

# Na<sup>+</sup>- and cGMP-induced Ca<sup>2+</sup> Fluxes in Frog Rod Photoreceptors

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**ABSTRACT** We have examined the Ca<sup>2+</sup> content and pathways of Ca<sup>2+</sup> transport in frog rod outer segments using the Ca<sup>2+</sup>-indicating dye arsenazo III. The experiments employed suspensions of outer segments of truncated, but physiologically functional, frog rods (OS-IS), intact isolated outer segments (intact OS), and leaky outer segments (leaky OS with a plasma membrane leaky to small solutes, but with sealed disk membranes). We observed the following. (a) Intact OS or OS-IS isolated and purified in Percoll-Ringer's solution contained an average of 2.2 mM total Ca<sup>2+</sup>, while leaky OS contained 2.0 mM total Ca<sup>2+</sup>. This suggests that most of the Ca<sup>2+</sup> in OS-IS is contained inside OS disks. (b) Phosphodiesterase inhibitors increased the Ca<sup>2+</sup> content to ~4.2 mM in intact OS or OS-IS, whereas the Ca<sup>2+</sup> content of leaky OS was not altered. (c) Na-Ca exchange was the dominant pathway for Ca<sup>2+</sup> efflux in both intact and leaky OS/OS-IS. The rate of Na-Ca exchange in intact OS/OS-IS was half-maximal between 30 and 50 mM Na<sup>+</sup>; at 50 mM Na<sup>+</sup>, this amounted to  $5.8 \times 10^7$  Ca<sup>2+</sup>/OS·s or 0.05 mM total Ca<sup>2+</sup>/s. This is much larger than the Ca<sup>2+</sup> component of the dark current. (d) Other alkali cations could not replace Na<sup>+</sup> in Na-Ca exchange in either OS-IS or leaky OS. They inhibited the rate of Na-Ca exchange (K ≥ Rb > Cs ≥ Li > TMA) and, as the inhibition became greater, a delay developed in the onset of Na-Ca exchange. The inhibition of Na-Ca exchange by alkali cations correlates with the prolonged duration of the photoresponse induced by these cations (Hodgkin, A. L., P. A. McNaughton, and B. J. Nunn. 1985. *Journal of Physiology*. 358:447-468). (e) In addition to Na-Ca exchange, disk membranes in leaky OS showed a second pathway of Ca<sup>2+</sup> transport activated by cyclic GMP (cGMP). The cGMP-activated pathway required the presence of alkali cations and had a maximal rate of  $9.7 \times 10^6$  Ca<sup>2+</sup>/OS·s. cGMP caused the release of only 30% of the total Ca<sup>2+</sup> from leaky OS. The rate of Na-Ca exchange in leaky OS amounted to  $1.9 \times 10^7$  Ca<sup>2+</sup>/OS·s. (f) Even though the ratio of plasma to disk membrane surface area in frog OS is ~1/100, the measured Ca<sup>2+</sup> flux across the plasma membrane would be expected to change the cytoplasmic Ca<sup>2+</sup> levels three times faster than Ca<sup>2+</sup> fluxes across the disk membrane.

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## INTRODUCTION

Recent observations on the effects of  $\text{Ca}^{2+}$  and cyclic GMP (cGMP) on the light-sensitive conductance in vertebrate rod photoreceptors have caused a dramatic shift in the thinking about the role of  $\text{Ca}^{2+}$  in visual transduction in vertebrate rod photoreceptors, from that of an excitatory messenger to a more indirect regulatory role (Fesenko et al., 1985; Matthews et al., 1985; Cobbs and Pugh, 1985; Yau and Nakatani, 1985*b*; Nicol et al., 1987). Changes in the extracellular  $\text{Ca}^{2+}$  concentration have strong effects on a number of important aspects of rod physiology and rod biochemistry, such as the magnitude of the dark current flowing into the outer segment (Hodgkin et al., 1984), the light sensitivity of rods (Bastian and Fain, 1982), and the rod cGMP content (Cohen et al., 1978; Woodruff and Fain, 1982; Cote et al., 1984). Indirect evidence suggests that changes in intracellular  $\text{Ca}^{2+}$  may influence the waveshape of the photoresponse, particularly its duration and falling phase (Hodgkin et al., 1985; Matthews et al., 1985). Some of the above effects are probably caused by changes in intracellular  $\text{Ca}^{2+}$ . To understand the involvement of  $\text{Ca}^{2+}$ , it is important to know how much  $\text{Ca}^{2+}$  is present in rods, where it is stored, and how fast and by what mechanism intracellular  $\text{Ca}^{2+}$  levels can change under experimental conditions that give rise to the above phenomena.

Reports on the "in situ"  $\text{Ca}^{2+}$  content of the rod outer segment in the intact retina yield strikingly different values (Hagins and Yosikami, 1975; Schröder and Fain, 1984; Fain and Schröder, 1985; Somlyo and Walz, 1985). Under normal conditions, internal  $\text{Ca}^{2+}$  is thought to be localized largely inside disks (Fain and Schröder, 1985), but after pretreatment of rods with a low- $\text{Ca}^{2+}$  Ringer's, large and sustained  $\text{Ca}^{2+}$  currents are observed that may load the cytoplasm with  $\text{Ca}^{2+}$  (Yau and Nakatani, 1984; Hodgkin et al., 1985). Two mechanisms have been described for rapid  $\text{Ca}^{2+}$  transport across the plasma membrane of the rod outer segment:  $\text{Ca}^{2+}$  currents through the light-sensitive conductance (Capovilla et al., 1983; Yau and Nakatani, 1984; Hodgkin et al., 1985) and Na-Ca exchange (Schnetkamp, 1980, 1981, 1986; Yau and Nakatani, 1984).  $\text{Ca}^{2+}$  fluxes were inferred from measurements of the membrane current in amphibian rods (Yau and Nakatani, 1984; Hodgkin et al., 1985) and from direct measurements of  $\text{Ca}^{2+}$  using  $^{45}\text{Ca}$  (Schnetkamp, 1980) or the  $\text{Ca}^{2+}$  indicator arsenazo III (Schnetkamp, 1986) in intact rod outer segments isolated from bovine retinas.

We have used a preparation of isolated frog outer segments with part of the ellipsoid still attached (OS-IS) and intact outer segments (OS) to measure the parameters of  $\text{Ca}^{2+}$  metabolism under conditions that are close to those under which normal physiological responses can be recorded (Biernbaum and Bownds, 1985). We used the  $\text{Ca}^{2+}$ -indicating dye arsenazo III to measure  $\text{Ca}^{2+}$  content and  $\text{Ca}^{2+}$  fluxes of OS in suspensions of OS or OS-IS. We sought to extend the results of electrophysiological observations with direct biochemical measurements of  $\text{Ca}^{2+}$ . We observed that Na-Ca exchange is the predominant mechanism of  $\text{Ca}^{2+}$  removal in isolated OS/OS-IS. Elevating external  $\text{K}^+$  had two effects on  $\text{Na}^+$ -stimulated  $\text{Ca}^{2+}$  from OS: the rate of Na-Ca exchange decreased and a lag

phase developed before Na-Ca exchange started working. The ion selectivity of these effects correlates with an increased duration of the photoresponse in a solution containing high  $K^+$  (Hodgkin et al., 1985). These results agree with the interpretation by these authors that high  $K^+$  slows down the removal of  $Ca^{2+}$  from the cell by Na-Ca exchange, and that this process is rate-limiting for the off phase of the photoresponse.

