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Effects of Cysteine Modification on the Activity of the cGMP-gated Channel from Retinal Rods

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Abstract. The effect of sulfhydryl reagents on the activity of the cGMP-gated channel from bovine retinal rods was studied by measurements of 8-Br-cGMP-(cGMP)-induced calcium efflux from rod membrane vesicles and records of 8-Br-cGMP-dependent sodium currents through channels incorporated into planar lipid bilayers. N-ethylmaleimide and mersalyl (thiol blockers) as well as diamide (dithiol-disulfide conversion agent) have a dual effect on the channels activity: at low concentration, they increase the apparent affinity for cyclic nucleotide (''activation'') at the same time inducing a loss of cooperativity for nucleotide binding; at higher concentration, N-ethylmaleimide and diamide produce a reduction of the amplitude and initial rate of the calcium release at saturating nucleotide concentration, while mersalyl is shown to reduce the activity of the channels in bilayer experiments (''inhibition''). Nitric oxide precursors have no effect. The results suggest that blocking at least 1 of the 3 cytoplasmic cysteine residues situated close to the cGMP-binding site in each channel subunit by N-ethylmaleimide, mersalyl, or diamide (forming a dimer between 2 subunits) increases the affinity for the nucleotide. Inhibition is produced by blocking at least one of the 2 other cytoplasmic sulfhydryl groups (N-ethylmaleimide, mersalyl, oxidized glutathione) or the 2 others (diamide, intrasubunit bridge), and may concern a process of channel inactivation. The 3 cytoplasmic sulfhydryl groups are accessible when the channels are in the open state, but not (or much less) accessible when the channels are in the closed state.

Key words: CNG channels — Photoreceptor — SH reagents — Calcium flux — Bilayers

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Introduction

Absorption of a photon by the photopigment molecule rhodopsin in retinal rod outer segments triggers an enzymatic cascade which leads to the closing of cyclic GMP-activated channels in the plasma membrane. Photoexcited rhodopsin catalyzes the binding of GTP to the retinal GTP-binding protein transducin which in turn activates a cGMP phosphodiesterase which rapidly hydrolyzes the cytoplasmic cGMP. This results in closing of the channels, reduction of the dark inward sodium current and hyperpolarization of the plasma membrane. Opening of these channels is cooperatively regulated by direct binding of at least 4 molecules of cGMP (*see* review by Eismann, Bönigk & Kaupp, 1993).

The rod photoreceptor channel is a polypeptide of apparent molecular mass of 63 kDa on SDS gels [Cook, Hanke & Kaupp, 1987]. Its amino acid sequence has been determined by cloning of cDNA and contains a single cyclic nucleotide binding site; expression of the 63 kDa subunit alone in *Xenopus* oocytes gives rise to cGMP-activated cationic currents with properties similar to those of the native channels [Kaupp et al., 1989]. The structure of the channel is not completely elucidated: from the cooperativity of cGMP binding, it must be an oligomer composed of at least four, and perhaps five, 63 kDa subunits. A distinct subunit (β subunit: Chen et al., 1993; or 240 kDa protein: Hsu & Molday, 1993), whose stoichiometry is unknown, has also been described.

The 63 kDa subunit contains 6 cysteine residues; according to the most recent model [Bönigk et al. 1993], 3 of them are close to the nucleotide binding site on the cytoplasmic side, one is on the external loop between helices H1 and H2, and the two others are situated within the membranous domain, in helices H1 and H4.

Sulfhydryl groups play essential roles in the structure or function of many proteins. Up to now, only two reports have suggested a possible role in the function of cGMP-activated channels. Silver ions at micromolar concentrations have been reported to stimulate a large Ca^{2+} release from rod outer segments with an intact plasma membrane [Schnetkamp & Szerencsei, 1989]; this action of Ag^+ is inhibited by the sulfhydryl reagent N-ethylmaleimide (NEM)[1] and by Hg^{2+} suggesting that Ag^+ may bind to a critical sulfhydryl group. Balakrishnan, Padgett & Cone (1990) reported that NEM potentiates the effects of low concentrations of cGMP on the calcium influx in rod outer segment membrane and suggested that –SH reagents act directly on the cGMP-activated ion channels.

We have investigated the role of –SH groups on bovine retinal cGMP-activated channel activity as measured with two different techniques: calcium efflux from calcium-loaded vesicles and sodium currents through single channels incorporated into planar lipid bilayers. Two types of thiol blockers have been used: the covalent reagent NEM, which is permeant, and the noncovalent reagent mersalyl, which is charged and considered as impermeant. We have also tested the effect of a disulfide bond reducing agent (DTT) and of sulfhydryl oxidizing reagents capable of forming disulfide bridges between two closely situated cysteine residues: diamide (permeant), oxidized glutathione (impermeant) and nitric oxide precursors.

Materials and Methods

Chemicals

A23187, N-ethylmaleimide, diamide, mersalyl, oxidized glutathione, sodium nitroprusside, 8-Br-cGMP and cGMP were from SIGMA. SIN-1 was a gift from Hoffmann-Laroche.

Preparation of Rod Outer Segment Membrane Vesicles

Bovine eyes were collected at a local slaughterhouse. Rod outer segments (ROS)[1] were isolated under red light as described by Sitaramayya (1986). Vesicles were obtained by washing the rod preparation three times in the dark with hypotonic buffer (HEPES 5 mM pH 7.4, PMSF 0.1 mM) at a rhodopsin concentration of 0.8–1 mg/ml in order to remove all soluble proteins as well as the peripheral transducin and cGMP-phosphodiesterase, which might cause cGMP hydrolysis. Pellets of washed membranes were resuspended at a rhodopsin concentration of about 10 mg/ml and 1 ml aliquots were frozen in liquid nitrogen. This preparation of washed ROS membrane vesicles was used for flux measurements and western blots. For incorporation into lipid bilayers, the membranes were further washed 3 times with hypotonic buffer.

[1] *Abbreviations:* ROS: rod outer segment; PMSF: phenylmethylsulfonyl fluoride; DTT: dithiotreitol; NEM: N-ethylmaleimide; GSSG: oxidized glutathione; NO: nitric oxide; SIN-1: 3-morpholinosydnonimine; CNG channels: cyclic nucleotide-gated channels

Modification and Calcium Loading of Outer Segment Membrane Vesicles

Frozen aliquots of washed dark membranes were diluted 3 times in 5 mM HEPES (adjusted to pH 7.4 with Arginine), 10 mM $CaCl_2$, 0.1 mM PMSF and centrifuged; the pellets were suspended in the same buffer at a rhodopsin concentration of 10 mg/ml. Incubation with –SH reagents (except mersalyl, *see* text) was carried out at room temperature in 1 ml of suspension, in the presence or absence of cGMP (8-Br-cGMP); the membranes were then diluted 3 times with cold buffer and immediately centrifuged for 10 min at 10^5 rpm in a Beckman TL-100 ultracentrifuge. Remaining reagent and nucleotide were removed by 3 washing steps: suspension of the pellet in 3 ml of buffer and centrifugation. After the last wash, the membrane pellet was resuspended in one ml of buffer by several passages through a syringe with a blunt needle firmly pressed onto the bottom of the tube. We found that this was an efficient and rapid way of loading the vesicles with calcium: the same amount of calcium could be released from vesicles resuspended like this in the presence of calcium as from vesicles resuspended in the absence of calcium and then dialysed overnight against calcium containing buffer. Vesicles thus loaded with calcium by mechanical breaking were used after 10 to 15 min. Control vesicles were treated exactly like modified vesicles (incubation with or without nucleotide, centrifugations, breaking with a syringe) except that –SH reagents were omitted. The concentration of control and modified membranes in a set of experiments was checked from the absorbance at 500 nm of solubilized aliquots, and adjusted to the same value. All steps were carried out under red light.

Calcium Efflux Measurements

Calcium release from calcium-loaded vesicles was measured in room light according to Bennett & Dupont (1979) in the presence of Ca^{2+} EGTA buffers: the release of 2 H^+ associated with Ca^{2+} binding to EGTA and the weak permeability of lipid membranes to EGTA allows transposition of a calcium efflux in the presence of EGTA outside the vesicles into a pH change. pH variations were measured with a combined electrode GK2421 C (Radiometer) connected to a pH-meter pHM 64 equipped with a REA 112 high-sensibility unit (Radiometer). The cuvette was equipped with a magnetic stirrer and maintained at 22°C by a circulating water bath.

Vesicles (100 µl) loaded with 10 mM $CaCl_2$ were diluted in 1.5 ml of 100 mM KCl, 1 mM $MgCl_2$, 1 mM HEPES and 1.2 mM EGTA (pH 7.00). The resulting free calcium concentration in the external medium, calculated according to Fabiato (1988) was ~5.10^{-7} M. The rhodopsin concentration in the cuvette ranged between 10 and 15 µM. cGMP- or 8-Br-cGMP-induced calcium release was initiated by injection of small volumes (1.6 to 8 µl) of concentrated nucleotide solutions (0.1 mM, 1 mM, 10 mM, 100 mM) with a Hamilton syringe under rapid stirring. Total releasable calcium was measured by addition of 7 µM of the calcium ionophore A23187; when A23187 was added after cGMP, the sum of cGMP-induced release and A23187-induced release was identical to the A23187-induced release in the absence of cGMP. When A23187 was added first, subsequent addition of cGMP induced no additional signal. The calibration of calcium-indicating signals was

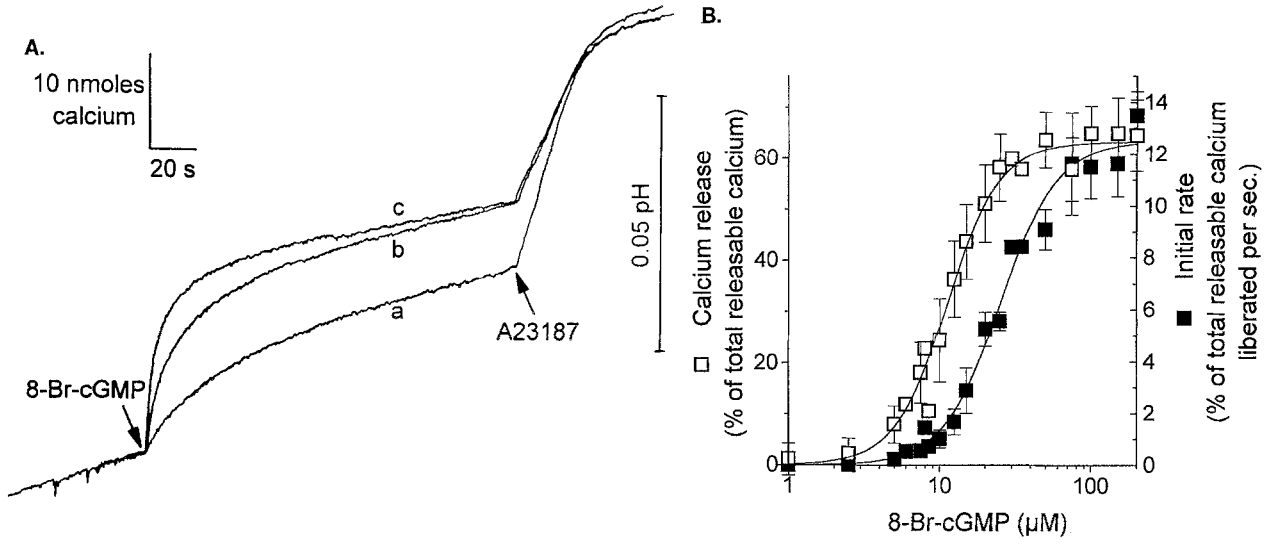


Fig. 1. 8-Br-cGMP-induced calcium release from calcium loaded vesicles from rod outer segment membranes. (*A*) Recordings of 8-Br-cGMP-induced and A23187-induced pH changes associated with calcium release (*see* Materials and Methods). Calcium loaded vesicles (20 nmoles rhodopsin) were suspended into EGTA-containing buffer, allowing measurement of calcium efflux as pH variations. Injection of 8-Br-cGMP induces a calcium release, the initial rate and amplitude of which depend on the nucleotide concentration: *a* 12.5 μM; *b* 25 μM; *c* 200 μM. Addition of the calcium ionophore A23187 (7 μM) then induces the release of the remaining internal calcium. The total releasable calcium is the sum of 8-Br-cGMP and of A23187-induced calcium release. (*B*) Variation of the amplitude (□) and initial rate (■) of the cyclic nucleotide-induced calcium release as a function of 8-Br-cGMP concentration, measured from pH recordings (as shown in *A*). Each data point is the mean from 8 measurements (different experiments), and error bars represent the standard deviation of the mean. The curves are the best fits of the data according to the Hill equation: the values calculated for the maximal amplitude (rate), EC_{50} for 8-Br-cGMP and n_H are given in Table 1.

obtained by several additions of 10 nanomoles of $CaCl_2$ in the cuvette after the measurement. Usually, the amount of total releasable calcium was of the order of 1 to 2 nanomoles of calcium per nanomole of rhodopsin, and total (nucleotide- and ionophore-induced) pH variation was less than 0.1 pH unit.

