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Silver Ions Induce a Rapid Ca^{2+} Release from Isolated Intact Bovine Rod Outer Segments by a Cooperative Mechanism

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Summary. Micromolar concentrations of silver ion activate large Ca^{2+} fluxes across the plasma membrane of intact rod outer segments isolated from bovine retinas (intact ROS). The rate of Ag^+-induced Ca^{2+} efflux from intact ROS depended on the Ag^+ concentration in a sigmoidal manner suggesting a cooperative mechanism with a Hill coefficient between 2 and 3. At a concentration of 50 μM Ag^+ the rate of Ca^{2+} efflux was 7×10^6 Ca^{2+}/outer segment/sec; this represents a change in total intracellular Ca^{2+} by 0.7 mM/outer segment/sec. Addition of the nonselective ionophore gramicidin in the absence of external alkali cations greatly reduced the Ag^+-induced Ca^{2+} efflux from intact ROS, apparently by enabling internal alkali cations to leak out. Adding back alkali cations to the external medium restored Ag^+-induced Ca^{2+} efflux when gramicidin was present. In the presence of gramicidin, Ag^+-induced Ca^{2+} efflux from intact ROS was blocked by 50 μM tetracaine or L-*cis* diltiazem, whereas without gramicidin both blockers were ineffective. Both L-*cis* diltiazem and tetracaine are blockers of one kinetic component of cGMP-induced Ca^{2+} flux across ROS disk membranes. The ion selectivity of the Ag^+-induced pathway proved to be broad with little discrimination between the alkali cations Li^+, Na^+, K^+, and Cs^+ or between Ca^{2+} and Mg^{2+}. The properties of the Ag^+-induced pathway(s) suggest that it may reflect the cGMP-dependent conductance opened in the absence of cGMP by silver ions.

Key Words photoreceptors · vision · ion channels · silver ions · sulfhydryl modification · Ca^{2+} transport

Introduction

The plasma membrane of the outer segments of vertebrate rod photoreceptors contains two known pathways for the transport of Ca^{2+}. The light-sensitive conductance of rods is a cGMP-dependent conductance (Fesenko, Kolesnikov & Lynbarsky, 1985; Yau & Nakatani, 1985; Zimmerman et al., 1985; Matthews, 1987), which can pass a current of Ca^{2+} (Capovilla et al., 1983; Yau & Nakatani, 1984*b*; Hodgkin et al., 1985). The second pathway for Ca^{2+} transport is Na-Ca exchange (Schnetkamp, 1980; Yau & Nakatani, 1984*b*; Schnetkamp, 1986; Hodgkin, McNaughton & Nunn, 1987; Schnetkamp & Bownds, 1987). Both pathways have a considerable capacity and can change total intracellular Ca^{2+} by as much as 0.1 (amphibian rods) −0.5 (bovine rods) mM/sec. The physiological relevance for such fast Ca^{2+} movements has yet to be established with certainty, but it has been suggested that Ca^{2+} controls light adaptation in vertebrate rod photoreceptors (Koch & Stryer, 1988; Matthews et al., 1988; Nakatani & Yau, 1988).

Silver ions at micromolar concentrations have been reported to stimulate Ca^{2+} release from skeletal sarcoplasmic reticulum; the data suggest that Ag^+ binds to a sulfhydryl group at the physiological site of the Ca^{2+} release channel (Abramson et al., 1983; Salama & Abramson, 1984; Palade, 1987). Ag^+ also induces tension development in frog skeletal muscle, which is thought to be caused by the binding of Ag^+ to a sulfhydryl group on membrane protein(s) in the T-tubules (Oba & Hotta, 1985*a*; *b*). Our study demonstrates that micromolar concentrations of Ag^+ stimulate a large Ca^{2+} release from rod outer segments (ROS) with an intact plasma membrane isolated from bovine retinas. We measured the ion selectivity of the Ag^+-induced pathway with the optical probe neutral red (Schnetkamp, 1985*a*; *b*); the ion selectivity was found to be rather broad with little discrimination between the alkali cations Li^+, Na^+, K^+, and Cs^+, and little discrimination between Ca^{2+} and Mg^{2+}.

Materials and Methods

Bovine eyes were purchased from a local abbatoir and collected in a light-tight box. Rod outer segments (ROS) with an intact plasma membrane and a high Ca^{2+} content were used throughout this study and were isolated and purified as described before (Schnetkamp, 1986). Intact ROS were stored in a medium containing 600 mM sucrose, 5% wt/vol Ficoll 400, and 20 mM HEPES (adjusted to pH 7.4 with arginine). The overall rhodopsin concentration of the stock suspension ranged between 150–

250 μM. Experiments were performed within 3 hr. The isolation procedure and all experimental manipulations were carried out in darkness or under dim red light illumination. In all calculations, isolated intact bovine ROS were assumed to be cylinders of 1 × 20 μm containing an overall rhodopsin concentration of 3 mM or 2.9×10^7 molecules of rhodopsin.

Ag^+-induced Ca^{2+} efflux was measured with the Ca^{2+}-indicating dye Arsenazo III in an SLM-Aminco DW2C dual-wavelength spectrophotometer. The cuvette house was equipped with a magnetic stirrer and was connected to a circulating bath to maintain a constant temperature of 25°C. In all measurements, the dual-wavelength mode was used with the wavelength pair of 650 and 750 nm and a bandwidth of 6 nm. The calibration of the Ca^{2+}-indicating signals was obtained by adding known amounts of Ca^{2+} to a ROS suspension in the cuvette. Arsenazo III was occupied by Ca^{2+} at levels of less than 20%. Validation of the experimental procedure and further details have been documented elsewhere (Schnetkamp, 1986).

In order to prevent precipitation of AgCl, all media were free of chloride; acetate salts of all the cations tested were used. In a few experiments we also tested the permeability of the plasma membrane to choline$^+$ and tetramethylammonium$^+$. In this case the respective chloride salts were used. We found that after a 2-min *preincubation* with $AgNO_3$ chloride salts were as effective as acetate salts, presumably because most of the Ag^+-induced modification of membrane permeability had been completed.

Measurement of Ag^+-Induced Cation Influx with the Dye Neutral Red

Ag^+-induced cation influx across the ROS plasma membrane was measured with the use of the dye neutral red as described previously (Schnetkamp, 1985*a*, *b*). The binding of the dye neutral red to the intracellular disk membranes depends on the surface potential at the disk membrane/water interface, which in turn depends on the internal cation concentration. The surface potential is an electrostatic potential generated by the presence of negatively charged residues (e.g., acidic phospholipids) at the surface of the membrane. Changes in the binding of the dye occur upon transport of cations across the plasma membrane when this transport causes a net increase in the intracellular cation concentration; changes in the binding of neutral red can be measured in real-time in a dual-wavelength spectrophotometer. We chose the wavelength pair of 540 and 650 nm (bandwidth 6 nm) to measure the amount of membrane-bound neutral red; at this wavelength pair changes in aqueous neutral red contribute little (Schnetkamp, Kaupp & Junge, 1981). Validation of the technique has been illustrated before with artificial bilayer membranes or intact ROS and well-defined ionophores such as gramicidin (Schnetkamp, 1985*a*,*b*). Here we illustrate the neutral red technique by showing the effect of ionophores on the alkali cation content of isolated intact ROS (Fig. 1). When intact ROS were suspended in a buffered sucrose medium with 50 μM neutral red, the difference in light absorption (A_{540}–A_{650}) gradually increased indicating an increase in the concentration of bound neutral red; this increase in bound neutral red was caused by a gradual loss of cations, which made the surface potential at the intracellular disk membranes more negative. Changes in light absorption were plotted upward to indicate a loss of cations. When we added the K^+-selective electrogenic carrier valinomycin, the leak of cations (presumably K^+) increased somewhat, but in the absence of a current loop to compensate for the outward K^+ current carried by valinomycin no large K^+ efflux was observed. We have demonstrated before that in isolated intact ROS the electrogenic protonophore FCCP can provide a current loop with another electrogenic carrier and act as an electrical shunt (Schnetkamp, 1985*a*). When both FCCP and valinomycin were added, a rapid and large change in light absorption was observed indicating a large K^+ efflux (addition of FCCP alone had no effect). Subsequent addition of the nonselective ionophore gramicidin caused a further change in light absorption indicating the release of another internal alkali cation different from K^+, i.e., Na^+. When gramicidin was added in the absence of other ionophores a large change in light absorption was observed, indicating the combined release of Na^+ and K^+. The absorption change observed by addition of gramicidin alone was very similar to that observed for the consecutive additions of FCCP/valinomycin and gramicidin. Addition of FCCP/valinomycin following a previous addition of gramicidin did not cause any further changes in light absorption, since K^+ was already released by the addition of gramicidin (*not illustrated*). After the release of internal cations was completed, consecutive steps of 0.2, 2 and 5 mM KCl were added at 60, 74 and 90 sec, respectively. In the absence of ionophores or in the presence of valinomycin, the K^+ additions caused only gradual and small changes in the binding of neutral red indicating little net transport of K^+. In contrast, rapid and large changes in light absorption indicating a rapid increase of intracellular K^+ were observed, when gramicidin was present or when both FCCP and valinomycin were present. The above results illustrate three points. First, internal surface potentials were sensitive to changes in K^+ concentration by as little as 0.2 or 2 mM after internal K^+ was lowered by the addition of gramicidin; this suggests that gramicidin caused the internal K^+ concentration to be lowered to < 1 mM, when no external K^+ was present. Second, the lack of a large and fast K^+ efflux by addition of the electrogenic K^+ carrier valinomycin indicates that the ROS plasma membrane in isolated intact bovine ROS does not contain endogenous conductances that can provide a current loop with the K^+ current carried by valinomycin. Third, the plasma membrane is expected to behave as a K^+ electrode in the presence of a single, dominant external conductance in the form of valinomycin; the binding of neutral red did not show any rapid changes when valinomycin was added or when the external K^+ concentration was changed in the presence of valinomycin demonstrating that binding of the dye neutral red was not affected by changes in membrane potential.

