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CYCLIC GMP AND THE PERMEABILITY OF THE DISKS OF THE FROG PHOTORECEPTORS

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SUMMARY

1. The diffusion of sodium, potassium and rubidium (not chloride) ions across the disk membrane is increased by cyclic guanosine monophosphate (cyclic GMP).

2. The increase is greater for sodium than for rubidium in the 0·01–0·1 mM concentration range.

3. Cyclic adenosine monophosphate (cyclic AMP) is less efficient than cyclic GMP; GMP and guanosine triphosphate are without effect.

4. The effect is present with either 1·8 mM calcium ions or 4 mM-EGTA in the perfusion fluid.

5. The presence of the cyclic GMP phosphodiesterase on the disk membranes is not needed for this effect.

6. The effect is present in both unbleached and fully bleached membranes.

INTRODUCTION

Two observations prompted this study. First, the isolated rod outer segments of the photoreceptors of the frog (*Rana catesbeiana*) have a high intracellular concentration of guanosine 3′, 5′-cyclic monophosphate (cyclic GMP) in darkness (Woodruff, Bownds, Green, Morrisey & Shedlovsky, 1977) and the disks of the rods have an enzyme that hydrolyses cyclic GMP in the light (Miki, Keirns, Marcus, Freeman & Bitensky, 1973; Chader, Herz & Fletcher, 1974; Goridis & Virmaux, 1974; Krishna, Krishnan, Fletcher & Chader, 1976; Bignetti, Cavaggioni & Sorbi, 1978). Secondly, no change of permeability of the visual membranes to light has been detected in broken photoreceptors, possibly because some essential cytoplasmic factor is lost in this condition (Sorbi & Cavaggioni, 1975). In this work the possibility is considered that cyclic GMP is one such factor and in particular that cyclic GMP increases the permeability of the visual membranes.

METHODS

The preparation. The experiments were performed in red light (Kodak safety light 1). The frogs (*Rana catesbeiana*) were fully adapted to darkness for a few hours before the experiments. The rods were isolated free of pigment epithelium, counted on a Bürker chamber and sedimented as previously described (Caretta & Cavaggioni, 1976); the pellet was resuspended in 1 ml.

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hypotonic KCl solution or mannitol solution (Table 1, solutions A and B), and the rods broken to disks or to stacks of disks by forcing the suspension through a 22-gauge syringe needle fifteen times. The suspension was sedimented (8000 *g*, 15 min) in a 1 ml. tube with conical bottom and the supernatant discarded. The pellet was resuspended in 20–40 μl. of one of the following radioactive solutions (Amersham): (i) isotonic (150 mM) ^{22}NaCl solution (100 μCi/ml.), (ii) ^{86}RbCl (0·1–1·0 mCi/mg Rb), ^{86}Rb being a good substitute of ^{42}K in the rods (Cavaggioni, Sorbi & Turini, 1973); this was diluted in isotonic KCl (Table 1, solution C) to an activity of 100 μCi/ml., (iii) isotonic (150 mM) ^{42}KCl (100 μCi/ml.) in a few experiments, and (iv) 300 mM H^{36}Cl (43 μCi/ml.) neutralized with KOH and Tris. The suspensions were loaded in darkness for 2 hr (in some cases also overnight) at room temperature (22–24 °C). After loading, aliquots of the preparation corresponding to about 4×10^5 rod outer segments (0·1 mg rhodopsin) were successively taken for the perfusions; each aliquot was resuspended in 0·5 ml. isotonic KCl solution and injected into a small cellulose column (80 μl. volume) resting over a Millipore filter (3 μm average pore diameter) and kept in a dark box.

