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Sulfhydryl Binding Reagents Increase the Conductivity of the Light-Sensitive Channel and Inhibit Phototransduction in Retinal Rods

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The mechanisms by which sulfhydryl (SH-) binding reagents modulate the light-sensitive conductance of retinal rods were investigated by current recording from single rods, by patch clamp recording from the plasma membrane of the rod outer segment (ROS), and by biochemical study of their effects on the light-induced hydrolysis of cyclic GMP. The electrophysiology, as well as measurements of the reagents' ability to traverse the ROS plasma membrane, was done on amphibian (*Rana* and *Ambystoma*) rods, and the biochemistry on bovine rods. The main SH-reagents used were *N*-ethyl-maleimide (NEM) and iodoacetamide (IAA). Both transiently increased rod current, but part of the large current could not be turned off by light. After a few minutes' exposure, NEM, but not IAA, caused a continuous decay of the rod's light sensitivity. In patch-clamp recordings from the ROS plasma membrane, the reagents increased conductivity both in the presence and absence of cGMP, consistent with the observation that the drug-induced current increase in intact rods involved both light-sensitive and light-*in*sensitive components. In vitro, NEM was found to be a powerful inhibitor of cGMP hydrolysis, which can explain the gradual loss of light sensitivity in the rod and could initially contribute to the increased dark current via elevated cGMP levels. Thus, SH-reagents act both by modifying the light-sensitive channel and by inhibiting phototransduction inside the rod.

Key words: photoreception; cyclic GMP; phosphodiesterase; SH-groups; oxidation; NEM; IAA; patch clamp.

1. Introduction

Retinal rods are sensitive to oxidation, losing their (osmotically measured) sodium permeability in oxygen-rich media (Bownds and Brodie, 1975; Wormington and Cone, 1978) and being transiently desensitized even by mildly oxidative treatments (Donner, Hemilä and Koskelainen, 1987). Conceivably, oxidation could act by altering the redox state of SH-groups in rod proteins, because the disulfide reducing agent DTT as well as SH-binding reagents are able to reverse the effects of oxidation (Cone, Usselman and Wu, 1984). Moreover, a high degree of SH-group oxidation is characteristic of the ageing tip part of the rod outer segment (ROS) as compared with the fresh base part (Derevyanchenko et al., 1985).

In an earlier study on amphibian retina (Donner, Hemilä and Koskelainen, 1989), all four SH-reagents tested (NEM, IAA, PCMP and PHMB) were found to initially increase rod current, but longer exposures to all except IAA finally abolished light sensitivity completely. These experiments led to the conclusion that the action of SH-binding agents involves at least two independent mechanisms—arguably, a direct channel effect and an internal action on the transduction machinery—but they did not have the resolution to decide the precise character and relative contribution of the mechanisms. In the present work, we investigate the mechanisms directly, studying channel effects by patch-clamp recording, and effects on phototransduction by biochemical methods. We find that part of the increase in light-sensitive conductance, as well as a leakage conductance which cannot be closed by light, is due to the direct modulation of channel conductivity by SH-reagents. The gradual destruction of light sensitivity by NEM (as well as PCMP and PHMB) is apparently due to powerful inhibition of tranduction enzymes, notably phosphodiesterase (PDE), after the drug has entered the rod.

2. Materials and Methods

Recording of the Outer Segment Current from Single Rods

The current recordings from isolated rods of the tiger salamander (*Ambystoma tigrinum*) were done according to the technique of Baylor, Lamb and Yau (1979) with details as described in Donner et al.