Results

Silver Ions Stimulate Ca^{2+} Efflux from Intact Bovine ROS

Micromolar amounts of silver ions added to a suspension of isolated intact bovine rod outer segments (ROS) stimulated Ca^{2+} release across the plasma membrane (Fig. 2*a*). Bovine ROS used in this and all other experiments were enriched in Ca^{2+} and contained 5–8 mol Ca^{2+}/mol rhodopsin (Schnetkamp, 1986). After a short lag phase of 1–5 sec, Ca^{2+} release was induced as a result of Ag^+ addition; both the rate and amount of Ca^{2+} release depended on the Ag^+ concentration in a sigmoidal

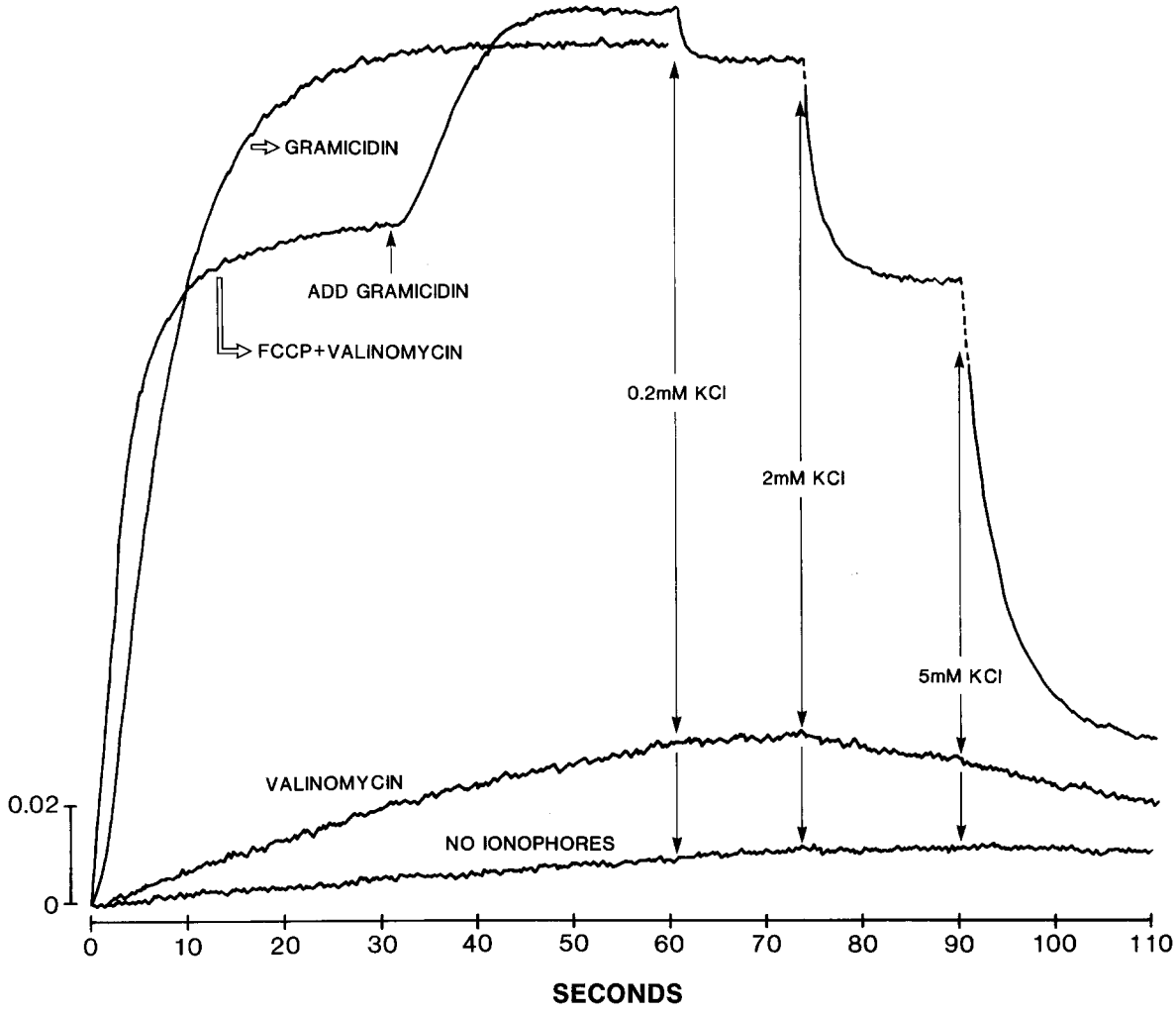

Fig. 1. Effect of ionophores on the alkali cation content of isolated intact ROS. Intact ROS were diluted 20-fold in a medium containing 600 mM sucrose, 20 mM HEPES (adjusted to pH 7.4 with arginine), 1 mM EDTA and 50 μM neutral red. The rhodopsin concentration was 7.0 μM. Dual-wavelength recordings are shown (A_{540}–A_{650}). The traces were started by addition of the indicated ionophores to a final concentration of 1 μM. Changes in light absorption prior to addition of ionophores is represented by the trace labeled "no ionophores." The arrows at 60, 74 and 90 sec, respectively, indicate the addition of consecutive steps of KCl to the indicated final concentration. Artifacts due to the opening of the slit during additions (duration 1 sec) were removed from the traces. The traces shown here and in all other figures were copied by hand from the original recordings. The noise in all traces was caused by the spinbar used to mix the suspension and did not reflect photometric sensitivity

fashion. Addition of 50 μM Ag^+ caused a Ca^{2+} release of 3.5 mol Ca^{2+}/mol rhodopsin after 90 sec and at a maximal rate of 7×10^6 Ca^{2+}/outer segment/sec. The latter number reflects a change in total intracellular Ca^{2+} of 0.7 mM/sec. In the absence of silver ions, Ca^{2+} transport across the plasma membrane of isolated intact bovine ROS is limited to Ca-Ca exchange and Na-Ca exchange as determined with ^{45}Ca fluxes (Schnetkamp, 1980) and the Ca^{2+}-indicating dye Arsenazo III (Schnetkamp, 1986); the maximal rate of Ca-Ca exchange and Na-Ca exchange is about 10^7 Ca^{2+}/outer segment/sec.

