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Ca^{2+} Buffer Sites in Intact Bovine Rod Outer Segments: Introduction to a Novel Optical Probe to Measure Ionic Permeabilities in Suspensions of Small Particles

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Summary. The nature of the Ca^{2+} buffer sites in intact rod outer segments isolated from bovine retinas (ROS) was investigated. The predominant Ca^{2+} buffer in intact ROS was found to be negatively charged groups confined to the surface of the disk membranes. Accordingly, Ca^{2+} buffering in ROS was strongly influenced by the electrostatic surface potential. The concentration of Ca^{2+} buffer sites was about 30 mM, 80% of which were located at the membrane surface in the intradiskal space. A comparison with observations in model systems suggests that phosphatidylserine is the major Ca^{2+} buffer site in ROS. Protons and alkali cations could replace Ca^{2+} as mobile counterions for the fixed negatively charged groups. At physiological ionic strength, the total number of these diffusible, but osmotically inactive, counterions was as large as the number of osmotically active cations in ROS. The surface potential is dependent on the concentration of cations in ROS and can be measured with the optical dye neutral red. Addition of cations to the external solution led to the release of the internally bound dye as the cations crossed the outer membrane. The chemical and spectral properties of the dye enable its use as a real-time indicator of cation transport across the outer envelope of small particles in suspension. In this study, the dye method is illustrated by the use of well-defined ionophores in intact ROS and in liposomes. In the companion paper this method is used to describe the cation permeabilities native to ROS.

Key Words calcium · rod photoreceptors · surface potentials

Introduction

Calcium ions play an important role in the functioning of rod photoreceptor cells; changes in Ca^{2+} can greatly alter the magnitude of the dark current flowing into the outer segment of the rod cell and changes in Ca^{2+} can alter the sensitivity of the rod cell to light (reviewed by Fain & Lisman, 1981; Kaupp & Schnetkamp, 1982; Korenbrot, 1985). Rod outer segments (ROS) in the living retina as well as isolated ROS contain substantial amounts of Ca^{2+} [1–3 mol Ca^{2+}/mol rhodopsin or 3–9 mM overall concentration (Hagins & Yoshikami, 1975; Schnetkamp, 1979; Schröder & Fain, 1984; but *see* Somlyo & Walz, 1985)]. In isolated ROS, the majority of the internal Ca^{2+} appears to be bound to the intradiskal surface of the disk membrane (Schnetkamp & Kaupp, 1985).

This study addresses the nature of the Ca^{2+} buffer sites in isolated intact ROS. Considering the molecular composition of ROS, obvious candidates for these buffer sites are negatively charged groups confined to the intracellular disk membranes. The overall rhodopsin concentration in the outer segment is about 3 mM, and the disk membranes contain about 65 molecules of phospholipid per molecule of rhodopsin (Daemen, 1973). Of these 65 phospholipids 10–11 are acidic phospholipids, 9–10 phosphatidylserine (PS) and 1 phosphatidylinositol (PI). These acidic phospholipids usually carry a single net negative charge at neutral pH. The surface of the rhodopsin molecule contains an excess of negatively charged residues (Ovchinnikov, 1982), and, therefore, rhodopsin carries a likely net negative surface charge at neutral pH. The negative charges on the phospholipids and rhodopsin are confined to the membrane surface (fixed charges) and are electrically neutralized by diffusible cations. These cations are either bound to the fixed charges or are located in the so-called diffuse double layer (McLaughlin, 1977). The results of this study suggest that the fixed negative charges on the disk membrane surface constitute the main Ca^{2+} buffer in intact ROS. The overall concentration of these fixed negative charges on the disk membranes and their associated cloud of diffusible (but osmotically inactive) counterions is comparable to that of the osmotically active salts in the cytoplasm.

The presence of fixed charges on a membrane surface is accompanied by an electrostatic potential, which is maximal at the surface of the mem-

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brane and drops to zero as the distance from the membrane increases (McLaughlin, 1977). The magnitude of this potential due to negative charges is a simple function of the cation concentration in the medium and can be used to measure cation concentrations. In a previous study we have used the dye neutral red to measure changes of the surface potential (Schnetkamp, Kaupp & Junge, 1981). The amphiphilic nature of neutral red allows it to bind to phospholipid bilayer membranes. When membranes contained a net negative charge the bound neutral red was predominantly in the positively charged form, causing its binding to be dependent on the surface potential. This study shows that in systems rich in internal membranes, such as liposomes or ROS, changes in the binding of neutral red to internal membranes can be used to measure with great accuracy changes in the concentration of internal cations upon changes in the concentration of external cations. The spectral properties of the dye allow its use as a real-time indicator of ionic permeabilities through the outer envelope of liposomes and ROS.

Materials and Methods

Preparations

ROS were isolated from bovine retinas according to procedures described before (Schnetkamp, Klompmakers & Daemen, 1979; Schnetkamp & Daemen, 1982; Schnetkamp & Kaupp, 1985). ROS were isolated with a sealed plasma membrane (intact ROS) or with a plasma membrane permeable to small solutes (leaky ROS); both intact and leaky ROS were isolated containing very little or no Ca^{2+} (Ca^{2+}-depleted ROS contained less than 0.1 mol Ca^{2+}/mol rhodopsin) or rich in Ca^{2+} (Ca^{2+}-enriched ROS containing more than 3 mol Ca^{2+}/mol rhodopsin). When the ionophores A23187 and gramicidin were added to Ca^{2+}-depleted ROS, these ROS contained a large pool of bound protons that could be exchanged against external Ca^{2+}. ROS were resuspended in 600 mM sucrose, 5% Ficoll 400, 20 mM HEPES, 8.8 mM arginine (pH 7.4) to a final rhodopsin concentration of about 150 μM. Experiments were performed within 6 hr after isolation of ROS. All experiments were carried out in darkness or under dim red illumination.

Liposomes were prepared from egg phosphatidylcholine (PC; isolated according to the procedure of Singleton, Gray, Brown & White, 1965). Phosphatidylserine (PS), phosphatidic acid (PA), and phosphatidylinositol (PI) were obtained from Avanti Polar Lipids (Birmingham, AL). The chloroform was evaporated in a rotary evaporator, and the thin film of phospholipids were resuspended in 600 mM sucrose, 10 mM HEPES, 4.4 mM arginine (pH = 7.4) to a final concentration of 30 mg phospholipid/ml. The liposomes were briefly sonicated (30 sec at 30 W).

Different ionophores were added from 1 mM stock solutions in methanol or ethanol (final concentration 0.2%). Methanol or ethanol additions of this magnitude were without any effect. Neither the organic solvents nor the ionophores themselves had any effect on the integrity of the plasma membrane in intact ROS.

Dual-Wavelength Measurements

Dual-wavelength measurements were carried out with a DW 2C SLM-Aminco spectrophotometer equipped with a cuvette holder having a magnetic stirring device. Changes in free proton concentration were followed with the pH-indicating dyes bromcresol purple (pH of about 6.3) and phenol red (pH of about 7.6), while changes in free Ca^{2+} concentration were monitored with the dye arsenazo III. The concentration of arsenazo III was 110 μM, and less than 20% of the arsenazo III was bound to Ca^{2+}. The dissociation constant for the Ca^{2+}-arsenazo III complex is about 3 μM at the ionic strength used (Kaupp, Schnetkamp & Junge, 1979), and, therefore, the free Ca^{2+} concentration was always less than 1 μM. The details and validity of these procedures were discussed previously (Schnetkamp & Kaupp, 1985).

