment) buffered at pH 7.8 with KOH. The suspension was sonicated on ice for 30 min (peak width of tip excursion = 6 μm) and passed through a G25 column (height = 50 cm; i.d. = 2 cm; 5 ml/min flux) to separate unincorporated dye from the vesicles. The eluting solution was 150 mM KCl, 1 mM EDTA, 30 mM Tris-HCl, pH 7.8. Arsenazo III incorporation into the vesicles was affected by phospholipid composition: 22 ± 3 nM AIII/mg phospholipids (mean ± SD, 3 preparations) for phosphatidylcholine vesicles, 25 ± 5 nM AIII/mg phospholipids for phosphatidylcholine-stearilamide (75 : 25 wt/wt) vesicles, 1.4 ± 0.6 nM AIII/mg phospholipids for phosphatidylcholine-phosphatidylserine (75 : 25 wt/wt) vesicles, 2 ± 1.2 nM AIII/mg phospholipids for phosphatidylcholine-phosphatidic acid (95 : 5 wt/wt) vesicles. The $T_{1/e}$ for calcium leakage was 6 hr for phosphatidylcholine-phosphatidylserine vesicles in KCl-EDTA solution, while it could not be measured for phosphatidylcholine or phosphatidylcholine-stearilamide vesicles (longer than 5 days).

Absorbance changes of AIII-loaded phospholipid vesicles were recorded as previously described [4] with a Beckman DB-GT spectrophotometer, while stirring the suspension in the cuvette. Mixing time (measured with bromophenol) was 0.4 sec. No absorbance change was observed at λ = 576, an AIII isosbestic point, thus ruling out turbidity changes.

Centrifugation of $BaCl_2$-loaded vesicles was employed to determine the homogeneity of the response of the vesicle population to diltiazem. This procedure relies upon the fact that barium-loaded vesicles precipitated after centrifugation for 3 hr at 50,000 rpm on a Beckman SW65 rotor, whereas vesicles from which barium was released did not precipitate under these conditions. For centrifugation experiments, AIII-$BaCl_2$-filled phosphatidylcholine vesicles (220 mg phospholipids) were loaded on top of 3 ml of 200 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 7.8, in the presence of increasing concentrations of diltiazem. The samples were centrifuged at 50,000 rpm with a SW65 Beckman rotor for 3 hr. The percentage of vesicles precipitated after treatment with the drug was determined by measuring the absorbance at λ = 576 (AIII isosbestic point) of the supernatant and of the pellet resuspended in 3 ml of 200 mM NaCl, 20 mM Tris-HCl, pH 7.8, in the presence of 1% emulphogene.

L-diltiazem was a gift from Seikagaku, Japan, and D-diltiazem was from Sigma or from Sigmatau. Nifedipine was from Sigma. The purity of D-diltiazem was checked by TLC on Silica Gel (Merck 7774) with butanol : acetone : acetic acid : 5% ammonia : water (35 : 25 : 15 : 14 : 16 vol/vol). Only one spot was detected with R_f = 0.67.

Arsenazo III and phosphatidylcholine were from Sigma. Stearilamide and phosphatidic acid were from Fluka. Phosphatidylserine was from Koch and Light.

Radiotracer ions (^{36}Cl, ^{45}Ca, ^{86}Rb and ^{22}Na) were from Amersham. ^{86}Rb was a cheaper substitute for K [3, 8].

Results

Addition of either diltiazem isomer to the perfusing solution caused an increase in the efflux of the tracer ions from photoreceptor membranes as is shown in Fig. 1. The magnitude of this effect depended on diltiazem concentration. The threshold was 12.5 μM. The ion effluxes from photoreceptor membranes showed similar sensitivity for both the L- and D-isomers of diltiazem, even at low drug concentration (Table 1). Diltiazem increased the efflux rates of ^{22}Na, ^{45}Ca, ^{86}Rb and ^{36}Cl from photoreceptor membranes and synaptosomes (*see below*) (Table 2). Toad photoreceptor membranes showed the same drug sensitivity as bovine membranes (Table 3). No effect of rhodopsin bleaching was observed.

These data suggest that diltiazem elicits a large ionic efflux by itself, acting like an ionophore. In fact, 5 ml of 1 mM diltiazem released all of the intravesicular ^{22}Na, and subsequent application of