The sigmoidal dependence of Ca^{2+} release on the Ag^+ concentration suggests a cooperative mechanism, whereby the binding of more than one silver ion is required in order to activate Ca^{2+} efflux. The data of Fig. 2*a* were analyzed in a Hill plot, which yielded a straight line with a dissociation constant of 31μM for Ag^+ and a Hill coefficient of 3.1 (Fig. 2*b*). In five different ROS preparations, the Hill coefficient varied between 2.2 and 3.1 with an average value of 2.6 ± 0.4 (SD). For these five preparations the average values (± SD) were: dissociation constant 33 μM (5); maximal rate of Ca^{2+} efflux at 50 μM Ag^+ 6.4×10^6 Ca^{2+}/outer segment/

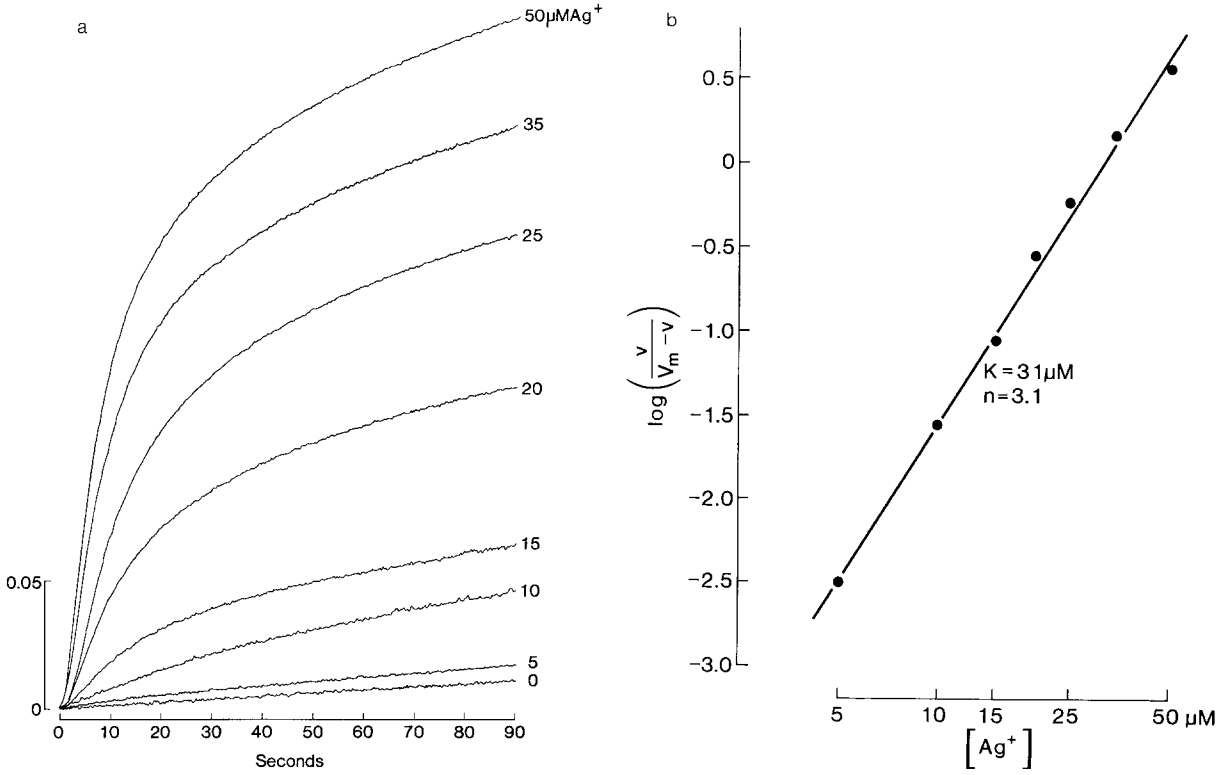

Fig. 2. Ag^+ activates Ca^{2+} release from intact ROS. Intact ROS were diluted 50-fold in a medium containing 600 mM sucrose, 50 mM KOAc, 1 μM gramicidin, 200 μM Arsenazo III, and 20 mM HEPES (adjusted to pH 7.4 with arginine). The final rhodopsin concentration was 3.4 μM. Ca^{2+} release was intitiated by addition of $AgNO_3$ at time zero to the indicated final concentrations. Ca^{2+} release was measured by an increase in the absorption difference A_{650}–A_{750}. The calibration bar of 0.05 absorbance unit represents 0.65 mol Ca^{2+}/mol rhodopsin. The optimal rate of Ca^{2+} release, v, developed after a short (1–5 sec) lag period and was calculated for each Ag^+ concentration. The maximal rate V_m of Ca^{2+} release at high Ag^+ concentration was extrapolated, and the results were plotted in a Hill plot. Temperature: 25°C

sec (0.6); amount of Ca^{2+} released 90 sec after addition of 50 μM Ag^+ 3.6 mol Ca^{2+}/mol rhodopsin (0.2).

Ionic Requirements for Silver-Induced Ca^{2+} Efflux

Addition of gramicidin D, a nonspecific ionophore for alkali cations and protons (Myers & Haydon, 1972), reduced the Ag^+-induced Ca^{2+} release by several-fold when the suspension medium contained only sucrose and buffer without alkali cations (Fig. 3); in a medium free of alkali cations, gramicidin caused a drastic depletion of intracellular K^+ and Na^+ (Fig. 1). Addition of external K^+ in the presence of gramicidin restored both intracellular K^+ levels (Fig. 1) and Ag^+-induced Ca^{2+} efflux, whereas addition of K^+ in the absence of gramicidin had little effect on the rate and amount of Ag^+-induced Ca^{2+} release (Fig. 3). The most prominent effect of KOAc in the absence of gramicidin is to increase the lag phase between Ag^+ addition and ensuing Ca^{2+} efflux from ROS (the lag phase was most conspicuous at reduced temperature, Fig. 3*b*). The above results suggest that Ag^+-induced Ca^{2+} release across the ROS plasma membrane requires internal, but not external alkali cations. In the absence of gramicidin, intact ROS contained sufficient alkali cations (40–50 mM Na^+ + K^+ were released by addition of gramicidin as measured by atomic absorption spectroscopy) to activate the Ag^+-induced Ca^{2+} efflux. Other alkali cations such as Li^+ and Cs^+ could replace K^+ in restoring Ag^+-induced Ca^{2+} efflux; the dependence of Ag^+-induced Ca^{2+} release on the concentration of the alkali cations is illustrated for Li^+ and K^+ in Fig. 4. In the absence of Ag^+, changes in the concentration of all alkali cations except for Na^+ did not cause any Ca^{2+} efflux from intact ROS irrespective of the presence of gramicidin; addition of Na^+ causes Ca^{2+} efflux by Na-Ca exchange (Schnetkamp, 1986).

Cation-depletion by addition of gramicidin could inhibit Ag^+-induced Ca^{2+} efflux in two ways. First, it could reduce the efficacy of Ag^+ to modify