Changes of the electrostatic potential at the surface of either the disk membrane or the liposome membrane were measured by the changes in the distribution of the amphiphile neutral red as described earlier (Schnetkamp et al., 1981). ROS samples were centrifuged in a table top centrifuge at 12,000 rpm for 30 sec. ROS formed a very tight pellet, while the supernatant did not contain any measurable amount of rhodopsin. The neutral red adsorbed to the disk membrane shows a red-shifted absorption peak and is predominantly in the protonated form at pH 7.4 in the suspension medium (Schnetkamp et al., 1981). The same spectral characteristics were observed for neutral red adsorbed to liposome membranes containing acidic phospholipids (either PA, PI or PS). At pH 7.4 the unprotonated basic form predominates in the suspension medium. These spectral properties of neutral red adsorbed to membranes allowed real-time monitoring of its binding and release in dual-wavelength recordings (wavelength pair: 540 and 650 nm; bandwidth 3 nm). A decrease in (A540-A650) indicates a release of neutral red from the membranes, since neutral red in aqueous solution (at pH 7.4) contributes little to the absorption at these wavelengths. In dual-wavelength recordings an empirical 'in situ' calibration is used as described in the experiments illustrated in Figs. 6 and 9.

Results

Throughout this study the following ionophores were used to induce specific ionic permeabilities to ROS or liposome membranes. Gramicidin is a nonselective channel for protons and alkali cations; FCCP and valinomycin are specific electrogenic carriers for protons and K^+, respectively; nigericin is an obligatory K-H exchanger; A23187 is an obligatory exchanger for protons and divalent cations (for a review on ionophores *cf.* Pressman, 1976).

Properties of Ca^{2+} and H^+ Buffering in Intact ROS

Intact ROS were prepared containing large amounts of exchangeable Ca^{2+} (Ca^{2+}-enriched ROS) or exchangeable H^+ (Ca^{2+}-depleted ROS) in the presence

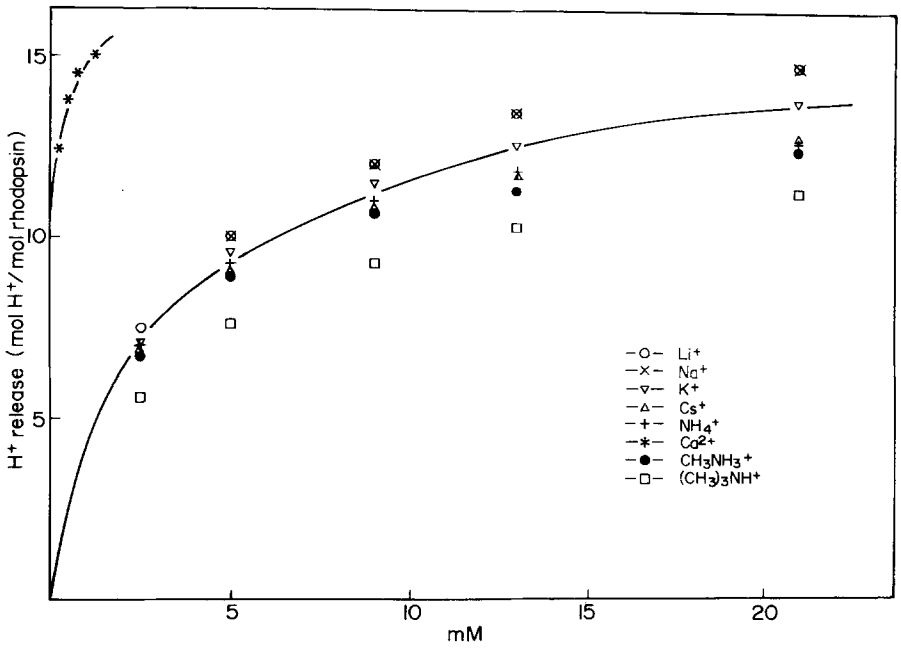

Fig. 1. Salt-dependent proton buffering in intact ROS. Intact Ca^{2+}-depleted bovine ROS were diluted in a medium containing 600 mM sucrose, 20 μM bromcresol purple, 0.4 mM HEPES, 0.18 mM arginine (pH 6.3) 2 μM gramicidin, 5.3 μM rhodopsin. Alkali cations had access to the intracellular compartments due to the presence of gramicidin. Cation-induced proton release was measured in the dual wavelength mode using the wavelength pair, 590 and 650 nm. Proton release was complete within 1 min. When Ca^{2+}-induced proton release was measured, 2 μM A23187 was also present. T = 26°C

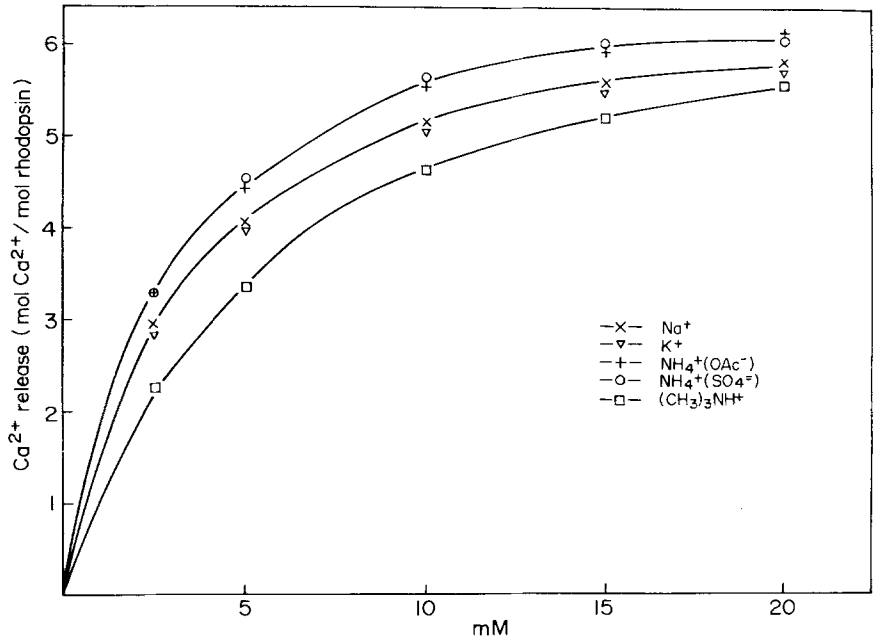

Fig. 2. Salt-dependent Ca^{2+} buffering in intact ROS. Intact Ca^{2+}-enriched ROS were suspended in 600 mM sucrose, 30 mM HEPES, 13.2 mM arginine, 110 μM arsenazo III, 2 μM gramicidin, 2 μM A23187, 3.9 μM rhodopsin, pH = 7.4. At the start of the experiment less than 10% of the arsenazo was occupied by Ca^{2+}, and the free Ca^{2+} concentration was less than 1 μM. Alkali cations had access to the intracellular compartments due to the presence of gramicidin, while amines are permeable due to the permeability of their neutral basic form. Cation-induced Ca^{2+} release was measured in the dual wavelength mode using the wavelength pair, 650 and 750 nm. In similar experiments other alkali cations like Li^+ and Cs^+ caused very similar Ca^{2+} release patterns as shown here for Na^+ and K^+. Ca^{2+} release was complete within 1 min. T = 26°C

of ionophores that shunt ROS membranes for Ca^{2+}, H^+, and alkali cations (Schnetkamp & Kaupp, 1985). In the absence of permeable monovalent cations in the suspension medium, intact ROS contained more than 6 mol Ca^{2+}/mol rhodopsin or more than 15 mol H^+/mol rhodopsin at a free concentration of less than 1 μM Ca^{2+} or H^+, respectively (Figs. 1 and 2). These observations were made in the presence of A23187 and gramicidin, suggesting that both Ca^{2+} and H^+ were predominantly bound in