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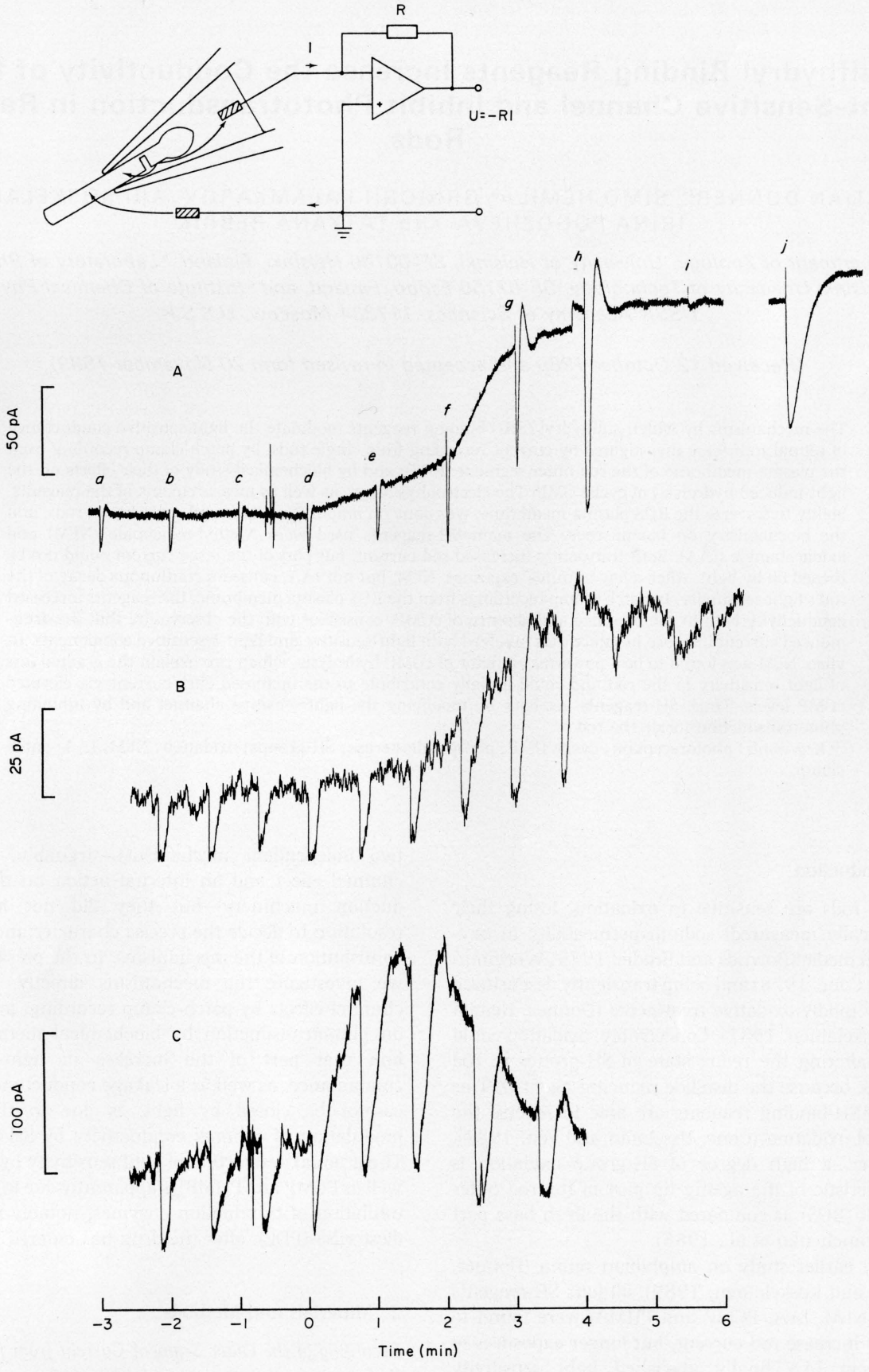

FIG. 1. The current of isolated rods, showing the effects of introducing SH reagents into the medium perfusing the outer segment. Ordinate: current; note the different vertical scale bars for the different records. The abscissa is a time axis, on which the records have been arbitrarily placed so that $t = 0$ approximately corresponds to the moment the drug took action (see text). The inset above the records shows the recording configuration. A, 0·5 mM NEM. The continuous record includes nine photoresponses, marked *a–i*, to 0·1-sec flashes; all except *h* were elicited by the same intensity, which was saturating before the drug took effect. Response *h* was obtained with a 2·8 log units higher flash intensity, as was the separate response *j* shown

(1989). The inner segment of the rod was drawn into a small, tight-fitting glass pipette. Thus, the current from the inner segment was constrained to flow through the recording system to reach the outer segment, which stuck out of the pipette and was perfused with Ringer solution (see Fig. 1, inset). The composition of the standard Ringer was (mM): NaCl, 95; KCl, 3; $CaCl_2$ 0·9; $MgCl_2$, 0·5; buffer: sodium phosphate 8 + $NaHCO_3$ 4 (pH 7·5). The drugs to be tested were added to this solution. The recordings were done at 21°C.

When assessing the character of current components not sensitive to light (cf. Fig. 1), it is important to note that the junction currents associated with the introduction of drug Ringers are small in these experiments (< 3 pA; see Donner et al., 1989). These insignificant currents have not been subtracted from the records.

Patch-Clamp Recording from the ROS Plasma Membrane

The techniques for recording the conductance of small patches of ROS plasma membrane were basically as described by Hamill et al. (1981). The patch pipettes, made of Pyrex glass (WPI, USA) had tip diameters below 0·5 μm and 10–20 MΩ resistance. They were filled with standard Ringer and the current was recorded with Ag/AgCl electrodes. The signal was recorded on tape and plotted on a chart recorder. The composition of the standard Ringer solution was (mM): NaCl, 90; KCl, 10; $MgCl_2$, 2; $CaCl_2$, 0·1; HEPES, 10 (pH 7·5). Before the experiment, the solution was filtered through a bacterial filter (0·22-μm pore diameter).