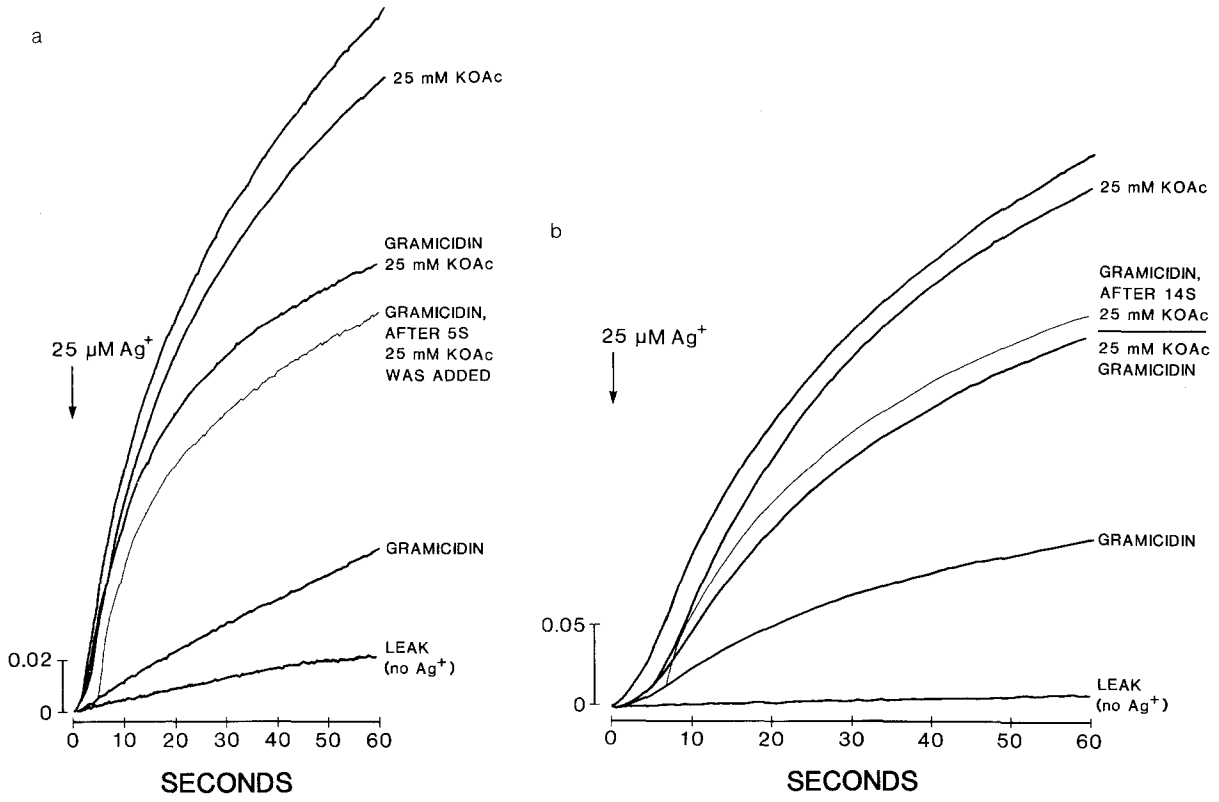

Fig. 3. Effect of K^+ on Ag^+-induced Ca^{2+} release. Intact ROS were diluted 50-fold in a medium containing 600 mM sucrose, 20 mM HEPES (adjusted to pH 7.4 with arginine), 100 μM Arsenazo III, 25 mM KOAc (as indicated), 1 μM gramicidin (as indicated). The rhodopsin concentration in the suspension amounted to 3.5 μM. Ca^{2+} release was initiated by addition of 25 μM $AgNO_3$ at time zero except for the leak trace where no silver was added (the leak in the absence of Ag^+ was not affected by additions such as gramicidin or KOAc). In two cuvettes, Ag^+ was added to intact ROS in the presence of gramicidin at time zero, followed by addition of 25 mM KOAc a few seconds after addition of Ag^+. The addition of KOAc caused an instantaneous increase in light absorption due to the effect of KOAc itself on the Arsenazo III spectrum; the magnitude of this absorption change was measured by adding KOAc in the absence of Ag^+ and was removed from the traces. The instantaneous absorption change upon addition of KOAc was followed by a time-resolved increase in the rate of absorption change due to Ca^{2+} release from ROS (illustrated by the thin traces); KOAc caused Ca^{2+} release only when both gramicidin and Ag^+ were present. Temperature 25°C (*a*), 12°C (*b*). The calibration bars represented a Ca^{2+} release of 0.25 mol Ca^{2+}/mol rhodopsin (*a*) and 0.61 mol Ca^{2+}/mol rhodopsin (*b*)

its target, or, second, it could affect the flux mechanism that results from modification by Ag^+; in the latter case, the modified flux mechanism requires internal alkali cations for Ca^{2+} efflux. We have investigated these possibilities in the experiment illustrated in Fig. 3. Ag^+-induced Ca^{2+} release always displayed a short lag phase between addition of Ag^+ and onset of Ca^{2+} release; this is most conspicuous in the experiment done at reduced temperature (Fig. 3*b*). In contrast, Na^+-activated Ca^{2+} release in isolated intact bovine ROS (Schnetkamp, 1986) and cGMP-induced Ca^{2+} release in fragmented ROS membranes (Schnetkamp, 1987) never showed a lag phase between addition of Na^+ or cGMP and ensuing Ca^{2+} release. We suggest that the lag phase observed in Ag^+-induced Ca^{2+} release reflects the time required for Ag^+ to modify its target on the ROS plasma membrane. In our experiment illustrated in Fig. 3, we initiated Ca^{2+} efflux by adding Ag^+ at time zero to a suspension of intact ROS in the presence of gramicidin and observed the reduced Ca^{2+} efflux as compared with that in the absence of gramicidin. In a separate cuvette, we repeated the experiment with the gramicidin-treated ROS, but now the addition of Ag^+ was followed after a few seconds by sufficient KOAc to reactivate Ag^+-induced Ca^{2+} efflux (thin traces). The KOAc addition restored Ca^{2+} release, but without any lag phase; this is most conspicuous in the experiment carried out at reduced temperature (Fig. 3*b*). The absence of a lag phase suggests that the reaction of Ag^+ with its target had taken place in the few seconds between the additions of Ag^+ and KOAc, respectively, but that in gramicidin-treated ROS the resulting Ca^{2+} efflux mechanism was inoperative in the absence of K^+; since addition of *external* KOAc

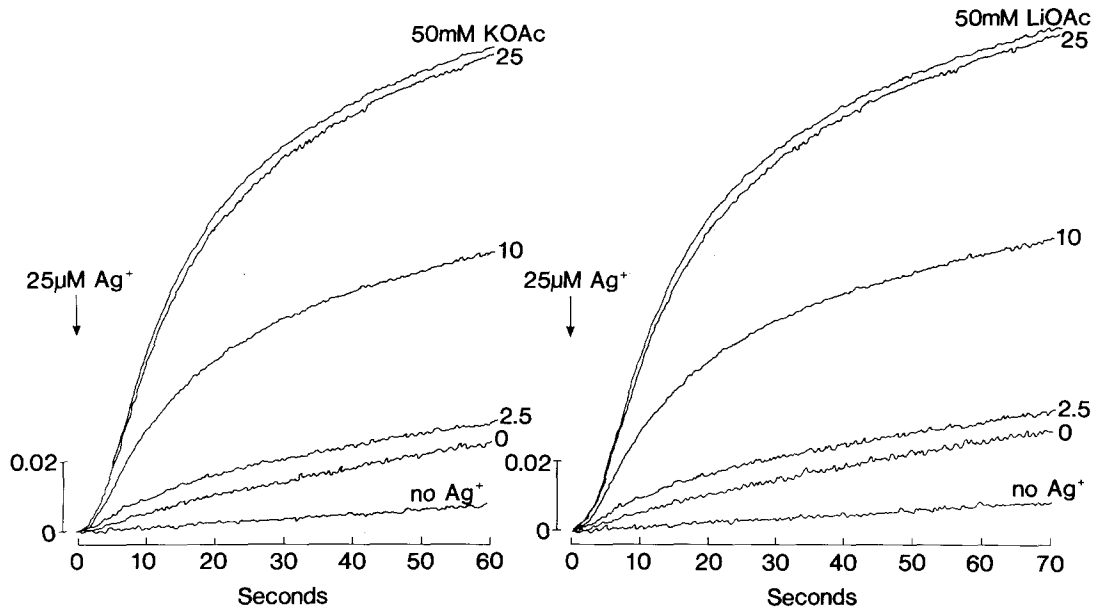

Fig. 4. Ag^+-induced Ca^{2+} release requires cytoplasmic alkali cations. Intact ROS were diluted 50-fold in a medium containing 600 mM sucrose, 200 μM Arsenazo III, 1 μM gramicidin D, 20 mM HEPES (adjusted to pH 7.4), and KOAc or LiOAc as indicated. The final rhodopsin concentration was 3.8 μM. Ca^{2+} release was initiated at time zero by addition of 25 μM $AgNO_3$ except for the trace labeled "*no* Ag^+". The latter indicates Ca^{2+} leakage in the absence of Ag^+. This leakage was the same at all concentrations of KOAc and LiOAc. The calibration bar of 0.02 absorbance unit represents 0.23 mol Ca^{2+}/mol rhodopsin. Temperature: 25°C

in the absence of gramicidin (and thus in the absence of changes in *internal* K^+) had no effect (Fig. 3), this result again suggests that *internal* K^+ was required for the Ca^{2+} efflux mechanism.

Blockers of Silver-Induced Ca^{2+} Efflux

The plasma membrane of isolated intact bovine ROS contains two ion transporting proteins, a cGMP-dependent conductance and a Na-Ca exchanger. Both tetracaine and L-*cis* diltiazem (at 50 μM) block cGMP-induced Ca^{2+} fluxes across ROS disk membranes (Koch & Kaupp, 1985; Schnetkamp, 1987) and cGMP-dependent currents in excised patches of bovine ROS plasma membrane (F.N. Quandt, G.D. Nicol, & P.P.M. Schnetkamp, *in preparation*), but have little effect on Na-Ca exchange (*not shown*). We have used both blockers of the cGMP-dependent conductance to address the question whether the Ag^+-induced Ca^{2+} release is more similar in this respect to cGMP-dependent Ca^{2+} fluxes than to Na^+-induced Ca^{2+} fluxes.