## MATERIALS AND METHODS

### *Preparations*

Intact OS, leaky OS (rod outer segments with a plasma membrane leaky to small solutes, but with sealed disk membranes), and OS-IS were prepared from isolated bullfrog retinas (*Rana catesbeiana*), essentially as described by Biernbaum and Bownds (1985). The integrity of the rod plasma membrane was assessed by the ability of the fluorescent dye didansylcysteine to gain access to the cytoplasm and stain the disk membranes (Yoshikami et al., 1974). Briefly, isolated retinas were removed from the eyecup and the retinas were transferred to a plastic weigh boat containing 500  $\mu$ l of isolation solution (105 mM NaCl, 2.5 mM KCl, 2 mM  $MgCl_2$ , 1 mM  $CaCl_2$ , 10 mM glucose, 5% Percoll, and 20 mM HEPES, adjusted to pH 7.5 with NaOH). For the experiments shown in Figs. 4–6, 100  $\mu$ M isobutylmethylxanthine (IBMX) was added to the isolation medium; this boosted the  $Ca^{2+}$  content (Table I) and allowed more precise measurements of  $Ca^{2+}$  fluxes in OS/OS-IS without altering their properties. The retinas were stretched gently with a pair of forceps, then gently shaken, and subsequently shredded with a pair of forceps. The tissue fragments were allowed to settle and the supernatant was layered on top of a Percoll linear density gradient in the above Ringer's solution (30–70% Percoll). The gradient was spun for 2 min at 11,000 rpm in a Sorvall SS34 rotor. Three bands were observed: the interface between 5 and 30% Percoll contained retinal debris; one-third down the gradient, a band contained largely leaky OS fragments that stained with didansylcysteine (Yoshikami et al., 1974); two-thirds down the gradient, a band contained a mixture of OS and OS-IS that excluded didansylcysteine, which indicated the presence of an intact plasma membrane. The percentage of OS could be increased by adopting more harsh procedures of shredding and shaking and by omitting the stretching. In some experiments, retinas were isolated and rod fragments were separated on Percoll gradients in an Na-free solution, in which NaCl was replaced by tetramethylammonium (TMA)-Cl (the pH was adjusted with arginine). The Percoll-TMA gradients gave equally satisfactory separations between the different bands, and sometimes allowed separation between intact OS and intact OS-IS. The various bands were taken from the gradient and stored on ice in the high-Percoll solutions until further use. All procedures up to this point were carried out under infrared illumination with the aid of image converters. Subsequent procedures could be carried out under infrared illumination or under dim red illumination without altering the results.

Immediately before use, the particles were diluted with 3 vol of a solution containing 125 mM TMA-Cl and 20 mM HEPES (adjusted to pH 7.4 with arginine). Rod particles were sedimented for 10 s at 5,000 rpm in a Sorvall SS34 rotor (Dupont Co., Wilmington, DE), the supernatant was removed, and the pellets were gently resuspended in a solution containing 125 mM TMA-Cl, 2.5 mM KCl, and 30 mM HEPES (adjusted to pH 7.4 with arginine). In some experiments, 250 mM sucrose replaced TMA-Cl without altering the results. The osmolarity of all the above solutions was adjusted to be between 230 and 240 mosmol.

### *Ca<sup>2+</sup> Measurements*

Ca<sup>2+</sup> measurements were made with the Ca<sup>2+</sup> indicator arsenazo III by dual-wavelength spectroscopy, essentially as described earlier (Schnetkamp and Kaupp, 1985; Schnetkamp, 1986). Spectra were recorded in an Aminco DW2 spectrophotometer (SLM Instruments Inc., Urbana, IL) equipped with a magnetic stirrer. The wavelength pair was 650 and 750 nm with a 5-nm bandwidth. A 100–200- $\mu$ l aliquot of the different OS or OS-IS suspensions was diluted to 2 ml with a medium containing 125 mM of a cation chloride (as indicated), 2.5 mM KCl, 100–200  $\mu$ M arsenazo III, 1  $\mu$ M carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), and 30 mM HEPES (adjusted to pH 7.4 with arginine). The rhodopsin concentration in the suspension was between 1 and 3  $\mu$ M. All experiments were carried out at room temperature (20–22°C). The absorbance difference at 650 and 750 nm ( $A_{650} - A_{750}$ ) was monitored in real time when different additions were made under continuous stirring. NaCl was added from 1- or 2.5-M stock solutions, and 8-bromo-cGMP and cGMP were added from 25-mM stock solutions. The traces were corrected for the instantaneous absorption changes observed upon addition of various electrolytes. These instantaneous absorption changes were primarily due to effects of the electrolytes on the arsenazo III spectrum proper and, to a lesser degree, to dilution and to Ca<sup>2+</sup> contaminants in the electrolytes. The instantaneous absorption changes induced by the addition of 50 mM NaCl (used in most experiments) depended on the electrolyte composition of the suspension medium. The mixing time was 2–3 s, which caused a maximal uncertainty of ~10% in the initial rate and maximal amplitude of Ca<sup>2+</sup> movements (in the case of Na-Ca exchange operating near the maximal rate). All measurements were performed under conditions such that 4–10% of the arsenazo III was complexed with Ca<sup>2+</sup> and there were minimal changes in free Ca<sup>2+</sup>. The free Ca<sup>2+</sup> concentration ranged between 0.5 and 2  $\mu$ M, assuming a dissociation constant of 15  $\mu$ M for the Ca<sup>2+</sup>–arsenazo III complex. The detection limit in our measurements was typically a change of ~0.04  $\mu$ M in total Ca<sup>2+</sup>. The absorption changes were calibrated *in situ* by adding known amounts of Ca<sup>2+</sup>. The validity of these analytical procedures has been demonstrated previously (Scarpa, 1979; Koch and Kaupp, 1985; Schnetkamp and Kaupp, 1985; Schnetkamp, 1986).

The total Ca<sup>2+</sup> content of OS-IS or OS was defined as the amount of Ca<sup>2+</sup> that could be released by the addition of 2  $\mu$ M A23187. The divalent cation ionophore A23187 (Pfeiffer and Lardy, 1976) makes all Ca<sup>2+</sup> in the OS accessible to release into the external medium and, under the conditions used in this study (high ionic strength and low free external Ca<sup>2+</sup>), releases >90% of all internal Ca<sup>2+</sup> from OS (Schnetkamp, 1979; Nicol et al., 1987). The subsequent addition of 0.1% Triton X-100 solubilized OS and caused the release of a further 0.1–0.15 mol Ca<sup>2+</sup>/mol rhodopsin in intact OS/OS-IS, but caused little further release in leaky OS. This Triton-induced Ca<sup>2+</sup> release probably originated from OS, but we cannot exclude some contribution by contaminating particles. The A23187-sensitive portion of the Ca<sup>2+</sup> content does not appear to be compromised by the possible presence of contaminating particles (predominantly pigment granules) since these particles (concentrated in the pellet after gradient centrifugation) do not release Ca<sup>2+</sup> upon addition of either A23187, NaCl, or cGMP (not illustrated). Also, nearly all sequestered Ca<sup>2+</sup> in OS/OS-IS suspensions could be released by Na-Ca exchange (intact OS/OS-IS) or the combination of Na-Ca exchange and the cGMP-dependent Ca<sup>2+</sup> flux (leaky OS), both quite specific transport pathways present in rod OS (see Results). George and Hagins (1983) describe significant Ca<sup>2+</sup> uptake by free mitochondria present in nonpurified crude suspensions of shaken retinas. For this reason, 1  $\mu$ M FCCP (a protonophore and mitochondrial uncoupler) was included in all our experiments. In addition to uncoupling mitochondria, FCCP also electrically shunts OS membranes and prevents a

possible back-pressure effect of electrogenic Na-Ca exchange (Schnetkamp, 1986). We tested the effect of addition of FCCP to suspensions of intact and leaky OS/OS-IS, and never observed any  $\text{Ca}^{2+}$  release. This illustrates that free mitochondria did not contribute to our  $\text{Ca}^{2+}$  measurements and that, under physiological conditions, mitochondria did not contain significant amounts of  $\text{Ca}^{2+}$ , in agreement with in situ measurements on single rod cells (Fain and Schröder, 1985; Somlyo and Walz, 1985).

At the end of each experiment, the integrity of the plasma membrane was assayed with didansylcysteine (Yoshikami et al., 1974), and <10% of intact OS or OS-IS had become leaky. It should be noted that this is in marked contrast to experiments that do not employ Percoll. The stability of intact OS or OS-IS purified on Percoll gradients is enhanced markedly as compared with those not exposed to Percoll.