The initial rate of release is expressed as a percentage of total releasable calcium liberated per second and the amplitude as a percentage of total releasable calcium. The data are fitted to the Hill equation:

$$Y = Y_{max}/[1 + (Kd/[ligand])^{n_H}]$$

with the MicroCal Origin software (Windows). The values given in the Tables for the maximal amplitude or initial rate (Y_{max}), EC_{50} (Kd) and n_H are those calculated by the program (±SE).

SDS Gel Electrophoresis and Western Blotting

Electrophoresis of washed membrane samples (20 μg rhodopsin) denatured in the presence or absence of 5% β-mercaptoethanol was carried out according to [Laemmli, 1970] (5 to 15% acrylamide gradient). The proteins were transferred to a nitrocellulose sheet by transverse electrophoresis for 2 hr at 1.5 A. After saturation of the remaining binding sites with bovine serum albumin, the nitrocellulose sheets were incubated overnight at 4°C with the monoclonal antibody PMc1F6 and then with ^{125}I-labeled antimouse IgG. Proteins were detected by autoradiography.

Incorporation into Planar Lipid Bilayer

Experiments were carried out as previously described [Ildefonse, Crouzy & Bennett, 1992]. Briefly: PE (phosphatidylethanolamine) and PS (phosphatidylserine) 70:30 (wt:wt), purified from bovine brain, or synthetic POPE (1-palmitoyl-2-oleoyl phosphatidyl-ethanolamine) and POPC (1-palmitoyl-2-oleoyl phosphatidylcholine) 50:50 (wt:wt) from Avanti Polar Lipids, Birmingham, AL, were dissolved in decane (30 mg/ml) and spread on a hole 200 μm in diameter. cGMP or 8-Br-cGMP was added to the *cis* chamber together with rod membrane vesicles (30 to 100 μg/ml) in order to allow immediate visualization of channel incorporation. Fusion of the vesicles was performed in the presence of 500 mM NaCl (*cis* chamber) with 100 mM NaCl in the *trans* chamber. All solutions contained contaminant Ca^{2+} and Mg^{2+}, and 10 mM HEPES pH 7.4. The experiments were carried out in room light at 20–22°C.

Currents were recorded with a Bio-Logic RK-300 patch-clamp amplifier equipped with a 10-GΩ feedback head stage. Bandwidth was 1 kHz. Potential values were defined as *cis* chamber minus *trans* chamber voltages, according to the physiological convention. Records were sampled at 2.4 kHz and then filtered (Gaussian digital filter) at 100 Hz.

Results

Characterization of the Calcium Efflux from Control Membrane Vesicles

Cyclic GMP (or 8-Br-cGMP) stimulates the release of a fraction of the total Ca^{2+} content of the membrane vesicles. As previously described [Koch & Kaupp, 1985;

Table 1. Characteristics of the 8-Br-cGMP-induced calcium efflux from control rod outer segment membrane vesicles

Amplitude			Initial rate		
Maximal signal (% of total calcium release)	EC_{50} for 8-Br-cGMP (μM)	Hill coefficient n_H	Maximal signal (% of total calcium release per sec)	EC_{50} for 8-Br-cGMP (μM)	Hill coefficient n_H
63.2 ± 2.2	11.2 ± 1.0	2.53 ± 0.37	12.3 ± 0.6	24.9 ± 1.4	2.47 ± 0.14

The values are obtained from the best fit of the data (±SE), according to the Hill equation (*see* Materials and Methods), from the 8 experiments shown in Fig. 1*B*.

Puckett & Goldin, 1986; Pearce et al., 1988; Bauer, 1988], the addition of the Ca^{2+} ionophore A23187 after nucleotide-induced release causes an additional release of Ca^{2+} (Fig. 1*A*). When the washed membranes suspensions (*see* Materials and Methods) were frozen in the presence of sucrose (5%), the nucleotide-induced calcium release saturated at 10–20% of the total releasable Ca^{2+}, suggesting that only 10 to 20% of the vesicles contained the cGMP-gated channel. However, we found that omission of sucrose before freezing the washed membranes suspensions increased the amplitude of the calcium release to 60–70% of the total released Ca^{2+}, probably due to increased breaking and fusion of plasma membrane (with high channel density) and disc membrane (without channel or with low channel density) [Cook et al., 1989; Bauer, 1988; Boesze-Battaglia, Albert & Yeagle, 1992]. All the experiments described below were carried out with membranes frozen in the absence of sucrose.

Both the rate and the amplitude of the nucleotide-induced Ca^{2+} release depend on the nucleotide concentration (Fig. 1*A,B*). At subsaturating nucleotide concentrations, part of the nucleotide-dependent calcium pool cannot be released, although it is released upon a second addition of saturating nucleotide concentration (*not shown*). The amplitude of the release is measured for each nucleotide concentration after 2 min 30 to 3 min, when the pH variation has returned to a linear variation parallel to the baseline observed before nucleotide addition. That this corresponds to a very low or nonconducting state of the channel has been checked by adding A23187 at various times (3 to 10 min) after addition of nucleotide: the amount of calcium released by the ionophore decreases only slightly with time, similarly to the decrease observed with control vesicles in the absence of nucleotide, indicating a slow leak unrelated to the channel. The same observation, that the amplitude of the calcium release varies with nucleotide concentration, has been reported by several authors (Koch & Kaupp, 1985; Puckett & Goldin, 1986; Koch et al., 1987; Pearce et al., 1988; Bauer, 1988). Addition of gramicidin, or valinomycin in order to abolish the membrane potential, does not modify the nucleotide dependence of the calcium release. As also previously described [Koch & Kaupp, 1985], the nucleotide concentration dependence of the calcium efflux is different for the rate and amplitude, the apparent affinity for the nucleotide being higher (EC_{50} lower) for the amplitude (Fig. 1*B*). Both can be fitted to the Hill equation with n_H between 2 and 3 indicating cooperative binding of the nucleotide. The values obtained for the fit of the data from the experiments shown in Fig. 1*B* are displayed in Table 1.

The nucleotide-induced calcium release signals (Fig. 1*A*) can be fitted with a single exponential, suggesting that the population of channel-containing vesicles is homogenous with respect to channel type, in contrast to the report by Koch et al. (1987), who describe the existence of two channel forms. The high proportion of channel-containing vesicles moreover suggests that most vesicles contain only a few channels[2]. When the release is induced by cGMP instead of 8-Br-cGMP from the same vesicle preparation, the amplitude at saturation is smaller by 1 to 2%: since 8-Br-cGMP is permeant while cGMP is not, this indicates that vesicles in which all the channels have their nucleotide binding site inside represent less than 2% of the total. The EC_{50} measured for cGMP from the amplitude or initial rate was 80 ± 6 μM and 160 ± 3 μM respectively. It can be noted that the EC_{50} measured for cGMP or 8-Br-cGMP from the open probability of a single channel incorporated into a lipid bilayer is significantly lower (18 μM and 3 μM respectively [Ilde-

[2] Assuming a channel density of 300 μ^{-2} on the plasma membrane [Cook et al., 1989], if a rod is 25μ long and has a diameter of 1μ, the number of channels per rod is 24×10^3. After fusion of plasma and disc membranes, the channels are diluted in mixed vesicles. If 60% of these vesicles contain a single channel while 40% contain no channel, there must be about 40×10^3 vesicles. Assuming that a rod contains 1000–2000 discs, each disc must be cleaved into 20–40 vesicles. The membrane surface of a flat disc being 1.5 μ^2, the surface of each vesicle is therefore between 0.04 and 0.08 μ^2, which corresponds to a diameter of 0.12–0.16 μ. If 60% of the vesicles contain an average of 2 (3) (10) (20) channels, there must be 20×10^3 (13×10^3) (4×10^3) (2×10^3) vesicles of diameter 0.16–0.22 μ (0.2–0.26 μ) (0.25–0.5 μ) (0.5–1 μ, which corresponds approximately to an intact disc). From an electron micrograph of the washed membranes vesicles, the diameter of most of the vesicles is between 0.1 and 0.4 μ, consistent with the presence of 1 to 10 channels per vesicle.

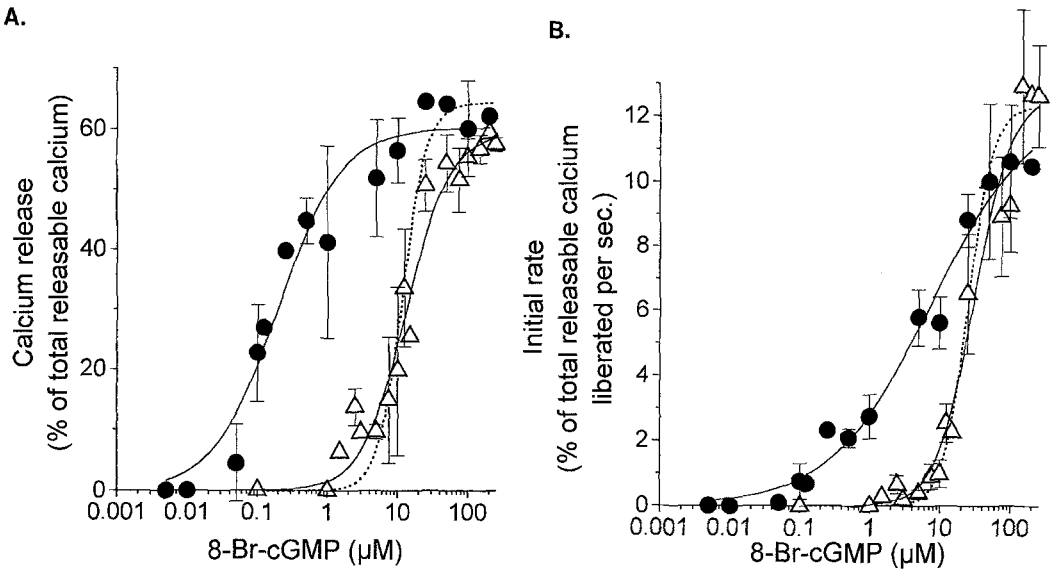


Fig. 2. Effect of N-ethylmaleimide on the amplitude (*A*) and initial rate (*B*) of the 8-Br-cGMP-induced calcium release. Vesicles were incubated in the dark for 15 min with 0.5 mM NEM in the presence (●) (channels in the open conformation) or absence (△) (channels in the closed conformation) of 250 μM 8-Br-cGMP, and then washed to remove unreacted NEM and nucleotide (*see* Materials and Methods). The data points are the mean from 2 (●) or 3 (△) different experiments, and error bars represent the standard deviation. The curves are the best fits of the data according to the Hill equation: the values calculated for the maximal signals, EC_{50} and n_H are given in Table 2 (NEM-treated membranes). The fits of the data shown in Fig. 1*B* for the control membranes are drawn with a dotted line (*see* Table 1 for the values of maximal signal, EC_{50} and n_H).