The Ag^+-induced Ca^{2+} release occurred regardless of the presence of gramicidin when 50 mM KOAc was present in the suspension medium (Fig. 3). However, the sensitivity of Ag^+-induced Ca^{2+} efflux to the blockers tetracaine and L-*cis* diltiazem was altered by gramicidin. In the absence of gramicidin neither L-*cis* diltiazem nor tetracaine (at 50 μM) had a significant effect on Ag^+-induced Ca^{2+} release, but in the presence of gramicidin both drugs were potent blockers (Fig. 5). In the case of diltiazem, the L-*cis* isomer was more effective than the D-*cis* isomer, similar as observed for the cGMP-induced Ca^{2+} release from ROS disks (Koch & Kaupp, 1985; Schentkamp, 1987).

Ion Selectivity of Silver-Induced Cation Influx in Intact ROS

The above experiments employed Arsenazo III to measure Ca^{2+} efflux from ROS. A more general technique to measure cation influx in intact ROS uses the dye neutral red (Schnetkamp, 1985*a*,*b*; Materials and Methods). The binding of neutral red to the intracellular disk membranes is dependent on the surface potential at the disk membrane/water interface. This surface potential is caused by the presence in the disk membrane of fixed negative charges such as acidic phospholipids (e.g., phosphatidylserine). In the experiment illustrated in Fig. 6, intact ROS were suspended in a sucrose medium to which at time zero 20 mM $Ca(OAc)_2$ or $Mg(OAc)_2$ was added. As Ca^{2+} or Mg^{2+} crossed the plasma membrane, they increased the internal cation concentration and lowered the surface potential. This was monitored by changes in absorption (A_{540}–A_{650}) illustrated in Fig. 6 (changes in light ab-

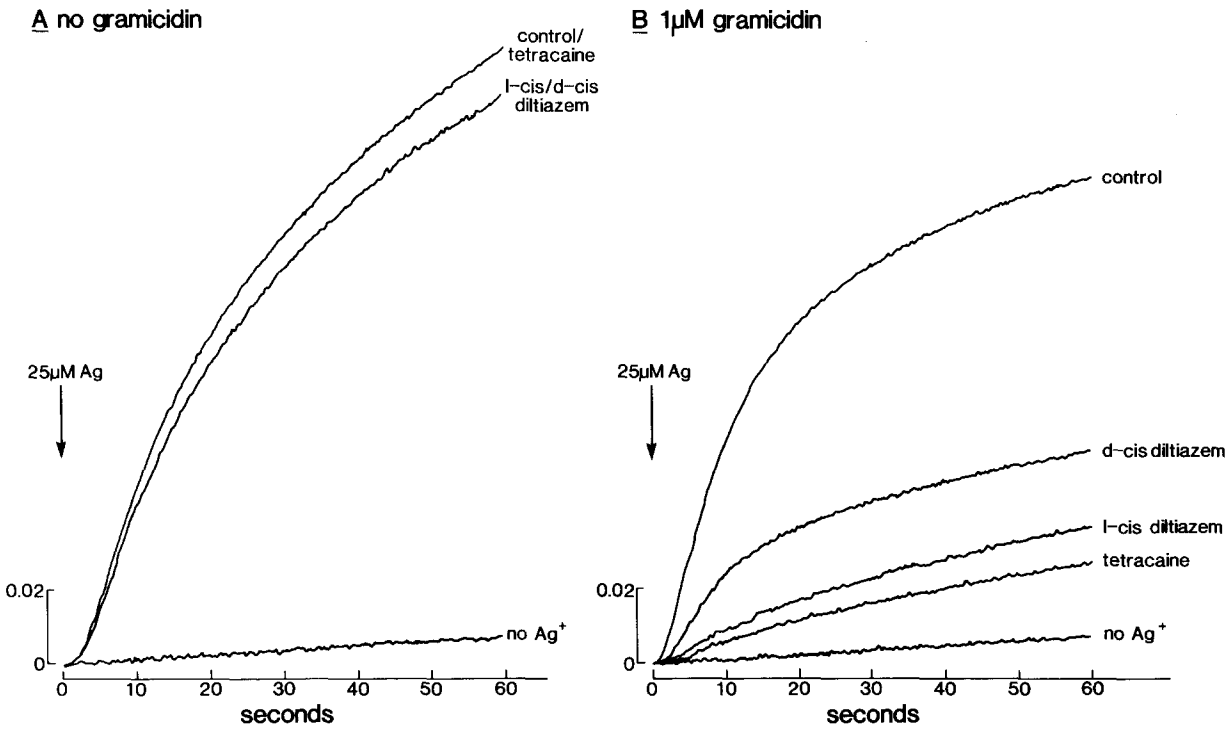

Fig. 5. Blockers of the Ag^+-induced Ca^{2+} release. Intact ROS were diluted 50-fold in a medium containing 600 mM sucrose, 50 mM KOAc, 200 μM Arsenazo III, 20 mM HEPES (adjusted to pH 7.4 with arginine), and gramicidin D as indicated. The rhodopsin concentration was 3.4 μM. The blockers L-*cis* diltiazem, D-*cis* diltiazem, and tetracaine were present at a concentration of 50 μM. Ca^{2+} release was initiated by addition of 25 μM $AgNO_3$ except for the trace labeled "*no* Ag^+." The latter indicates Ca^{2+} leakage prior to the addition of Ag^+. The different blockers added did not alter the leakage rate. The calibration bar of 0.02 absorbance unit represents 0.26 mol Ca^{2+}/mol rhodopsin. Temperature: 25°C

sorption indicating an increase in internal cation concentration are plotted upward). In the absence of Ag^+, the absorption changes upon addition of Ca^{2+} or Mg^{2+} were identical and very slow, indicating that the ROS plasma membrane was not very permeable to these cations. Addition of the divalent cation ionophore A23187 caused a rapid absorption change indicating a rapid influx of Ca^{2+} that equilibrated the Ca^{2+} gradient. When ROS were preincubated with 25 μM Ag^+, subsequent addition of Ca^{2+} or Mg^{2+} caused a rapid change in absorption indicating a greatly increased permeability of the plasma membrane to Ca^{2+} and Mg^{2+}. Addition of A23187 equilibrated the Ca^{2+} gradient and the total absorption change was about the same as that observed in the absence of Ag^+. These results indicate that Ag^+ increases both Ca^{2+} influx (Fig. 6) and efflux (Figs. 2 and 3).

Ag^+-induced Ca^{2+} efflux was blocked by gramicidin in the absence of alkali cations (Fig. 3). In the experiment illustrated in Fig. 6, the trace labeled "gramicidin" reflects the Ag^+-induced Ca^{2+} influx when gramicidin was added prior to the addition of Ag^+; this resulted in a reduced Ca^{2+} influx as judged from the smaller and slower absorption change. The above results demonstrate that both methods for measuring the Ca^{2+} permeability of the ROS plasma membrane produced consistent results.

The "neutral red" technique is not limited to divalent cations, but can be applied to alkali cations as well. The experiment illustrated in Fig. 7 investigates the effect of Ag^+ on the permeability of the ROS plasma membrane for alkali cations. The experimental protocol is the same as that discussed for the experiment shown in Fig. 6, except that at time zero 50 mM of the indicated acetate salts were added. In the absence of Ag^+ the plasma membrane showed significant permeabilities to all alkali cation tested as judged by the rate of change in light absorption; the pattern observed is similar to that described before (Schnetkamp, 1985*b*). Preincubation of intact ROS with 25 μM Ag^+ increased the rate of influx for all alkali cations tested as judged from the rate of the change in absorption; in the presence of Ag^+ little ion selectivity among alkali cations was observed (Fig. 7*b*). Addition of gramicidin caused the equilibration of all alkali cation gradients, and the total absorption changes were similar with or without Ag^+. The total absorption changes (0.11–0.13 absorbance units) differed somewhat between