The perfusion. The disks were perfused with the isotonic KCl solution (with Tris-HCl buffer added to equalize the concentration in the test solution) driven by a peristaltic pump. The initial 5–8 ml. were discarded and then samples of ten drops (420 μl.) were collected every 12–18 sec with an automatic drop collector into scintillation vials. Without modifying the flow the test solution replaced the KCl solution in the perfusion gradually in *ca.* 40 sec (about three samples) as judged from the appearance of the test substance in the effluent. The test solutions were prepared by adding a concentrated solution of the nucleotide to the KCl solution. The concentrated solution contained 20 m-mole/l of the nucleotide (determined spectrophotometrically) in 175 mM-Tris-HCl buffer solution (pH 7·4). The local concentration of cyclic GMP effectively in contact with the disks was not defined in the present conditions because of the spontaneous activity of the disk phospodiesterase that hydrolysed about 5 μmole cyclic GMP/min per mg of rhodopsin in darkness when rods broken in KCl were used; this hydrolysis decreased considerably the effective concentration and obscured the significance of the results when the concentration of cyclic GMP in the perfusion was less than 1 mM. At similar concentrations rods broken in mannitol were preferred because the phosphodiesterase was nearly absent and the spontaneous hydrolysis was thus minimized (Bignetti *et al.* 1978).

Loading experiments. The mannitol-washed disks of two frogs have been used for each experiment. The disks in 2·5 ml. KCl solution were mixed with 125 μl. concentrated solution of cyclic GMP and 60 μl. isotonic ^{22}NaCl solution (100 μCi/ml.) at time zero. Successively, 0·5 ml. samples were filtered rapidly under vacuum on fibreglass prefilters and immediately washed twice with 5 ml. cold KCl solution. Controls were made without cyclic GMP and a blank was prepared without disks. The prefilters were soaked overnight in 1 ml. 3% Triton X-100 solution.

The assay of radioactivity. For the perfusion samples, 3 ml. scintillation solution (Lumagel) was added to each collection vial and to a vial with the cellulose column taken at the end of the perfusion. For the loading experiments, the prefilters were added with 7 ml. of the scintillation solution and counted a few days later when the prefilters had become clear. The radioactivity was counted with a liquid scintillation counter with optimal windows and gain; the precision was 2% unless otherwise stated. In some experiments double-labelling was made with ^{86}Rb or ^{42}K and ^{22}Na or ^{36}Cl and the radioactivities separated on the basis of the different decays.

RESULTS

Results with ^{22}Na

Cyclic GMP increased the efflux of ^{22}Na from the loaded disks into the perfusion fluid. With 1 mM-cyclic-GMP the increase, measured by taking the ratio of the peak to the base-line efflux, was 4·7 ± 2·8 (mean ± S.D. of sixteen perfusions) in KCl-washed disks (Fig. 1). Cyclic GMP also increased the rate of loading of the disks placed in a radioactive solution of ^{22}Na (Fig. 2) in two experiments.

The effect of 1 mM-cyclic GMP did not depend on the ionic composition of the

perfusing solution, which contained either KCl or NaCl (Table 1, solutions C and D), nor on the presence of 1·8 mM-Ca^{2+} or 4 mM-EGTA (Table 1, solutions E and F; Fig. 3). The effect was present in disks washed either in KCl or in mannitol solution, i.e. in disks with or without the cyclic GMP phosphodiesterase, and in fully bleached disks as well as in disks kept in darkness (Table 2, KCl and Mannitol, and Dark and Bleached, respectively). We failed to modulate sizeably with light (0·40 % bleaching) the efflux from disks with the enzyme.

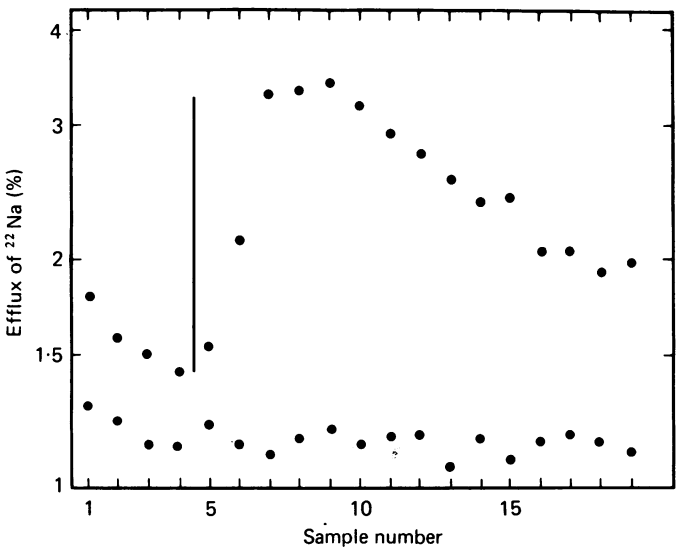

Fig. 1. Per cent efflux of ^{22}Na. Upper trace: test perfusion; lower trace: control perfusion of the same preparation. 1 mM-cyclic GMP and 0·1 mM-GTP were introduced with the test perfusion (vertical bar). One sample every 17 sec.