fonse et al., 1992]). The EC_{50} measured from the rate of calcium efflux or from the open probability is not a measure of the dissociation constant for the nucleotide, but rather an apparent constant, including the affinity for the nucleotide and the equilibrium constant for the open-closed transitions. It is possible that one of these constants (or both) is affected by the nature of the cation. The fact that the amplitude of the calcium release also depends on the nucleotide concentration has been proposed to be due to an inactivation of the channel [Puckett & Goldin, 1986; Koch et al., 1987; Pearce et al., 1988] and, indeed, cannot be understood otherwise, since at nonsaturating nucleotide concentrations, part of the nucleotide-sensitive Ca^{2+} pool cannot be released. As previously described [Ildefonse & Bennett, 1991], the channel can take at least 4 open conformations, corresponding to the binding of one, two, three or four cGMP molecules, and having discrete conductance levels (about 25, 50, 75 and 100% respectively). The rate of calcium release is expected to reflect the proportion of channels in each of the four states, with lower rates when the lower conducting states are predominant. It is suggested that, at each conducting state of the channel, corresponds a different level of calcium release, which depends on both the rate of inactivation and the rate of calcium release. This inactivation is not observed in patch clamp or bilayers studies however, and has remained unexplained. Although it seems unlikely that such inactivation occurs in vivo, since the existence of the dark current implies that the channels remain open, understanding the mechanism which underlies this phenomenon might give indications concerning the structure of the channel. We have studied below the effect of –SH reagents on both the rate and amplitude of the nucleotide-induced calcium release in order to also investigate the possible role of –SH groups in the mechanism of inactivation of the channel.

Disulfide Bond Reducing Agents

Incubating membrane vesicles with DTT up to 40 mM during several hours, or addition of DTT (up to 10 mM) directly in the cuvette before measurement does not modify the relations between nucleotide concentration and amplitude or rate of the nucleotide induced calcium release. No effect of DTT (10 mM, incubation ≥6 min, 3 experiments) was observed on the activity of channels incorporated into a lipid bilayer.

Thiol-Group Reagents

Effects of N-ethylmaleimide (NEM) on Calcium Efflux

The experiments illustrated in Fig. 2 show that the channel activity is notably modified by treatment of membrane vesicles with NEM (0.5 mM, 15 min), only however if a saturating concentration of cyclic nucleotide is present during incubation (i.e., if the channels are mostly in the open state): for both the amplitude (*A*) and the

Table 2. Modification of the 8-Br-cGMP-induced calcium release by treatment of the rod outer segment membrane vesicles with NEM in the presence or absence of nucleotide (channels in the open or closed state respectively)

Incubation		Amplitude			Initial rate		
NEM	8-Br-cGMP 250 μM	Maximal signal (% of total calcium release)	EC_{50} for 8-Br-cGMP (μM)	Hill coefficient n_H	Maximal signal (% of total calcium release per sec)	EC_{50} for 8-Br-cGMP (μM)	Hill coefficient n_H
0.5 mM	–	59.2 ± 1.3	13.5 ± 1.8	1.34 ± 0.13	12.4 ± 1.3	33.4 ± 5.0	1.74 ± 0.19
15 min	(n = 3)						
	+	60.2 ± 2.4	0.2 ± 0.1	0.96 ± 0.20	12.4 ± 1.1	7.4 ± 0.9	0.61 ± 0.04
	(n = 2)						
50 mM	–	29.5 ± 3.0	4.6 ± 2.0	0.71 ± 0.21	3.8 ± 1.1	14.4 ± 3.1	0.48 ± 0.07
30 min	+	26.7 ± 1.6	0.2 ± 0.2	0.83 ± 0.47	2.8 ± 0.5	1.5 ± 1.1	0.63 ± 0.63

The vesicles were incubated in the dark with different concentrations of NEM ± 250 μM 8-Br-cGMP (*see* Materials and Methods) before measurements of the calcium efflux. The values are obtained from the fits to the Hill equation of the data from the experiments shown in Fig. 2 in which maximal "activation" is observed (NEM 0.5 mM, 15 min), and from a similar experiment where maximal "inhibition" is observed (NEM 50 mM, 30 min). The number of experiments (when > 1) is indicated between brackets. Compare with the values given in Table 1 for the control membranes.

initial rate (*B*) of the calcium efflux, the apparent affinity for the nucleotide is increased (EC_{50} decreased) and the Hill coefficient is reduced to values lower than 1 or close to 1 (9 experiments), indicating a loss of cooperativity for binding of the nucleotide. The experimental conditions in Fig. 2 (0.5 mM NEM, 250 μM nucleotide, 15 min incubation) were found to produce maximal activation. In the absence of nucleotide during incubation, the activity of the channel is not (initial rate), or only slightly modified (amplitude), suggesting that the site(s) for binding of NEM which regulates the activity is not accessible (or much less accessible) when the channel is in a closed state. The values of the maximal rate or amplitude, EC_{50} and n_H from the fits of the data from the experiments shown in Fig. 2 (incubation with 8-Br-cGMP) are displayed in Table 2 (compare with Table 1 for the control experiment). The same effect is observed when saturating concentrations of cGMP instead of 8-Br-cGMP are present during incubation with NEM (*not shown*).

At higher concentrations of NEM or after longer incubation, the apparent affinity for the nucleotide is significantly increased even when the vesicles are incubated in the absence of nucleotide (channels in the closed state), but still much less than when incubation is carried out in the presence of nucleotide (channels in the open state). In addition, independently of whether the incubation is carried out in the presence or absence of nucleotide, the maximum rate and amplitude of the calcium release are significantly reduced, indicating that at least one other –SH group regulates the activity of the channel. This reduction of the maximal signal ("inhibition") is perhaps already visible under the conditions of Fig. 2 (0.5 mM NEM), although the difference with the control experiment might not be significant. There is almost no increase of inhibition between 10 mM and 50 mM NEM (30 min incubation). Values obtained with 50 mM NEM, which are therefore assumed to correspond to maximal inhibition, are given in Table 2.

Effects of Mersalyl[3] on Calcium Efflux

Similar, although less pronounced, activation is observed with the noncovalent reagent mersalyl up to 30 μM, which is also associated with a loss of cooperativity for nucleotide binding (Fig. 3). Since mersalyl is not covalent, it is added directly in the pH-meter cuvette before addition of nucleotide. The same rate and amplitude are measured whether mersalyl is added several minutes before addition of nucleotide or immediately before (or after). The fact that activation is less pronounced than activation by NEM may be due to the fact that the –SH group may become accessible only after addition of the nucleotide (*see* below and Fig. 4), so that when the initial rate is measured, mersalyl has not had time to bind to all the sites. Fitting the dependence of the amplitude or rate of the nucleotide-induced calcium release on mersalyl concentration (8-Br-cGMP 10 μM) with the Hill equation gives an EC_{50} of 19.4 ± 1.5 μM with a Hill coefficient of 3.3 ± 0.4 (Fig. 3*C*), suggesting that at least 3 or 4 molecules of mersalyl bind to the channel in the activation mechanism. This result suggests that there is at least one binding site for mersalyl on each channel subunit. Higher concentrations of mersalyl induce a calcium release in the absence of nucleotide; the totality of the internal calcium can be released at 300 μM mersalyl, suggesting that this process does not (or not only) concern the channels, which do not release more than 70% of the total calcium. Inhibition by higher concentrations of mersalyl is however unambiguously observed in bilayers experiments (*see* below, Fig. 6).

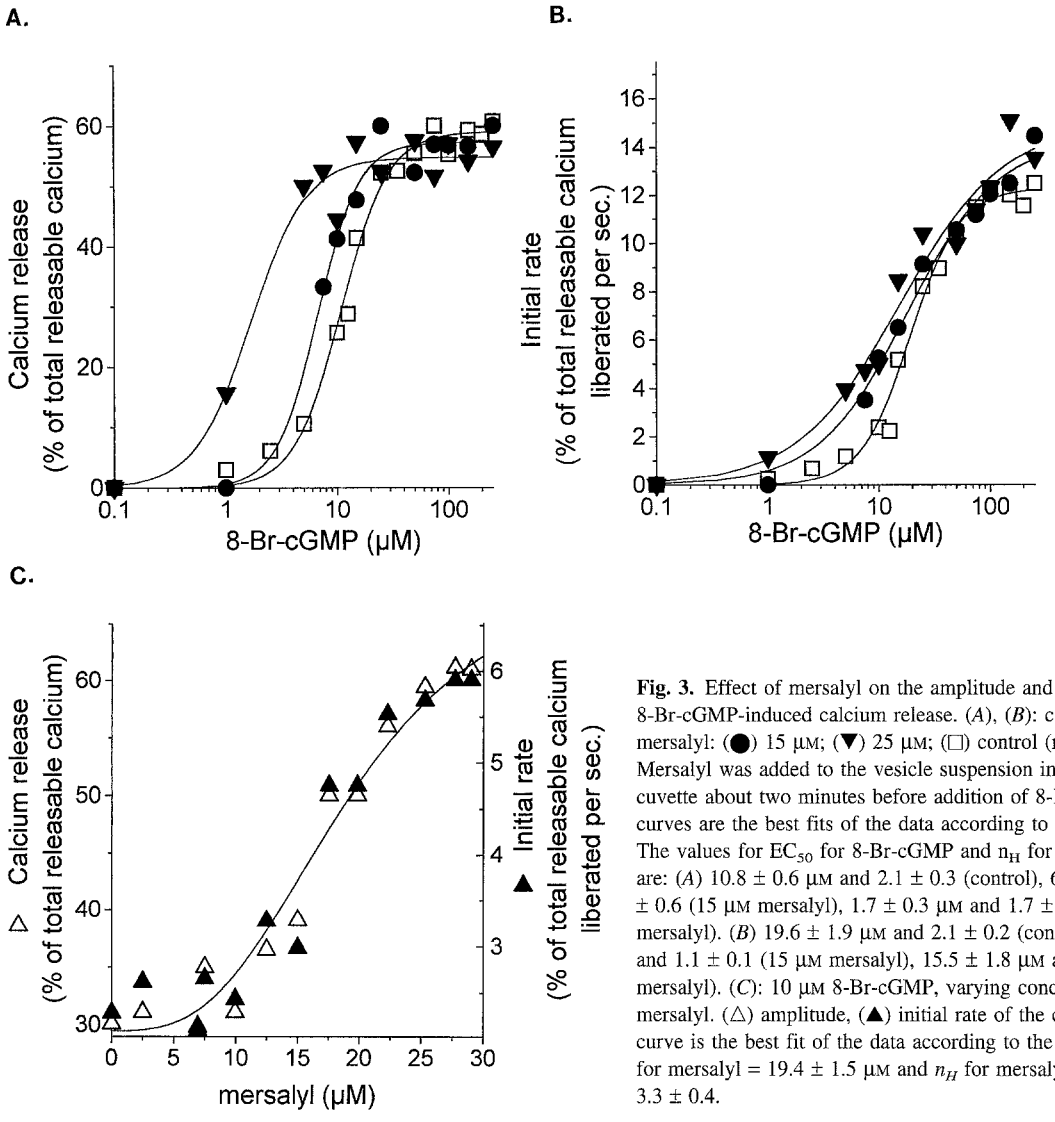


Fig. 3. Effect of mersalyl on the amplitude and initial rate of the 8-Br-cGMP-induced calcium release. (*A*), (*B*): concentration of mersalyl: (●) 15 μM; (▼) 25 μM; (□) control (no mersalyl). Mersalyl was added to the vesicle suspension in the pH-meter cuvette about two minutes before addition of 8-Br-cGMP. The curves are the best fits of the data according to the Hill equation. The values for EC_{50} for 8-Br-cGMP and n_H for 8-Br-cGMP binding are: (*A*) 10.8 ± 0.6 μM and 2.1 ± 0.3 (control), 6.6 ± 0.6 μM and 2.3 ± 0.6 (15 μM mersalyl), 1.7 ± 0.3 μM and 1.7 ± 0.9 (25 μM mersalyl). (*B*) 19.6 ± 1.9 μM and 2.1 ± 0.2 (control), 18 ± 1.2 μM and 1.1 ± 0.1 (15 μM mersalyl), 15.5 ± 1.8 μM and 0.9 ± 0.1 (25 μM mersalyl). (*C*): 10 μM 8-Br-cGMP, varying concentration of mersalyl. (△) amplitude, (▲) initial rate of the calcium release. The curve is the best fit of the data according to the Hill equation: EC_{50} for mersalyl = 19.4 ± 1.5 μM and n_H for mersalyl binding = 3.3 ± 0.4.