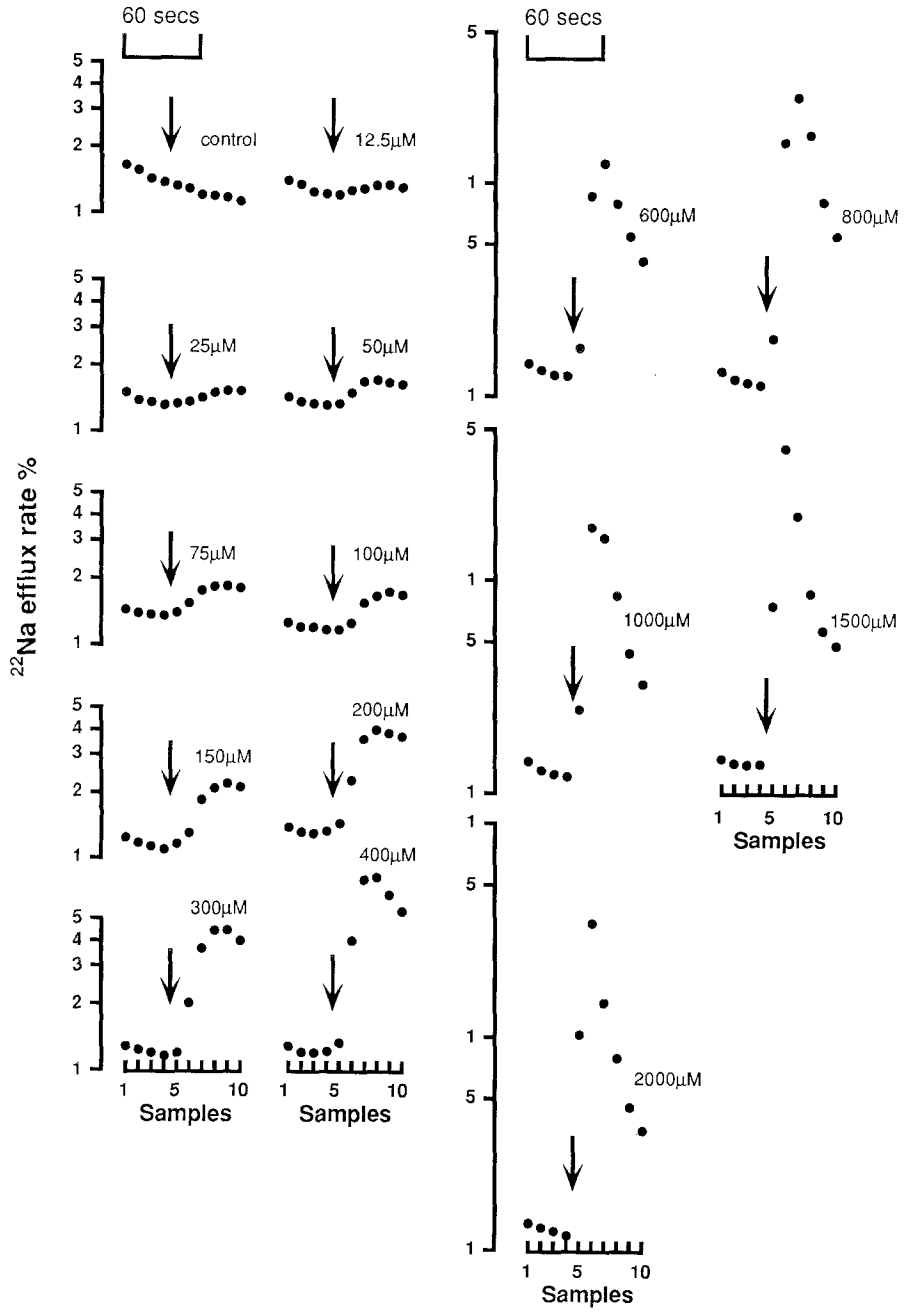

Fig. 1. Effect of increasing diltiazem concentrations on ^{22}Na efflux rate from photoreceptor membranes. The arrows indicate when diltiazem has been added to the membranes. Diltiazem concentrations (μM) are indicated above the traces. There were 0.6 mg photoreceptor membranes per test; one sample (0.5 ml) was taken every 10 sec

gramicidin (5 ml, 3 μg/ml) was without effect. Conversely, a pretreatment with gramicidin (5 ml, 3 μg/ml) abolished the diltiazem-induced release of ions. A similar diltiazem-dependent release was obtained when photoreceptor membranes or synaptosomes (*see below*) were loaded with ^{45}Ca and the ionophore employed was A23187 (Table 4). Under these conditions, diltiazem reduced the cGMP-dependent efflux as previously described [9, 11, 15], but, because of the large ionic efflux activated by diltiazem, interpretation of these results is not straightforward (Table 5). These experiments confirmed that diltiazem, like ionophores, acted upon the total internal ionic pool, while cGMP exerted its effect on a subpopulation of vesicles.

To test the possibility that the increased ionic permeability resulting from application of the drug might induce the generation of a diffusion potential, we altered the composition of the perfusate from NaCl to either KCl or choline chloride. This change

had no effect on diltiazem-dependent increase in ionic permeability. Moreover, we determined that diltiazem did not saturate an ionic mechanism by changing the specific activity of the intravesicular radiotracer ions. Incubation of ^{22}Na in either KCl or NaCl did not alter the diltiazem-induced efflux (*data not shown*). Hence, between 0 and 150 mM NaCl or KCl, diltiazem does not saturate an ionic mechanism. Finally, we observed that application of 100 μM nifedipine, an alternative calcium channel blocker, had no effect on ionic fluxes from photoreceptor membrane vesicles (or from the other membrane preparations studied).

Table 1. Effect of L and D-diltiazem on ^{22}Na efflux rate of bovine photoreceptor membranes (mean ± SD of three membrane preparations)

Drug conc. (μM)	Efflux rate increase	
	L-diltiazem	D-diltiazem
12.5	0.20 ± 0.08	0.16 ± 0.06
100	0.57 ± 0.16	0.43 ± 0.10
200	3.76 ± 0.41	2.06 ± 0.25
400	6.81 ± 0.69	5.29 ± 0.19
800	22.14 ± 5.46	19.54 ± 3.26
2000	24.65 ± 6.34	29.18 ± 4.33

Efflux studies on intact dark-adapted retina revealed that the effect of diltiazem was different from that observed in purified photoreceptor membranes. It has to be pointed out that the retinas were perfused with Ringer-Aspartate (*see* Materials and Methods) in order to block the synaptic transmission between photoreceptor cells and the rest of the retina. Figure 2 shows that light inhibited the efflux of ^{86}Rb from the intact retina, as previously described [3, 8]. Under similar ionic conditions, diltiazem reduced the efflux of ^{86}Rb, mimicking the effect of light. Moreover, the effect of light was abolished during diltiazem perfusion (Fig. 2, trace *g*). The amplitude of the effect of diltiazem was affected by the ionic composition of the perfusing solution. The largest effect was observed in the presence of choline-EDTA, the condition in which it is possible to observe the largest light effect on the efflux of ^{86}Rb, while the effect of diltiazem was almost (but not completely) abolished under ionic conditions in which the light effect was completely abolished (*see* Table 6).