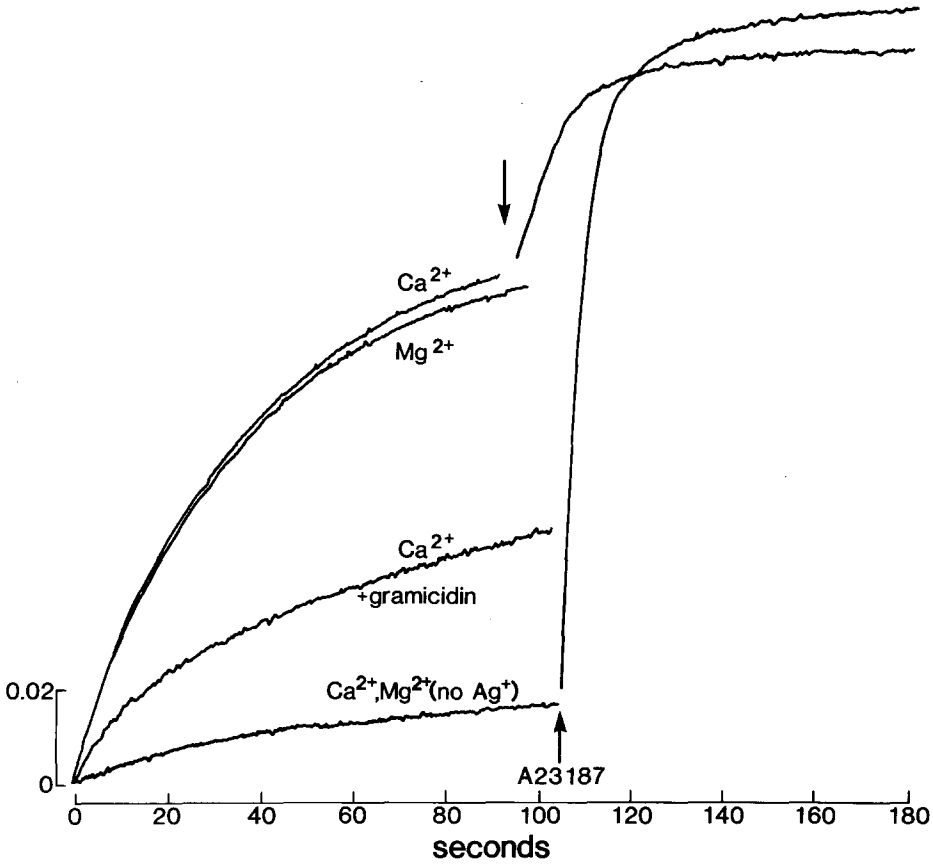

Fig. 6. Ag^+ increases the permeability of the ROS plasma membrane to Ca^{2+} and Mg^{2+}. Intact ROS were diluted 20-fold in a medium containing 600 mM sucrose, 2 mM KOAc, 1 μM FCCP, 50 μM neutral red, and 20 mM HEPES (adjusted to pH 7.4 with arginine). The final rhodopsin concentration was 6.7 μM. Except for the traces labeled *no* Ag^+ the suspension was preincubated for 2 min with 25 μM $AgNO_3$ prior to addition of FCCP and the start of the trace. The traces were started upon addition of 20 mM (final concentration) of the indicated acetate salts. The addition of the acetate salts caused an instantaneous absorption change caused by the dilution. For clarity, these instantaneous absorption changes are edited out. Transport of Ca^{2+} or Mg^{2+} across the ROS plasma membrane was monitored by the amount of the dye neutral red adsorbed to the intracellular disk membranes as monitored by the absorption difference (A_{540}–A_{650}) (described under Materials and Methods). The calibration bar represents a change in absorbance by 0.02 absorbance unit. In the absence of Ag^+, the traces observed after addition of 20 mM $Ca(OAc)_2$ and $Mg(OAc)_2$, respectively, were indistinguishable. In the trace labeled *Ca^{2+} and gramicidin* 1 μM gramicidin D replaced 1 μM FCCP. The gramicidin was added prior to addition of Ag^+ in order to block the efficacy of Ag^+ (*cf.* Fig. 2). At the arrows, about 100 sec after the start of the traces, 10 μM A23187 was added, which rapidly equilibrated the Ca^{2+} and Mg^{2+} gradients as illustrated for Ca^{2+}. The equilibrium levels observed in the presence of Mg^{2+} and A23187 were within 0.01 absorbance unit of those observed with Ca^{2+} and A23187 present, but took longer to establish. Temperature: 25°C

different alkali cations in a similar pattern ($Li^+ > Na^+ = K^+ > Cs^+$) as that observed for the Ag^+-induced absorption changes (for clarity the gramicidin-induced absorption changes are not illustrated). Simple electrostatic theory predicts that the changes in surface potential are dependent on the valency of the cation, but independent of the nature of the cation (McLaughlin, 1977).

Silver ions also increase the permeability of the plasma membrane to larger organic cations such as $choline^+$ and $tetramethylammonium^+$, although the rates were about one-third of those observed for alkali cations; in the absence of Ag^+ the plasma membrane was virtually impermeable to either $choline^+$ or tetramethylammonium (*not shown*). At Ag^+ concentrations up to 100 μM, the ROS plasma membrane did not become permeable to solutes such as glucose-6-phosphate and NADP (tested by the ability of cytoplasmic enzymes of the pentose phosphate pathway to utilize these externally added substrates; Schnetkamp & Daemen, 1981). However, higher concentrations of Ag^+ ions (100 μM–1 mM) made the plasma membrane permeable to the above solutes.

Sulfhydryl Reagents Block Ag^+-Induced Ca^{2+} Release

We investigated the involvement of a sulfhydryl group in the Ag^+-induced Ca^{2+} release from intact ROS by using two other sulfhydryl reagents, i.e., N-ethylmaleimide (NEM) and Hg^{2+}. Neither NEM

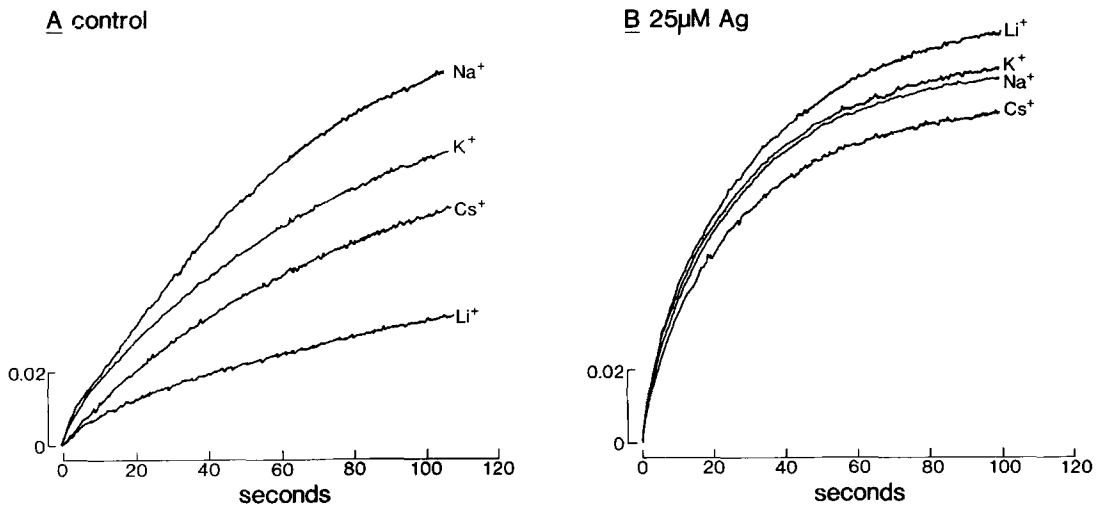

Fig. 7. Ag^+ increases the permeability of the ROS plasma membrane for alkali cations. Experimental conditions and incubation with Ag^+ are identical to the protocol described in the legend of Fig. 4, except that at time zero 50 μM of the indicated alkali cation acetates were added. Temperature: 25°C