The preparation was done under dim red light but the recordings in normal room illumination. Isolated ROSs were obtained by shaking pieces of frog (*Rana temporaria*) retina in Ringer. The pipette was brought close to a ROS lying on the bottom of a Petri dish and a gigaseal was established by transient suction. The patch of plasma membrane was excised by movement of the pipette, yielding an 'inside out' configuration. The conductance of the whole patch was determined by recording complete voltage-current functions. (The electrical conductance of the ROS plasma membrane is almost entirely due to the light-sensitive channels, although in the presence of divalent cations single-channel currents are too small to be detected, cf. Haynes, Kay and Yau, 1986; Zimmerman and Baylor, 1986.)

PDE Activity Measurements

PDE activity was measured as pH changes associated with the hydrolysis of cyclic GMP, as described by Liebman and Evanczuk (1982). The experiments were done on bovine ROS discs isolated by the method of Smith, Stubb and Litman (1975). In dark-adapted ROSs, the reaction was initiated with a light flash of 5 msec duration bleaching 1% of the rhodopsin. In bleached outer segments, the reaction was initiated by addition of 140 μM cGMP. The pH changes were recorded with a PHM-84 pH meter in a medium containing 10 mM HEPES (pH 8), 2 mM $MgCl_2$ and 0·1 mM EDTA.

Measurements of the Permeability of the Plasma Membrane to SH-Reagents

The permeability of the ROS plasma membrane to NEM and IAA was estimated by measuring the degree of modification of rhodopsin SH-groups in intact retinas that had been exposed to the reagent for various durations. The rhodopsin molecule is known to contain one to two SH-groups which are easily modified by NEM or IAA (Robertson et al., 1974; Fung and Hubbell, 1978). Thus the rate of SH-group modification gives an estimate of membrane permeability to the drug.

Isolated frog retinas were incubated in a medium containing 110 mM NaCl, 2 mM, KCl, 1·8 mM $CaCl_2$, 2·2 mM $NaHCO_3$ and 5·6 mM glucose. To this, 10 mM NEM or 10 mM IAA was added. After 20 or 120 min incubation, the retinas were washed four times to remove free SH-reagents and homogenized in 45% sucrose with 50 mM Tris-HCl at pH 7·5. After 1 hr of centrifugation at 5500 ***g*** in a step gradient of sucrose (2 ml 45% + 2 ml 35% + 2 ml 28% + 2 ml Tris-HCl), pure ROSs were collected between the 35% and 28% sucrose layers. The pellet was washed twice (10 ml 75 mM NaCl, 50 mM Tris-HCl, pH 7·5) by 30 min centrifugation at 20000 rpm (IA-21 rotor). SH-group modification was measured spectrophotometrically in 0·1% CTAB extracts as described by Ellman (1959).

to the right. B, 0·5 mM PCMP. The record includes 12 photoresponses. The first 11 were obtained with 0·1-sec flashes of the same intensity, which was saturating until the cell lost its light sensitivity very rapidly after ca. 4 min drug exposure. The last response, barely visible at the end of the record, was elicited by a 2 log units stronger flash. C, 2 mM IAA. The record includes eight photoresponses to 0·1-sec flashes of the same intensity. All responses are saturated, as is evident from the long plateau-like peak (see especially the last response). The decrease in the amplitude of the saturated response after the peak of the IAA-induced transient, as well as the rapid relaxation of the transient, is due to the decreased driving force for Na^+ as this ion carries most of the large current (> 200 pA at the peak) into the rod (cf. Yau, McNaughton and Hodgkin, 1981).
The noise 'spikes' seen in A and C around time $t = -1$ are artifacts produced by the manual switch to drug perfusion. Also note the baseline artifact (jump) in panel A, which has not quite returned at the moment the large response *h* peaks; thus the current not sensitive to light appears slightly exaggerated in the figure (the corrected value is 16 pA, see text).