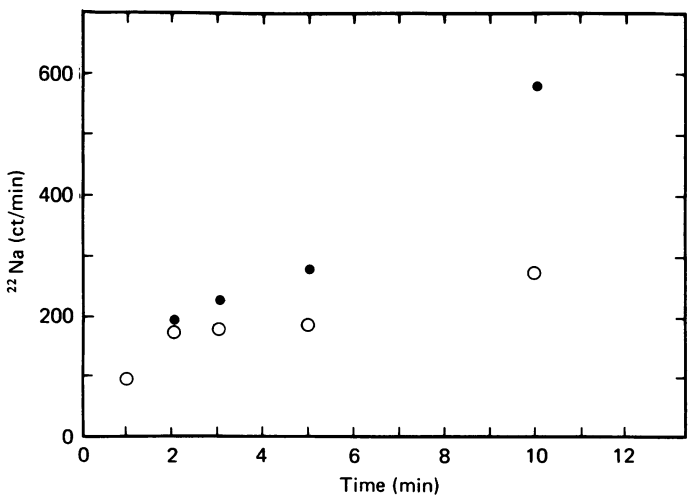

Fig. 2. Loading of ^{22}Na into the disks. ●, with 1 mM-cyclic GMP; ○, without cyclic GMP.

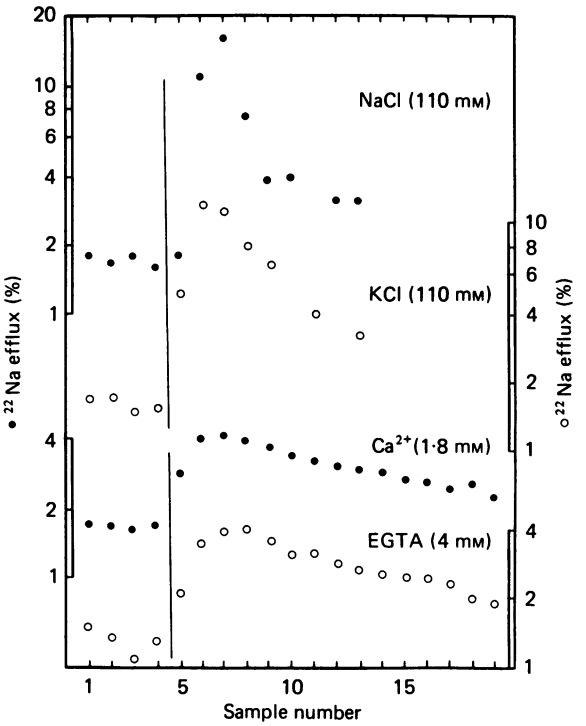

Fig. 3. Invariance of the effect in perfusions of different composition. From above: ●, perfusion with NaCl (Table 1, solution D), and ○, perfusion with KCl (Table 1, solution C), same preparation; ●, perfusion with 1·8 mM Ca^{2+}, and ○, perfusion with 4 mM-EGTA, same preparation (Table 1, solutions E and F). 1 mM-cyclic GMP and 0·1 mM-GTP after the vertical bar. Two upper traces from counts with 5% precision. One sample every 17 sec.

TABLE 1. Composition of the solutions (mM)

	A	B	C	D	E	F
KCl	55	—	110	—	110	110
NaCl	—	—	—	110	—	—
Mannitol	—	220	—	—	—	—
Tris-HCl*	4·3	0·9	8·7	8·7	8·7	8·7
$MgCl_2$	1·1	—	2·3	2·3	2·3	2·3
$CaCl_2$	—	—	—	—	1·8	—
EGTA†	—	—	—	—	—	4
pH	7·4	7·4	7·4	7·4	7·4	7·4

* Tris(hydroxymethyl)-amino-methane.
† Ethylene glycol *bis*(β-aminoethyl ether) N, N, N′-tetra-acetic acid.