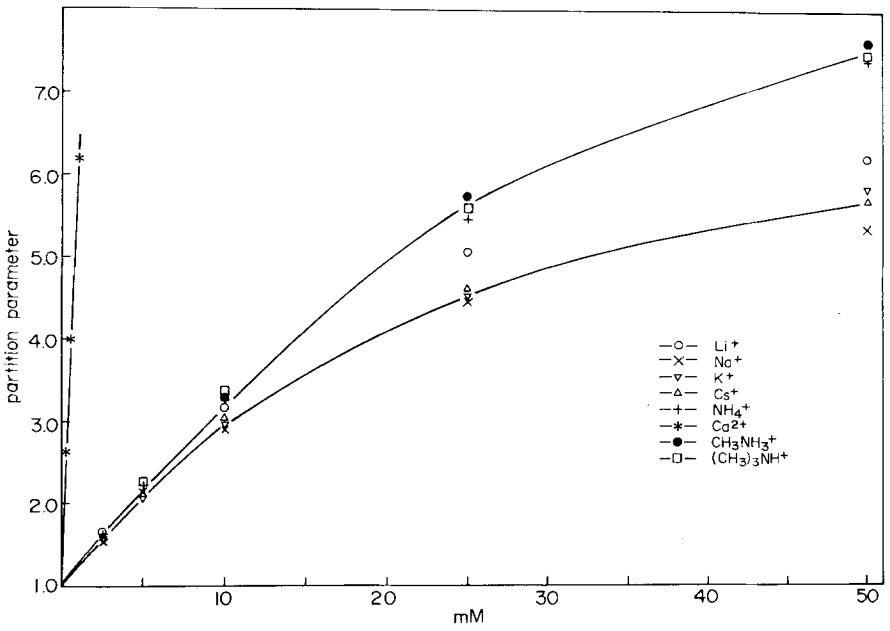

Fig. 3. Cation-induced release of neutral red from intact ROS. Intact Ca^{2+}-enriched ROS were incubated for 5 min at 21°C in a medium containing 600 mM sucrose, 20 mM HEPES, 8.8 mM arginine, 1 mM NH_4OAc, 50 mM choline chloride, 1 μM gramicidin, 65 μM neutral red and 19.2 μM rhodopsin (pH = 7.4). Cations were added by isotonic replacement of choline chloride by chloride salts of the indicated cations. Neutral red binding to ROS was measured by the sedimentation method. When Ca^{2+}-induced release of neutral red was measured, 2 μM A23187 were also present

ROS. Both Ca^{2+} and H^+ were released in increasing amounts as the concentration of monovalent cations able to enter intact ROS increased in the external medium. Assuming that the overall rhodopsin concentration in ROS amounts to 3 mM and that the aqueous compartments comprise 60% of the total volume, a change in the internal cation concentration by 5 mM represents a change in the amount of osmotic cations by 1 mol/mol rhodopsin. This change by only 1 mol/mol rhodopsin caused the release of 10 mol H^+/mol rhodopsin (Fig. 1) or 4 mol Ca^{2+}/mol rhodopsin (Fig. 2). This result shows that at low ionic strength the number of bound cations greatly exceeded that of osmotic cations. Two observations demonstrate that the Ca^{2+} (and H^+) buffer sites are located predominantly inside ROS. First, impermeable cations such as choline, tetramethylammonium, or arginine had no effect on Ca^{2+} (and H^+) binding. Second, monovalent cations did not cause any Ca^{2+} release in the absence of A23187 (except for Na^+ which causes Ca^{2+} release via the Na-Ca exchanger in ROS, *cf.* Schnetkamp, 1980).

The release patterns observed in Figs. 1 and 2 were nonspecific for monovalent cations, i.e., the bulky trimethylamine was as effective as small cations such as Li^+ or Na^+. The most straightforward interpretation of this finding is that the binding of Ca^{2+} and H^+ is controlled by the surface potential (Davis & Rideal, 1963; McLaughlin, 1977). In most biological membranes the surface potential is due to the presence of (a net excess of) negatively charged groups confined to the membrane surface. This surface potential causes an increase in the concentration of diffusible cations near the surface of the membrane as compared with the concentration in the suspension medium. As a consequence, binding sites located at the surface of the membrane are occupied. The surface potential is made less negative as the salt concentration in the medium increases. For a negative surface potential this essentially depends, as observed, on the concentration and valency of the cation, not on the type. A reduction of the surface potential causes a decrease of the surface concentration of Ca^{2+} or H^+, which then dissociate from their respective binding sites. This interpretation is supported also by the observation that divalent cations were much more effective than monovalent cations in releasing H^+ (as illustrated in Fig. 1 for Ca^{2+}).

In a previous study the binding of the dye neutral red to disk membranes was used as a measure of the electrostatic surface potential in ROS (Schnetkamp et al., 1981). In accordance with this, the absorption of neutral red to ROS membranes depended in a nonspecific manner on the concentration of permeable monovalent cations, while divalent cations were much more effective than monovalent cations in causing the release of neutral red (Fig. 3). The partition parameter reflects changes in the adsorption of the dye. A value of unity indicates no change, while a value of n indicates an n-fold change in absorption. No change in dye adsorption was observed when impermeable cations like choline, tetramethylammonium, or arginine were added. Differences between the divalent cations Mg^{2+}, Ca^{2+}, and Mn^{2+} were not more pronounced than those between the monovalent

cations. The cation-induced changes in the adsorption of neutral red were reversible. Addition of 1 mM Ca^{2+} (A23187 present) caused a large release of neutral red from ROS membranes, which was completely reversed after subsequent addition of 1 mM EDTA.

Stoichiometry of Cation-Induced Ca^{2+} Release from Internal Buffer Sites

This section addresses the question of the overall stoichiometry of the cation-induced Ca^{2+} release from internal buffer sites. The surface of the internal membranes in intact ROS is not freely accessible to cations added to the external medium. Access to the disk membranes in intact ROS can be controlled by choosing appropriate ionophores. The experiment shown in Fig. 4 illustrates the usefulness of specific ionophores to determine the ion fluxes that accompanied K^+-induced Ca^{2+} release; valinomycin is an electrogenic K^+ carrier, while nigericin is an obligatory K-H exchanger (Pressman, 1976).

1) K^+-induced Ca^{2+} release was not observed unless A23187 was present to make ROS membranes permeable to Ca^{2+}.
2) A small and slow Ca^{2+} release was observed when, in addition to A23187, valinomycin was present. K^+ transport through valinomycin most likely established a K^+ diffusion potential with little mass transport of K^+. A23187 is an electroneutral Ca-H exchanger and is unlikely to be affected by changes in the transmembrane potential. The slow release observed is probably a measure of the intrinsic proton permeability of ROS membranes.
3) A rapid and large K^+-induced Ca^{2+} release (A23187 present) was observed when both valinomycin and the proton carrier FCCP were added, or when nigericin was added. No Ca^{2+} release was observed when these ionophores were added in the absence of K^+.

These results demonstrate that K^+-induced Ca^{2+} release was not induced by changes in the transmembrane potential, but instead required bulk transport of K^+ into ROS. The efficiency of the combination of the obligatory exchangers A23187 and nigericin suggests that this release reflects an electroneutral Ca-K exchange. The proton fluxes via the different ionophores (e.g., A23187 and nigericin) are used to couple Ca^{2+} and K^+ fluxes. The same conclusion can be reached by considering the flow of charge through the plasma membrane in this experiment. The large Ca^{2+} release observed in Fig. 4 amounted to more than 10^8 Ca^{2+}/outer segment. This large efflux must have been electrically compensated by an equally large influx of K^+.