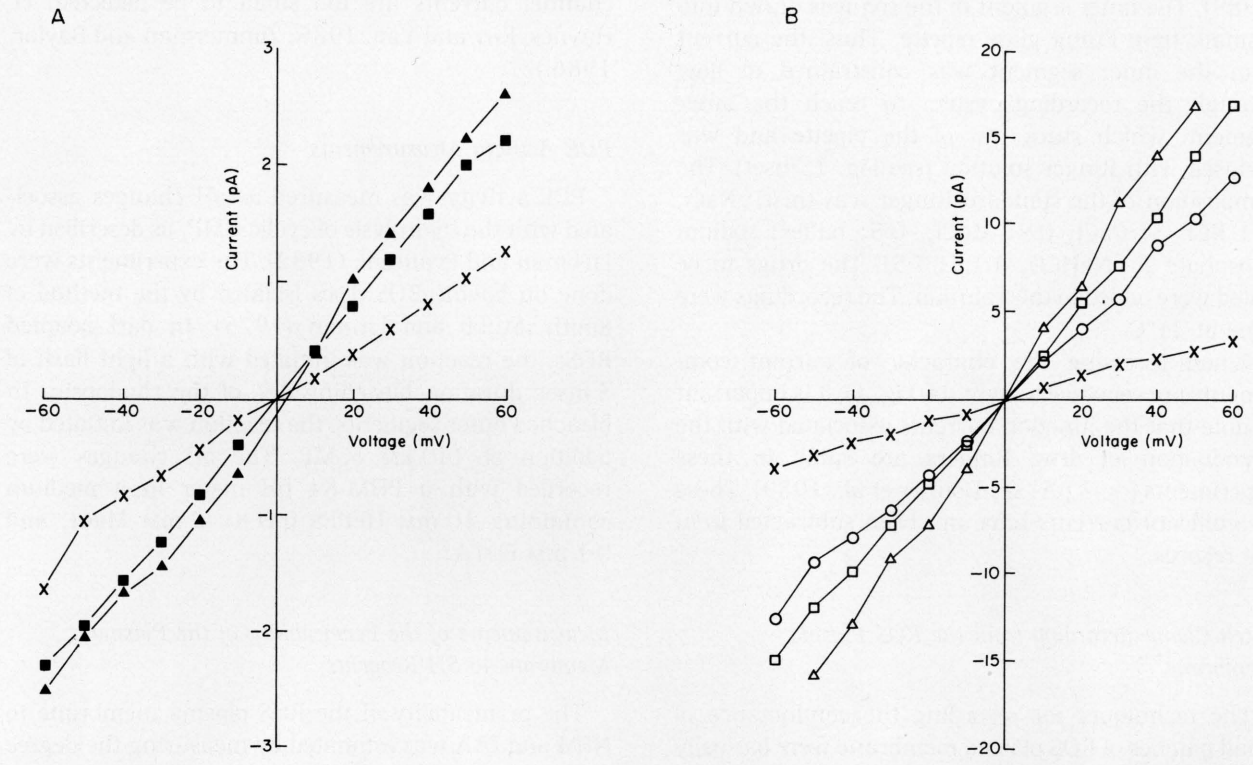

FIG. 2. Effect of NEM on the conductance of patches of outer segment plasma membrane, in the absence (A) and in the presence (B) of cyclic GMP. The curves are voltage-current relations recorded from inside-out membrane patches perfused from the outside with different solutions. Abscissa: applied voltage; ordinate: current through the whole patch. A, Results obtained with cGMP-free Ringer solution. (×) control; (■) 0·5 mM NEM; (▲) 2 mM NEM. B, Results obtained with 100 μM cGMP added to the standard Ringer solution. (×) control without cGMP; (○) 100 μM cGMP added; (□) 0·5 mM NEM; (△) 2 mM NEM.

The SH-Reagents Used

In a previous study (Donner et al., 1989), four sulfhydryl binding reagents: N-ethylmaleimide (NEM), iodoacetamide (IAA), *p*-hydroxymercuribenzoate (PHMB) and *p*-chloromercuriphenylsulfonic acid (PCMP; all reagents by Sigma) were used. Since NEM and the two mercury-containing compounds had basically similar effects on both frog ERG and the current of isolated salamander rods (Donner et al., 1989, and Fig. 1), we chose to focus mainly on the effects of NEM and IAA, while PHMB or PCMP were used only in a few control experiments.

3. Results

Effects of Different SH-Reagents on the Current of Single Rods

Figure 1 shows three current records from isolated salamander rods, each taken around the time of application of one SH-reagent: NEM (A), PCMP (B) and IAA (C). The reagent was added to the standard Ringer solution perfusing the ROS. Due to the comparatively large volume of the chamber (turnover time ca. 2 min) and the varying position of the pipette therein, there was some uncertainty regarding the time the drug effectively reached the rod. Therefore, the records have been provided with a common (relative) time axis, where the moments when the drug takes visible effect in each case coincide at time zero.