TABLE 2. Ratio of the per cent efflux of ^{22}Na (or ^{42}K) after to before the nucleotide in paired experiments

Expt.	KCl	Mannitol	Dark	Bleached	Na	K	cyclic GMP	cyclic GMP
1	4·00	2·50	1·85	2·00	2·8	2·8	2·68	1·42
2	1·85	1·75	3·60	3·50	2·5	3·2	3·18	1·78
3	2·75	3·80	—	—	—	—	2·25	1·70
Mean	2·87	2·68	2·73	2·75	2·7	3·0	2·70	1·63
S.D.	1·08	1·04	1·23	1·06	0·2	0·3	0·47	0·19

Ionic specificity

^{22}Na, ^{86}Rb and in a few experiments ^{42}K and ^{36}Cl have been considered. In experiments with double labelling an effect of about the same size was obtained with either ^{22}Na or ^{86}Rb (increase ratio 1·8 and 2·3 respectively in one perfusion), or with ^{22}Na and ^{42}K (Table 2, Na and K) in KCl- washed disks and with 1 mM-cyclic-GMP in the

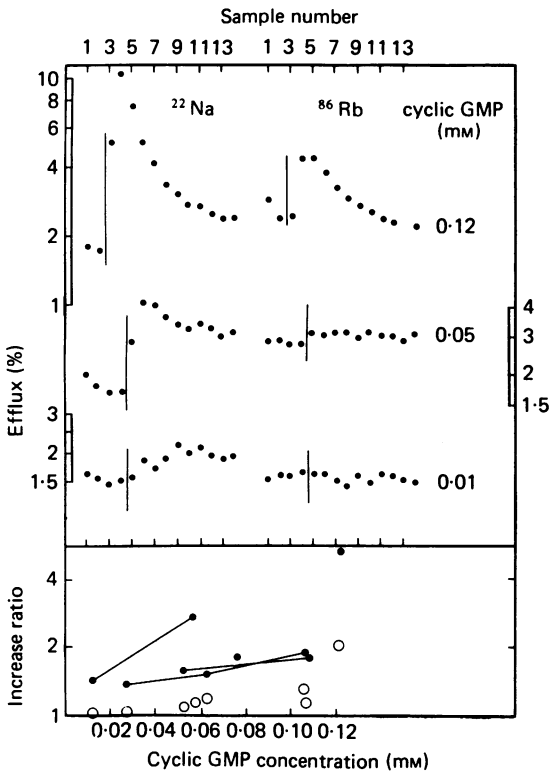

Fig. 4.

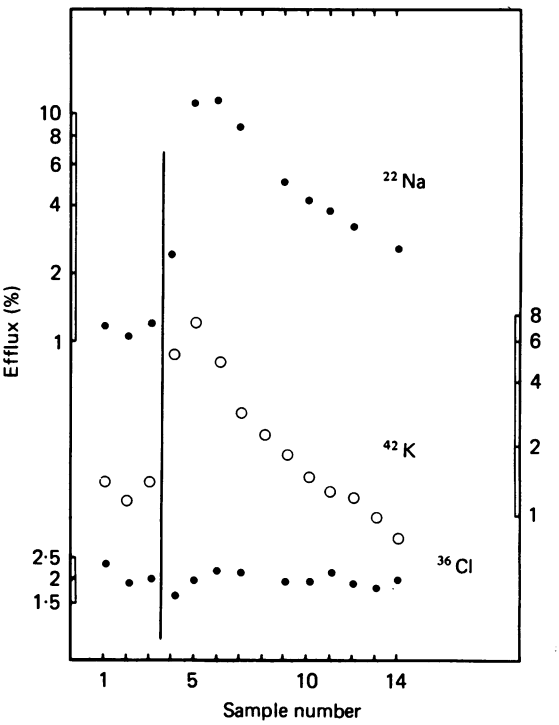

Fig. 5.