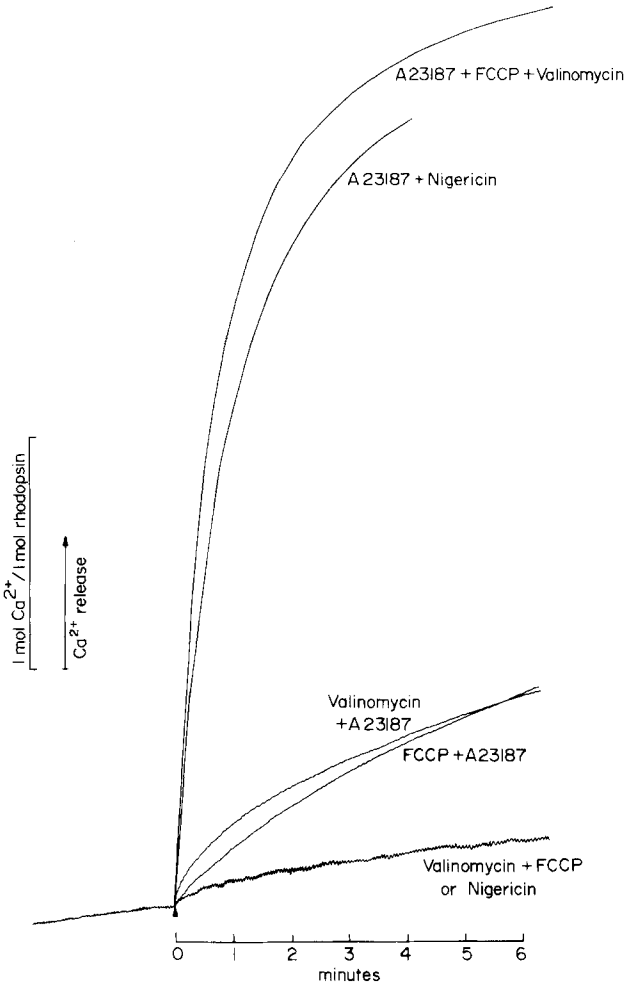

Fig. 4. Ionic fluxes associated with K^+-induced Ca^{2+} release. Ca^{2+}-enriched intact ROS were suspended in 600 mM sucrose, 30 mM HEPES, 13.2 mM arginine, 110 μM arsenazo III, 3.3 μM rhodopsin (pH = 7.4). Ionophores were present at a concentration of 1 μM. Ca^{2+} release was measured in the dual-wavelength mode by an increase in (A650-A750). At time zero, 10 mM KCl was added, and at that point less than 10% of the arsenazo III was bound to Ca^{2+}. T = 13°C

In a separate experiment the ionophore dependence of the K^+-induced release of neutral red from ROS was determined. Valinomycin alone had no effect, whereas the combination of valinomycin plus FCCP, or addition of nigericin, were fully effective. This result demonstrates that transmembrane potentials do not contribute to the binding of neutral red to disk membranes.

Compartmentalization of Ca^{2+} Buffering in ROS

In this section the distribution of salt-dependent Ca^{2+} buffer sites between the two sides of the disk

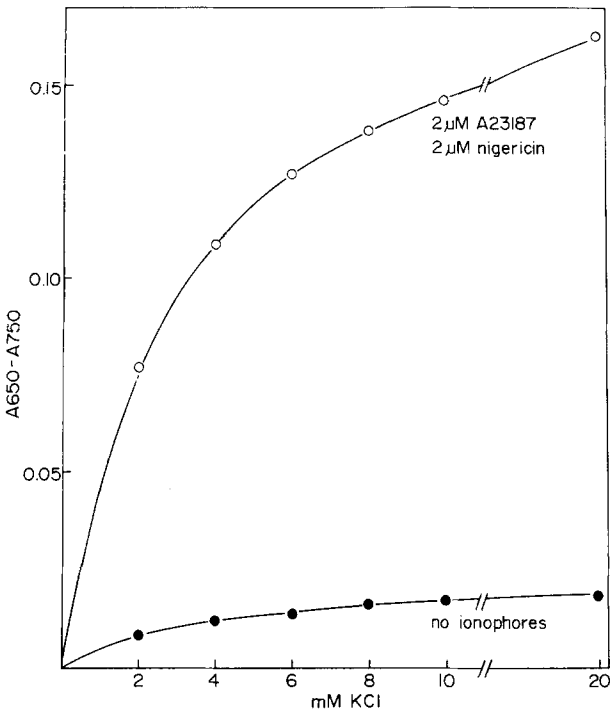

Fig. 5. Sidedness of Ca^{2+} binding in leaky ROS. Ca^{2+}-enriched leaky ROS were suspended in 600 mM sucrose, 10 mM HEPES, 4.4 mM arginine, 110 μM arsenazo III, 13.4 μM rhodopsin (pH = 7.4). K^+-induced Ca^{2+} release was measured in the dual-wavelength mode and indicated by an increase in (A650-A750). Ionophores (2 μM) were present as indicated. Ca^{2+} release upon addition of KCl was followed until completion (about1 min), and the final amount of Ca^{2+} release was plotted as a function of KCl concentration. T = 26°C

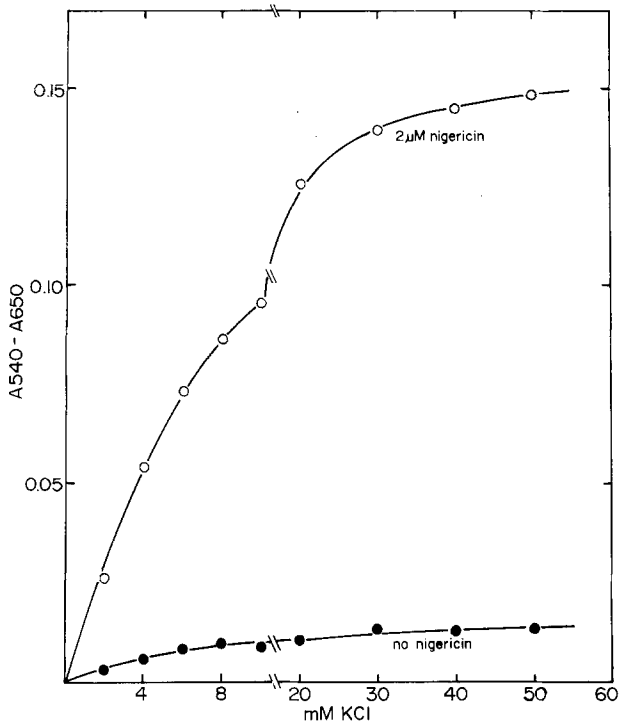

Fig. 6. Sidedness of neutral red binding in leaky ROS. Leaky ROS were suspended in 600 mM sucrose, 10 mM HEPES, 4.4 mM arginine, 10 μM neutral red, 200 μM EDTA, 2 μM A23187 (pH = 7.4). Neutral red release was measured in the dual-wavelength mode by the decrease in (A540-A650). A decrease in absorption was plotted upward. Neutral red release is plotted as a function of the external K^+ concentration. Ionophores were present as indicated. The K^+-induced release of neutral red was completed within 1 min after addition of KCl. In the absence of nigericin only the instantaneous (less than 2 sec) release of neutral red is plotted. This instantaneous release reflected release from the cytoplasmic surface and was followed by a slow and sustained release caused by K^+ transport across the disk membrane. T = 26°C

membrane is addressed. The K-H exchange ionophore nigericin was used to discriminate between Ca^{2+} buffer sites in the intradiskal space and those in the cytoplasmic space. For this purpose ROS with a leaky plasma membrane were used. In leaky ROS externally added K^+ has access to the cytoplasmic space without the need for ionophores, but ionophore is required to gain access to the intradiskal space. The results of a typical experiment are shown in Fig. 5. The K^+-induced Ca^{2+} release in the absence of nigericin was about 11% of that observed in its presence. This suggests that 89% of the salt-dependent Ca^{2+} buffer sites were located in the intradiskal space, or, more precisely, at the intradiskal surface of the disk membrane (Fig. 5).

In a separate experiment, the same protocol was used to measure the sidedness of the K^+-induced release of neutral red from disk membranes (Fig. 6). As described in Materials and Methods the absorption difference A540-A650 is a direct measure of the amount of neutral red adsorbed to membranes. The K^+-induced absorption change in the absence of nigericin was about 10% of that observed in its presence (Fig. 6). This suggests that 90% of the neutral red released by K^+ was previously bound at the intradiskal surface of the disk membrane. The same asymmetric binding of neutral red to the two sides of the disk membrane was found when the Ca^{2+}-induced release of neutral red was measured in Ca^{2+}-depleted leaky ROS. The accessibility of the intradiskal space to Ca^{2+} was regulated by addition of A23187. In experiments similar to those shown in Figs. 5 and 6, consistently 85–90% of the Ca^{2+} or neutral red associated with ROS was located in the intradiskal space, most likely bound to the surface of the membrane.