First consider in detail the NEM record [Fig. 1(A)], which is typical of a total of seven similar experiments where 0·5 mM NEM was applied. Superposed on the baseline are nine photoresponses to brief light flashes (marked *a–i*). For all except response *h* flashes were of the same high intensity (denoted logI = 0). Response *h* on the peak of the drug-induced transient, as well as the separate response *j*, was obtained with a 2·8 log units higher flash intensity. In the predrug situation, the responses to logI = 0 (responses *a–d*) were saturated, implying that their peaks mark the level of zero current, and their amplitudes give the size of the rod's dark current. When the drug takes effect, the baseline is deflected in a direction opposite to photoresponses (upwards in the figure), indicating that the dark current of the rod grows. Indeed, the previous strong flash (logI = 0) is now seen to elicit much larger responses (*f–g*), showing that the current which can be modulated by light has grown. However, response *g* no longer reaches the level of zero current marked by the predrug responses. The next response (*h*), evoked by a very strong flash (logI = 2·8), shows that this is due to two factors. On one hand, this response peaks closer to the currentless level, implying that logI = 0 was no longer enough to produce a truly saturated response—the relative sensitivity of the rod (percentage of current turned off by one photoisomer-

ization) has decreased. But on the other hand, even response *h* in fact fails to reach the level of zero current. This indicates that there remains a current component which can not be turned off by light [16 pA in Fig. 1(A), and 14 and 15 pA in two other experiments with the same concentration of NEM and sufficiently stable DC recording].

Sensitivity continues to fall quite rapidly: in Fig. 1(A) the next response to intensity logI = 0 (marked *i*) is already very small. The size of the light-sensitive current, however, has not decreased nearly as dramatically, as exemplified by the saturated response *j* shown separately at the right-hand edge of the NEM record. This response was recorded at time $t = 12$ min. The light-sensitive current at that moment was still ca. three times larger than before the application of NEM, but the fall in sensitivity had made it necessary to use much stronger stimuli than before to turn off all that current.

The picture obtained with PCMP [Fig. 1(B)] is, in all essentials, similar to that seen with NEM. IAA, however [Fig. 1 (C)] differs in the important respect that there is no decay of the relative sensitivity of the rod. Throughout the record, saturated responses were elicited by the same flash intensity as before the drug was applied, and we have followed rods in 2 mM IAA perfusion for up to 12 min without observing any significant decay of light sensitivity. Similarly, in ERG recordings (Donner et al., 1989), no significant loss of sensitivity was observed during 20 min perfusion with 2 mM IAA.

To summarize, all the reagents: (1) initially increased the dark current of the rod, (2) increased the amplitude of saturating responses by an amount correlated to, but smaller than, the increase in dark current, and (3) correspondingly induced a light-*in*sensitive current component, which persisted however saturating the stimulus flash. With two of the drugs shown in Fig. 1 (NEM and PCMP), as well as with PHMB (see Donner et al., 1989), the initial effects were followed by a fast decay of light sensitivity. Common to all the SH-reagents was the tendency to produce response overshoots after acting for a couple of minutes. This effect will not, however, be considered further in this work.

The experiments on the photocurrent of intact rods indicate that SH-reagents exert at least two independent types of effects on the rod. The hypothesis we propose to investigate is that the SH-reagents: (1) directly increase the conductivity of the ROS channels (part of the initial current increase and, in particular, the component not sensitive to light), and (2) inhibit phototransduction inside the rod (the continuous decay of light sensitivity, but also part of the initial increase in light-sensitive current). The latter effects would be weak or absent in IAA.

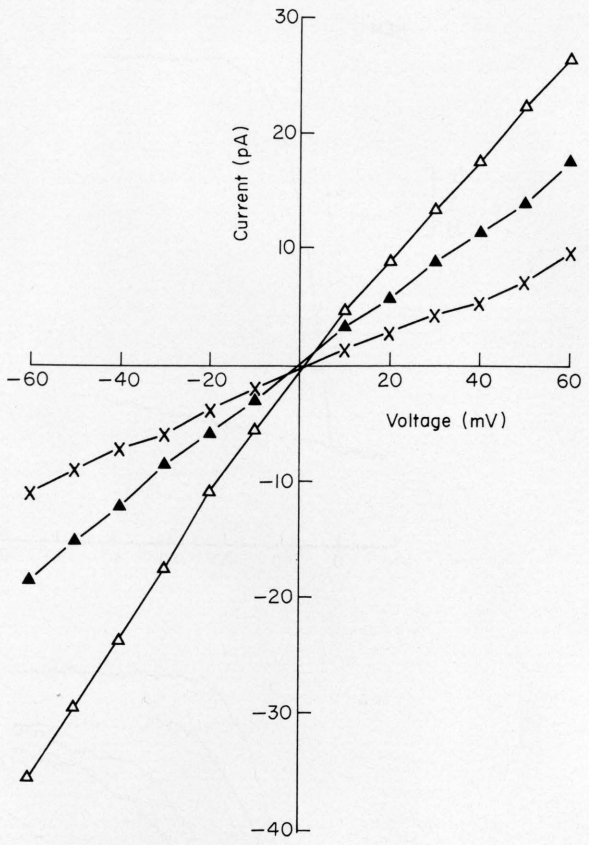

FIG. 3. Effect of IAA on the conductance of a patch of outer segment plasma membrane. (×) control (standard Ringer without cGMP); (▲) 2 mM IAA; (△) 2 mM IAA + 100 μM cGMP.