## RESULTS

### *Frog OS/OS-IS Isolated in Normal Ringer's Contain ~2.2 mM Total $\text{Ca}^{2+}$ Contained Predominantly Inside Disks*

When intact OS/OS-IS were isolated and purified in normal Ringer's solution (including 1 mM  $\text{CaCl}_2$ ), they contained ~0.9 mol  $\text{Ca}^{2+}$ /mol rhodopsin as judged from the  $\text{Ca}^{2+}$  released from OS/OS-IS upon addition of the  $\text{Ca}^{2+}$  ionophore A23187 (Table I). An additional 0.1–0.15 mol  $\text{Ca}^{2+}$  rhodopsin could be released in intact OS/OS-IS by adding Triton X-100, and part or all of this  $\text{Ca}^{2+}$  may be located within OS (in the remainder of this study, the ionophore-sensitive portion of the  $\text{Ca}^{2+}$  content will be reported). The overall rhodopsin concentration in frog OS amounts to 2.5 mM (Liebman and Entine, 1968), and thus OS contain ~2.2–2.5 mM total  $\text{Ca}^{2+}$ . This value is similar to that observed by Nicol et al. (1987), who used the same photoreceptor preparation. Little difference was obvious between preparations of various OS/OS-IS contents, which is consistent with the data of Fain and Schröder (1985) and Nicol et al. Most of the  $\text{Ca}^{2+}$  appeared to be located inside OS disks, since OS with a plasma membrane leaky to didansylcysteine (largely fragmented OS and very few OS-IS) contained as much  $\text{Ca}^{2+}$  as intact OS or OS-IS (Table I). These results are in agreement with the in situ localization of Ca in toad retinas (Schröder and Fain, 1984; Fain and Schröder, 1985). These authors conclude that the majority of Ca (4–5 mM total Ca) is located within rod OS disks and very little Ca is contained in mitochondria located in the ellipsoid. We did not observe any noticeable  $\text{Ca}^{2+}$  release when the mitochondrial uncoupler FCCP was added to a suspension of OS-IS; FCCP causes a nearly complete release of  $\text{Ca}^{2+}$  stored in mitochondria (see Materials and Methods).

### *Phosphodiesterase Inhibitors Double the $\text{Ca}^{2+}$ Content of Intact OS/OS-IS, But Do Not Change That of Leaky OS*

When either of the phosphodiesterase inhibitors IBMX or 2-*o*-propoxyphenyl-8-azapurin-6-one (Popap) was added to the Ringer's solution during isolation and purification, the  $\text{Ca}^{2+}$  content of intact OS or OS-IS, but not that of leaky OS, was significantly increased (Table I). This could be caused by (a) increased  $\text{Ca}^{2+}$  loading in the cytoplasm (free and bound), or (b) increased loading of the disks, caused by an increase in the free cytoplasmic  $\text{Ca}^{2+}$  concentration and/or an increase in cGMP-dependent  $\text{Ca}^{2+}$  loading of the disks.

An increase in cytoplasmic  $\text{Ca}^{2+}$  levels induced by phosphodiesterase inhibitors could be the result of an increased  $\text{Ca}^{2+}$  current through the light-sensitive conductance (e.g., Capovilla et al., 1983). This could disturb a pre-existing equilibrium of a  $\text{Ca}^{2+}$  influx through the light-sensitive conductance and  $\text{Ca}^{2+}$  removal via Na-Ca exchange (as proposed by Yau and Nakatani, 1985a). Alternatively,  $\text{Ca}^{2+}$  homeostasis in rods could be dominated by Na-Ca exchange; an increased  $\text{Na}^+$  current through the light-sensitive conductance could result in a decrease of the electrochemical  $\text{Na}^+$  gradient. This in turn would cause an increase in the free cytoplasmic  $\text{Ca}^{2+}$  concentration owing to the altered equilibrium conditions of Na-Ca exchange. It is difficult to distinguish between these two models since an increase in the dark current is probably always accompanied by a decrease in the electrochemical  $\text{Na}^+$  gradient. Although it is reasonable to interpret the effect of phosphodiesterase inhibitors as being due to an increase in

TABLE I  
*Ca<sup>2+</sup> Content of Isolated Frog OS/OS-IS*

Isolation conditions	Intact	Leaky
Ringer + 1 mM $\text{CaCl}_2$	0.87±0.16 (7)	0.81±0.18 (11)
	2.2 mM	2.0 mM
Ringer + 1 mM $\text{CaCl}_2$ + 100 $\mu\text{M}$ IBMX	1.68±0.32 (12)	0.83±0.19 (11)
	4.2 mM	2.1 mM
Ringer + 1 mM $\text{CaCl}_2$ + 0.01% Popap	1.68±0.26 (5)	0.89±0.25 (5)
	4.2 mM	2.2 mM

The total  $\text{Ca}^{2+}$  content of isolated frog OS/OS-IS purified on Percoll gradients was determined by addition of the  $\text{Ca}^{2+}$  ionophore A23187 as described in the Materials and Methods and discussed in the text. The data are expressed as moles of  $\text{Ca}^{2+}$  per moles of rhodopsin, and can be converted to millimolar total  $\text{Ca}^{2+}$  (second row) with the use of an overall rhodopsin concentration of 2.5 mM in OS (Liebman and Entine, 1968). The data are the pooled results of preparations with a variable ratio of OS/OS-IS (range, <10% OS-IS to >80% OS-IS). When phosphodiesterase inhibitors were present, there appeared to be a slight trend toward higher values for the  $\text{Ca}^{2+}$  content in OS-rich preparations (1.9 mol Ca/mol rhodopsin) as compared with preparations rich in OS-IS (1.4 mol Ca/mol rhodopsin).

rod cGMP levels, some caution is warranted. While 100  $\mu\text{M}$  IBMX has strong effects on the physiology of rods (Capovilla et al., 1983) and on the OS  $\text{Ca}^{2+}$  content (Table I), similar IBMX concentrations induce only small changes in rod cGMP levels (Cote, R. H., unpublished observations).

#### *Ca<sup>2+</sup> Fluxes in Intact and Leaky OS/OS-IS*

Proposals for the role of  $\text{Ca}^{2+}$  in rods, for example in light adaptation, critically depend on the ability of the cell to change the internal  $\text{Ca}^{2+}$  concentration on a time scale of seconds. Therefore, we investigated the pathways in both the plasma and disk membrane that may contribute to rapid changes in  $\text{Ca}^{2+}$ . When isolated intact or leaky OS/OS-IS were transferred to a TMA-based solution (free  $\text{Ca}^{2+}$  concentration, <0.3  $\mu\text{M}$ ), they lost (or in a few cases gained) internal  $\text{Ca}^{2+}$  at a slow to negligible rate, as indicated by the  $\text{Ca}^{2+}$ -indicating dye arsenazo III in

the external medium (Figs. 1–6). The slow loss of  $\text{Ca}^{2+}$  in a TMA-based solution probably reflected nonspecific leakage, and was in most cases faster in leaky OS than in intact OS. We observed two specific pathways for  $\text{Ca}^{2+}$  transport across OS membranes, and these results were not altered by the different isolation and purification media used in this study. Neither phosphodiesterase inhibitor tested had a direct effect on the  $\text{Ca}^{2+}$  transporters observed, i.e., Na-Ca exchange and a cGMP-induced pathway (see below). The different incubation media used in this study could give rise to different values for the membrane voltage. For this reason, all experiments reported in this study were carried out in the presence of the electrogenic proton carrier FCCP (1  $\mu\text{M}$ ), which should prevent major changes of the membrane voltage and should also prevent any back-pressure effect (see Materials and Methods).

*Na-Ca exchange in intact and leaky OS/OS-IS.* Independently of the isolation history of OS (i.e., with or without phosphodiesterase inhibitor, purified on TMA as opposed to Na Ringer's gradients), the addition of external  $\text{Na}^+$  invariably resulted in a rapid release of most internal  $\text{Ca}^{2+}$ . This was observed in both intact and leaky preparations and is illustrated in Fig. 1 for OS/OS-IS isolated in standard Ringer's solution. The initial rates of  $\text{Na}^+$ -stimulated  $\text{Ca}^{2+}$  release were  $3.8 \times 10^7$  and  $1.0 \times 10^7$   $\text{Ca}^{2+}/\text{OS}\cdot\text{s}$ , respectively, for intact and leaky OS. No difference was observed between intact preparations of varying OS-IS content (ranging between <10 and 80% OS-IS). The addition of  $\text{Na}^+$  resulted within 3 min in the release of 70–80% of internal  $\text{Ca}^{2+}$  in intact OS or OS-IS.  $\text{Ca}^{2+}$  release was observed only when  $\text{Na}^+$ , as opposed to any other alkali cation, was added;  $\text{Na}^+$ -induced  $\text{Ca}^{2+}$  release indicates the operation of Na-Ca exchange. A very similar Na-Ca exchange has been described for bovine OS (Schnetkamp, 1980, 1981, 1986; see also Table III).