Comparison between ROS and Liposomes Containing Acidic Phospholipids

In this section the molecular basis for the salt-dependent Ca^{2+} and H^+ buffer sites in ROS is considered. Only two components in the disk membrane

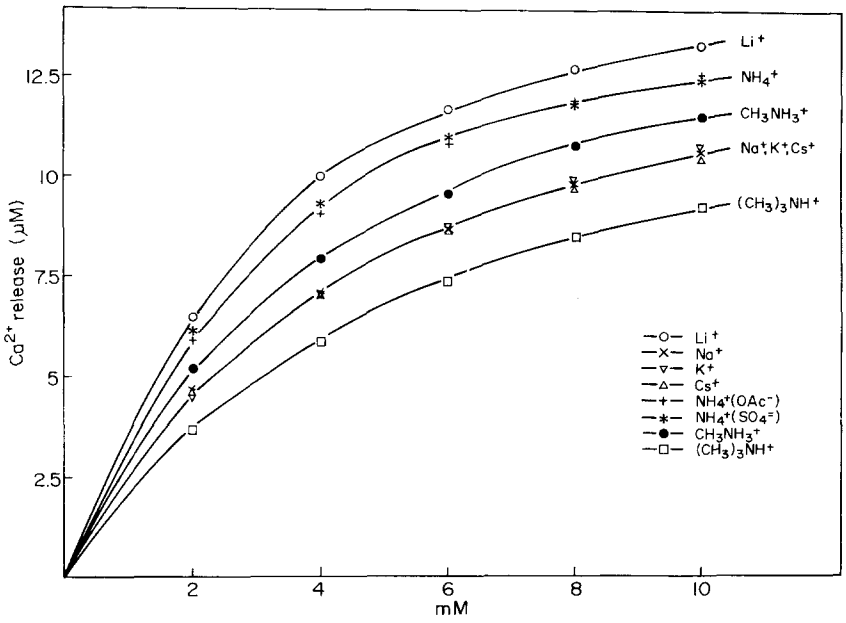

Fig. 7. Salt-dependent Ca^{2+} buffering in PC-PS (5 : 1) liposomes. Liposomes were suspended in 600 mM sucrose, 10 mM HEPES, 4.4 mM arginine, 110 μM arsenazo III, 2 μM A23187, 2 μM gramicidin, 120 μM PS. Total Ca^{2+} present was 19 μM of which about 16 μM was bound to PS and about 3 μM to arsenazo III (pH = 7.4). Free Ca^{2+} was less than 1 μM. Chloride salts (except when indicated otherwise) of the indicated cations were added and Ca^{2+} release was measured in the dual-wavelength mode (A650-A750). Cation-induced Ca^{2+} release was completed within 1 min. T = 26°C

are present in sufficient concentration and carry negative charges, acidic phospholipids (mainly PS) and rhodopsin. Therefore, the binding of Ca^{2+}, H^+, and neutral red to ROS was compared with their binding to PC liposomes containing acidic phospholipids, or with PC liposomes containing rhodopsin. Multiwalled liposomes rather than unilamellar vesicles were used, so that ionophores could control the accessibility of cations to the large majority of the membrane surface in the same way as was done in the experiments with ROS (Figs. 4, 5 and 6).

At first, the binding of Ca^{2+} and its subsequent release induced by monovalent cations was measured in Ca^{2+}-depleted intact and leaky ROS, and in PC liposomes containing 15% acidic phospholipids (PS, PA or PI). The suspension medium contained only impermeable organic cations (other experimental conditions such as ionophores were as described in the legend of Fig. 7). When 16 μM Ca^{2+} was added to any of the above preparations containing 120 μM PS (PA or PI), Ca^{2+} was quantitatively removed from the suspension medium lowering its free Ca^{2+} concentration to less than 1 μM as determined by arsenazo III. Subsequent addition of permeable monovalent cations caused the release of part of the Ca^{2+} bound (illustrated for intact ROS in Fig. 2 and for PC-PS liposomes in Fig. 7). Similar results were obtained for PC-PA, for PC-PI liposomes, and for leaky ROS. In contrast to the above results, PC liposomes or rhodopsin-PC recombinants did not bind any noticeable amount of Ca^{2+} regardless of the salt concentration.

Figure 8 illustrates a different example of the similarity between ROS (broken line) and PC-PS liposomes (solid line) with respect to Ca^{2+} binding. A23187 and FCCP were present at the start of the traces. First, addition of valinomycin was accompanied by the uptake of Ca^{2+}, probably caused by the release of internal K^+. Subsequent addition of gramicidin caused the uptake of some more Ca^{2+}, probably reflecting the release of internal Na^+ (note that the liposomes were prepared in a solution containing both Na^+ and K^+). This Ca^{2+} uptake represents the reverse process of the cation-induced Ca^{2+} release illustrated in Figs. 2 and 7. Next, 10 mM NH_4OAc was added. This caused a step increase in the internal ionic strength and a large Ca^{2+} release was observed. Ammonium acetate is a permeant electrolyte due to the permeation of the neutral species ammonia and acetic acid. Finally, addition of 0.2% Triton X-100 solubilized the membranes and caused a complete release of Ca^{2+}. The dispersed components of both ROS and liposomes showed little Ca^{2+} buffering at the low free Ca^{2+} concentration used. This suggests that the large majority of the Ca^{2+} buffer sites in ROS were groups located at the membrane surface. The pattern of changes in Ca^{2+} binding induced by the different perturbations was very similar in ROS as compared with PC-PS liposomes.

PC liposomes containing 15% acidic phospholipids (PS, PA, or PI) bound neutral red in a manner very similar to that observed in ROS. The protonated form of neutral red predominated in the membrane, and the absorption peak was shifted to 540 nm as observed in ROS (Schnetkamp et al., 1981). In PS-PC liposomes the spectrum of bound neutral red was identical to that observed in ROS; broader and less red-shifted spectra were observed in PC-PA or PC-PI liposomes (not illustrated). The cation-induced release of neutral red in liposomes containing acidic phospholipids did not depend on the type

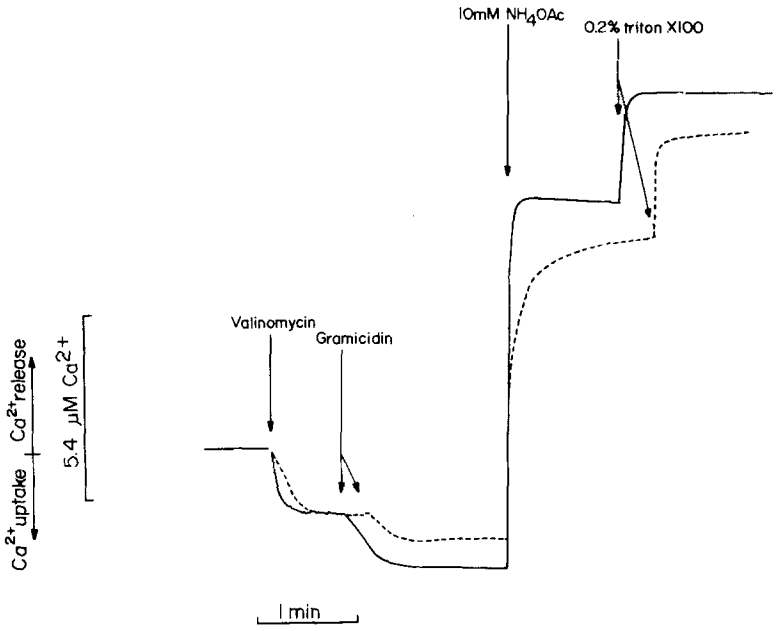

Fig. 8. Properties of Ca^{2+} buffering in PC-PS liposomes and in intact ROS. PC-PS (5 : 1) liposomes were prepared in 600 mM sucrose, 10 mM HEPES, 4.4 mM arginine, 2 mM KCl, 2 mM NaCl, 750 μM $CaCl_2$, 6 mM PS (pH = 7.4). The liposomes were diluted in 600 mM sucrose, 10 mM HEPES, 4.4 mM arginine, 110 μM arsenazo III, 2 μM FCCP, 2 μM A23187 to a final concentration of 120 μM PS (pH = 7.4). Ca^{2+}-enriched ROS were suspended in 600 mM sucrose, 20 mM HEPES, 8.8 mM arginine, 2 μM A23187, 2 μM FCCP, 3.2 μM rhodopsin (about 30 μM acidic phospholipids), 110 μM arsenazo III (pH = 7.4). Both in ROS and liposomes less than 10% of the arsenazo III was bound to Ca^{2+}, and the free Ca^{2+} concentration was less than 1 μM. Ca^{2+} uptake and release was monitored in ROS (broken line) and the liposomes (solid line) by changes in (A650-A750) recorded in the dual-wavelength mode. At the arrows the indicated additions were made; both valinomycin and gramicidin were added to a final concentration of 2 μM. T = 26°C