Effects of SH-Reagents on the Conductivity of the Channels in the ROS Plasma Membrane

To reveal a possible direct action of SH-reagents on channel conductivity, we perfused inside-out patches of the ROS plasma membrane with Ringer containing NEM or IAA in the concentrations we had previously used in experiments on the current of intact rods (0·5 mM, as in the experiments illustrated in Fig. 1, or 2 mM as in ERG experiments reported by Donner et al., 1989). These patch-clamp experiments were done both without cGMP (mimicking the physiological situation after a strongly saturating flash) and in the presence of 100 μM cGMP, which is a concentration that opens about 80% of the cGMP-dependent conductance in frog rods (Fesenko, Kolesnikov and Lyubarsky, 1985). Figure 2 displays voltage-current relations from two experiments before and during NEM perfusion. Panel A shows results obtained without cGMP and panel B with 100 μM cGMP.

In the experiment of Fig. 2(A) (no cGMP), the conductance of the patch is seen to be almost doubled by the introduction of 0·5 mM NEM. A further moderate increase is seen to occur when the NEM concentration is raised to 2 mM.

In Fig. 2(B) the introduction of 100 μM cGMP (○) into an initially cGMP-free medium (×) is seen to increase the conductance of the patch ca. fourfold. In

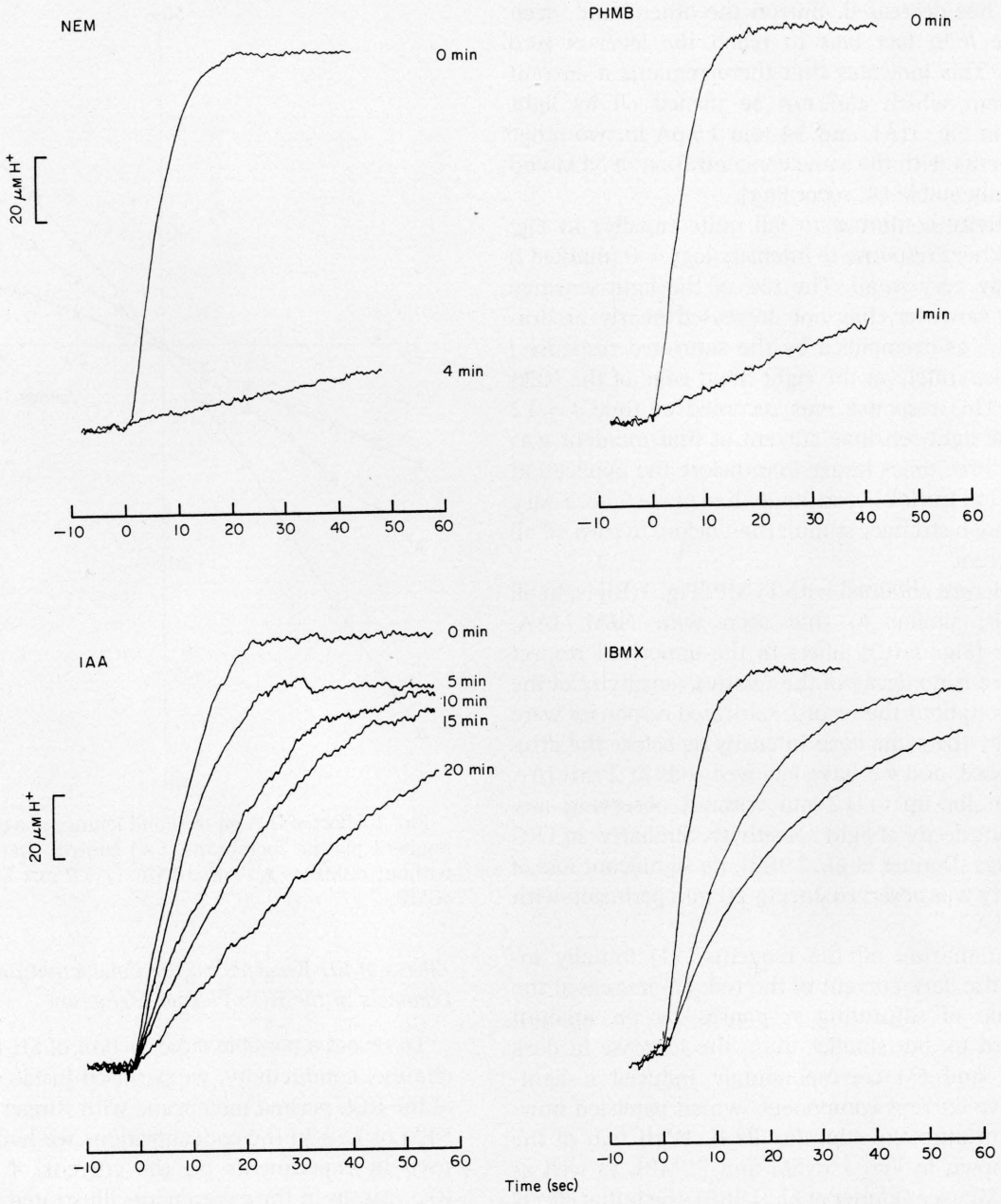

FIG. 4. The effect of NEM, PHMB, IAA and IBMX on cGMP hydrolysis in vitro. Abscissa: time, where $t = 0$ indicates the time of a strong flash of light. Before the flash, 140 μM cGMP was added to the medium. Ordinate: H^+ concentration (which indicates the amount of cGMP hydrolysis); the vertical scale bar corresponds to 20 μM H^+. For further details, see Materials and Methods. In the panels for NEM, PHMB and IAA, the time that had passed from the addition of the drug is shown beside each record; 0 min signifies control without drug. In the IBMX panel, the top trace is a control, while both the lower traces were recorded 4 min after the addition of drug: 0·1 mM (middle trace) and 0·25 mM (bottom trace).

this situation, the subsequent application of 0·5 mM (□) and 2 mM (△) NEM further increased the conductance by factors 1·3 and 1·6, respectively. The absolute increase caused by 2 mM NEM was in fact five times larger in the cGMP-activated than in the cGMP-free situation (130 pS compared with ca. 25 pS).

The results of an experiment with IAA are shown in Fig. 3 (one of three similar experiments). As in Fig. 2, the crosses show the voltage-current relation in the initial state when the membrane patch is perfused with standard Ringer without cGMP. The filled triangles show the relation after the addition of 2 mM IAA to the medium: the conductance has been roughly doubled. Finally, the open triangles mark data recorded in perfusion containing 2 mM IAA + 100 μM cGMP, indicating that the conductance is still cGMP-sensitive in the presence of IAA.