Successive Treatment with NEM and Mersalyl (Calcium Efflux)

Figure 4 shows that no significant additional activation by mersalyl is observed after previous incubation of the membranes with 0.5 mM NEM in the presence of nucleotide (compare with Fig. 2). However, if the membranes are incubated with NEM in the absence of nucleotide (blocking the –SH groups which are accessible in the closed state, but inducing no activation), then addition of mersalyl after removal of unreacted NEM produces an activation which is similar to that observed with untreated membranes (compare with Fig. 3). These results suggest that the same thiol groups, which are not accessible in the closed state, are involved in the activation by NEM and mersalyl. It can be noted in Fig. 4 that after incubation with NEM, and mainly after incubation with NEM + 8-Br-cGMP, inhibition (reduction of the amplitude and rate of the calcium release at saturating nucleotide concentration) as well as activation is observed at low mersalyl concentration which do not induce inhibition in the absence of previous treatment by NEM (Fig.3).

Single Channel Recordings in the Presence of NEM or Mersalyl

The effects of NEM (Fig. 5) and mersalyl (Fig. 6) have been studied on channels incorporated into a lipid bilayer. In the experiment shown in Fig. 5, where a single channel was incorporated, the open probability of the channel is increased about 90 sec after addition of 1 mM NEM, and the conductance increases step by step, successively underlining the 4 sublevels previously described [Ildefonse & Bennett, 1991] until the maximum level is reached (about 8 min after addition of NEM),

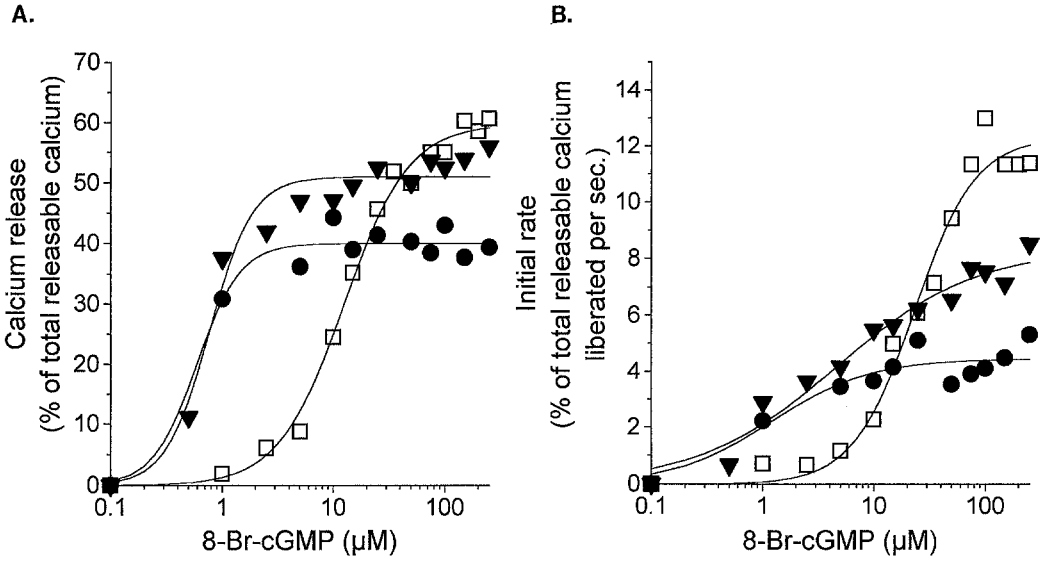


Fig. 4. Modification by mersalyl of the 8-Br-cGMP-induced calcium efflux from vesicles previously treated with NEM. The membranes were first treated with NEM (0.5 mM, 15 min) as in the experiments shown in Fig. 2, in the presence (●) or absence (▼) of 250 μM 8-Br-cGMP. Mersalyl (25 μM) was added directly to the NEM-treated membrane suspension in the pH meter cuvette about 2 min before addition of 8-Br-cGMP. (□): control membranes (no treatment with NEM, no mersalyl). The curves are the best fits of the data according to the Hill equation. The values for EC_{50} for 8-Br-cGMP and n_H for 8-Br-cGMP binding are: (*A*) 12.4 ± 0.7 μM and 1.6 ± 0.1 (control), 0.8 ± 0.1 μM and 2.3 ± 1.6 (NEM then mersalyl), 0.6 ± 0.2 μM and 2.3 ± 1.5 (NEM + 8-Br-cGMP, then mersalyl). (*B*) 23.2 ± 2.8 μM and 1.6 ± 0.3 (control), 4.5 ± 1.5 μM and 0.7 ± 1.3 (NEM then mersalyl), 1.2 ± 0.6 μM and 1.0 ± 0.4 (NEM + 8-Br-cGMP, then mersalyl).

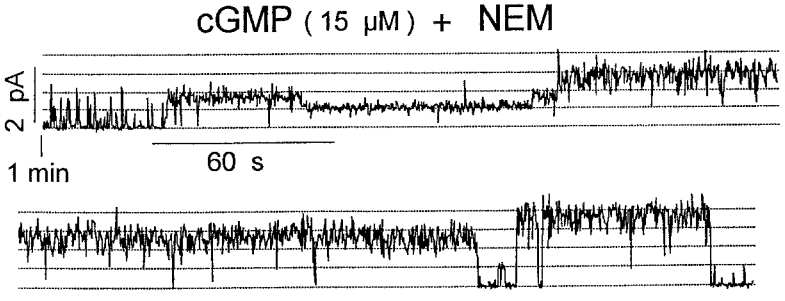


Fig. 5. Effect of NEM on a single channel incorporated into planar lipid bilayer (outward current). NEM (1 mM) was added directly into the *cis*-chamber after incorporation of a channel. Record starts 1 min after NEM addition. cGMP: 15 μM. Voltage: +60 mV. Lipids: PE/PS from bovine brain (*see* Materials and Methods). The dotted lines correspond to the 4 conductance levels previously described (25, 50, 75 and 100% of the maximal conductance) [Ildefonse & Bennett, 1991].

suggesting that 4 molecules of NEM successively bind to each of the 4 channel subunits. Similar behavior was observed in more than 10 experiments with a single channel, activation starting between 30 sec and 2 min after addition of NEM, although the 4 sublevels are not always so clearly underlined. No inhibition could be observed at 30 mM NEM after 20–40 min incubation in the presence of 100 μM 8-Br-cGMP (3 experiments).

The effect of mersalyl is shown in Fig. 6. Addition of mersalyl on the external side of the channel (*trans* chamber, corresponding to the inside of the vesicles) had no effect during at least 3 min (4 experiments), while addition of mersalyl on the cytoplasmic side of the channel (*cis* chamber) induces maximal activation almost immediately (from less than 1 to a few seconds after 10 sec of rapid stirring, 12 experiments), confirming that mersalyl cannot cross the membrane within the time course of our experiments. Prolonged incubation at low concentrations (<30 μM), or higher concentrations of mersalyl, result in decreasing the conductance although the open probability remains high (6 experiments). In the experiment shown in Fig. 6*B* (110 μM 8-Br-cGMP, 200 μM mersalyl), where two channels are incorporated, the conductance is decreased by steps corresponding to the sublevels, suggesting that there is one inhibition site on each subunit.

It is concluded, therefore, that the –SH groups which react with NEM, inducing activation of the channel, and also react with mersalyl, are situated on the cytoplasmic side of each channel subunit. Blocking at least another –SH group by mersalyl on the cytoplasmic side of each channel subunit induces a reduction of the conductance of the channel. It is not possible to know whether inhibition by NEM in flux measurements concerns the same cysteine residue(s); in both cases, however, inhibition requires higher concentration of reagent or longer incubation.

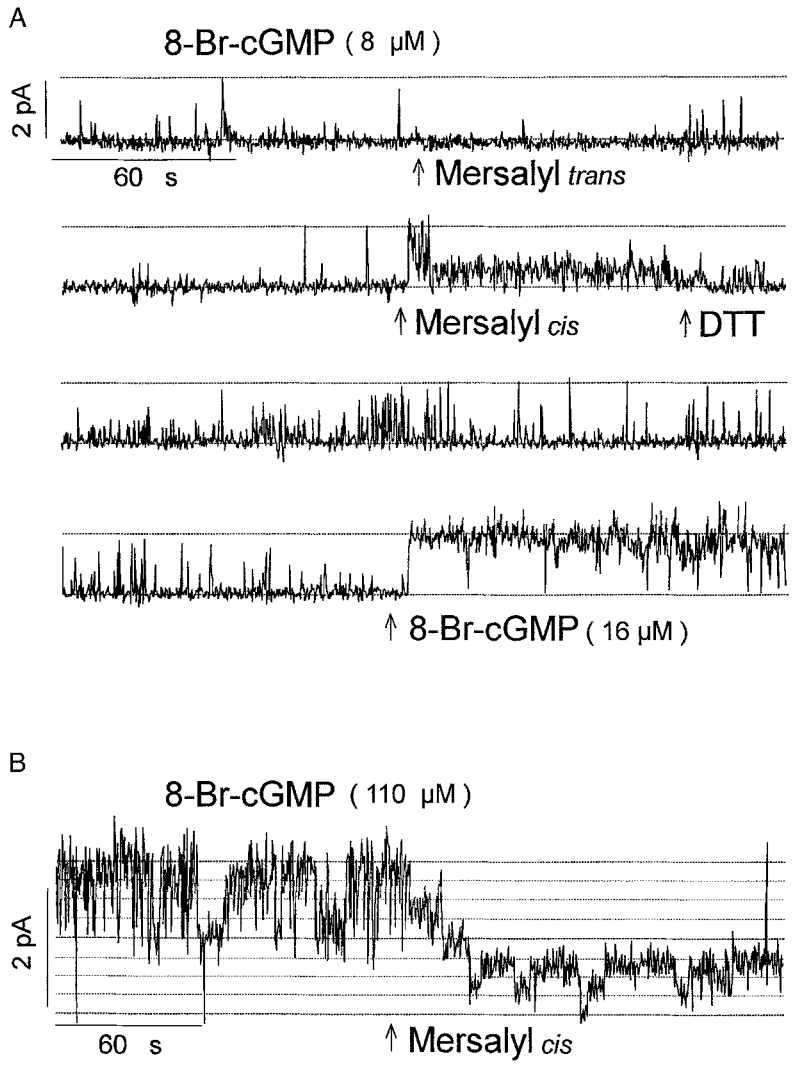


Fig. 6. Effect of mersalyl on channels incorporated into planar lipid bilayer (outward current). (*A*) single channel, 8 μM 8-Br-cGMP, +40 mV. Addition of mersalyl (60 μM) in the *trans*-chamber (corresponding to the external side of the channel) did not modify the channel activity for 3 min; a second addition (60 μM) in the *cis*-chamber induces a rapid increase of both the conductance and open probability of the channel; after about 30 sec, the conductance of the channel then decreases by steps. A few minutes after addition of excess –SH groups (1 mM DTT) to displace mersalyl from the channel sites, the activity of the channel returns to its initial level, and can be increased to the maximal current level by further addition of 8-Br-cGMP. Dotted lines: closed state and maximal conductance level. (*B*) 2 channels incorporated, 110 μM 8-Br-cGMP. Addition of 200 μM mersalyl in the *cis*-chamber induces progressive stepwise reduction of the conductance, which is successively stabilized at each conductance sublevel. Dotted lines underline the closed state and the 4 sublevels of each of the 2 incorporated channels. Lipids: synthetic POPE/POPC.