To establish if the diltiazem effect on intact photoreceptors could be due to inhibition of cGMP binding, we measured the influence of diltiazem on SAF-cGMP binding to two photoreceptor membrane preparations (their permeability data are shown in Table 5). No effect of diltiazem (up to 2 mM) on SAF-cGMP binding was detectable in the concentration

Table 2. Effect of diltiazem on efflux rates of different ions from bovine photoreceptor membranes (A_{280}/A_{500} = 2.02 + 0.08; n = 3) and synaptosomes (mean ± SD of three preparations)

Diltiazem conc. (μM)	Photoreceptor membranes Efflux rate increase			
	^{22}Na	^{45}Ca	^{86}Rb	^{36}Cl
12.5	0.13 ± 0.04	0.23 ± 0.06	0.11 ± 0.04	0
25	0.21 ± 0.09	0.31 ± 0.10	0.16 ± 0.08	0.12 ± 0.05
50	0.26 ± 0.12	0.38 ± 0.09	0.31 ± 0.11	0.18 ± 0.06
100	0.44 ± 0.09	0.56 ± 0.14	0.39 ± 0.09	0.32 ± 0.12
200	1.94 ± 0.32	2.71 ± 0.86	1.46 ±.0.37	1.09 ± 0.12
400	6.20 ± 0.57	7.30 ± 0.58	4.63 ± 1.26	3.12 ± 0.84
600	7.84 ± 1.22	8.61 ± 0.92	6.24 ± 0.76	4.94 ± 1.08
800	13.64 ± 3.22	15.41 ± 2.91	10.8 ± 1.73	7.23 ± 1.42
1000	19.54 ± 3.26	21.03 ± 3.24	16.21 ± 2.64	11.28 ± 2.18
2000	31.18 ± 6.43	28.73 ± 5.43	22.81 ± 4.74	18.64 ± 3.73
	Synaptosomes Efflux rate increase			
	^{22}Na	^{45}Ca	^{86}Rb	^{36}Cl
250	0.21 ± 0.07	0.35 ± 0.14	0.21 ± 0.04	0.11 ± 0.04
500	1.62 ± 0.24	2.34 ± 0.40	1.12 ± 0.37	0.81 ± 0.14
800	4.22 ± 0.52	6.40 ± 1.04	4.10 ± 0.69	3.24 ± 0.83
2000	10.38 ± 0.71	12.49 ± 1.27	8.24 ± 1.32	6.74 ± 0.65

range (0.3–10 μM) at which SAF-cGMP activates the channel (*data not shown*). Therefore, competition for cGMP binding (as previously suggested, [15]) cannot be the mechanism by which diltiazem reduces the effect of cGMP.

Since diltiazem expressed opposing effects on ionic fluxes from intact retina compared with those observed in photoreceptor membrane preparations, we tested other intact and fragmented membrane systems, intact bovine erythrocytes and brain synaptosomal membranes. It is important to note that neither preparation has a cGMP-activated permeability (*data not shown*).

Table 3. Effect of diltiazem on ^{22}Na efflux rate from bovine and toad photoreceptor membrane preparations (mean ± SD of three bovine and two toad membrane preparations)

Diltiazem conc. (μM)	Efflux rate increase	
	Toad	Bovine
50	0.34 ± 0.18	0.29 ± 0.11
100	0.55 ± 0.14	0.43 ± 0.10
200	1.88 ± 0.61	2.06 ± 0.25
400	7.20 ± 1.81	5.29 ± 0.19
800	14.23 ± 5.41	19.54 ± 3.26
2000	27.38 ± 7.04	29.18 ± 4.33

Table 4. Effect of sequential perfusion with ionophores (either gramicidin or A23187) and diltiazem on the release of ^{22}Na or ^{45}Ca from biological membranes

	Photoreceptor membranes	Synaptosomes
A		
^{22}Na		
Gramicidin	88 ± 3	96 ± 2
Diltiazem	3 ± 2	0
^{45}Ca		
A23187	92 ± 3	85 ± 5
Diltiazem	0	5 ± 2
B		
^{22}Na		
Diltiazem	91 ± 6	81 ± 4
Gramicidin	0	6 ± 2
^{45}Ca		
Diltiazem	84 ± 6	81 ± 10
A23187	3 ± 2	0

[a] Membranes were perfused with 5 ml of a solution containing either gramicidin (3 μg/ml) or A23187 (10 μg/ml) and diltiazem (1 mM). The amount of ions released by each treatment is expressed as percentage of the total amount of radiotracer ions present on the celite column before starting the perfusion with the test solutions. (A) The ionophore was added before diltiazem. (B) Diltiazem was added before the ionophore.

Diltiazem induced ^{22}Na effluxes from rat brain synaptic vesicles (Fig. 3). In this preparation no effect of diltiazem was observed below 200 μM, a concentration substantially above that required for photoreceptor membrane vesicles.

We observed that diltiazem increased the permeability to Na, Rb, Ca and Cl ions with a similar concentration dependence, as was the case for photoreceptor membrane vesicles (Table 2). Divalent cations (Ca, Sr and Ba), at 1 mM in the perfusing solution, reduced the effect of high diltiazem concentration (above 800 μM) but had no effect on the permeability increase induced by lower drug concentration (*data not shown*). In fact, the effect of 2 mM diltiazem in the presence of divalent cations is 65% of the one that can be observed with 1 mM EDTA in the perfusing solution.

In intact bovine erythrocytes diltiazem increased ^{86}Rb efflux rate (Fig. 4) with a sensitivity slightly higher than in synaptosomes. We were unable to load intact erythrocytes or photoreceptors with ^{45}Ca or ^{22}Na, most likely because these ions are actively extruded, and ^{36}Cl basal efflux rate was 2.6 times higher than ^{86}Rb basal efflux rate, thus making a comparison between diltiazem effects difficult to interpret.