Fig. 4. Effect of different concentrations of cyclic GMP. On the left: efflux of ^{22}Na; on the right: efflux of ^{86}Rb. From above: 0·12, 0·05, and 0·01 mM cyclic GMP. Mannitol-washed discs. Two upper traces are two perfusions from the same preparation, four lower traces are from another preparation. Counts with 3 % precision. One sample every 12 sec. The inset at the bottom shows the relationship between the concentration of cyclic GMP and the increase ratio of the efflux for ^{22}Na (●) and ^{86}Rb (○) obtained in pairs of perfusions. The points for ^{22}Na from the same preparations are connected. Loading in 150 mM-NaCl with ^{22}Na or ^{86}Rb (1 mM-Rb).

Fig. 5. Effect of cyclic GMP on ^{22}Na, ^{42}K and ^{36}Cl efflux. From above: efflux of ^{22}Na, ^{42}K and ^{36}Cl. 1 mM-cyclic GMP and 0·1 mM-GTP. Three perfusions of the same preparation. One sample every 17 sec.

perfusion solution. When 0·1 mM-cyclic GMP was used to explore a more physiological concentration, the results were irregular probably because the nucleotide was spontaneously hydrolysed by the disks to a different extent in different preparations. For this reason the 0·12–0·01 mM concentration range has been studied with mannitol-washed discs in which hydrolysis is nearly absent. In this range the effect

was proportional to the concentration of cyclic GMP, although there were considerable differences of amplitude in different preparations. In pairs of experiments with the same preparation the effect was always greater with ^{22}Na than with ^{86}Rb (Fig. 4). The efflux of ^{36}Cl was not increased by 1 mM-cyclic GMP (Fig. 5); three experiments with double labelling, ^{86}Rb or ^{42}K and ^{36}Cl, showed that although the

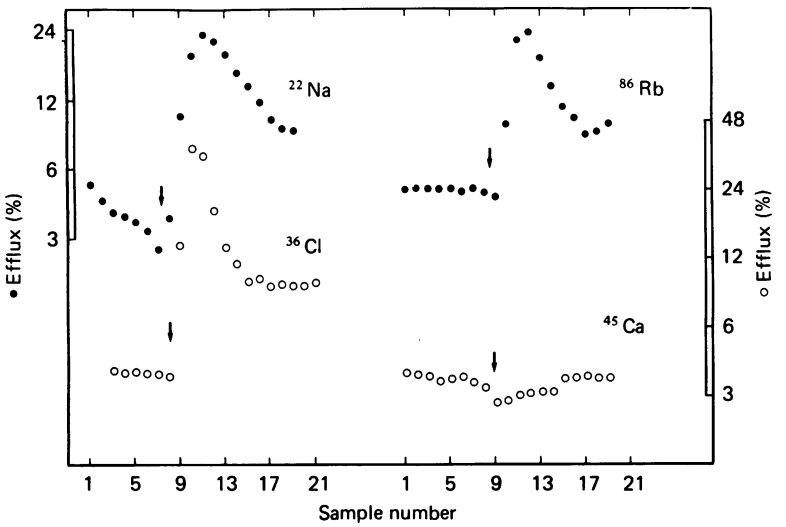

Fig. 6. Effect of PIMB. From left to right: upper traces, efflux of ^{22}Na and ^{36}Rb; bottom traces, efflux of ^{36}Cl and ^{45}Ca. 200 μl. 1·5 mM-PIMB (pH 7·4) are injected in the perfusion (arrow). One sample every 22 sec.

cation efflux was increased by 1 mM-cyclic GMP, the efflux of ^{36}Cl from the same disks was not increased. On the other hand a release of ^{36}Cl could be induced from the disks by means of unspecific permeabilizing agents, the mercurials, e.g. the *p*-hydroxy mercuribenzoic acid (PIMB) or the *p*-hydroxymercuribenzene sulphonic acid (PIMBS), thus indicating that ^{36}Cl had diffused to some extent into the disks during the loading period (Fig. 6). The efflux of ^{45}Ca has not been considered since the PIMB test was negative and there was no evidence of sizeable intradisk accumulation (Fig. 6).

Nucleotide specificity

Nucleotides others than cyclic GMP have been tested and the effects on the efflux of ^{22}Na have been compared. In the same preparation 1 mM-cyclic AMP was less efficient than cyclic GMP at the same concentration (Table 2, cyclic GMP and cyclic AMP), and 0·1 mM-cyclic AMP did not give a sizeable effect (two experiments). With 1 mM-GMP or 1 mM-guanosine only a very slow increase was obtained, whereas 1 mM-GTP was without effect (Fig. 7) and 0·1 mM-GTP in addition to 1 mM-cyclic GMP did not modify the effect.