*cGMP-dependent  $\text{Ca}^{2+}$  flux in leaky OS.* In leaky OS, an additional  $\text{Ca}^{2+}$  transport pathway gated by cGMP or 8-bromo-cGMP was observed (Figs. 1 and 2). This was not seen in intact OS or OS-IS, probably because the added 8-bromo-cGMP did not gain access (on a time scale of seconds) to the gating site located in the cytoplasm (cf. Koch and Kaupp, 1985; Fesenko et al., 1985; Schnetkamp, 1986). The cGMP-dependent  $\text{Ca}^{2+}$  flux was not observed in the TMA medium, but required the presence of alkali cations, as observed in bovine disks (Koch and Kaupp, 1985). This dependence did not arise from the fact that these alkali cations could compensate electrically for an electrogenic  $\text{Ca}^{2+}$  efflux from disks. cGMP-dependent  $\text{Ca}^{2+}$  efflux was not observed in a TMA medium even in the presence of FCCP. The electrogenic proton carrier FCCP plus a  $\text{Ca}^{2+}$  channel (induced by cGMP) should provide transport pathways equivalent to the Ca-H exchanger A23187. A23187 rapidly released all  $\text{Ca}^{2+}$  from disks (Fig. 3). In the experiments documented in this article, we used 8-bromo-cGMP rather than cGMP since the former is not as rapidly hydrolyzed by the OS phosphodiesterase (Zimmerman et al., 1985). At physiological pH, the hydrolysis of each cGMP results in the production of one proton. The resulting acidification can cause  $\text{Ca}^{2+}$  release from pH-sensitive  $\text{Ca}^{2+}$  chelators such as EGTA or arsenazo III (see below). Both cGMP and 8-bromo-cGMP (at 250  $\mu\text{M}$ ) give a maximal and indistinguishable  $\text{Ca}^{2+}$  release (Koch and Kaupp, 1985), which

indicates that, under our assay conditions (no GTP present), the results are not contaminated by pH artifacts.

*Separate  $Ca^{2+}$  pools available to cGMP and  $Na^+$ ?* The combined addition of  $Na^+$  and cGMP caused the release of most  $Ca^{2+}$  in leaky OS, but cGMP alone

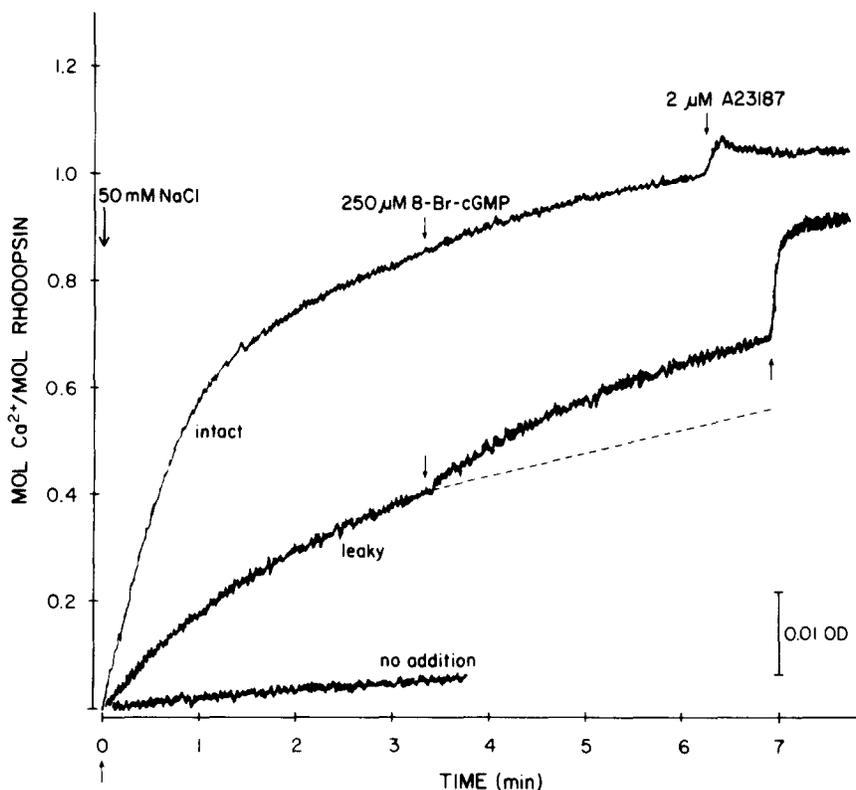


FIGURE 1.  $Na^+$ -stimulated  $Ca^{2+}$  efflux from intact and leaky frog OS/OS-IS. Intact and leaky OS/OS-IS were resuspended in a solution containing 125 mM TMA-Cl, 2.5 mM KCl, 30 mM HEPES, and 13.2 mM arginine (pH 7.4), to which was added after resuspension 7.5 mM KCl (bringing total KCl to 10 mM), 200  $\mu$ M arsenazo III, and 1  $\mu$ M FCCP. KCl was required to optimize the rate of cGMP-induced  $Ca^{2+}$  efflux, whereas KCl inhibited Na-Ca exchange (Fig. 6). As a compromise, a concentration of 10 mM KCl was used.  $Ca^{2+}$  efflux was initiated by the addition of 50 mM NaCl at time zero. At the second arrow, 250  $\mu$ M 8-bromo-cGMP was added; at the third arrow, 2  $\mu$ M A23187 was added. The control trace labeled "no addition" represents the leak of  $Ca^{2+}$  before the addition of NaCl. The traces shown in Fig. 1 and in all other figures were copied by hand from the original recordings.

could release only 30% of total  $Ca^{2+}$ . The  $Na^+$ -dependent  $Ca^{2+}$  pool appeared complementary to the cGMP-dependent  $Ca^{2+}$  pool, as clearly shown in Fig. 2. The cGMP-dependent  $Ca^{2+}$  release was hardly reduced, even if most  $Ca^{2+}$  was released previously by  $Na^+$ . This suggests that there are separate pools of  $Ca^{2+}$  available for the cGMP- and  $Na^+$ -dependent release of  $Ca^{2+}$  from rods.

cGMP-induced  $\text{Ca}^{2+}$  release in leaky OS was optimal in a medium containing high  $\text{K}^+$ , whereas this medium inhibited Na-Ca exchange (Fig. 3A, compare traces 1 and 3). Fig. 3A compares the rate and amount of Na-Ca exchange and cGMP-induced  $\text{Ca}^{2+}$  release under conditions optimal for each process. The amount of cGMP-induced  $\text{Ca}^{2+}$  release was 0.23 mol  $\text{Ca}^{2+}$ /mol rhodopsin (SD = 0.06; four observations), or ~30% of the total  $\text{Ca}^{2+}$  present in leaky OS. The

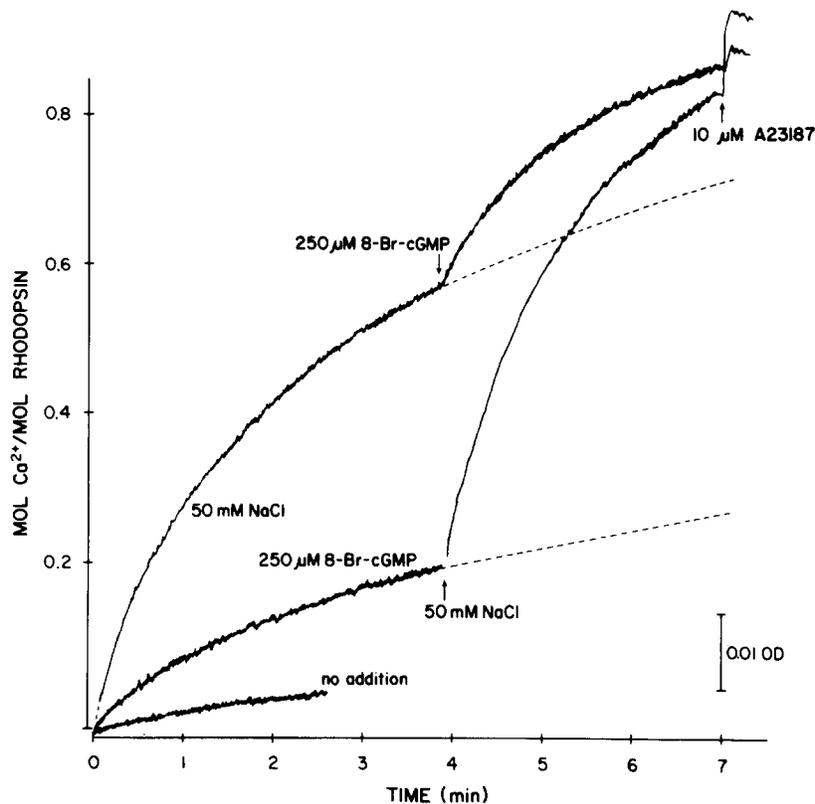


FIGURE 2. cGMP- and  $\text{Na}^+$ -stimulated  $\text{Ca}^{2+}$  efflux in leaky OS. Leaky OS were suspended in a solution containing 250 mM sucrose, 10 mM KCl, 30 mM HEPES, and 13.2 mM arginine (pH 7.4), to which was added after resuspension 200  $\mu\text{M}$  arsenazo III and 1  $\mu\text{M}$  FCCP.  $\text{Ca}^{2+}$  efflux was initiated at time zero by the addition of 250  $\mu\text{M}$  8-bromo-cGMP or 50 mM NaCl as indicated. At the first arrow, 250  $\mu\text{M}$  8-bromo-cGMP and 50 mM NaCl were added as indicated. At the second arrow, 10  $\mu\text{M}$  A23187 was added. The control trace labeled "no addition" represents the leak of  $\text{Ca}^{2+}$  before the addition of 8-bromo-cGMP or NaCl.

initial rates of cGMP- or  $\text{Na}^+$ -induced  $\text{Ca}^{2+}$  release are summarized in Table II. The rates are expressed as either  $\text{Ca}^{2+}$  per outer segment per second or as picomoles per square centimeter per second.