The relationship between diltiazem concentration and Na (or Rb) permeability increase in different membrane preparations is shown in Fig. 5. It is apparent that different membranes present different thresholds and different saturating levels. All of the curves displayed a sigmoid shape, suggesting a cooperative binding of diltiazem. An exact analysis of cooperativity was not possible because of our uncertainty on the exact value of saturation. That is, at the highest tested concentrations, all the ions were released from the membranes, therefore it was not

Table 5. Effect of sequential perfusion with cGMP and either gramicidin or diltiazem on the release of intravesicular ^{22}Na from photoreceptor membranes[a]

	^{22}Na		
A			
cGMP	18 ± 5	cGMP	21 ± 3
Diltiazem	76 ± 6	Gramicidin	70 ± 8
B			
Diltiazem	88 ± 3	Gramicidin	92 ± 3
cGMP	1	cGMP	0

[a] Photoreceptor vesicles were perfused first with 5 ml of a solution 130 mM KCl, 25 mM Tris-HCl, 1 mM EDTA, pH 7.7, containing 3 μg/ml gramicidin or 1 mM diltiazem and then with a solution containing 500 μM cGMP. Results are expressed as in Table 1. (A) cGMP was added before gramicidin or diltiazem. (B) diltiazem or gramicidin were added before cGMP.

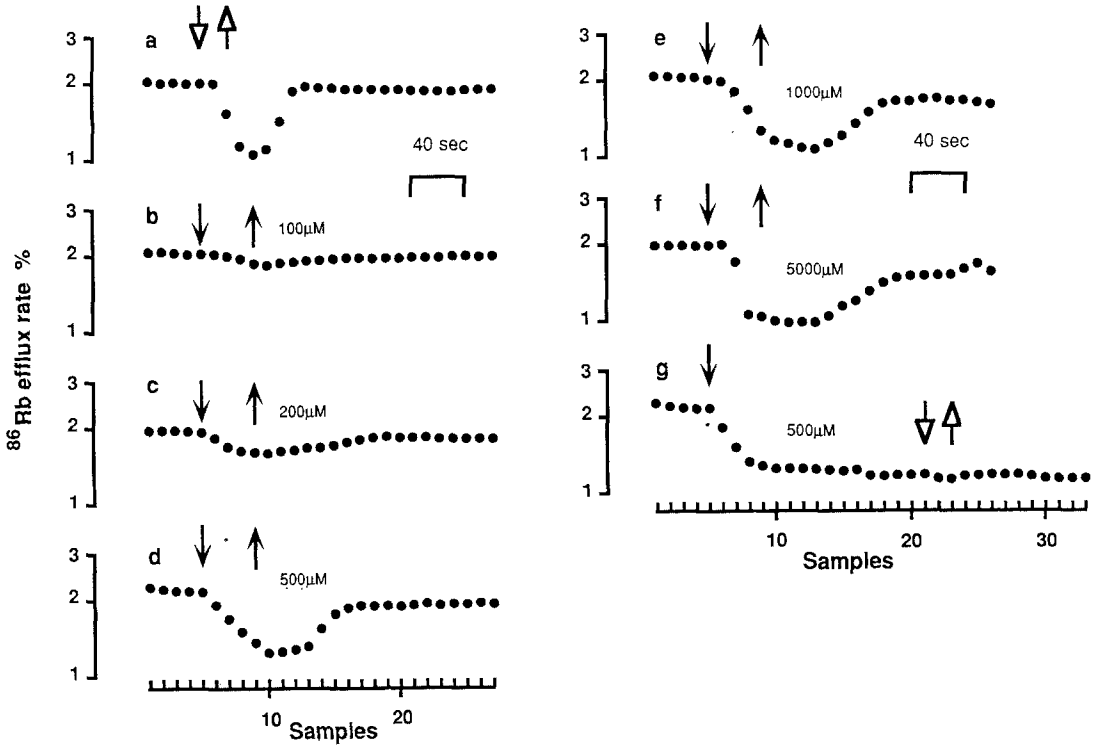

Fig. 2. Effect of light and diltiazem on the ^{86}Rb efflux from toad retina. Trace *a*: light effect; trace *b*: diltiazem: 100 μM; trace *c*: 200 μM; trace *d*: 500 μM; trace *e*: 1 mM; trace *f*: 5 mM; trace *g*: 500 μM, from the first arrowhead until the end of the perfusion, 2nd and 3rd arrowhead indicating light period. One sample (800 μl) was taken each 1 min. The intensity of the light stimulus (traces *a* and *g*) was 2.4 × 10^5 photons/rod/sec. The downward arrowheads indicate the onset, while the upward ones indicate the offset of diltiazem (black arrowheads) and of light (open arrowheads)

Table 6. Light and diltiazem-dependent decrease of ^{86}Rb efflux rate from isolated toad retina under different ionic conditions[a]

	Light	Diltiazem
Choline EDTA	46 ± 7	52 ± 4
Ringer	23 ± 7	38 ± 8
K-$CaCl_2$	0	7 ± 3

[a] The values: mean ± SD of three experiments, expressed as % decrease from the control value. Light: 2.4 × 10^5 photons/sec/rod; λ = 510. Diltiazem = 500 μM.

possible to determine whether the apparent saturating level resulted from saturation of diltiazem binding or from exhaustion of the intravesicular ionic compartment.

Since we observed such a variability in the diltiazem sensitivity of the different preparations, we investigated whether phospholipids were an important factor in the mechanism of the drug's action. We loaded phospholipid vesicles of different composition with AIII-$CaCl_2$. Figure 6 shows the effect of diltiazem on membrane permeability of phosphatidylcholine vesicles filled with AIII-$CaCl_2$. Addition of diltiazem (1 and 2 mM final concentration, first two arrows) caused a small decrease in absorbance (respectively 4 and 8% of AIII absorbance). The time course of these initial absorbance changes appeared step-like: after an initial fast small decrease, the absorbance change could remain unchanged for hours. At higher diltiazem concentration (3 or 4 mM) different kinetics were observed: the initial rapid decrease (whose time course is lost in the mixing time of the cuvette, 0.4 sec), was followed by a slow and continuous decrease. On the right, the effect of increasing concentrations of the ionophore A23187 on the kinetics of Ca efflux are shown for comparison.