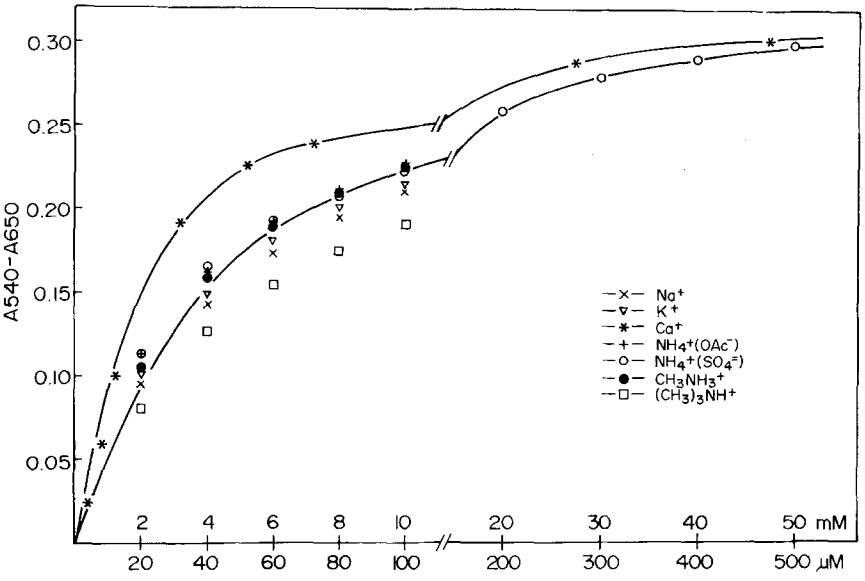

Fig. 9. Cation-induced neutral red release in PC-PS (5 : 1) liposomes. Liposomes were suspended in 600 mM sucrose, 10 mM HEPES, 4.4 mM arginine, 10 μM neutral red, 120 μM PS, 2 μM nigericin, 2 μM A23187 (pH = 7.4). Cation-induced neutral red release was followed in the dual-wavelength mode with the wavelength pair, 540 and 650 nm. A decrease in (A540-A650) was plotted upward. Release of neutral red was induced by the indicated cations (chloride salts unless indicated otherwise) and was completed within 1 min. The μM scale applies to Ca^{2+}; the mM scale applies to the monovalent cations. T = 26°C

of cation, but on the valency (illustrated in Fig. 9 for PC-PS liposomes). In contrast, no absorption of protonated neutral red could be detected in PC liposomes or in rhodopsin-PC recombinants regardless of the salt concentration.

Cation-induced proton release was also observed in PC-PS liposomes, but differed quantitatively from that in ROS. At pH 6.3 in the suspension medium Ca^{2+} caused a maximal release of 0.4 mol H^+/mol PS from PC-PS (5 : 1) liposomes (monitored with bromcresol purple, gramicidin and A23187 present). Half-maximal H^+ release occurred at 40 μM Ca^{2+} similar as observed in ROS (about 50 μM, *see* Schnetkamp & Kaupp, 1985). At pH 7.6 in the suspension medium Ca^{2+}-induced H^+ release was reduced to 0.15 mol H^+/mol PS (monitored with fenol red). In contrast, Ca^{2+}-induced proton release in intact ROS increased slightly from pH 6.3 to 7.6 and amounted to 1.5–2.0 mol H^+/mol PS (Schnetkamp & Kaupp, 1985).

Is Cation-Induced Ca^{2+} Release Caused by Internal pH Changes?

In the previous sections the case was advanced that the cation-induced release of Ca^{2+} from disk membranes or from PC-PS liposomes reflected an over-

all electroneutral exchange controlled by the surface potential of these intracellular membranes. Changes in the external cation concentration were communicated to intracellular compartments either by the use of amines or by the use of ionophores. The use of cation-proton exchangers as ionophores and the efficiency of amines may suggest that the observed changes in Ca^{2+} binding were caused by pH changes rather than by changes of the surface potential.

This alternative explanation was investigated in two ways. First, different ammonium salts were used, sulfate as an impermeant anion and acetate as a salt of a weak acid. In the case of ammonium acetate the neutral species rapidly cross membranes and recombine in the intracellular compartments. This causes parallel changes of the external and internal ionic strength without giving rise to pH changes. In the case of ammonium sulfate only ammonia permeates through membranes and recombines in the intracellular space with protons originating from intracellular buffers. This could result in an increase in the intracellular pH. The two ammonium salts were tested in both ROS and PC-PS liposomes (Figs. 2, 7, and 9); they caused identical patterns of Ca^{2+} release and of neutral red release, suggesting that these release patterns were not caused by putative changes of the intracellular pH.

In the second set of experiments different potassium salts were used; chloride as an impermeant anion, thiocyanate as a permeant anion, and acetate as an anion of a weak acid. The rationale for this experiment is schematically depicted in Fig. 10. In the presence of the electrogenic K^+ carrier, valinomycin transport of KSCN through bilayer membranes takes place due to the permeable anion SCN^-. K^+ transport is limited to K-H exchange when chloride is the anion and both valinomycin and the electrogenic proton carrier FCCP are present (with only valinomycin present little mass transport of K^+ occurs, but instead an inside-positive diffusion potential develops). KOAc is transported when both valinomycin and FCCP are present, while in the presence of valinomycin alone the internal pH becomes acidic (due to the permeation of HOAc) and a K^+ diffusion potential develops. Figure 11 illustrates the results of an experiment on K^+-induced Ca^{2+} release in PC-PS liposomes containing the Ca^{2+} ionophore A23187. No net Ca^{2+} movements were observed unless a K^+ ionophore was present. At the first arrow 1 μM valinomycin was added, which caused a prominent Ca^{2+} release, but only in the case of KSCN. In the case of KCl or KOAc a diffusion potential is expected to be established by the electrogenic transport of K^+, preventing a significant equilibration of the chemical K^+ gradient. No large Ca^{2+} release was observed in this

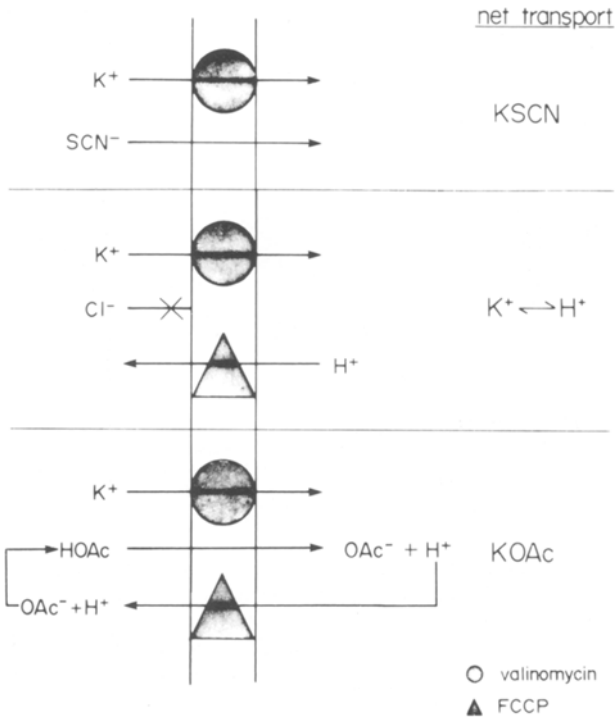

Fig. 10. K^+ transport across biological membranes as a function of different ionophores and anions. The vertical lines represent the diffusion barrier constituted by a phospholipid bilayer membrane. Net transport of potassium salts in the presence of the electrogenic carrier valinomycin (filled circle) can occur when a permeable anion (thiocyanate) is present or when the potassium salt of a weak acid (acetic acid) is used in combination with the electrogenic proton carrier FCCP (filled triangle)

case, suggesting that Ca^{2+} release could not be elicited by changes of the membrane potential across the liposome membrane in agreement with the electroneutral operation of A23187. At the second arrow FCCP was added, which caused a rapid Ca^{2+} release for all three potassium salts. The final amounts of Ca^{2+} release showed little dependence on the nature of the anion. These results suggest that K^+-induced Ca^{2+} release reflected the increase in the internal K^+ concentration and that putative pH changes did not contribute significantly to this Ca^{2+} release. The proton fluxes through FCCP and A23187 were required to couple Ca^{2+} and K^+ fluxes.