The  $\text{Ca}^{2+}$  pools accessible to cGMP and to  $\text{Na}^+$  were found to have a different sensitivity to hypotonic shocks. A fivefold hypotonic shock reduced the  $\text{Ca}^{2+}$  content of leaky OS from ~0.9 mol  $\text{Ca}^{2+}$ /mol rhodopsin to 0.40 mol  $\text{Ca}^{2+}$ /mol

rhodopsin ( $\pm 0.06$ ; five observations).  $\text{Na}^+$ -induced  $\text{Ca}^{2+}$  efflux was strongly reduced under these conditions (Fig. 3B, trace 1), whereas both the rate (Table II) and amount ( $0.29 \pm 0.04$  mol  $\text{Ca}^{2+}$ /mol rhodopsin or  $\sim 75\%$  of the remaining  $\text{Ca}^{2+}$ ; three observations) of cGMP-induced  $\text{Ca}^{2+}$  release were not affected by the osmotic shock (Fig. 3B, trace 2). This reinforces the above suggestion of separate pools of  $\text{Ca}^{2+}$  accessible to cGMP and  $\text{Na}^+$ .

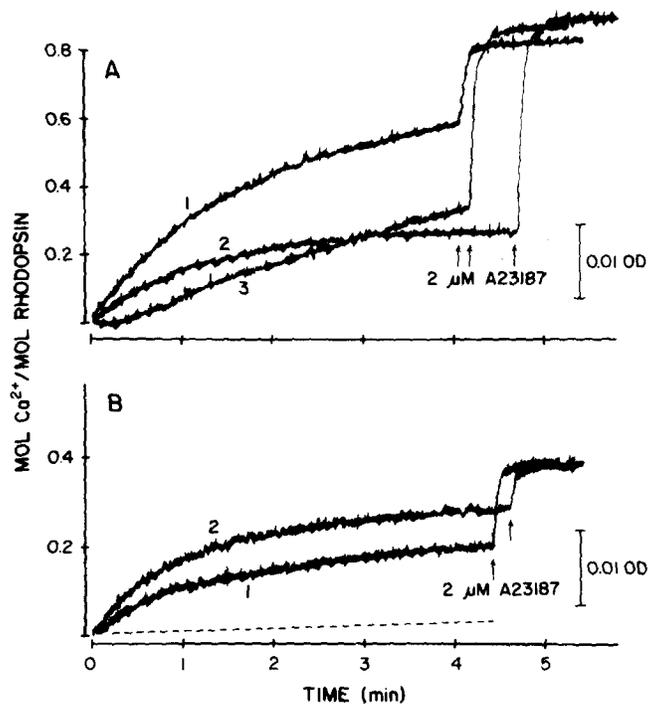


FIGURE 3. Compartmentation of  $\text{Ca}^{2+}$  in leaky OS. Leaky OS were suspended in 125 mM TMA-Cl, 2.5 mM KCl, 30 mM HEPES, and 13.2 mM arginine (pH 7.4). (A)  $\text{K}^+$  inhibits  $\text{Na}^+$ -stimulated  $\text{Ca}^{2+}$  release, but not cGMP-stimulated  $\text{Ca}^{2+}$  release. Leaky OS were diluted 10-fold in the above suspension medium containing in addition 200  $\mu\text{M}$  arsenazo III and 1  $\mu\text{M}$  FCCP (trace 1), or in a medium in which 125 mM KCl replaced TMA-Cl (traces 2 and 3).  $\text{Ca}^{2+}$  efflux was initiated at time zero by the addition of 250  $\mu\text{M}$  8-bromo-cGMP (trace 2) or 50 mM NaCl (traces 1 and 3). At the arrow, 2  $\mu\text{M}$  A23187 was added.  $\text{Ca}^{2+}$  leakage before the addition of NaCl or 8-bromo-cGMP was negligible on the time scale of this experiment. (B) Hypotonic shocks deplete the  $\text{Na}^+$ -sensitive, but not the cGMP-sensitive,  $\text{Ca}^{2+}$  compartment. Leaky OS were hypotonicly shocked by 10-fold dilution into 20 mM HEPES, 8.8 mM arginine, and 200  $\mu\text{M}$  arsenazo III (pH 7.4). After 30 s, either 50 mM TMA-Cl (trace 1) or 50 mM KCl (trace 2) was added.  $\text{Ca}^{2+}$  efflux was initiated at time zero by the addition of 50 mM NaCl (trace 1) or 250  $\mu\text{M}$  8-bromo-cGMP (trace 2). At the arrow, 2  $\mu\text{M}$  A23187 was added.  $\text{Ca}^{2+}$  leakage before the addition of NaCl or 8-bromo-cGMP was negligible on the time scale of this experiment.

TABLE II  
*Ca<sup>2+</sup> Fluxes in Intact and Leaky Frog OS/OS-IS*

Intact OS/OS-IS: Na <sup>+</sup> -stimulated Ca <sup>2+</sup> efflux in different media				
TMA-Cl	LiCl	KCl	RbCl	CsCl
5.8±1.5 × 10 <sup>7</sup> (24)	3.2±0.5 × 10 <sup>7</sup> (4)	1.0±0.15 × 10 <sup>7</sup> (9)	1.2±0.25 × 10 <sup>7</sup> (3)	2.7±0.8 × 10 <sup>7</sup> (4)
Leaky OS: Na <sup>+</sup> - and cGMP-stimulated Ca <sup>2+</sup> efflux				
Na <sup>+</sup> -stimulated Ca <sup>2+</sup> efflux in TMA medium			1.9±0.6 × 10 <sup>7</sup> (12)	
cGMP-stimulated Ca <sup>2+</sup> efflux in KCl medium			10.2±2.8 × 10 <sup>6</sup> (7)	
cGMP-stimulated Ca <sup>2+</sup> efflux after lysis			9.0±2.5 × 10 <sup>6</sup> (6)	

Initial rates of Na<sup>+</sup>- and cGMP-stimulated Ca<sup>2+</sup> efflux from intact OS-IS/OS and leaky OS are tabulated. Na<sup>+</sup>-stimulated Ca<sup>2+</sup> efflux was measured at 50 mM NaCl, and cGMP-induced Ca<sup>2+</sup> efflux was measured at 250 μM 8-bromo-cGMP. The free Ca<sup>2+</sup> concentration in the external medium was between 0.5 and 2 μM. Results are expressed in Ca<sup>2+</sup> per OS per second (± SD; the number of observations is given in parentheses). Frog OS dimensions were taken to be 6.5 × 60 μm and the number of rhodopsin molecules per outer segment was taken to be 3 × 10<sup>9</sup> (2.5 mM total rhodopsin).