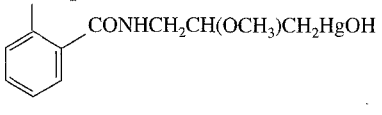
Disulfide Bridge Formation

Effects of Diamide[3] on Calcium Efflux and Single Channel Activity

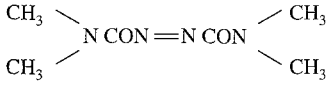
Diamide is a thiol oxidizing agent promoting dithiol-disulfide conversion, which, in contrast to oxidized glutathione, does not itself contain a disulfide bridge [Kosower & Kosower, 1969]. Like NEM, it has two effects on the nucleotide-induced calcium release: increase of the apparent affinity for the nucleotide (''activation''), and reduction of the amplitude and initial rate of the calcium release at saturating nucleotide concentrations (''inhibition'') (Fig. 7 and Table 3). As in the experiments with NEM, the Hill coefficient is reduced to values lower than 1, indicating a loss of cooperativity for nucleotide binding. While the activation is similar when incubation with diamide is carried out in the presence of cGMP or 8-Br-cGMP, the inhibitory effects of diamide is more pronounced when membrane vesicles are incubated with 8-Br-cGMP than with cGMP (Table 3). The presence of nucleotide during incubation is again necessary for activation, and facilitates inhibition which is observed to a lesser extent in the absence of nucleotide for the initial rate (Fig. 7*B*), but not for the amplitude (Fig. 7*A*). However, in contrast to the effect of NEM, both effects are observed simultaneously for a given diamide

[3]

mersalyl: OCH_2COONa



diamide (azodicarboxylic acid bis(dimethylamide)):



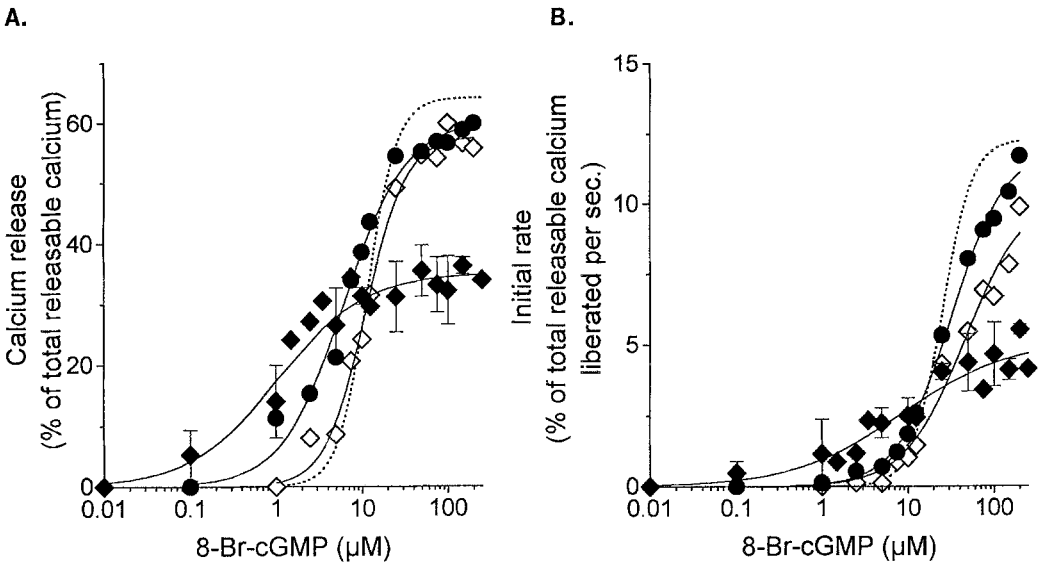


Fig. 7. Effect of diamide on the amplitude (A) and initial rate (B) of the 8-Br-cGMP-induced calcium release. Vesicles were incubated for 10 min in the dark with 5 mM diamide, in the absence (◇) or presence (♦) of 250 μM 8-Br-cGMP; the fits of the data from control membranes (Fig. 1*B*) are indicated with a dotted line. Part of the membranes treated with diamide in the presence of 8-Br-cGMP and then washed as described in Materials and Methods were incubated for 15 min with 10 mM DTT before measuring the calcium efflux (●). Data points are from one experiment (◇ and ●) or the mean from 3 different experiments (♦), and error bars represent the standard deviation. The curves are the best fits of the data according to the Hill equation; the values for the maximal signal, EC_{50} and n_H for the nonreduced membranes are given in Table 3. The values of EC_{50}/n_H from the fit of the data for the membranes treated with DTT (●) are for the amplitude: 6.2 ± 0.6 μM/1.2 ± 0.14, and for the initial rate: 31.8 ± 1.2 μM/1.4 ± 0.1. *See* Table 1 for the control membranes.

concentration, and it was not possible to find conditions where only activation could be observed. The conditions in Fig. 7 (5 mM, 10 min) were chosen because they give maximal activation but limited inhibition. Maximal activation by diamide is not significantly different from maximal activation by NEM. Maximal inhibition is achieved at 50 mM diamide (almost complete at 20 mM, not shown) (30 min incubation in the presence of nucleotide) and is also equivalent to maximal inhibition by NEM (compare Tables 2 and 3).

Values obtained from the fit of the data from the experiments shown in Fig. 7 (5 mM diamide, 10 min) and from similar experiments at higher diamide concentration (50 mM, 30 min) corresponding to maximal inhibition are displayed in Table 3 (compare with Table 1 for control membranes). Note that when incubation with diamide 50 mM is carried out in the presence of cGMP instead of 8-Br-cGMP, the amplitude is reduced to the same extent, whereas the initial rate is not.

The two effects of diamide, activation (increase of the apparent affinity for the nucleotide) and inhibition (reduction of the maximum rate and amplitude of the calcium release) are almost totally reversed by incubation of the diamide-treated vesicles with DTT (10 mM, 15 mn) (Fig. 7*A* and *B*) confirming that the two effects are due to disulfide bond formation. As stated above, incubation of untreated membranes with DTT has no effect on the amplitude nor on the rate of the efflux.

The fact that, under the conditions used in Fig. 7, the activation is maximal while the inhibition is only partial (at least as far as the amplitude is concerned, Table 3), suggests that two distinct S–S bridges can be formed: the first one, which induces "activation", would be formed in all the channels (or channel subunits) since activation is maximal, while the second, which induces "inhibition", would only concern part of the channels (or channel subunits) under the conditions used (5 mM diamide).

The activating effect of diamide (5 mM) on channels incorporated in a planar lipid bilayer was observed in all experiments performed (5). A single channel record is shown in Fig. 8: in the 3 experiments where a single channel was incorporated, only the second sublevel and the maximal level were underlined, as in the example shown. No inhibition at all was observed in 4 single channel records in the presence of saturating 8-Br-cGMP concentrations, where incubation could be carried out for 36 min (6 mM diamide), 20 min (10 mM diamide), 10 and 35 min (20 mM diamide).

Successive Treatment with NEM and Diamide (Calcium Efflux)

If the membranes are first treated with NEM (0.5 mM, 15 min) in the absence of nucleotide in order to block the –SH groups which are not involved in activation (induc-

Table 3. Modification of the 8-Br-cGMP-induced calcium release by treatment of the membranes with diamide in the presence or absence of nucleotide (channels in the open or closed state respectively)

Incubation		Amplitude			Initial rate		
Diamide	Nucleotide 250 μM	Maximal signal (% of total calcium release)	EC_{50} for 8-Br-cGMP (μM)	Hill coefficient n_H	Maximal signal (% of total calcium release per sec)	EC_{50} for 8-Br-cGMP (μM)	Hill coefficient n_H
5 mM	–	57.7 ± 1.4	11.1 ± 0.7	1.85 ± 0.22	10.4 ± 1.4	46.3 ± 4.0	1.24 ± 0.20
10 min.	cGMP (n = 5)	47.3 ± 2.7	0.2 ± 0.1	0.79 ± 0.17	8.0 ± 1.1	4.6 ± 2.5	0.59 ± 0.05
	8-Br-cGMP (n = 3)	35.2 ± 1.1	1.0 ± 0.4	0.86 ± 0.17	4.9 ± 0.2	6.7 ± 1.7	0.97 ± 0.30
50 mM	cGMP	29.9 ± 1.2	0.2 ± 0.1	1.48 ± 0.40	9.5 ± 0.9	10.0 ± 2.0	0.87 ± 0.08
30 min	8-Br-cGMP	24.8 ± 2.2	0.4 ± 0.5	0.91 ± 0.75	3.3 ± 0.2	2.0 ± 1.6	0.86 ± 0.37

The vesicles were incubated in the dark with diamide in the presence or absence of cGMP or 8-Br-cGMP (250 μM). The values are the fits of the data from the experiments shown in Fig. 7 (diamide 5 mM, 10 min: "activation") and from similar experiments (diamide 50 mM, 30 min: "inhibition"). The number of experiments (when > 1) is indicated between brackets. Compare with the values given in Table 1 for the control membranes and in Table 2 for the membranes treated with NEM.

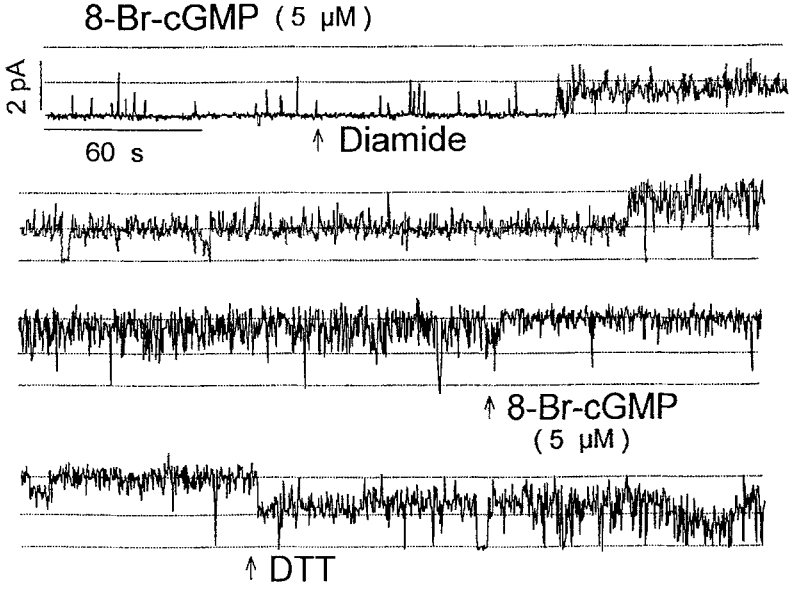


Fig. 8. Effect of diamide on a single channel incorporated into a planar lipid bilayer (outward current). Diamide (5 mM) was added in the *cis*-chamber after incorporation of a channel (8-Br-cGMP 5 μM). Note the stabilization of the second sublevel and then of the maximal level. A second addition of 5 μM 8-Br-cGMP stabilized the current at the maximal level, and addition of DTT (10 mM) induced partial reversion of the activation before the film broke. Note that reversion by DTT also underlines the second sublevel. Voltage + 60 mV. Lipids: synthetic POPE/POPC. Dotted lines: closed state, second sublevel and maximal conductance level.

ing no activation), and then treated with diamide in the presence of nucleotide, both activation and inhibition are observed, as in the absence of NEM treatment (Table 4, compare with Table 3). This indicates that none of the two –SH groups of each disulfide bridge is blocked by NEM in the closed state under the conditions used (therefore they are not or weakly accessible in the closed state). On the other hand, activation caused by treatment with NEM (0.5 mM, 15 min) in the presence of nucleotide is not further modified by additional treatment with diamide in the presence of nucleotide (Table 4, compare with Table 2). Indeed: (i) diamide does not further activate the 8-Br-cGMP-induced calcium release, and (ii) it does not reduce its maximal rate and amplitude, although, under the conditions used, NEM itself does not induce inhibition (indicating that at least one –SH group, which is responsible for inhibition by NEM, is still free). These results indicate that at least one of the –SH groups involved in the "activation" bridge, which is not accessible in the closed state, is the same as the –SH group(s) which is (are) involved in activation by NEM. They also indicate that at least one of the –SH groups involved in "inhibition" by diamide is also blocked by NEM in the open state under the conditions used (0.5 mM, 15 min), and that this –SH group is distinct from that involved in inhibition by NEM.