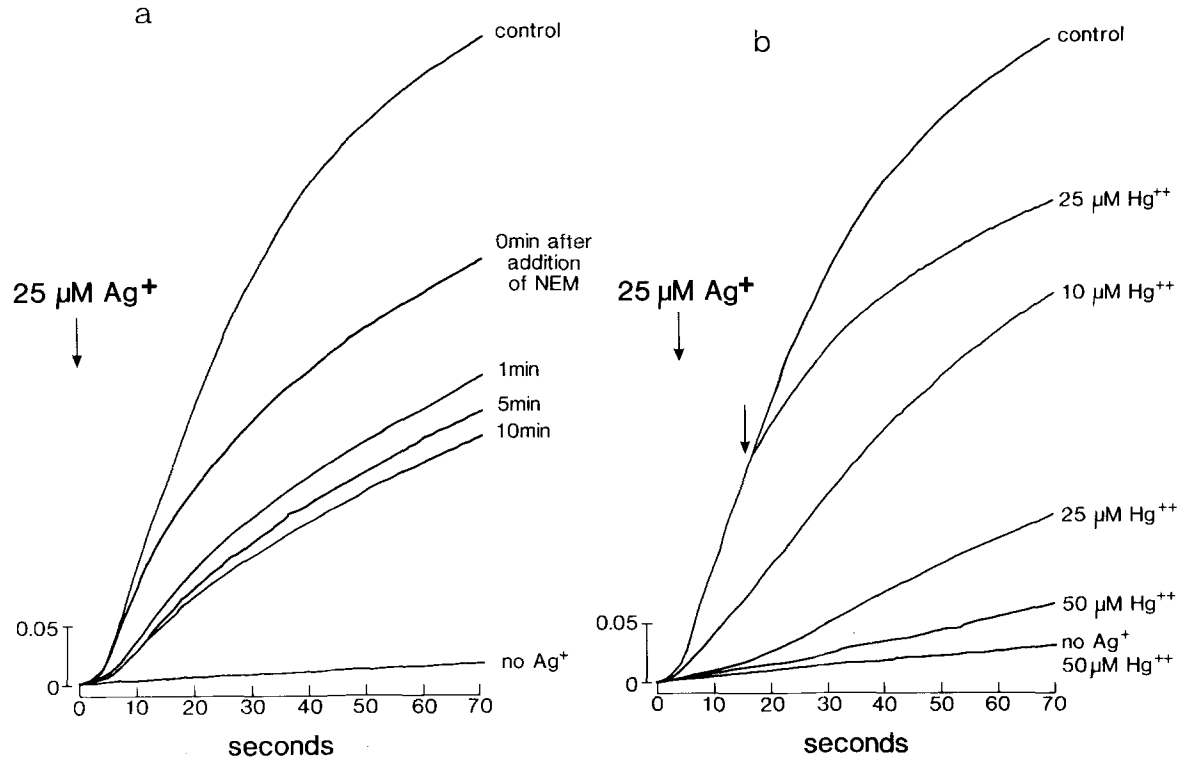

Fig. 8. Ag^+-induced Ca^{2+} release is inhibited by other sulfhydryl reagents. Intact ROS were diluted 50-fold in a medium containing 600 mM sucrose, 200 μM Arsenazo III, and 20 mM HEPES (adjusted to pH 7.4 with arginine). At time zero, Ca^{2+} release was initiated by addition of 25 μM $AgNO_3$ from a 10-mM stock solution. (*a*) Intact ROS were incubated with 0.5 mM N-ethylmaleimide (NEM) for the indicated times prior to addition of Ag^+. The trace labeled *no* Ag^+ represents the Ca^{2+} leak from intact ROS in the absence of Ag^+. (*b*) Ag^+-induced Ca^{2+} release was measured in the presence of increasing concentrations of $HgCl_2$ at the indicated concentrations. The arrow at about 15 sec indicates the addition of 25 μM $HgCl_2$. The trace labeled *no* Ag^+, *50* μM Hg^{++} represents the Ca^{2+} leak from intact ROS in the absence of Ag^+, but in the presence of 50 μM $HgCl_2$

nor Hg^{2+} caused any Ca^{2+} release by themselves. Preincubating intact ROS with 0.5 mM NEM, prior to addition of Ag^+, caused a progressive decrease of the rate of Ag^+-induced Ca^{2+} release as the preincubation time was increased (Fig. 8*a*). In a similar fashion, addition of increasing concentrations of Hg^{2+} caused a progressive decrease of the rate of Ag^+-induced Ca^{2+} release (Fig. 8*b*). When Hg^{2+} or

NEM were added during the rising phase of the Ag^+-induced Ca^{2+} release, this release was gradually aborted. Addition of 0.5 mM of dithiothreitol or β-mercaptoethanol immediately aborted Ag^+-induced Ca^{2+} release (*not shown*). These results suggest strongly that Ag^+-induced Ca^{2+} release was caused by the binding of Ag^+ to a sulfhydryl group on the rod plasma membrane.

Discussion

Silver ions induced a rapid Ca^{2+} efflux or influx (depending on the direction of the Ca^{2+} gradient) across the plasma membrane of intact rod outer segments isolated from bovine retinas. Ca^{2+} fluxes across the ROS plasma membrane were measured with two different techniques, which gave consistent results (*cf.* Figs. 3 and 6). Under all conditions a lag period was observed between addition of Ag^+ and subsequent Ca^{2+} release suggesting that activation of the flux mechanism is preceded by modification of some target group by silver ions. Lowering the temperature and addition of external KOAc were two factors that increased the duration of the lag period; the latter can be explained by the effect of KOAc on the surface potential on the outer leaflet of the plasma membrane reducing the effective concentration of Ag^+ at the membrane surface. The observation that the action of Ag^+ that was inhibited by the sulfhydryl reagents NEM and Hg^{2+} (Fig. 8) suggests that Ag^+ may bind to a critical sulfhydryl group similar as suggested for the Ag^+-induced Ca^{2+} release from sarcoplasmic reticulum (SR) vesicles (Abramson et al., 1983; Salama & Abramson, 1984). In three other aspects, Ag^+-induced Ca^{2+} release differed markedly between SR vesicles and ROS: (*i*) A number of other sulfhydryl reagents including NEM and Hg^{2+} were effective in promoting Ca^{2+} release in SR vesicles, but not in ROS. (*ii*) Ag^+-induced Ca^{2+} release showed a sigmoidal dependence on the Ag^+ concentration in ROS, but not in SR vesicles. (*iii*) Ag^+-induced Ca^{2+} release in ROS displayed a distinct lag phase in ROS, but not in SR vesicles. These differences suggest significant differences in targets.

The Ca^{2+} efflux induced by 50 μM Ag^+ amounted to 3.6 mol Ca^{2+}/mol rhodopsin (equivalent to 10.8 mM total Ca^{2+}) and had a maximal rate of 6.4×10^6 Ca^{2+}/outer segment/sec (equivalent to a change of 0.7 mM total Ca^{2+}/sec or a current of about 2 pA/outer segment). These values can be compared with Na^+-dependent Ca^{2+} fluxes measured in bovine ROS with the same Arsenazo III technique. Na^+-stimulated Ca^{2+} efflux (at 50 mM Na^+) can release about 4 mol Ca^{2+}/mol rhodopsin at a rate of 5×10^6 Ca^{2+}/outer segment/sec (Schnetkamp, 1986).

The Ag^+-induced flux mechanism could either reflect a different way to activate one of the two known Ca^{2+} transporters, i.e., the Na-Ca exchanger or the cGMP-dependent conductance, or it could activate another cation-selective transporter not observed before. The Ca^{2+} fluxes through the Ag^+- and Na^+-dependent mechanism are of similar magnitude under the conditions of the Arsenazo III experiments (*see above*), but other properties are very dissimilar such as the ion selectivity: Na-Ca exchange shows an absolute discrimination between Na^+ and all other alkali cations and between Ca^{2+} and Mg^{2+} (Schnetkamp, 1980), whereas the Ag^+-induced pathway shows little discrimination between these cations (Figs. 6 and 7).

Several points of similarity can be noted for the cGMP- and for the Ag^+-induced pathways:

(*i*) The ion selectivity of both is similar; the cGMP-dependent/light-sensitive conductance has been shown to be permeable to alkali cations and to Ca^{2+} and Mg^{2+} (Capovilla et al., 1983; Hodgkin, McNaughton & Nunn, 1985; Woodruff, Fain & Bastian, 1982; Yau & Nakatani, 1984*a*).

(*ii*) Both the cGMP- and the Ag^+-induced pathway show a sigmoidal dependence on the ligand concentration with Hill coefficients ranging between 2 and 3 (Fig. 2; Fesenko et al., 1985; Koch & Kaupp, 1985; Yau & Nakatani, 1985; Zimmerman et al., 1985). This may suggest that both pathways/channels are composed of at least three interacting subunits that each have to bind the ligand in order for the channel to open.