#### *Dependence of Na-Ca Exchange on External Na<sup>+</sup> Concentration*

The rate of Na-Ca exchange increased as the Na<sup>+</sup> concentration in the external medium was increased from 0 to 100 mM (Fig. 4). The rates observed in Fig. 4 fit reasonably well with a model in which the binding of two Na ions is rate-limiting for Na-Ca exchange and the dissociation constant for Na<sup>+</sup> is 30 mM, as observed in bovine OS (Schnetkamp, 1986). At 100 mM, the observed rate is

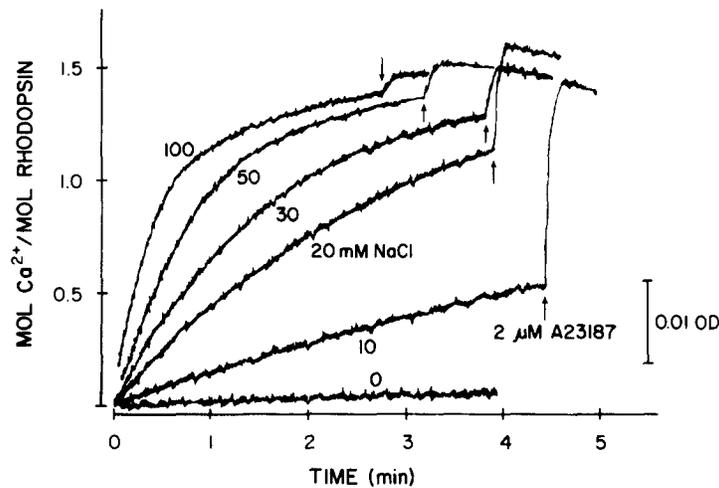


FIGURE 4. Dependence of Na-Ca exchange on external Na<sup>+</sup> concentration in intact OS-IS/OS. Intact OS/OS-IS were suspended in 125 mM TMA-Cl, 2.5 mM KCl, 30 mM HEPES, and 13.2 mM arginine (pH 7.4), to which was added after resuspension 100 μM arsenazo III and 1 μM FCCP. Ca<sup>2+</sup> efflux was initiated at time zero by the addition of NaCl to the indicated final concentrations. At the arrows, 2 μM A23187 was added.

~50% too high; this physiological  $\text{Na}^+$  concentration was not used consistently since a very fast initial phase was frequently observed, which we interpret to indicate that part of the OS was broken by the hypertonic shock. The experiments illustrated in Figs. 4–6 were performed with OS/OS-IS isolated in IBMX-containing Ringer's to increase the  $\text{Ca}^{2+}$  of intact OS/OS-IS and allow more measurements to be made on a single preparation. IBMX had no effect on the rate of Na-Ca exchange as measured with our protocol.

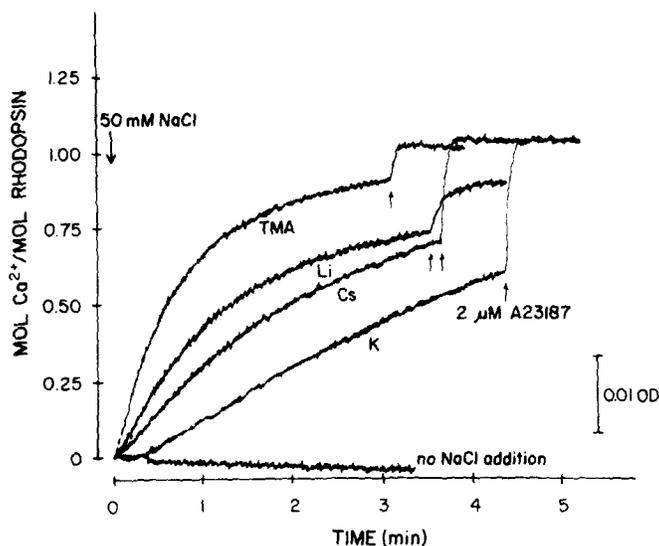


FIGURE 5. Na-Ca exchange in media of different ionic compositions. Intact OS/OS-IS were resuspended in a medium containing 125 mM TMA-Cl, 2.5 mM KCl, 30 mM HEPES, and 13.2 mM arginine (pH 7.4). Immediately before use, the suspension was diluted 20-fold in this suspension medium, to which was added 100  $\mu\text{M}$  arsenazo III and 1  $\mu\text{M}$  FCCP, and in which TMA-Cl was replaced by equimolar amounts of the indicated chloride salts.  $\text{Ca}^{2+}$  efflux was initiated at time zero by the addition of 50 mM NaCl. At the arrows, 2  $\mu\text{M}$  A23187 was added. The trace labeled "no NaCl addition" represents the  $\text{Ca}^{2+}$  leakage before the addition of  $\text{Na}^+$ . Leakage varied slightly among the different media, but never exceeded 10% of the slowest efflux rate observed after the addition of NaCl.

#### *K<sup>+</sup> Inhibits the Rate of Na-Ca Exchange*

Partial replacement of  $\text{Na}^+$  in the extracellular solution by  $\text{K}^+$  causes a dramatic lengthening of the photoresponse of toad rods ( $\text{K}^+ > 20 \text{ mM}$ );  $\text{Rb}^+$ , but not  $\text{Li}^+$  or  $\text{Cs}^+$ , could replace  $\text{K}^+$  in this effect (Hodgkin et al., 1985). Hodgkin et al. interpret this result as indicating that high external  $\text{K}^+$  inhibits Na-Ca exchange and that changes in intracellular  $\text{Ca}^{2+}$  somehow control the duration of the photoresponse. We have measured  $\text{Na}^+$ -stimulated  $\text{Ca}^{2+}$  efflux from isolated OS/OS-IS in media of different ionic composition, and observed that different cations inhibited Na-Ca exchange in the order  $\text{K}^+ \geq \text{Rb}^+ > \text{Cs}^+ \geq \text{Li}^+ > \text{TMA}$  (Fig. 5 and Table II). Incubating rods with the different cations tested did not lower their total  $\text{Ca}^{2+}$  content, which confirms that these cations could not replace  $\text{Na}^+$

in exchange transport with  $\text{Ca}^{2+}$  (the apparently lower  $\text{Ca}^{2+}$  content observed in  $\text{Li}^+$  medium can be accounted for by the reduced sensitivity of arsenazo III in this medium). The experiment illustrated in Fig. 6 shows that the inhibition of Na-Ca exchange (measured as  $\text{Na}^+$ -stimulated  $\text{Ca}^{2+}$  efflux) gradually increased as a function of the external  $\text{K}^+$  concentration.

It is noteworthy that as inhibition of Na-Ca exchange became stronger, a pronounced lag phase was observed before  $\text{Na}^+$ -stimulated  $\text{Ca}^{2+}$  efflux developed fully (Figs. 5 and 6). At the highest  $\text{K}^+$  concentration, it took 10–20 s before

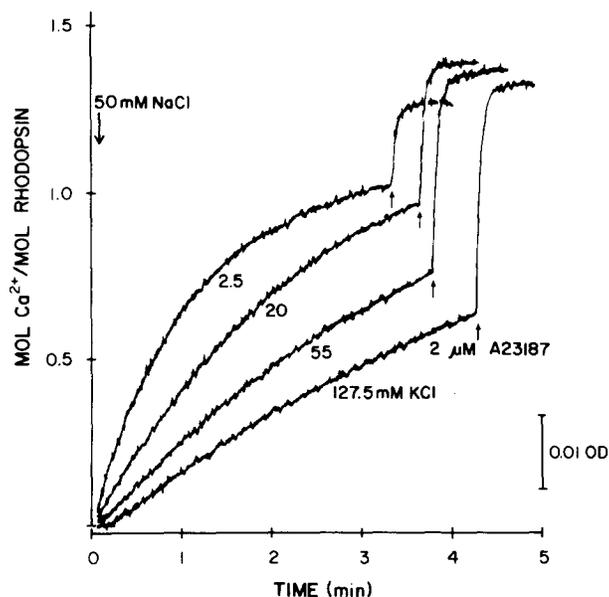


FIGURE 6. Inhibition of Na-Ca exchange by KCl. Intact OS/OS-IS were resuspended in a medium containing 125 mM TMA-Cl, 2.5 mM KCl, 30 mM HEPES, and 13.2 mM arginine (pH 7.4). Immediately before use, the suspension was diluted 10-fold in the same medium, to which was added 200  $\mu\text{M}$  arsenazo III and 1  $\mu\text{M}$  FCCP, and in which TMA-Cl was isotonicity replaced by KCl as indicated.  $\text{Ca}^{2+}$  efflux was initiated at time zero by the addition of 50 mM NaCl. At the arrows, 2  $\mu\text{M}$  A23187 was added. For clarity, a trace representing leakage before the addition of  $\text{Na}^+$  is not included. Leakage increased slightly as the KCl concentration increased, but never exceeded 10% of the slowest efflux rate observed after the addition of NaCl.

$\text{Ca}^{2+}$  efflux became noticeable, and the maximal rate of Na-Ca exchange developed only after 30–60 s. The obvious interpretation of such a phenomenon is that  $\text{Ca}^{2+}$  release is a consequence of a consecutive reaction (for example, the consecutive action of Na-Ca exchange across the disk and plasma membrane). However, such a model seems unlikely, since a similar lag phase was observed in OS with a leaky plasma membrane (Fig. 3A).