Effects of SH-Reagents on the Light-Induced Hydrolysis of cGMP

When NEM had acted for a few minutes on the intact rod, the (still large) photoresponses slowed down, the intensity-response curve steepened, and flash responses tended to be facilitated by a preceding

flash (cf. Donner et al., 1989). Similar changes have been described in connection with the inhibition of PDE (Capovilla, Cervetto and Torre, 1983; Cervetto and McNaughton, 1986), which emerges as a likely 'internal' target for the SH-reagents. If so, it should be feasible in biochemical experiments to demonstrate the inhibition of phototransduction by at least NEM and the other destructive SH-reagents. IAA would then be expected to be ineffective and/or unable to enter the rod (see further below).

We tested the effects of 0·1 mM NEM and 0·2 mM IAA on PDE activity by monitoring the pH changes attending cGMP hydrolysis in vitro (see Materials and Methods). For comparison, similar experiments were carried out with 0·1 mM PHMB, and also with 0·1 and 0·25 mM IBMX, the most commonly used phosphodiesterase inhibitor. The results are summarized in Fig. 4.

It is seen that NEM even in 0·1 mM concentration is an extremely powerful inhibitor of cGMP hydrolysis, suppressing the light-induced reaction almost completely and immediately. The same holds true for PHMB, but not for IAA. The thorough destruction of phototransduction by NEM and PHMB is especially striking beside the effects of IBMX, which appear moderate by comparison. Yet these concentrations of IBMX are sufficient to produce dramatic physiological effects (Cervetto and McNaughton, 1986).

The Permeability of the Plasma Membrane to NEM and IAA

It is obvious from Fig. 4 that if NEM is able to traverse the cell membrane even to a small degree, its inhibition of phototransduction enzymes is quite sufficient to account for the sensitivity decay seen in physiological experiments. And on the other hand, the inhibition effected by IAA is small but not negligible. A contributing reason for the relative physiological harmlessness of IAA could be that it is unable to enter the rod. To clarify these points, we studied the permeability of the ROS plasma membrane to NEM and IAA. We did this by measuring the rate of modification of rhodopsin SH-groups in CTAB extracts from retinas that had been exposed to 10 mM NEM or 10 mM IAA for various periods (see Materials and Methods).