The fact that activation by diamide and by NEM (and therefore mersalyl) seems to concern the same sites

Table 4. Modification by diamide of the 8-Br-cGMP-induced calcium efflux from vesicles previously treated with NEM (0.5 mM, 15 min) in the presence or absence of 250 μM 8-Br-cGMP) (channels in the open or closed state respectively)

Incubation 1		Incubation 2		Amplitude			Initial rate		
NEM 0.5 mM 15 min	8-Br-cGMP 250 μM	Diamide 5 mM 10 min.	Nucleotide 250 μM	Maximal signal (% of total calcium release)	EC_{50} for 8-Br-cGMP (μM)	Hill coefficient n_H	Maximal signal (% of total calcium release per sec)	EC_{50} for 8-Br-cGMP (μM)	Hill coefficient n_H
+	−	+	8-Br-cGMP	35.7 ± 5.3	0.6 ± 0.2	0.40 ± 0.07	5.6 ± 0.5	7.1 ± 3.7	0.38 ± 0.09
+	+	+	8-Br-cGMP (n = 2)	54.8 ± 2.0	0.2 ± 0.1	1.09 ± 0.08	9.8 ± 0.4	2.4 ± 0.4	0.76 ± 0.08

The membranes were first treated with NEM (±8-Br-cGMP) as in the experiments shown in Fig. 2, and then treated with diamide + 250 μM 8-Br-cGMP (channels in the open state) as in Fig. 7. The values are the fits of the data according to the Hill equation. The number of experiments (when >1) is indicated between brackets. Compare with the values given in Tables 2 and 3 for the membranes treated with NEM only or with diamide only.

suggests that there is also at least one site for diamide-induced activation in each subunit. Again, it can be noted that inhibition by diamide, as inhibition by NEM and mersalyl, requires higher reagent concentrations than activation.

Effects of Oxidized Glutathione (GSSG)

The experimental data shown in Table 5 illustrate the effect of GSSG on 8-Br-cGMP-induced calcium release for concentrations from 2 mM to 7 mM (45 min incubation). Whatever the concentration, only an inhibitory effect is observed, on the amplitude and rate as well as on the EC_{50} (see Table 1 for comparison with control membranes). Incubation of membrane vesicles with GSSG alone gives the same modification of channel activity as incubation with GSSG and nucleotide (cGMP or 8-Br-cGMP). It must be noted that we cannot be sure that GSSG induces the formation of a disulfide bridge between 2 cysteine residues from the channel: it could also react with a single cysteine as a simple sulfhydryl reagent forming a channel-S-S-G molecule. Nevertheless, since GSSG is impermeant, this experiment, like the experiment with mersalyl shown in Fig. 6, indicates that blocking at least one –SH group on the cytoplasmic side of the channel induces inhibition. This (or these) –SH group may be the same as those which react with NEM, mersalyl or diamide under inhibiting conditions.

Attempts to observe the effect of GSSG on a single channel were all unsuccessful due to GSSG-related instability of the lipid bilayer.

Effects of Nitric Oxide Precursors

The NO precursors sodium nitroprusside (up to 1 mM) and SIN-1 (up to 1 mM) had no effect on the channel activity, neither in calcium flux measurements nor in bilayer experiments. This indicates that disulfide bridge formation by action of NO is not likely to be a physiological way of channel regulation in vivo.

Western Blots of Diamide and GSSG-treated Membranes

These experiments have been carried out in order to determine whether the disulfide bridges formed in the presence of diamide (and perhaps GSSG) join cysteine residues situated within the same subunit (in that case the molecular weight of the protein labeled with the antichannel antibody is expected to be 63 kDa), or of different subunits (in that case, several higher molecular weight complexes, corresponding to oligomers of the 63 kDa subunit, could be labeled under nonreducing conditions). Different samples were analyzed: (i) no activation nor inhibition (control membranes, membranes treated with diamide (5 mM) in the absence of nucleotide); (ii) maximal activation + weak inhibition (diamide 5 mM + cGMP, 10 min); (iii) maximal activation + intermediate inhibition (diamide 5 mM + 8-Br-cGMP, 10 min; membranes treated with NEM in the absence of nucleotide followed by diamide 5 mM + 8-Br-cGMP); (iv) maximal activation + maximal inhibition (diamide 50 mM + 8-Br-cGMP, 30 min); (v) inhibition without activation (GSSG 7 mM, 45 min). For each condition, one sample was denatured in SDS buffer containing β-mercaptoethanol (Fig. 9*A*) and another one in denaturing buffer in the absence of reducing agent (Fig. 9*B*). Western blots were labeled with the anti-63 kDa channel monoclonal antibody PMc 1F6, directed against the C-terminal region [L. Molday, *personal communication*]. All the reduced samples (Fig. 9*A*) show a single band corresponding to the 63 kDa monomer. In the non reduced samples (Fig. 9*B*), additional bands of higher molecular weight are also labeled, although with different

Table 5. Modification of the 8-Br-cGMP-induced calcium release by treatment of the membranes with oxidized glutathione

Incubation		Amplitude			Initial rate		
GSSG 45 min.	8-Br-cGMP 250 μM	Maximal signal (% of total calcium release)	EC_{50} for 8-Br-cGMP (μM)	Hill coefficient n_H	Maximal signal (% of total calcium release per sec)	EC_{50} for 8-Br-cGMP (μM)	Hill coefficient n_H
2 mM	+	60.6 ± 1.0	8.7 ± 0.4	2.28 ± 0.24	10.7 ± 0.6	30.3 ± 3.3	2.00 ± 0.30
4 mM	+	40.5 ± 1.1	12.9 ± 0.9	1.92 ± 0.25	5.3 ± 0.3	32.2 ± 2.5	2.00 ± 0.16
7 mM	+	29.9 ± 2.6	17.2 ± 4.5	1.22 ± 0.28	2.9 ± 0.1	26.6 ± 2.3	2.06 ± 0.31

The values are obtained from the fit of the data according to the Hill equation. Compare with Table 1 for the control membranes.

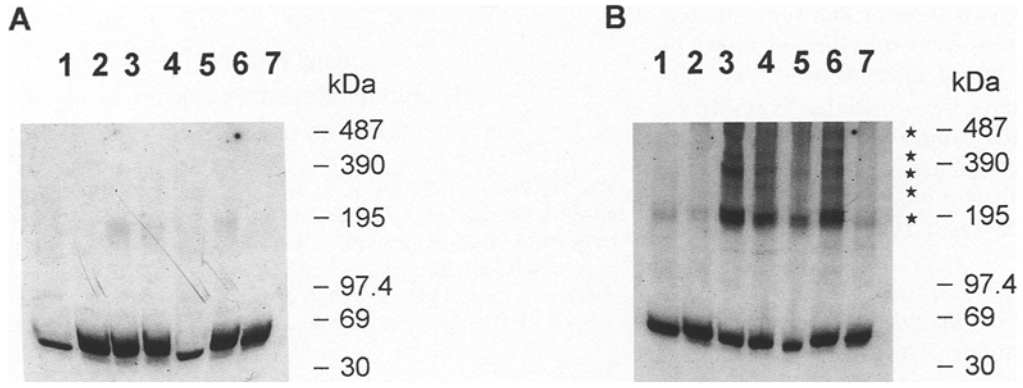


Fig. 9. Western blots of (*A*) reduced or (*B*) nonreduced SDS samples of ROS membranes. (1): control membranes; (2) membranes treated with 5 mM diamide in the absence of nucleotide (channel in the closed state); (3) membranes treated with 5 mM diamide + 250 μM cGMP (10 min); (4) membranes treated with 5 mM diamide + 250 μM 8-Br-cGMP (10 min); (5) membranes treated with 50 mM diamide + 250 μM 8-Br-cGMP (30 min); (6) membranes treated with diamide 5 mM + 250 μM 8-Br-cGMP (10 min) after previous treatment with NEM (0.5 mM, 15 min) in the absence of nucleotide; (7) membranes treated with GSSG 7 mM (45 min) + 250 μM 8-Br-cGMP. Conditions (3), (4) and (6) correspond to maximal activation by diamide, with partial inhibition (more pronounced for (4) and (6) than for (3)); (5) corresponds to maximal activation and maximal inhibition. In (6), previous treatment with NEM blocks the –SH groups which are accessible in the closed state, indicating that formation of the 180 kDa complex in (*B*) lanes (3) and (4) is due to –SH groups which are accessible only in the open state. Weaker labeling in (*A*) lanes (1) and (5) and in (*B*) lane (5) is due to incomplete transfer of the protein to the nitrocellulose sheet. Calibration: bovine carbonic anhydrase (30 kDa), bovine serum albumin (69 kDa), and cross-linked phosphorylase B (97.4 kDa; 195 kDa; 292 kDa; 390 kDa; 487 kDa).

intensity. The first band, with an approximate MW of 180 ± 10 kDa, appears as a double band when faintly labeled: it is present in low amounts in the control membranes (lane 1), membranes incubated with diamide in the absence of nucleotide (lane 2, conditions under which there is no activation nor significant inhibition), and membranes incubated with GSSG (lane 7, no activation). It is however strongly labeled when the membranes are incubated with diamide in the presence of cGMP or 8-Br-cGMP (lanes 3, 4 and 6: conditions under which activation is maximal and similar for the two nucleotides, while inhibition is much less pronounced for cGMP than for 8-Br-cGMP). Four other bands of higher MW (approximately 290, 350, 430 and 480 kDa) are also visible, but faintly labeled, in all samples. It is difficult to identify the stoichiometry of complexes of cross-linked subunits solely from their molecular weight, which is often different from (higher than) the sum of the molecular weights (*see* for example [Clerc, Catty & Bennett, 1992]); some high molecular weight complexes may also correspond to complexes of 63 kDa and 240 kDa subunits. Nevertheless, the first band above the monomer (which appears at 180 kDa in all samples including the control membranes) is in any case expected to be the dimer: indeed, formation of a dimer by cross-linking is statistically more probable than formation of a trimer, and it does not seem possible to obtain a trimer without any trace of dimer. Since the 180 kDa complex (which is therefore proposed to be a dimer) is formed in similar amounts whether cGMP or 8-Br-cGMP is present during incubation (conditions of maximal activation), these results therefore suggest that activation due to incubation with diamide is associated with the formation of a dimer. The fact that maximal activation gives rise to higher amounts of dimer (rather than of tetramer or pentamer) moreover suggests that the diamide-induced disulfide

bridge joins two cysteine residues situated at the same position in two adjacent subunits: indeed, formation of a disulfide bridge between two identical cysteine residues of adjacent subunits can only produce dimers whatever the level of activation, while formation of a disulfide bridge between two different residues of adjacent subunits is expected to produce dimers for minimal activation but tetramers (or pentamers according to the number of subunits per functional channel molecule) for maximal activation. It is interesting to note that in the single channel recordings in the presence of diamide (Fig. 8), only the second and fourth (maximal) levels are stabilized. If, as previously described [Ildefonse & Bennett, 1991], the four conductance sublevels correspond to binding of cGMP to one, two, three or four subunits, and if diamide increases the sensitivity to cGMP (as shown in Fig. 7), then the formation of a dimer between two subunits is expected to modify the sensitivity to cGMP of these two subunits at the same time. Thus, at cGMP concentrations where the channel is mostly in the closed state, formation of one (then of two) dimer(s) would lead to major expression of the second (then of the maximal) conductance level.