Considering the interpretation by Hodgkin et al. (1985) that different cations in the external medium exert their effect on the waveform of the photoresponse

by inhibiting Na-Ca exchange, our results corroborate their interpretation. However, our data may suggest that inhibition must exceed 50% to have a noticeable effect on rod physiology, since it appears that Na-Ca exchange has some spare capacity before the rate of  $\text{Ca}^{2+}$  transport via Na-Ca exchange becomes limiting (compare Figs. 7 and 9 from Hodgkin et al., 1985, with Figs. 5 and 6 in our study). Alternatively, the effect of  $\text{K}^+$  on the physiological response could be caused by a delay in the onset of Na-Ca exchange, i.e., the capacity of Na-Ca exchange is still sufficient, but it takes several seconds after a flash of light for Na-Ca exchange to start operating.

Neither inhibition of Na-Ca exchange by  $\text{K}^+$  nor the lag phase in the onset of Na-Ca exchange in  $\text{K}^+$  media has been reported in other systems where Na-Ca exchange has been measured, including isolated bovine OS (Schnetkamp, 1986, and references therein). At present, it is unclear whether this represents a true difference between the amphibian and the mammalian systems, or rather reflects differences in the isolation procedures.

#### *Effects of High-Energy Phosphates, Inositol Trisphosphate, and Light*

Other compounds of interest were tested on  $\text{Ca}^{2+}$  fluxes in purified leaky OS, including MgATP, MgGTP, and inositol 1,4,5-trisphosphate. None proved effective in causing either release or accumulation of  $\text{Ca}^{2+}$  or in modifying  $\text{Ca}^{2+}$  release induced by cGMP or  $\text{Na}^+$  (not shown). It should be noted that in more fragmented and  $\text{Ca}^{2+}$ -depleted disk membrane preparations, an ATP-dependent  $\text{Ca}^{2+}$  uptake has been described (Schnetkamp et al., 1977; Puckett et al., 1985). The protocols for measuring cGMP- and  $\text{Na}^+$ -induced  $\text{Ca}^{2+}$  release were designed to give maximal net rates of  $\text{Ca}^{2+}$  transport. Under these conditions, the results were not affected by light once the purified OS/OS-IS were transferred to the TMA medium. These observations on frog OS/OS-IS are very similar to those described for intact and leaky bovine OS (Caretta, 1985; Koch and Kaupp, 1985; Schnetkamp, 1980, 1981, 1986).

George and Hagins (1983) report two aspects of  $\text{Ca}^{2+}$  transport in crude suspensions of broken OS using  $\text{Ca}^{2+}$  mini-electrodes: a cGMP- and ATP-dependent  $\text{Ca}^{2+}$  uptake by disks, and a light-induced  $\text{Ca}^{2+}$  release from disks in the presence of cGMP. Using  $\text{Ca}^{2+}$  mini-electrodes, we did not observe the  $\text{Ca}^{2+}$  uptake in our purified leaky OS preparation, but under certain conditions, we did observe an apparent light-induced  $\text{Ca}^{2+}$  release from disks in the presence of cGMP. In our hands, however, this turned out to be an artifact of the  $\text{Ca}^{2+}$  chelators added to bind the  $\text{Ca}^{2+}$  contaminants present in the solution. The ligand-binding properties of  $\text{Ca}^{2+}$  chelators such as EGTA or ATP are strongly dependent on the pH, and even the minor acidification caused by the rapid light-induced hydrolysis of cGMP in the presence of 30 mM HEPES (pH 7.4) proved sufficient to give a large  $\text{Ca}^{2+}$  release originating from added  $\text{Ca}^{2+}$  chelators. In contrast to the cGMP-induced  $\text{Ca}^{2+}$  release described above, the light-induced  $\text{Ca}^{2+}$  release in the presence of cGMP was not observed when 8-bromo-cGMP was added (see also George and Bitensky, 1984). This result suggests that great caution should be exercised under conditions in which stray  $\text{Ca}^{2+}$  is chelated by ATP, GTP, or EGTA; these conditions are particularly prone to causing artificial

Ca<sup>2+</sup> movements owing to the pH dependence of these Ca<sup>2+</sup> chelators. That such artifacts may indeed explain some of the cited observations is suggested by George and Bitensky (1986), who report that some of their Ca<sup>2+</sup> movements correlate with pH changes.

#### DISCUSSION

##### *Ca<sup>2+</sup> Content of Frog OS*

This study describes Ca<sup>2+</sup> transport through both the plasma membrane and disk membranes of isolated and purified frog OS/OS-IS. Ca<sup>2+</sup> fluxes through the plasma membrane could change the total Ca<sup>2+</sup> concentration in OS by 50 μM/s and could alter the total Ca<sup>2+</sup> content by 1.5 mM within 1 min. Ca<sup>2+</sup> removal from OS can be attributed unambiguously to Na-Ca exchange in view of its absolute dependence on external Na<sup>+</sup>. We also observed a Ca<sup>2+</sup> influx of 1.2–2 mM in intact OS/OS-IS when they were isolated in Ringer's solution containing phosphodiesterase inhibitors (Table I). This result is consistent with a Ca<sup>2+</sup> influx via the light-sensitive conductance, since Ca<sup>2+</sup> currents through the light-sensitive conductance have been observed in the presence of IBMX (Capovilla et al., 1983). Another pathway for Ca<sup>2+</sup> influx is reverse Na-Ca exchange (Schnetkamp, 1986), but it is difficult to assess its contribution to total Ca<sup>2+</sup> influx.

When contemplating possible roles for the rapid and large Ca<sup>2+</sup> movements in OS/OS-IS via Na-Ca exchange reported in this study, it is necessary to assess the in situ Ca<sup>2+</sup> content of OS in the functioning retina. Unfortunately, reports on the in situ Ca<sup>2+</sup> content of rod OS in the vertebrate retina disagree. For rods in the amphibian retina, Schröder and Fain (1984) and Hagsins and Yoshikami (1975) give values between 3 and 5 mM, whereas Somlyo and Walz (1985) report a much lower value of 0.25 mM. OS/OS-IS isolated and purified from frog retinas in normal Na<sup>+</sup> Ringer's contained 2.2 mM Ca<sup>2+</sup> (Table I) and gave normal electrophysiological responses to illumination (Birnbaum and Bowns, 1985). From this we conclude that physiologically functional OS-IS can contain significant amounts of Ca<sup>2+</sup>, and that most of this Ca<sup>2+</sup> can be removed quite quickly from OS by Na-Ca exchange. We cannot exclude the possibility that the OS in OS-IS have accumulated Ca<sup>2+</sup> after separation from the retina, although it does not seem to impair their physiological performance.

##### *Comparison of Ca<sup>2+</sup> Fluxes across the Plasma and Disk Membrane in Bovine and Frog OS*

Table III summarizes the rates of Ca<sup>2+</sup> transport across OS membranes reported in this study for frog, and compares them with similar observations on isolated and purified bovine OS (Koch and Kaupp, 1985; Schnetkamp, 1980, 1986). Qualitatively, the observations are very similar in both species. (a) Na-Ca exchange is observed in both plasma and disk membranes. It should be noted that physical separation of disks by osmotic lysis strongly reduced Na-Ca exchange across the disk membrane. (b) A cGMP-dependent pathway is observed in the disk membrane, which has access to only a 20–30% fraction of the total Ca<sup>2+</sup> in

disks. The  $\text{Ca}^{2+}$  fluxes in frog rods are compared with that portion of the dark current ( $\sim 1.5$  pA) thought to be carried by  $\text{Ca}^{2+}$  under normal conditions (Yau and Nakatani, 1985a); a 1.5-pA  $\text{Ca}^{2+}$  current would contribute a  $\text{Ca}^{2+}$  flux of 1/10 the capacity of Na-Ca exchange. The maximal rates of light-induced  $\text{Ca}^{2+}$  release from rat and toad retinas are  $2 \times 10^4$  and  $1.2 \times 10^6$   $\text{Ca}^{2+}/\text{OS}\cdot\text{s}$ , respectively (Yoshikami et al., 1980; Gold and Korenbrot, 1980). These values are quite small compared with the capacity of Na-Ca exchange, and may therefore reflect only small changes in the equilibrium conditions of the Na-Ca exchanger.