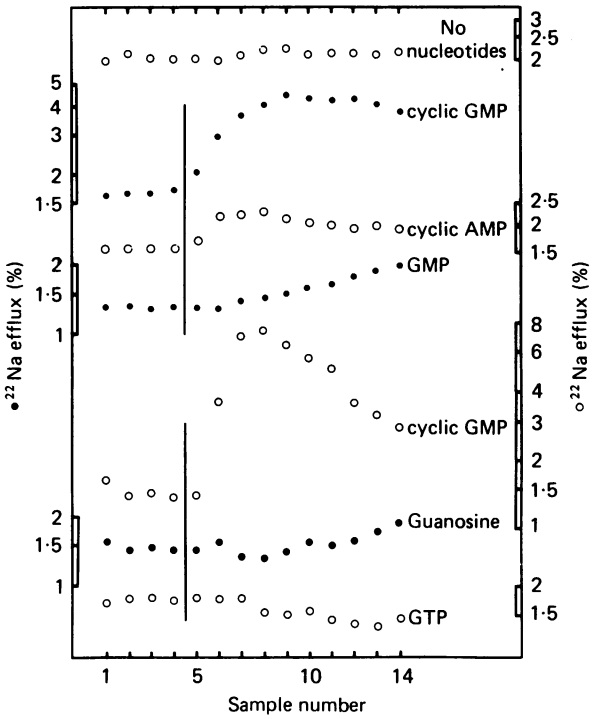

Fig. 7. Nucleotide specificity. From above: control without nucleotide, 1 mM-cyclic GMP, 1 mM-cyclic AMP, 1 mM-GMP, same preparation; 1 mM-cyclic GMP, 1 mM-guanosine, 1 mM-GTP, another preparation.

DISCUSSION

A greater diffusion of cations and in particular sodium ions across the disk membranes is observed in the presence of cyclic GMP. The fact that both the efflux and influx of sodium are increased by cyclic GMP excludes the possibility that the effect is due to a change of the electrical potential across the disk membranes, or to a different affinity of the membranes to superficially bound sodium, and indicates that a permeability change is the cause. The ionic specificity shows that this effect is not a generic increase of the membrane permeability to cations and anions of the kind observed in the disks with strong illuminations (Sorbi & Cavaggioni, 1975). In addition the nucleotide specificity is not a function of the hydrophobic character of the molecule, cyclic GMP being intermediate in this aspect between guanosine and GMP which have been shown to be two ineffective molecules; it can thus be ruled outthat the cyclic nucleotides interfered with the lipid bilayer of the membrane in an unspecific way. The greater specificity of cyclic GMP compared to cyclic AMP adds substance to the notion that the guanosine phosphates are involved in the biochemical aspects of photoreceptor physiology (Wheeler & Bitensky, 1977; Bitensky, Wheeler, Aloni, Vetury & Matuo, 1978). Cyclic GMP between 0·01 and 0·1 mM is sufficient to increase the sodium permeability, i.e. the effect is present with cyclic GMP in the range of the intracellular concentration of *ca.* 0·04 M measured in the isolated frog *in darkness* (Woodruff *et al.* 1977). Interestingly, on the basis of osmotic

measurements a correlation has been postulated between photically or pharmacologically induced changes of cyclic GMP within the rods and the sodium permeability of the rod cell membrane (Bownds & Brodie, 1975; Brodie & Bownds, 1976). On the other hand the rubidium permeability is scarcely affected by cyclic AMP in this range of concentration, and the same is likely for the potassium permeability. The simplest inference from the present observations *in vitro* to the *in vivo* physiology is that the intracellular concentration of cyclic GMP may contribute to keeping high the sodium permeability of the disks; it will require further experiments to know whether this applies also to the cell membrane. Our inability to modulate the efflux with light by activating the hydrolysis of cyclic GMP may be due to the high spontaneous activity (probably artifactual) of this enzyme in the present preparation and does not exclude a physiological modulation in the cell.

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