The results illustrated in Fig. 6 could be accounted for either by the diltiazem-induced release of a small percentage of the calcium ions from each vesicle or by the complete release of calcium from a small percentage of the vesicles.

To distinguish between these two alternative hypotheses, we performed both centrifugation and permeability experiments on similarly treated AIII-$BaCl_2$-loaded vesicles. Barium-loaded vesicles were precipitable after centrifugation, as described in Materials and Methods. The results showed that Ba-loaded vesicles could be completely sedimented. In-

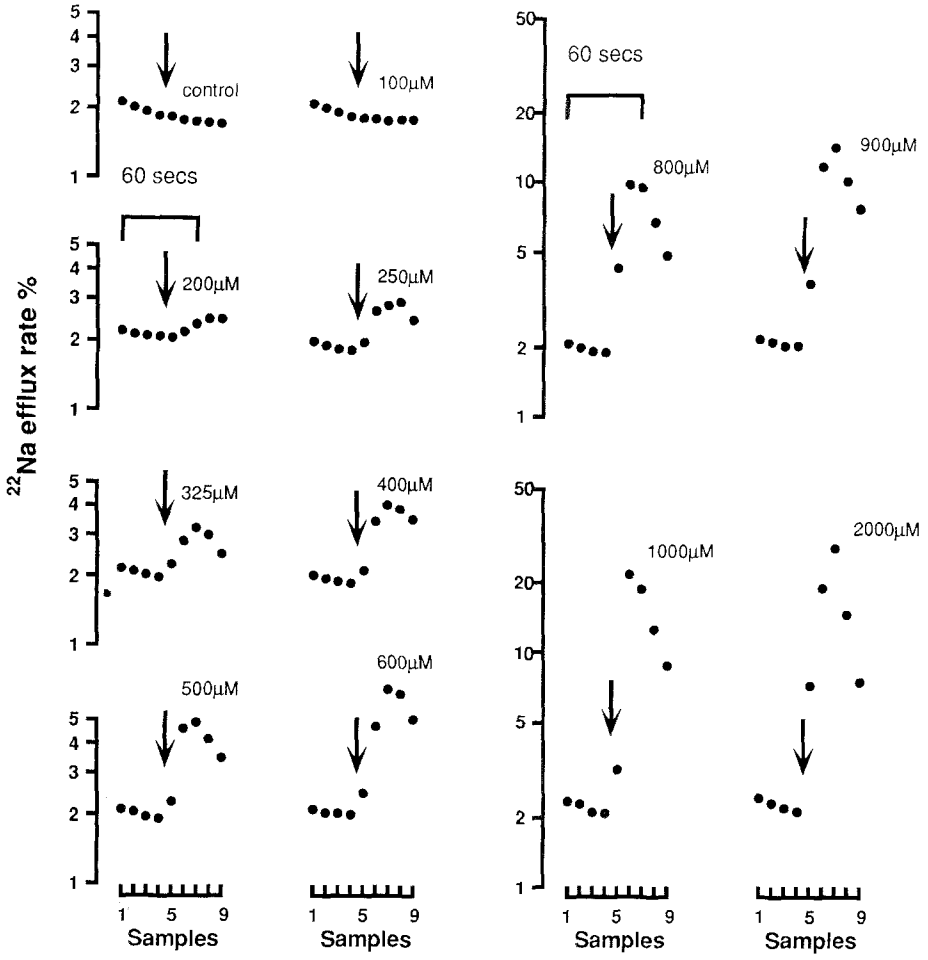

Fig. 3. Effect of increasing diltiazem concentration on the efflux of ^{22}Na from synaptosomes. The arrows indicate when diltiazem has been added to the membranes. Diltiazem concentrations (μM) are indicated above the traces. There were 0.6 mg membranes per test; one sample (0.5 ml) was taken every 10 sec

creasing diltiazem concentration up to 2 mM does not affect sedimentation of the vesicles: no absorbance could be detected in the supernatant, suggesting thus that the small rapid decrease in absorbance observed after addition of 1–2 mM diltiazem did not depend on releasing all the ions from a small fraction of vesicles but rather on an increase of permeability (rapidly inactivated) in the total vesicle population. Addition of 4 mM diltiazem, which caused complete desaturation of the AIII absorbance spectrum, resulted in a failure of any vesicles to pellet, indicating complete release of barium from the intravesicular pool. The fact that diltiazem caused a change in the permeability of phospholipid vesicles, suggested that the composition of the vesicles might be important to the expression of the drug effect. We tested the influence of vesicle composition and net membrane charge on the diltiazem-induced release of calcium. Figure 7 shows the effect of diltiazem on both negatively and positively charged vesicles. On the left the absorbance spectra of phosphatidylcholine-phosphatidylserine vesicles in the presence of 400 μM diltiazem are shown; the kinetics present a continuous trend; also at lower concentration no step-like effect can be observed. Similar effects were observed with phosphatidic acid vesicles, at a lower ratio (phosphatidic acid 5 mg, phosphatidylcholine 95 mg). On the other side, in the presence of positively charged vesicles, repeated additions of diltiazem caused only small, step-like decreases in absorbance (Fig. 7, up to 6 mM final diltiazem concentration).