Since there is no additional band labeled under conditions where inhibition by diamide is either partial (10 min incubation with diamide 5 mM in the presence of 8-Br-cGMP, lane 4 and 6) or maximal (30 min incubation with 50 mM diamide in the presence of 8-Br-cGMP, lane 5), compared to lane 3 where inhibition is weak, it can be suggested that inhibition by diamide is associated with the formation of an intrasubunit bridge. This implies that it concerns 2 cysteine residues situated on the same side of the membrane, and close enough to react with each other: the only possible candidates are 2 of the 3 cysteine residues close to the nucleotide binding site. The absence of additional band in the sample incubated with GSSG does not allow to conclude whether GSSG forms an intrasubunit bridge (the same as diamide ?) or reacts with a single –SH group (*see above*).

Relation between Rate and Amplitude

The relation between the rate and amplitude of the calcium release measured for each 8-Br-cGMP concentration in experiments with control membranes, membranes treated with NEM (in the presence of 8-Br-cGMP) and membranes treated with diamide (in the presence of 8-Br-cGMP) is shown in Fig. 10. The data obtained from NEM-treated membranes, under conditions where activation but no inhibition is observed, are not distinct from those obtained from control membranes. The data obtained from diamide-treated membranes, which present both activation (at low nucleotide concentration) and inhibition (at higher nucleotide concentration), fall in the same range at lower rate/amplitude values (which correspond to activation) but exhibit a significantly different relation between rate and amplitude at higher rate/amplitude values (corresponding to inhibition).

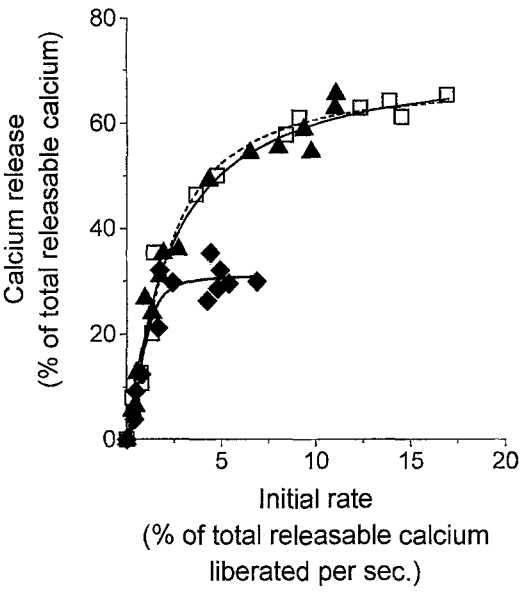


Fig. 10. Relation between initial rate and amplitude of the 8-Br-cGMP-induced calcium release from control vesicles, and vesicles treated with NEM and diamide. (□) control; (▲) membranes treated with NEM 0.5 mM in the presence of 250 μM 8-Br-cGMP (15 min); (♦) membranes treated with diamide 5 mM in the presence of 250 μM 8-Br-cGMP (10 min). Data from one experiment.

This suggests that the NEM- or diamide-induced decrease of EC_{50} for nucleotide observed for the variations of amplitude and rate of the calcium release are two consequences of the same modification. In the first paragraph (characterization of the calcium efflux), we have suggested that at each conducting state of the channel (1, 2, 3 or 4 cGMP bound) corresponds a different level of calcium release due to inactivation. That NEM and diamide (lower nucleotide concentrations) induce corresponding acceleration of the efflux and shift of the inactivation level towards that of higher conducting states suggests that their effect is to increase the association constant for the nucleotide, which will result in increasing the proportion of higher conducting states for a given nucleotide concentration. In the diamide-induced inhibition, on the contrary, the reduced rate of release at nucleotide saturation does not correspond to the amplitude level expected from the rate/amplitude relation of the control membranes: therefore the reduction of amplitude cannot be solely accounted for by a shift in the proportion of the different conducting states. Rate/amplitude relations obtained from experiments with inhibiting NEM concentrations or with GSSG are also distinct from that of control membranes, the data points being situated below those of control membranes as in the case of diamide-dependent inhibition (*not shown*). Thus, inhibi-

tion by cysteine modification seems to affect the channel activity at a level which is not (or not only) the affinity for the nucleotide: either at the level of its conductance or equilibrium constant for the open/closed transition, or at the level of the mechanism of inactivation. The fact that inhibition by NEM and diamide was not observed in bilayer experiments may suggest that this effect concerns the inactivation process (which is similarly absent in bilayers experiments). The fact that the amplitude/rate relation is similar in the case of inhibition by diamide, NEM and GSSG suggests in addition that inhibition by all the reagents tested is achieved through the same mechanism, and is likely to involve the same –SH group(s): therefore 1 or 2 of the 3 cytoplasmic cysteine residues (at least one of these being involved in activation, *see* above).

Discussion

We have described the effect of –SH reagents (NEM and mersalyl) and of thiol-oxidizing reagents (diamide, GSSG, NO precursors) on the activity of cGMP-gated channels, using two different approaches: measurement of nucleotide-induced calcium efflux from calcium loaded vesicles, and records of nucleotide-dependent sodium currents by incorporation of native vesicles into planar lipid bilayers. The results obtained from the two sets of experiments are not necessarily strictly comparable, but rather complementary. In flux measurements, a large number of experiments can be carried out and each measurement concerns a large number of channels; on the opposite, bilayer experiments allow direct time-resolved observation of the effect of a reagent on the activity of a single channel molecule.

Dual Effect of Cysteine Modification

NEM and diamide have a dual effect on the channel activity measured from calcium efflux: an activating effect, which is also observed with mersalyl (increase of the apparent affinity for the nucleotide), and an inhibitory effect (reduction of the amplitude and initial rate of the calcium release at saturating nucleotide concentration). Under the conditions used, activation by NEM and diamide, and inhibition by diamide, are observed when incubation with the reagent is carried out in the presence of nucleotide (channel mostly in the open state), but not (or much less) when incubation is carried out in the absence of nucleotide, indicating that the cysteine residues involved are not accessible, or much less accessible, in the closed state. The extent of activation and of inhibition is similar for NEM and diamide. In bilayer experiments, activation by NEM, mersalyl and diamide is also observed, but only mersalyl clearly induces inhibition (reduction of the channel conductance).

Activation

The activating effects of NEM and mersalyl, and of NEM and diamide are not additive. Moreover, previous treatment with NEM in the absence of nucleotide, in order to block the cysteine residues which are not involved in NEM activation, does not prevent activation by mersalyl or diamide. This suggests that the same –SH groups are involved in the 3 reactions. Bilayer experiments show that mersalyl is impermeant and acts on the cytoplasmic side, indicating that these –SH groups are situated on the cytoplasmic side of the protein. Of the 6 cysteine residues, only 3 are cytoplasmic according to the model of Bönigk et al. (1993): Cys481, Cys505 and Cys573, the first one being situated just before the cGMP binding site, and the two others within the binding pocket, in sheets β2 and β8 respectively [Kumar & Weber, 1992; Weber et al., 1987]. Cooperative effect of mersalyl for activation (Fig. 3*C*) suggests that binding sites are present on each channel subunit. This can also be concluded, for the activation site, from the single channel recording in the presence of NEM (Fig. 5) in which successive stabilization of each of the 4 conductance sublevels previously described [Ildefonse & Bennett, 1991] which correspond to nucleotide binding to 1, 2, 3 or 4 subunits, is observed. This suggests successive binding of a molecule of NEM to each of the 4 channel subunits, thus successively increasing the sensitivity to cGMP of each subunit, leading to an increased open probability of each conductance sublevel. Western blots of unreduced SDS samples of membranes incubated in the presence of diamide (Fig. 9*B*), which induces the formation of disulfide bridges ([Kosower & Kosower, 1969], and reversion by DTT: *see* Fig. 7), suggest that the activating disulfide bridge is formed between two –SH groups of cysteine residues at the same position in two adjacent subunits, thus forming dimers. Steric hindrance could explain the lack of activation (and even slight increase of EC_{50} for the nucleotide) by GSSG, which is much larger than diamide, and might not be able to reach the activation site even in the open conformation.

Inhibition

The fact that higher concentrations of NEM, mersalyl or diamide are required for maximal inhibition than for maximal activation suggests that there is one site for activation and one site for inhibition. In the case of diamide, the results therefore suggest the existence of two distinct bridges, one which activates, and one which in-

hibits. Western blots of unreduced samples of membranes incubated in the presence of diamide (Fig. 9*B*) suggest that the inhibitory bridge would join two –SH groups situated within the same subunit, which must be therefore 2 of the 3 cytoplasmic cysteine residues (the only ones situated close enough to form a disulfide bridge). From the molecular model of the cGMP-binding domain [Kumar & Weber, 1992; Weber et al., 1987], it can be suggested that the 2 cysteine residues are either Cys505 and Cyst573 which face each other on both sides of the binding pocket, or Cys481 and Cys573 which are both situated underneath the binding site in the model, while Cys481 and Cys505 seem to be further apart. The fact that inhibition by diamide is more pronounced when the incubation is carried out in the presence of 8-Br-cGMP rather than cGMP suggests that steric hindrance due to the bromo substituent favors the "inhibition" bridge. Experiments with labeled NEM are underway in order to determine precisely which cysteine residues are responsible for activation and inhibition by NEM and diamide. The 2 impermeant reagents mersalyl and GSSG also induce inhibition when added on the cytoplasmic side. It is not known whether the same –SH group(s) is (are) involved in inhibition by the 4 reagents (NEM, mersalyl, diamide and GSSG), but several close similarities can be noted: (i) inhibition by NEM, mersalyl or diamide requires higher concentrations than activation, (ii) the extent of inhibition by NEM and diamide is equivalent, (iii) the rate/amplitude relation is similar for membranes treated with NEM, diamide and GSSG at inhibiting concentrations. It appears thus likely that inhibition by the 4 reagents concerns at least one common –SH group. The –SH group(s) involved in inhibition by NEM would therefore also be cytoplasmic. Experiments with successive treatment by NEM and diamide indicate that at least one of the –SH groups implicated in the diamide-induced inhibiting bridge (which is proposed to be cytoplasmic, *see above*) reacts with NEM in the open state without inducing inhibition (Table 4); since also at least one cytoplasmic –SH group is responsible for activation by NEM, it can be deduced that blocking the third cytoplasmic –SH residue (which is necessarily the second –SH group involved in the diamide-induced inhibiting bridge) with NEM would induce inhibition.