The above experiment was repeated with intact ROS and the same results were obtained except for that KSCN did not cause any Ca^{2+} release when only valinomycin was present (*see also* Fig. 4). This is consistent with the observation that the large majority of Ca^{2+} buffer sites are located in the intradiskal space (Fig. 5), where the counterions for the fixed anionic sites greatly outnumber those of osmotic anions.

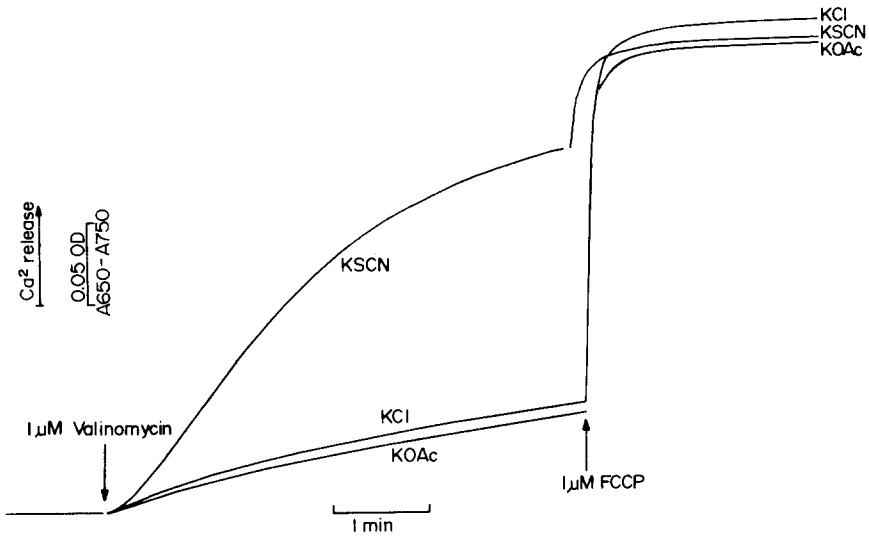

Fig. 11. Anion-dependence of K^+-induced Ca^{2+} release in PC-PS (5 : 1) liposomes. Liposomes were suspended in 600 mM sucrose, 10 mM HEPES, 4.4 mM arginine, 110 μM arsenazo III, 2 μM A23187, 120 μM PS (pH = 7.4). The liposomes contained 16 μM bound Ca^{2+}. Ca^{2+} release was monitored in the dual-wavelength mode by the increase in (A650-A750). The potassium salts were present at 20 mM and their addition did not cause any Ca^{2+} release from liposomes when no potassium ionophore was present. This is indicated by the horizontal trace prior to addition of valinomycin. Ionophores were added at the arrows as indicated. Total K^+-induced Ca^{2+}-release after addition of both valinomycin and FCCP was about 13.5 μM. T = 26°C

Discussion

Properties and Localization of Ca^{2+} Buffer Sites in ROS

As illustrated in the first two figures of this paper, ROS membranes can adsorb large amounts of Ca^{2+} or H^+ at rather low free concentrations in the suspension medium. For example, 6 mol Ca^{2+}/mol rhodopsin represents an overall concentration of 18 mM at a free Ca^{2+} concentration of about 1 μM (Fig. 2); 15 mol H^+/mol rhodopsin equates to an overall H^+ concentration in ROS of 45 mM at pH 6.3 (Fig. 1). These results were obtained in the presence of ionophores that shunt ROS membranes for protons, alkali cations, and Ca^{2+}. The amount of Ca^{2+} or H^+ associated with ROS membranes depended on the internal ionic strength and was greatest at low ionic strength. As discussed in the context of Figs. 1 and 2, at low ionic strength the number of cations associated with ROS membranes could outnumber those in the aqueous compartments by as much as a factor of ten.

Ca^{2+} and protons associated with the disk membranes served to electrically neutralize the negative charges confined to the surface of the disk membrane. This explanation is suggested in particular by the observation that the monovalent cations tested were equally effective to release Ca^{2+} or H^+, and that divalent cations were much more effective than monovalent cations in releasing protons. The observations are consistent with the theory of electrostatic surface potentials (Davis & Rideal, 1963; McLaughlin, 1977). The distribution of the dye neutral red is a measure of this surface potential (Schnetkamp et al., 1981). The binding of neutral red to disk membranes displayed a similar sidedness and cation dependence as observed for the binding of Ca^{2+} (*compare* Figs. 5 and 6). Ca^{2+} associated with negatively charged residues at the surface of the disk membrane constituted the predominant Ca^{2+} buffer in intact ROS (Figs. 2 and 8). The buffer sites could rapidly exchange Ca^{2+} for H^+ (Schnetkamp & Kaupp, 1985) or for monovalent cations (Figs. 2 and 8). The accessibility of these sites to exchange Ca^{2+} depended on the permeability of the disk and plasma membrane (Figs. 4 and 5), not on the properties of the buffer sites. At physiological pH the overall concentration of these membrane-associated cations was about 60 mEq (20 charge eq/mol rhodopsin). On the assumption that the cytoplasmic volume in ROS is 50% of the total volume, this concentration is equivalent to a cytoplasmic concentration of 120 mM. Accordingly, at normal ionic strength the pool of cations that serves to neutralize fixed negative charges on the disk membranes is as large as the pool of osmotically active cations in the cytoplasm; membrane-associated cations do not contribute to the osmotic pressure.

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constitutes at most 5% of the total membrane surface area and did not contribute significantly to the Ca^{2+} buffer sites. The large majority of the Ca^{2+} buffer sites appeared to be associated with the intradiskal surface of the disk membranes. This was concluded from the observation that externally added K^+ could only release Ca^{2+} from the disk membranes in leaky ROS when an appropriate K^+ ionophore made the intradiskal space accessible (Fig. 5). The intradiskal aqueous volume amounts to about 10% of the total volume of ROS (Chabre & Cavaggioni, 1975; Yeager, Schoenborn, Engelman & Stryer, 1981). Therefore, the net concentration of single negatively charged residues on the membrane surface in the intradiskal space is about 0.5 M. This value is most likely several times higher than that of osmotically active salts, and implies that the large majority of cations in the intradiskal space have an immobile anion as counterion. As a result, transport of cations in and out of disks is confined to cation-cation exchange. This exchange can take place by obiligatory exchangers such as A23187 and nigericin (Figs. 5, 6 and 9) or by net compensating currents carried by electrogenic ionophores such as valinomycin and FCCP (Figs. 4 and 11).

Exchange between Ca^{2+} and Other Cations of the Ca^{2+} Buffer Sites

Electrical neutralization of surface charges can be achieved in two ways: first by charge compensation (i.e., binding or adsorption), and second by screening. Screening means that the neutralizing cations are located in the so-called diffuse double layer. This is a layer of diffusible counterions typically extending some tens of Å from the membrane surface. This diffuse double layer is a result of the electrostatic potential generated by the fixed negative charges (Davis & Rideal, 1963; McLaughlin, 1977). The following arguments favor the notion that Ca^{2+}, H^+, and neutral red are not diffuse double-layer ions, but instead are predominantly adsorbed or bound to the disk membrane.