Discussion

The results reported above indicate that diltiazem has opposite effects on ionic permeability of disrupted photoreceptor membranes and intact retinal photoreceptor cells. Although it is possible that these data are relevant for the interpretation of contradictory electrophysiological results [20], we cannot provide a convincing explanation for this discrepancy; there are no data on diltiazem

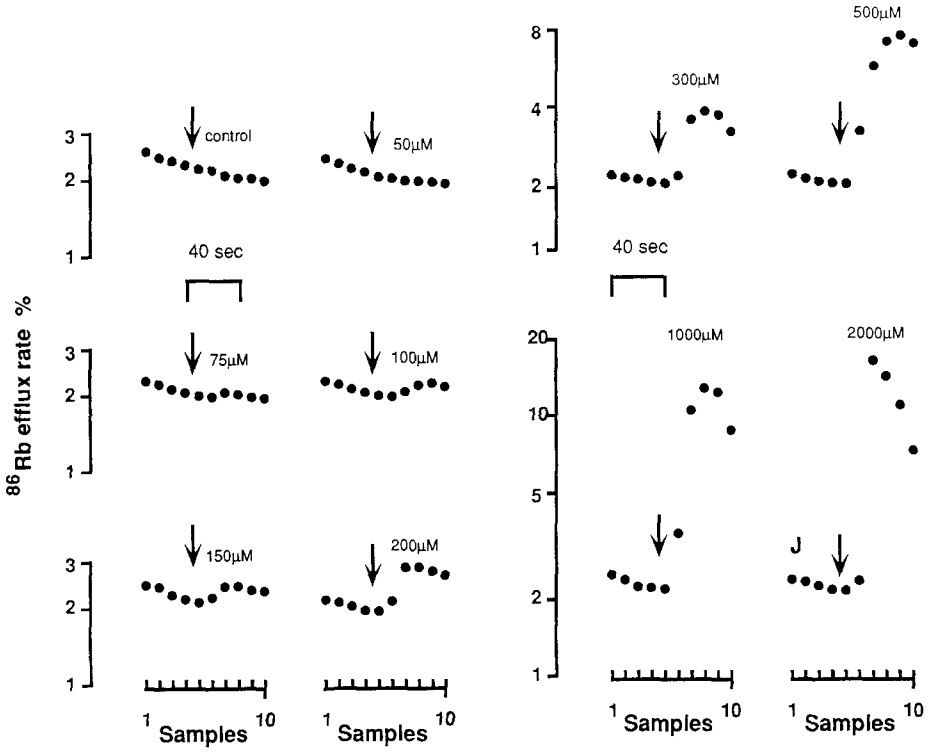

Fig. 4. Effect of increasing diltiazem concentration on the efflux of ^{86}Rb from erythrocytes. The arrows indicate when diltiazem has been added to the membranes. Diltiazem concentrations (μM) are indicated above the traces. One sample was taken every 10 sec. Perfusion was carried out in 150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 7.8, 8 mM glucose

permeability through photoreceptor plasma membranes, but, since diltiazem can cross phospholipid membranes, it is reasonable to assume that it can move through photoreceptor plasma membranes. An important difference between the two preparations is that disrupted photoreceptor membranes are inside out (glucidic groups are not available for concanavalin binding), while glucidic groups in plasma membrane have the opposite orientation. Moreover, photoreceptor membrane preparations are a mixture of disk and plasma membranes. These two kinds of membranes have a different protein composition [14, 16] and particularly the distribution of the cGMP- and light-sensitive channel is restricted to the plasma membrane [7, 10]. The diltiazem inhibition of ^{86}Rb efflux from intact photoreceptor cells is likely to depend (as previously described) on the closure of the cGMP- and light-sensitive channel because of similar amplitude and ionic sensitivity as the light effect. On the other hand, the experiments on phospholipid vesicles show that proteins are not necessarily involved in the diltiazem-dependent permeability increase and that membrane phospholipid composition or charge may be a critical factor (although in an unpredictable way because of the asymmetric distribution of phospholipids within the membranes; *see* [2, 17]).

The similarity between the inhibition curve of diltiazem on cGMP channel on photoreceptor plasma membrane patches and the diltiazem activation curve of disk membrane ionic permeability (Fig. 3 in [19] and Fig. 5 in this paper) may suggest that these two apparently opposite effects have a common molecular origin. It should be considered that purified cGMP-sensitive channels reconstituted in lecithin vesicles become insensitive to diltiazem [11], suggesting that phospholipid composition may be relevant also for the inhibition of the cGMP-sensitive channel.

On the other side, diltiazem increases ionic membrane permeability in erythrocytes, still intact as judged by their hemoglobin content, and in synaptosomes. These experiments suggest that the ability of diltiazem to increase membrane permeability may be a wide-spread phenomenon, and present also in intact cells.

Although we have not been able to estimate the thresholds for diltiazem effect on phospholipid vesicles, the experiments shown in Figs. 6 and 7 demonstrate that the diltiazem-activated increase of membrane permeability depends on the phospholipid composition (and/or on the net electrical charge) of the membranes. We suggest that these differences may be related to the different thresholds for activating ion permeability observed in preparations of biological membranes.