The upper part of Table I gives the degree of SH-group modification after the specified periods of incubation in NEM. After 20 min, the content of intact SH-groups had fallen to 85% of control, and after 120 min it was down to 70%. In view of the power of NEM as an inhibitor of phototransduction, this rate of entering the rod appears quite sufficient to achieve the observed physiological effects.

The picture with IAA is different (lower part of Table I). After 20 min, no change in SH-group content per rhodopsin molecule could be seen. Only after 120 min incubation a significant decrease of intact SH-groups was observed. Thus, even in 10 mM concentration (i.e. five times higher than in our electrophysiological experiments), the penetration of IAA into the cell is slow. This reinforces our conclusion that IAA acts from the outside of the rod directly on the channel.

TABLE I

Percentages of intact (unmodified) sulfhydryl groups in rhodopsin extracted from retinas that had been exposed to 10 mM NEM or 10 mM IAA for different durations. 100% unmodified SH-groups indicates that the drug has hardly penetrated into the rod at all. The error limits are standard deviations of two independent determinations

Reagent	Incubation time (min)	% of unmodified SH-groups/rhodopsin
NEM (10 mM)	0	100
	20	85±1
	120	70±3
IAA (10 mM)	0	100
	20	100
	120	78±3

4. Discussion

Modification of the Channel Properties by SH-Reagents

The ROS plasma membrane contains at least two protein complexes in which the binding of SH-reagents could have dramatic physiological consequences. One is the light-sensitive channel, the other is the Na/Ca exchange mechanism. However, inhibition of the Na/Ca exchange is known to abolish the light-sensitive current (Yau and Nakatani, 1984a,b), and so it is difficult to reconcile with at least the relative harmlessness of IAA.

Thus, it seems that the initial increase of membrane conductance can be ascribed to the binding of the reagents to the channel proteins. Conductivity was increased by SH-reagents both in the cGMP-activated ('channels open') state and in the cGMP-free ('channels closed') state. Correspondingly, in intact rods, the current increase induced by SH-reagents was in part reflected as a growth of saturating photoresponses, but also involved a component which could not be turned off by light. Schnetkamp and Szerencsei (1989) have recently reported the opening of ROS Ca^{2+} conductance by silver ions and suggest that this is due to the binding of Ag^{+} to SH-groups of the light-sensitive channel. Though seemingly in agreement with the increases in Na^{+} conductance we report here, the Ag^{+}-induced Ca^{2+} release could paradoxically be blocked with NEM and other SH-reagents. Moreover, the latter reagents were unable to induce Ca^{2+} release. If it is accepted that Ag^{+} acts by SH-binding, this emphasizes that the functional changes of the channel are quite sensitive to the exact nature of the agent bound.

The fact that the impermeant IAA is effective both from the cytoplasmic side of the membrane (as in the patch-clamp experiments) and from the outside (as in the experiments on intact rods) indicates that there is at least one SH-group accessible on each side of the membrane. The SH-groups on the outer surface of the cell offer the interesting possibility of isolating channel molecules from the plasma membrane by means of high-affinity chromatography.

The Inhibition of Phosphodiesterase Activity

We think that PDE inhibition is the most likely explanation for the inhibition of light-induced cGMP hydrolysis (Fig. 4). The α subunit of the PDE molecule is known to contain 16 SH-groups (Ovchinnikov et al., 1987), so it is rather a trivial truth that SH-binding reagents should be able to modify it. The significance of the present results then lies in showing: (1) that there are SH-groups at the active site of the enzyme molecule, and (2) that the modification of these groups inhibits the enzymatic activity extremely efficiently. In view of the easy penetration of NEM into the cell, the inhibition of PDE is sufficient to account for the irreversible inactivation of phototransduction under prolonged perfusion. The fact that IAA does not destroy photosensitivity in the intact rod is consistent with the observation that this drug enters the rod only slowly and is a relatively inefficient inhibitor of the phototransduction cascade.

It is possible that the SH-reagents act not only directly on PDE, but also on the G-protein, which is another potential target in the transduction cascade (Ho and Fung, 1984; Reichert and Hofmann, 1984). However, at least the effect of NEM cannot be due mainly to G-protein modification, because the binding of NEM to the SH-groups of the G-protein seems too slow (Ho and Fung, 1984, and our unpubl. res.) to accomplish the very fast inhibition of cGMP hydrolysis (Fig. 4).

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