It is puzzling that inhibition by NEM and diamide was never observed in sodium current recordings (like the inactivation process), whereas it is always observed with mersalyl (Fig. 6). Although it cannot be totally excluded that the absence of inhibition in bilayer records may be due to particular conditions of these experiments, this may indicate that the action of mersalyl is different. Sodium current record showing the stepwise inhibition by mersalyl (Fig. 6*B*), underlining neighboring sublevels from the maximum to lower levels, suggests that mersalyl binds successively to each channel subunit (therefore

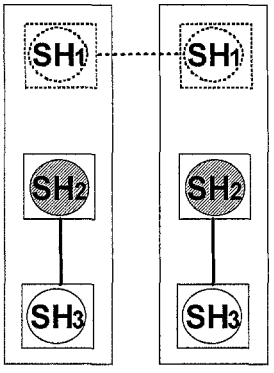


Fig. 11. Schematic representation of the sites of action of NEM and diamide. The diagram shows the cytoplasmic domain of 2 adjacent subunits, each containing 3 cysteine residues: SH1, SH2 and SH3. Attached squares represent diamide-induced disulfide bridges: the activating bridge (between the 2 subunits) is drawn with a dotted line, and the inhibiting bridges (intrasubunit) with full line. Circles represent binding of NEM: dotted line for activation and full line for inhibition; the grey circle indicates that SH2 also reacts with NEM, without inducing inhibition, but it is not known whether it participates in activation.

that there is at least one site on each subunit, rather than a single site within the pore).

Conclusion

Thus, altogether, our results suggest that blocking at least one of the 3 cytoplasmic cysteine residues by NEM or mersalyl, or by diamide (forming a disulfide bridge between 2 adjacent subunits) produces activation, while blocking at least one of the 2 others (NEM, mersalyl, GSSG) or the 2 others (diamide, formation of an intrasubunit bridge) produces inhibition. Interpretation of the action of NEM and diamide is schematically represented in Fig. 11.

Channel Inactivation

The result shown in Fig. 1 support the existence of a cyclic nucleotide-dependent inactivation process, as previously proposed from cation flux measurements [Puckett & Goldin, 1986; Koch et al., 1987; Pearce et al., 1988], and which is observed neither in patch-clamp studies nor in bilayer experiments. As stated above, this inactivation probably does not occur in vivo, but it would be interesting to understand the reason for its occurrence in flux measurements. Inactivation has been shown previously to be independent of the nature of the cation and of the gradient across the membrane: Pearce et al. (1988) have described the same phenomenon with calcium flux from calcium-loaded vesicles (as in our experiments) and sodium flux in the absence of a gradient. The existence

of a calcium gradient across the vesicle membrane gives rise, upon addition of cGMP (which allows calcium efflux), to a diffusion potential which is positive outside and therefore could oppose to the cation efflux; this diffusion potential will be dissipated at the same time as the calcium gradient, only if charge equilibration is allowed. In our experiments, charge equilibration is allowed by K^+ influx through the channel. On the opposite, sodium currents shown in Figs. 5, 6 and 8 are determined by the direction of the imposed voltage (cations moving from the positive side to the negative side, a situation which cannot be reproduced in flux measurements). However, in agreement with the results of Pearce et al. (1988) with sodium flux, it seems unlikely that inactivation is related to this difference, since addition of valinomycin or gramicidin, which abolish the diffusion potential across the vesicle membrane, does not modify the nucleotide dependence of the calcium release. Moreover, no inactivation is observed in bilayer experiments, if sodium currents are determined by the ionic gradient, imposing the voltage at 0 (*not shown*). Schnetkamp (1990) on the other hand measured sodium influx into ROS vesicles from absorbance change of the dye neutral red in the absence of any other cation, so that cGMP-induced sodium influx can only take place in the presence of a proton ionophore which allows charge equilibration. Addition of the proton ionophore at varying time after addition of the nucleotide induces identical sodium fluxes, demonstrating that there is no inactivation due to a ligand-induced conformation change (desensitization) as in the case of the acetylcholine receptor. Although the mechanism underlying inactivation remains unexplained, our results (Fig. 1) suggest that at each open state of the channel (1, 2, 3 or 4 molecules of cGMP bound) corresponds a different level of calcium release due to inactivation. The fact that the amplitude/rate relation (Fig. 10) is not modified under activating conditions (0.5 mM NEM and 5 mM diamide at low nucleotide concentrations) indicates that the effect of these reagents is to increase the affinity for the nucleotide, so that the equilibrium is shifted towards higher conducting states of the channel, without modifying the inactivation process. Inhibition by NEM, diamide and GSSG, which modifies the amplitude/rate relation, seems to affect the channel activity at another level, which might be the mechanism of inactivation (since neither inhibition by NEM or diamide, nor inactivation is observed in bilayer records).

Conformation Change on Open/Closed Transition

It has been shown recently that two peptides from the C-terminal domain (amino acids 580-611 and 636-662) close to the cGMP-binding domain (485-610) are homolog to the "ball" of the voltage dependent K^+ channels, which binds to a receptor domain in the mouth of the channel [Kramer, Goulding & Siegelbaum, 1994]. This suggests that the C-terminal part of cyclic nucleotide-gated channel subunits could block the pore in the absence of nucleotide like a quadruple plug, and that binding of nucleotide would induce a conformation change which would unplug the pore [Goulding et al., 1992]. Our finding that the 3 cysteine residues close to the nucleotide binding site are weakly (or not) accessible in the closed state and become accessible in the open state could be consistent with this hypothesis.

Loss of Cooperativity for cGMP Binding

Another important result of the present study is the loss of cooperativity for nucleotide binding after treatment with NEM (as also reported by Balakrishnan et al., 1990) and diamide under activating conditions. One simple hypothesis is that, in the closed state, the four (five ?) channel subunits are linked by disulfide bridges at the level of the cGMP-binding site. Binding of the first cGMP molecule would break the bridge between one subunit and its neighbor, facilitating nucleotide binding to this adjacent subunit; this would break the bridge between the second and third subunit, and so on. These disulfide bridges would have to be very unstable, and, indeed, the major band labeled on the western blot of unreduced membranes (after SDS denaturation) is the monomer of 63 kDa, although the dimer and several higher MW bands are also present (Fig. 9*B*). The fact that DTT does not modify the cooperativity for nucleotide binding suggests that the disulfide bridges would be buried inside the protein in the closed state (which is also consistent with the "plug" hypothesis, *see above*). This model explains the cooperativity for nucleotide binding and the loss of cooperativity after –SH modification; it also explains the cooperativity for mersalyl binding, since binding of one molecule of mersalyl to one subunit with cGMP bound increases the affinity of the other subunits for the nucleotide, which allows another molecule of mersalyl to bind, etc.

Further experiments are however needed to resolve this question.

No Physiological Role for NO!

Finally, the absence of effect of nitric oxide precursors suggests that the activity of the channel is not regulated by nitric oxide in vivo.

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References

Balakrishnan, K., Padgett, J., Cone, R.A. 1990. Calcium flux in rod outer segment membranes: NEM potentiates the effects of cGMP. *Biophys. J.* **57**:371a (*Abstr.*)

Bauer, P.J. 1988. Evidence for two functionally different membrane fractions in bovine retinal rod outer segments. *J. Physiol.* **401:**309–327

Bennett, N., Dupont, Y. 1979. Use of Ca-EGTA buffers for transposing Ca^{2+} efflux into pH changes: application to rod outer segment membrane vesicles. *FEBS Lett.* **98:**229–232

Boesze-Battaglia, K., Albert, A.D., Yeagle, P.L. 1992. Fusion between disc membranes and plasma membrane of bovine photoreceptor cells is calcium dependent. *Biochemistry* **31:**3733–3738

Bönigk, W., Altenhofen, W., Müller, F., Dose, A., Illing, M., Molday, R.S., Kaupp, U.B. 1993. Rod and cone photoreceptor cells express distinct genes for cGMP-gated channels. *Neuron* **10:**865–877

Chen, T.-Y., Peng, Y.-W., Dhallan, R.S., Ahamed, B., Reed, R.R., Yau, K.-W. 1993. A new subunit of the cyclic nucleotide-gated cation channel in retinal rods. *Nature* **362:**764–767

Clerc, A., Catty, P., Bennett, N. 1992. Interaction between cGMP-phosphodiesterase and transducin α-subunit in retinal rods. A cross-linking study. *J. Biol. Chem.* **267:**19948–19953

Cook, N.J., Hanke, W., Kaupp, U.B. 1987. Identification, purification and functional reconstitution of the cyclic GMP-dependent channel from rod photoreceptors. *Proc. Natl. Acad. Sci. USA.* **84:**585–589

Cook, N.J., Molday, L.L., Reid, D., Kaupp, U.B., Molday, R.S. 1989. The cGMP-gated channel of bovine photoreceptors is localized exclusively in the plasma membrane. *J. Biol. Chem.* **264:**6996–6999

Eismann, E., Bönigk, W., Kaupp, U.B. 1993. Structural features of cyclic nucleotide-gated channels. *Cell. Physiol. Biochem.* **3:**332–351

Fabiato, A. 1988. Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *In:* Methods in Enzymology. S. Fleischer and B. Fleischer, editors. Vol. 157. pp. 378–417. Academic Press, San Diego

Goulding, E.H., Ngai, J., Kramer, R.H., Colicos, S., Axel, R., Siegelbaum, S.A., Chess, A. 1992. Molecular cloning and single-channel properties of the cyclic nucleotide-gated channel from catfish olfactory neurons. *Neuron* **8:**45–58

Hsu, Y.-T., Molday, R.S. 1993. Modulation of the cGMP-gated channel of rod photoreceptor cells by calmodulin. *Nature* **361:**76–79

Ildefonse, M., Bennett, N. 1991. Single-channel study of the cGMP-dependent conductance of retinal rods from incorporation of native vesicles into planar lipid bilayers. *J. Membrane Biol.* **123:**133–147

Ildefonse, M., Crouzy, S., Bennett, N. 1992. Gating of retinal rod cation channel by different nucleotides: comparative study of unitary currents. *J. Membrane Biol.* **130:**91–104

Kaupp, U.B., Niidome, T., Tanabe, T., Terada, S., Bönigk, W., Stühmer, W., Cook, N.J., Kangawa, K., Matsuo, H., Hirose, T., Miyata, T., & Numa, S. 1989. Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic GMP-gated channel. *Nature* **342:**762–766

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 behavior. *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

Kosower, E.M., Kosower, N.S. 1969. Lest I forget thee, glutathione . . . *Nature* **224:**117–120

Kramer, R.H., Goulding, E., Siegelbaum, S.A. 1994. Potassium channel inactivation peptide blocks cyclic nucleotide-gated channels by binding to the conserved pore domain. *Neuron* **12:**655–662

Kumar, V.D., Weber, I.T. 1992. Molecular model of the cGMP-binding domain of the cyclic GMP-gated ion channel. *Biochemistry* **31:**4643–4649

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680–685

Pearce, L.B., Calhoon, R.D., Burns, P.R., Vincent A., Goldin, S.M. 1988. Two functionally distinct forms of guanosine cyclic 3′,5′-phosphate stimulated cation channels in a bovine rod photoreceptor disk preparation. *Biochemistry* **27:**4396–4406

Puckett, K.L., Goldin, S.M. 1986. Guanosine 3′,5′-cyclic monophosphate stimulates release of actively accumulated calcium in purified disks from rod outer segments of bovine retina. *Biochemistry* **25:**1739–1746

Schnetkamp, P.P.M. 1990. Cation selectivity of and cation binding to the cGMP-dependent channel in bovine rod outer segment membranes. *J. Gen. Physiol.* **96:**517–534

Schnetkamp, P.P.M., Szerencsei, R.T. 1989. Silver ions induce a rapid Ca^{2+} release from isolated intact bovine rod outer segments by a cooperative mechanism. *J. Membrane Biol.* **108:**91–102

Sitaramayya, A. 1986. Rhodopsin kinase prepared from bovine rod disk membranes quenches light activation of cGMP phosphodiesterase in a reconstituted system. *Biochemistry* **25:**5460–5468

Weber, I.T., Steitz, T.A., Bubis, J., Taylor, S.S. 1987. Predicted structure of cAMP binding domains of Type I and II regulatory subunits of cAMP-dependent protein kinase. *Biochemistry* **26:**343–351