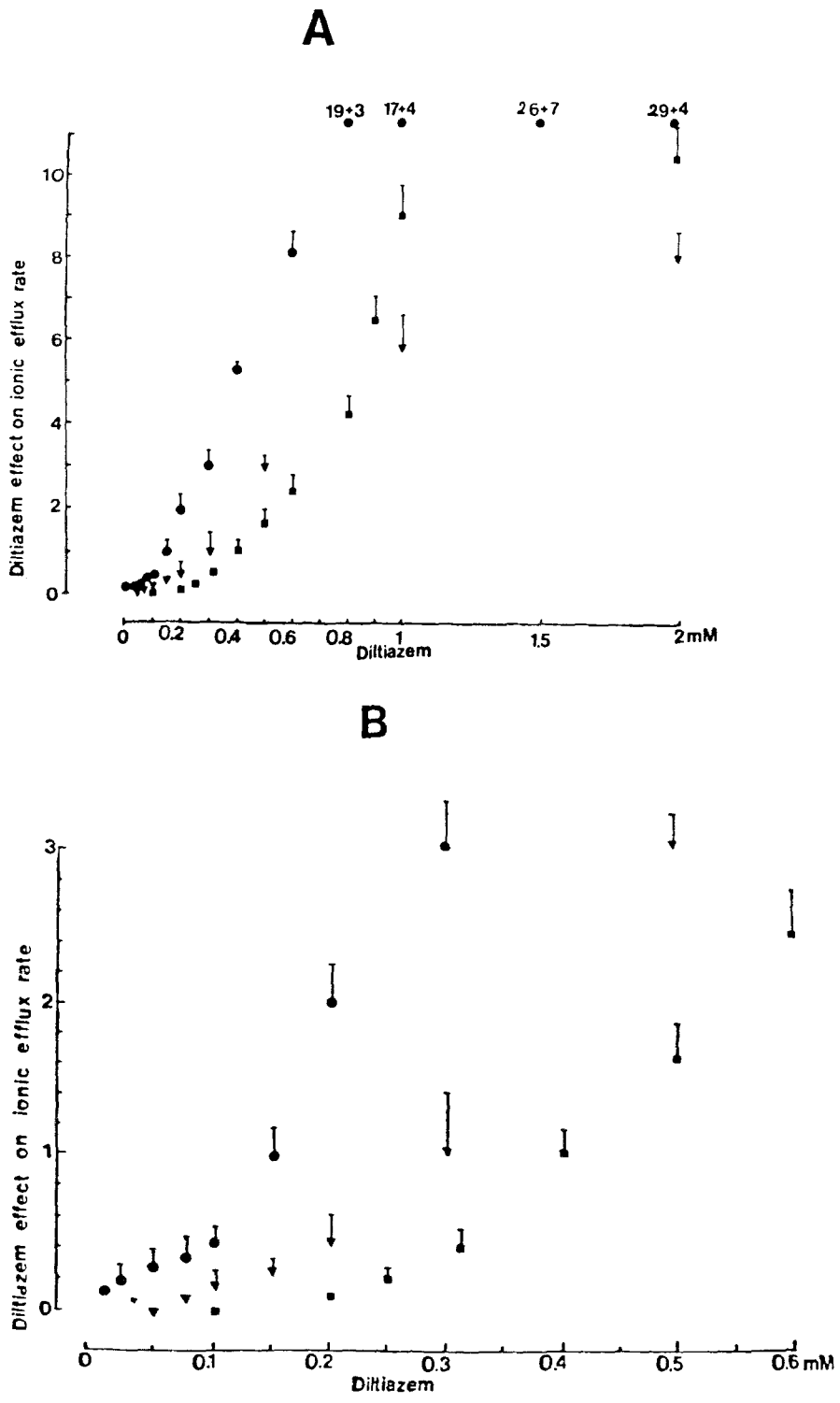

Fig. 5. Relationship between diltiazem concentration and permeability increase in three different preparations. (*A*) Every point is the mean ± SD of three experiments. Circles: bovine photoreceptor membranes (A_{280}/A_{500} = 1.99 + 0.11, n = 3). Triangles: erythrocytes. Squares: synaptosomes. (*B*) Expanded representation of the initial part of *A*

Our observations of the diltiazem effect (at concentration above 12.5 μM) on ionic permeabilities suggest that, at these concentrations which are much higher than the clinically useful concentration (0.3–1 μM), the drug cannot be considered specific for either the photoreceptor cGMP-dependent channel or the Ca slow channel. It appears most likely that the effects of diltiazem reported in many systems at concentrations higher than 10 μM (for example, action potential duration and

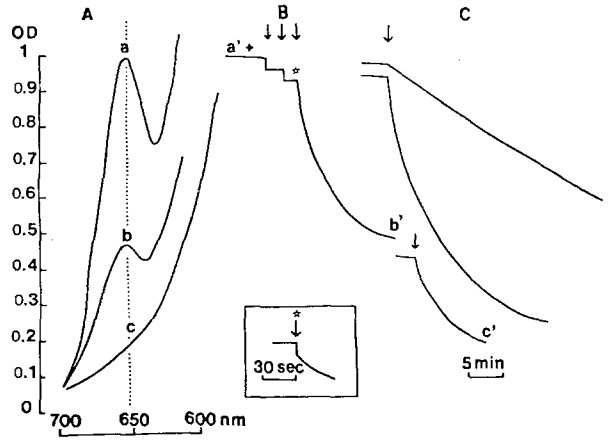

Fig. 6. Effect of diltiazem on Ca permeability of phospholipid vesicles. Phosphatidylcholine vesicles loaded with Ca-AIII. (*A*) spectra (*a*, *b*, *c*) recorded at different times (*a'*, *b'* and *c'*) are presented. The dotted line indicates 652 nm. (*B*) Continuous recording of absorbance changes at $\lambda = 652$ showing the effect of diltiazem addition (1 mM final concentration after each arrow). The cross after *a'* indicates the effect of a control injection. The inset shows, on an expanded time scale, the effect of the third diltiazem addition. The break on the trace after *b'* depends on the time required to record the AIII spectrum *b*. AIII concentration = 50 μM, 1 mg phospholipids/ml. (*C*) Traces *x* and *y* show the effect of ionophore A23187 addition (1 and 5 μg/ml, respectively) on AIII titration. The conditions were the same as middle traces