1) Mn^{2+} could substitute for Ca^{2+} in experiments such as that shown in Fig. 2. Binding of Mn^{2+} to PS vesicles can be monitored by electronparamagnetic resonance (EPR) due to broadening of the absorption lines (Puskin, 1977; Puskin & Coene, 1980). The EPR spectrum of Mn^{2+} associated with the disk membranes was completely broadened out (data not shown), and this suggests that Mn^{2+} and, likewise Ca^{2+} are predominantly adsorbed or bound to the disk membrane.
2) If cation-induced proton release involved protons located in the diffuse double layer, unreasonably large surface potentials of −300 mV or more negative have to be postulated. Therefore, cations release protons from disk membranes that were previously bound.
3) The change in the spectral properties of neutral red associated with disk membranes indicates that the dye is adsorbed at the membrane surface.

Monovalent cations caused the release of Ca^{2+}, H^+, and neutral red in a nonspecific way (Figs. 1–3) consistent with a screening action. Bound cations are released from binding sites and replaced by screening cations located in the diffuse double layer. In this scenario the charge density of the disk membrane must change dramatically in our experiments because up to 15 positive charges bound per rhodopsin molecule can be released from the membrane and replaced by diffuse double layer cations; the initial charge density is estimated to be only 1.5 e^-/rhodopsin (Schnetkamp et al., 1981; Kitano et al., 1983). The total number of cations associated with the disk membranes does not change, only their distribution between bound cations and diffuse double layer cations.

A difficulty with this description arises upon a closer inspection of the K^+-induced release of Ca^{2+} illustrated in Fig. 4. Electroneutrality in this K-Ca exchange is illustrated by its ionophore dependence and is enforced by the fact that no large amount of uncompensated charge transport through the plasma membrane can occur. If each bound Ca^{2+} was replaced by 2 K^+ located in the diffuse double layer, a charge separation would take place at the surface of the membrane. The surface potential would become more negative, and no Ca^{2+} release should occur. However, Ca^{2+} release was observed, suggesting that all diffusible counterions for the fixed negative charges are bound to these anionic sites and are not located in a diffuse double layer.

Molecular Basis of the Ca^{2+} Buffer Sites in ROS

The quantity and localization of Ca^{2+} buffer sites in ROS limit their possible molecular source to two components of the disk membrane, the carboxyl groups of phosphatidylserine [about 9 mol PS/mol rhodopsin (Daemen, 1973; Stone, Farnsworth & Dratz, 1979)] and surface residues on the rhodopsin molecule. Considering the pK's of the different groups of PS (reviewed by Cevc, Watts & Marsh, 1981), it is unlikely that the phosphate groups of PS are protonated at neutral pH. According to the rhodopsin structure proposed by Ovchinnikov (1982) both the part of the rhodopsin molecule protruding into the cytoplasm and that protruding into the in-

tradiskal space contain 9–10 glutamate or aspartate groups. The observed release of 15 mol H^+/mol rhodopsin can be accounted for only when a significant part of the carboxyl groups of either PS or rhodopsin or of both are protonated at low ionic strength. Increasing the ionic strength decreases the surface potential, increases the surface pH, and the carboxyl groups deprotonate.

If one assumes that rhodopsin is the main contributor to the Ca^{2+} and proton binding sites, the observed sidedness of these sites is consistent with the strongly asymmetric charge distribution on the rhodopsin molecule. The cytoplasmic surface of rhodopsin has a net charge of +3, whereas the intradiskal surface carries a net charge of −7 (Ovchinnikov, 1982). On the other hand, rhodopsin reconstituted in PC liposomes did not show any of the Ca^{2+} or neutral red binding characteristics displayed by the disk membrane.

The case for PS as the major contributor to the Ca^{2+} binding sites can be advanced from the observations that the PS in PC-PS liposomes mimicked disk membranes in several characteristic properties such as the binding of Ca^{2+}, the biniding of neutral red, and the change of the spectral properties of this dye. In order to obtain the observed stoichiometry of proton release both the carboxyl and the amino proton of PS have to contribute to cation-induced proton release. Ca^{2+}-induced proton release from ROS quantitatively differed from that in PC-PS liposomes, but no fundamentally new properties of PS have to be postulated. The amino proton of PS is released by Mn^{2+} and other transition metal ions (Puskin & Coene, 1980; McLaughlin, 1981), whereas at lower pH values in the solution an increase of the ionic strength causes the release of the carboxyl proton from PS (McLaughlin, 1982; this study). As a result, Mn^{2+} binding to PS vesicles shows a strong pH dependence between pH 7 and 9 (Puskin & Coene, 1980). A similar pH dependence has been observed for Ca^{2+} binding in ROS (Kaupp, Schnetkamp & Junge, 1981). These results suggest that the known properties of PS in model systems can account for the basic observations in ROS if one allows for shifts in the pK of the carboxyl group. A possible cause for this shift could be the close proximity of the intradiskal membrane surfaces, which impedes a proper development of a diffuse double layer. This particular molecular model requires that about 85% of the PS in stacked disks is located in the inner leaflet of the disk membrane. This is in conflict with reports on the PS distribution in isolated disk preparations, where 50% (Drenthe, Klompmakers, Bonting & Daemen, 1980) to more than 80% (Miljanich, Nemes, White & Dratz, 1981) of the PS was found to be located on the outer leaflet of the disk membrane. It is possible that destacking and swelling of disks causes a redistribution of phospholipids in the disk membrane. Such a redistribution is suggested by the change in sidedness of the rapid light-induced Ca^{2+} release observed in different types of disk preparations (Kaupp et al., 1981). The striking similarities between ROS and PC-PS liposomes with respect to the binding of Ca^{2+} and neutral red suggest that PS is a serious candidate for the Ca^{2+} buffer sites in ROS.

Neutral Red Distribution as a Probe to Measure Ionic Permeabilities

The data shown in Figs. 3 and 9 demonstrate that the binding of neutral red to ROS or PC-PS liposomes was dependent in a simple manner on the internal cation concentration. In both systems the greater part of the membrane surface is inaccessible to the external solution and changing the internal cation concentration requires addition of cations to the external solution together with appropriate ionophores to move the cations across the membrane. The changes in neutral red binding were dependent on the valency, but not on the type of cation. These changes can be used to quantitate cation movements across the ROS plasma membrane or across the liposome membrane. Changes in neutral red binding can be measured in two ways. First, the distribution of neutral red can be measured by sedimenting the particles and measuring the concentration of neutral red remaining in the supernatant (illustrated in Fig. 3). Secondly, at neutral pH the spectral properties of neutral red in the aqueous phase are quite different from those of neutral red adsorbed to the membrane. Changes in the binding of neutral red can be monitored in real-time with a dual-wavelength spectrophotometer (the data shown in Figs. 6 and 9 are taken from such measurements). The repartitioning of neutral red offers the following properties as a method to measure ionic permeabilities in suspensions of small particles like ROS or liposomes containing acidic phospholipids:

1) Neutral red rapidly partitions and repartitions in the particles due to the permeability of its neutral form. Changes of the partition coefficient by fivefold were induced by addition of 25 mM NH_4OAc and were complete within 1 sec.
2) Neutral red does not respond to diffusion potentials.
3) Intrinsic calibration curves can be obtained easily by applying the permeant electrolyte ammonium acetate or by using appropriate ionophores.

4) Permeable monovalent cations (alkali cations in the presence of gramicidin; amines) are all equally effective in releasing neutral red, while permeable divalent cations (earth alkali cations in the presence of A23187) are 20–100× more effective than monovalent cations.
5) To measure ionic permeabilities, a step change in the concentration of a particular cation is made in the external medium. In order to allow equilibration of the thus-formed chemical gradient, the membrane has to be electrically shunted. In membrane-rich systems such as ROS or multiwalled liposomes this can be achieved by adding the protonophore FCCP (*cf.* Figs. 4 and 11). In other systems permeant anions such as SCN^{-1} may be used (*cf.* Fig. 11).
6) The partitioning of neutral red in ROS membranes can be measured with a typical precision better than 5%. Within the linear range of the neutral red response the partition coefficient changed by 20–25% when the concentration of monovalent cations was increased by 1 mM, while a 65% change is observed when Ca^{2+} was raised from 1 to 10 μM.
7) The method has the disadvantage of working optimally at relatively low ionic strength (up to 50 mM monovalent cations).

In the companion paper this method has been used to measure the ionic permeabilities in the plasma membrane of isolated intact ROS.

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