TABLE III  
*Ca<sup>2+</sup> Fluxes in Intact and Leaky Bovine and Frog Rod OS*

	Frog	Bovine	
Intact (plasma membrane)			
Na-Ca exchange	$6.0 \times 10^7$	$5 \times 10^6$	$\text{Ca}^{2+}/\text{OS}\cdot\text{s}$
	0.050	0.52	mM total $\text{Ca}^{2+}\cdot\text{s}$
	8.2	13.3	pmol/cm <sup>2</sup> ·s
Ca flux of 1.5 pA, via dark current	$4.7 \times 10^6$		$\text{Ca}^{2+}/\text{OS}\cdot\text{s}$
	0.004		mM total $\text{Ca}^{2+}/\text{s}$
	0.64		pmol/cm <sup>2</sup> ·s
Leaky (disk membrane)			
Na-Ca exchange	$1.9 \times 10^7$	$3 \times 10^6$	$\text{Ca}^{2+}/\text{OS}\cdot\text{s}$
	0.016	0.31	mM total $\text{Ca}^{2+}/\text{s}$
	0.024	0.41	pmol/cm <sup>2</sup> ·s
cGMP dependent	$9.5 \times 10^6$	$8.5 \times 10^4$	$\text{Ca}^{2+}/\text{OS}\cdot\text{s}$
	0.008	0.009	mM total $\text{Ca}^{2+}/\text{s}$
	0.012	0.012	pmol/cm <sup>2</sup> ·s

$\text{Ca}^{2+}$  fluxes observed for intact frog OS/OS-1S in this study are compared with those observed for intact bovine OS (Koch and Kaupp, 1985; Schnetkamp, 1986). The latter two studies were chosen because they used protocols very similar to the ones adopted in our study. Na-Ca exchange was observed at 50 mM external  $\text{Na}^+$ , and cGMP-induced  $\text{Ca}^{2+}$  release was observed at 250  $\mu\text{M}$  cGMP or 8-bromo-cGMP. The free  $\text{Ca}^{2+}$  concentration in the external medium was between 0.1 and 2  $\mu\text{M}$ . Other relevant data for fluxes across bovine disk membranes are reported by Caretta (1985). A Ca current of 1.5 pA through the light-sensitive conductance was taken from Yau and Nakatani (1985a). The dimensions of frog OS were taken to be  $6.5 \times 60$   $\mu\text{m}$  containing 2.5 mM total rhodopsin and 2,000 disks. The dimensions of bovine OS were taken to be  $1 \times 20$   $\mu\text{m}$  containing 3 mM total rhodopsin and 800 disks.

The  $\text{Ca}^{2+}$  flux through the Na-Ca exchanger in the plasma membrane of both frog and bovine OS is comparable when expressed as flux per surface area. This value is also similar to the flux calculated from the Na-Ca exchange current in toad rods reported by Yau and Nakatani (1984). In view of the different dimensions of these photoreceptors, it is obvious that mammalian OS can change their total internal  $\text{Ca}^{2+}$  in a much more dramatic fashion than amphibian OS when expressed in millimolar units per second. The inhibitory effect of  $\text{K}^+$  on Na-Ca exchange in frog rods marks a clear difference from the effects of  $\text{K}^+$  on Na-Ca exchange in bovine rods. These are intriguing findings considering the

suggestion that the rate of Na-Ca exchange can control in some way the duration of the photoresponse (Hodgkin et al., 1985) and may be involved in light adaptation. Adapting lights have different effects on the kinetics of flash-induced photoresponses for the amphibian and mammalian species documented so far (cf. Baylor et al., 1984).

The cGMP-dependent flux across the disk membrane measured with leaky OS is also similar in bovine and frog OS when expressed as flux per surface area. However, Na-Ca exchange in leaky OS is different in frog and bovine no matter how it is expressed. It is curious that, in both species, Na-Ca exchange in the plasma membrane has roughly the same capacity (in  $\text{Ca}^{2+}$  per outer segment per second) as compared with Na-Ca exchange in disk membranes, although the ratio of the surface area plasma membrane to disk membrane is quite different.

A few studies have described ATP-dependent  $\text{Ca}^{2+}$  uptake into disks (Schnetkamp et al., 1977; George and Hagins, 1983; Puckett et al., 1985). The first two used bovine disks, but more disrupted and  $\text{Ca}^{2+}$ -depleted preparations were used to observe an ATP effect; the rates observed were  $\sim 2 \times 10^4 \text{ Ca}^{2+}/\text{OS}\cdot\text{s}$ , which is much smaller than the ATP-independent  $\text{Ca}^{2+}$  fluxes in less disrupted bovine disk preparations (Schnetkamp, 1979, 1986; Table III). Unfortunately, it is difficult to extract quantitative information from the study employing frog disks (George and Hagins, 1983).

*Implications of the Dynamics of  $\text{Ca}^{2+}$  Fluxes across Plasma and Disk Membranes for the Regulation of  $\text{Ca}^{2+}$  in Rod Photoreceptors*

The observation that the flux through the Na-Ca exchanger in the plasma membrane in both frog and bovine OS is higher than any flux reported across the disk membrane has implications for the relevance of the latter. The majority of the evidence suggests that, under normal conditions, most of the  $\text{Ca}^{2+}$  in OS is contained inside disks (Schnetkamp, 1979; Schnetkamp and Kaupp, 1985; Fain and Schröder, 1985; Table I and its discussion). However, the addition of external  $\text{Na}^+$  results in the appearance of  $\text{Ca}^{2+}$  in the extracellular space without a lag phase and at rates higher than any  $\text{Ca}^{2+}$  flux observed across the disk membrane in leaky OS. The lack of a lag phase suggests that  $\text{Na}^+$ -stimulated  $\text{Ca}^{2+}$  efflux does not require the build-up of internal  $\text{Na}^+$ . Two explanations could be considered. First, Na-Ca exchange could involve  $\text{Na}^+$  and  $\text{Ca}^{2+}$  that do not equilibrate in the cytoplasm, for example, since the Na-Ca exchangers in disk and plasma membranes may be located in close apposition. Second,  $\text{Ca}^{2+}$  fluxes in intact rods are rate-limited by Na-Ca exchange across the plasma membrane; the disks respond to changes of the free cytoplasmic  $\text{Ca}^{2+}$  concentration by adjusting their internal  $\text{Ca}^{2+}$  much faster than Na-Ca exchange across the plasma membrane. This explanation requires the presence of a  $\text{Ca}^{2+}$  transport system in disk membranes of quite different properties that has yet to be described, and, more importantly, has a capacity significantly higher than any reported  $\text{Ca}^{2+}$  flux across the disk membrane in leaky OS or other disk membrane preparations. The second explanation appears to be refuted by the observation that  $^{44}\text{Ca}$ - $^{40}\text{Ca}$  exchange in functional rods in the intact retina appears to be limited to  $\text{Ca}^{2+}$  in the cytoplasm, whereas  $\text{Ca}^{2+}$  in the disks does not exchange (Fain and Schröder,

1985). If one assumes that the observed  $\text{Ca}^{2+}$  fluxes across the disk membrane are representative of those in intact rod cells, another consequence arises. All  $\text{Ca}^{2+}$  fluxes across the disk membrane as measured in leaky OS are much smaller than Na-Ca exchange across the plasma membrane. This means that  $\text{Ca}^{2+}$  fluxes across the plasma membrane can change intracellular  $\text{Ca}^{2+}$  much faster than  $\text{Ca}^{2+}$  fluxes across the disk membrane. Na-Ca exchange is a bidirectional transporter subjected to the equilibrium conditions imposed by the  $\text{Ca}^{2+}$  and  $\text{Na}^+$  gradients. Under such conditions,  $\text{Ca}^{2+}$  fluxes across the disk membrane are unlikely to contribute to changes in the free cytoplasmic  $\text{Ca}^{2+}$  concentration, but are limited to controlling the intradiskal  $\text{Ca}^{2+}$  content.

In summary, the results of this study document for frog OS/OS-IS the  $\text{Ca}^{2+}$  fluxes associated with Na-Ca exchange, and their inhibition by high external  $\text{K}^+$  in two respects, rate and lag phase, which correlates with a prolonged duration of the photoresponse as documented by Hodgkin et al. (1985). Our results raise questions on the issue of the dynamics of  $\text{Ca}^{2+}$  fluxes in rods. The dynamics of  $\text{Ca}^{2+}$  fluxes across the plasma membrane imply that  $\text{Ca}^{2+}$  fluxes across the disk membrane as observed in disk membrane preparations either do not contribute to the control of cytosolic  $\text{Ca}^{2+}$  levels or that these observed fluxes are not representative of those occurring in intact OS.

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