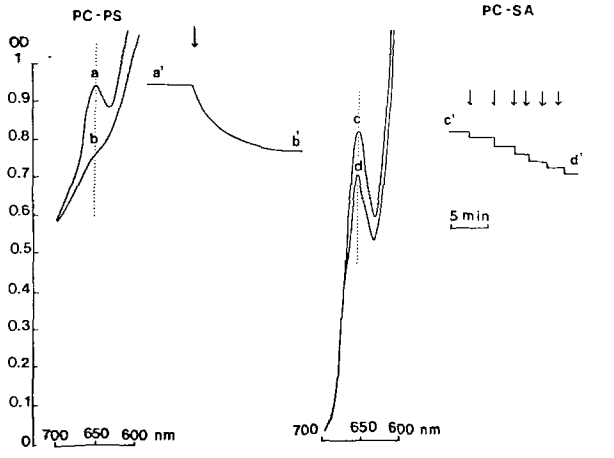

Fig. 7. Effect of diltiazem addition on charged phospholipid vesicles loaded with AIII-Ca. The AIII spectra *a–d* were recorded at times *a'–d'*, while recording continuous absorbance changes. Shown on the left are phosphatidylcholine (75 mg)-phosphatidylserine (25 mg) vesicles (PC-PS); diltiazem = 400 μM after the arrow; AIII = 12 μM, 9 mg phospholipids/ml. Note the increased level of light scattering, because of the high amount of phospholipids. Shown on the right are phosphatidylcholine (75 mg)-stearilamide (25 mg) vesicles (PC-SA); diltiazem = 1 mM after each arrowhead (final concentration = 6 mM). AIII = 50 μM, phospholipids = 1 mg/ml

amplitude and maximum upstroke velocity in human heart cell [13] may at least in part depend on the nonspecific permeability increase we reported above.

References

1. Booth, R.F.G., Clark, J.B. 1978. A rapid method for the preparation of relatively pure metabolically competent synaptosomes from rat brain. *Biochem. J.* **176:**365–370
2. Bretscher, M.S. 1972. Phosphatidylethanolamine: Differential labeling in intact cells and cell ghosts of human erythrocytes by a membrane-impermeable reagent. *J. Mol. Biol.* **71:**523–528
3. Capovilla, M., Caretta, A., Cervetto, L., Torre, V. 1983. Ionic movements through light sensitive channels of toad rods. *J. Physiol.* **343:**295–310
4. Caretta, A., Cavaggioni, A. 1983. Fast ionic flux activated by cyclic GMP in the membrane of cattle rod outer segments. *Eur. J. Biochem.* **132:**1–8
5. Caretta, A., Cavaggioni, A., Sorbi, R.T. 1979. cGMP and the permeability of the disk of the frog photoreceptors. *J. Physiol.* **295:**171–178
6. Caretta, A., Cavaggioni, A., Sorbi, R.T. 1985. Binding stoichiometry of a fluorescent cGMP analogue to membranes of rod outer segments. *Eur. J. Biochem.* **153:**49–53
7. Caretta, A., Saibil, H. 1989. Visualization of cyclic nucleotide binding sites in the vertebrate retina by fluorescence microscopy. *J. Cell Biol.* **108:**1517–1522
8. Cavaggioni, A., Sorbi, R. T., Turini, S. 1972. Efflux of potassium from the isolated frog retina: A study of the photic effect. *J. Physiol.* **222:**427–445
9. Cook, N.J., Hanke, W., Kaupp, U.B. 1987. Identification, purification and functional reconstitution of cyclic GMP dependent channel from rod photoreceptors. *Proc. Natl. Acad. Sci. USA* **84:**585–589
10. Cook, N.J., Molday, L.L., Reid, D., Kaupp, U.B., Molday, R.S. 1989. The cGMP gated channel of bovine photoreceptors is localized exclusively in the plasma membrane. *J. Biol. Chem.* **264:**6996–6999
11. Cook, N.J., Zeilinger, C., Koch, K.W., Kaupp, U.B. 1986. Solubilization and functional reconstitution of the cGMP-dependent channel from rod photoreceptors. *J. Biol. Chem.* **261:**17033–17039
12. Fesenko, E.E., Kolesnikov, S.S., Lyubarsky, A.L. 1984. Induction by cGMP of cationic conductance in plasma membrane of retinal outer segments. *Nature* **313:**310–313
13. Hirth, C., Borchard, U., Hafner, D. 1983. Effect of the calcium antagonist diltiazem on action potentials, slow response and force of contraction in different cardiac tissues. *J. Mol. Cell. Cardiol.* **15:**799–809
14. Kamps, K.M.P., De Grip, W.J., Daemen, F.J.M. 1982. Use of a density modification technique for isolation of the plasma membrane of rod outer segments. **687:**296–302
15. Koch, K.W., Kaupp, U.B. 1985. Cyclic GMP directly regulated a cationic conductance in membranes of bovine rods by a cooperative mechanism. *J. Biol. Chem.* **260:**6788–6800
16. Molday, R.S., Molday, L.L. 1987. Differences in protein composition of bovine retinal rod outer segment disk and plasma membranes isolated by a ricin-gold-dextran density perturbation method. *J. Cell Biol.* **105:**2589–2601
17. Op den Kamp, J.A.F. 1979. Lipid asymmetry in membranes. *Annu. Rev. Biochem.* **48:**47–71

18. Pearce, L.B., Calhoon, R.D., Burns, P.R., Vincent, A., Goldin, S.M. 1988. Two functionally distinct forms of guanosine 3'-5'-phosphate stimulation cation channels in bovine rod photoreceptor disk preparation. *Biochemistry* **27:**4396–4406
19. Rispoli, G., Menini, A. 1988. The blocking effect of L-*cis*-diltiazem on the light sensitive current of isolated rods of the tiger salamander. *Eur. Biophys. J.* **16:**65–71
20. Stern, J.K. Kaupp, U.B., MacLeish, P.R. 1986. Control of the light regulated current in rod photoreceptors by cyclic GMP, calcium and L-*cis*-diltiazem. *Proc. Natl. Acad. Sci. USA* **83:**1163–1167

Received 26 July 1990; revised 15 February 1991