(*iii*) cGMP-dependent Ca^{2+} fluxes across ROS disk membranes require the presence of alkali cations (Koch & Kaupp, 1985; Schnetkamp, 1987), not unlike the Ag^+-induced Ca^{2+} efflux (Fig. 3). In both cases it is not clear what the origin of the alkali cation requirement is except for that it does not reflect charge compensation for the Ca^{2+} current; the alkali cation requirement of both Ag^+- and cGMP-induced Ca^{2+} fluxes was not removed by addition of gramicidin, a competent electrical shunt by itself (Fig. 1 and its discussion; Schnetkamp, 1987).

(*iv*) L-*cis* diltiazem and tetracaine have been described as blockers for cGMP-induced Ca^{2+} fluxes across bovine ROS disk membranes (Koch & Kaupp, 1985; Schnetkamp, 1987), and cGMP-induced currents across excised patches of bovine ROS plasma membrane (F.N. Quandt, G.D. Nicol, & P.P.M. Schnetkamp, *in preparation*), whereas these drugs had little effect on Na-Ca exchange. Both L-*cis* diltiazem and tetracaine were effective blockers of Ag^+-induced Ca^{2+} ef-

flux from intact ROS, but only under certain conditions (Fig. 5); perhaps in a similar vein, cGMP-dependent fluxes display two components, one blocked by L-*cis* diltiazem and one not blocked (Koch, Cook & Kaupp, 1987; Schnetkamp, 1987). These results could be interpreted to mean that both cGMP-dependent conductance and the Ag^+-induced flux mechanism exist in two states, which in both cases can be distinguished by blockers such as L-*cis* diltiazem. It is presently not clear what the significance or the molecular mechanism of the two pharmacologically distinct cGMP-dependent conductances are. Similarly, it is puzzling how the ionophore gramicidin produces such a starkly different sensitivity to blockers for the Ag^+-induced pathway.

From the above similarities, we suggest that Ag^+ binds to a critical sulfhydryl group on the extracellular side of multiple subunits of the cGMP-dependent conductance(s) opening the channel, perhaps in a similar fashion as binding of cGMP does from the cytoplasmic side. One should note, however, that the Ag^+-induced pathway was somewhat permeable to organic cations, whereas the cGMP-dependent conductance, measured in the same preparation of bovine ROS, showed no permeability to organic cations (P.P.M. Schnetkamp, *in preparation*). The Ag^+-induced permeability to small organic cations may reflect a different process of non-selective permeabilization of the plasma membrane by Ag^+ ions, which can be noticed for larger molecules such as NADP at higher Ag^+ (> 0.1 mM) concentrations.

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References

Abramson, J.J., Trimm, J.L., Weden, L., Salama, G. 1983. Heavy metals induce rapid calcium release from sarcoplasmic reticulum vesicles isolated from skeletal muscle. *Proc. Natl. Acad. Sci. USA* **80:**1526–1530

Capovilla, M., Caretta, A., Cervetto, L., Torre, V. 1983. Ionic movements through light-sensitive channels of toad rods. *J. Physiol. (London)* **343:**295–310

Fesenko, E.E., Kolesnikov, S.S., Lynbarsky, A.L. 1985. Induction by cyclic GMP of cationic conductance in plasma membrane of retinal rod outer segment. *Nature (London)* **313:**310–313

Hodgkin, A.L., McNaughton, P.A., Nunn, B.J. 1985. The ionic selectivity and calcium dependence of the light-sensitive pathway in toad rods. *J. Physiol. (London)* **358:**447–468

Hodgkin, A.L., McNaughton, P.A., Nunn, B.J. 1987. Measurment of sodium-calcium exchange in salamander rods. *J. Physiol (London)* **391:**347–370

Koch, K.-W., Cook, N.J., Kaupp, U.B. 1987. The cGMP-dependent channel of vertebrate rod photoreceptors exists in two forms of different cGMP sensitivity and pharmacological behaviour. *J. Biol. Chem.* **262:**14415–14421

Koch, K.-W., Kaupp, U.B. 1985. Cyclic GMP directly regulates a cation conductance in membranes of bovine rods by a cooperative mechanism. *J. Biol. Chem.* **260:**6788–6800

Koch, K.-W., Stryer, L. 1988. Highly cooperative feedback control of retinal rod guanylate cyclase by calcium ions. *Nature (London)* **334:**64–66

Matthews, G. 1987. Single-channel recordings demonstrate that cGMP opens the light-sensitive ion channel of the rod photoreceptors. *Proc. Natl. Acad. Sci. USA* **84:**299–302

Matthews, H.R., Murphy, R.L.W., Fain, G.L., Lamb, T.D. 1988. Photoreceptor light adaptation is mediated by cytoplasmic calcium concentration. *Nature (London)* **334:**67–69

McLaughlin, S. 1977. Electrostatic potentials at membrane-solution interfaces. *Curr. Top. Membr. Transp.* **9:**71–144

Myers, V.B., Haydon, D.A. 1972. Ion transfer across lipid membranes in the presence of gramicidin A. *Biochim. Biophys. Acta* **274:**313–322

Nakatani, K., Yau, K.-W. 1988 Calcium and light adaptation in retinal rods and cones. *Nature (London)* **334:**69–71

Oba, T., Hotta, K. 1985*a*. Silver ion-induced tension development and membrane depolarization in frog skeletal muscle fibres. *Pfluegers Arch.* **405:**354–359

Oba, T., Hotta, K. 1985*b*. Tension development in frog skeletal muscle induced by silver ions. *Jpn. J. Physiol.* **35:**841–851

Palade, P. 1987. Drug-induced Ca^{2+} release from isolated sarcoplasmic reticulum. II: Releases involving a Ca^{2+}-induced Ca^{2+} release channel. *J. Biol. Chem.* **262:**6142–6148

Salama, G., Abramson, J.J. 1984. Silver ions trigger Ca^{2+} release by acting at the apparent physiological release site in sarcoplasmic reticulum. *J. Biol. Chem.* **259:**13363–13369

Schnetkamp, P.P.M. 1980. Ion selectivity of the cation transport system of isolated intact cattle rod outer segments: Evidence for a direct communication between the rod plasma membrane and the rod disk membranes. *Biochim. Biophys. Acta* **598:**66–90

Schnetkamp, P.P.M. 1985*a*. 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. *J. Membrane Biol.* **88:**249–262

Schnetkamp, P.P.M. 1985*b*. Ionic permeabilities of the plasma membrane of isolated intact bovine rod outer segments as studied with a novel optical probe. *J. Membrane Biol.* **88:**263–275

Schnetkamp, P.P.M. 1986. Sodium-calcium exchange in the outer segments of bovine rod photoreceptors. *J. Physiol. (London)* **373:**25–45

Schnetkamp, P.P.M. 1987. Sodium ions selectively eliminate the fast component of guanosine cyclic 3′, 5′-phosphate induced Ca^{2+} release from bovine rod outer segment disks. *Biochemistry* **26:**3249–3253

Schnetkamp, P.P.M., Bownds, M.D. 1987. Na^+- and cGMP-induced Ca^{2+} fluxes in frog rod photoreceptors. *J. Gen. Physiol.* **89:**481–500

Schnetkamp, P.P.M., Daemen, F.J.M. 1981. Transfer of high-energy phosphate in bovine rod outer segments: A nucleotide buffer system. *Biochim. Biophys. Acta* **672:**307–312

Schnetkamp, P.P.M., Kaupp, U.B., Junge, W., 1981. Interfacial potentials at the disk membranes of isolated intact cattle rod

outer segments as a function of the occupation state of the intradiskal cation-exchange binding sites. *Biochim. Biophys. Acta* **642:**213–230

Woodruff, M.L., Fain, G.L., Bastian, B.L. 1982. Light-dependent ion influx into toad photoreceptors. *J. Gen. Physiol.* **80:**517–536

Yau, K.-W., Nakatani, K. 1984*a*. Cation selectivity of light-sensitive conductance in retinal rods. *Nature (London)* **309:**352–354

Yau, K.-W., Nakatani, K. 1984*b*. Electrogenic Na-Ca exchange in retinal rod outer segment. *Nature (London)* **311:**661–663

Yau, K.-W., Nakatani, K. 1985. Light-suppressible, cyclic GMP-sensitive conductance in the plasma membrane of a truncated rod outer segment. *Nature (London)* **317:**252–255

Zimmerman, A.L., Yamanaka, G., Eckstein, F., Baylor, D.A., Stryer, L. 1985. Interaction of hydrolysis-resistant analogs of cyclic GMP with the phosphodiesterase and light-sensitive channel of retinal rod outer segments. *Proc. Natl. Acad. Sci. USA* **82:**8813–8817

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