Accelerated Publications

Sodium Ions Selectively Eliminate the Fast Component of Guanosine Cyclic 3′,5′-Phosphate Induced Ca^{2+} Release from Bovine Rod Outer Segment Disks†

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ABSTRACT: Guanosine cyclic 3′,5′-phosphate (cGMP) induced Ca^{2+} release from bovine rod outer segment (ROS) disks showed two kinetic components that could be distinguished in three ways: (1) The slow component (half-rise time of about 30 s) was blocked by 1-cis diltiazem [cf. Koch, K. W., & Kaupp, U. B. (1985) *J. Biol. Chem. 260*, 6788–6800], whereas the fast component (half-rise time of <1 s) was not affected by 1-cis diltiazem. (2) The slow component required the presence of alkali cations, whereas the fast component did not. (3) Preincubation with Na^+ (50 mM) selectively eliminated the fast component, whereas the slow component was not affected. The action of Na^+ appeared to be caused by Na–Ca exchange removing Ca^{2+} from a pool that can also be accessed by cGMP. The slow component of cGMP-induced Ca^{2+} release was not affected by Na^+ and, hence, appears to reside in disks that do not contain a functional Na–Ca exchanger. The local anesthetic tetracaine blocked both the slow and fast component of cGMP-induced Ca^{2+} release from bovine ROS disks.

The plasma membrane of the outer segments of vertebrate rod photoreceptors contains a cation-selective conductance. This conductance is dependent on the presence of guanosine cyclic 3′,5′-phosphate (cGMP) on the cytoplasmic side of the membrane and has properties very similar to those of the light-sensitive conductance in rods (Fesenko et al., 1985; Yau & Nakatani, 1985; Matthews, 1987). In the absence of divalent cations single-channel currents can be recorded showing that ionic channels underly the cGMP-dependent conductance (Haynes et al., 1986; Zimmerman & Baylor, 1986). Cationic fluxes that require the presence of cGMP have also been demonstrated across rod outer segment (ROS)[1] disk membranes (Caretta et al., 1979; Caretta & Cavaggioni, 1983; Koch & Kaupp, 1985; Puckett & Goldin, 1986; Schnetkamp & Bownds, 1987).

Both the currents through the plasma membrane and the fluxes across disk membranes depend on the cGMP concentration in a sigmoidal manner (above references), and both are blocked by 1-cis diltiazem (Stern et al., 1986) and by 3,4-dichlorobenzamil (Nicol et al., 1987). These results suggest that both the rod outer segment disk and plasma membrane contain a very similar cGMP-dependent conductance. This simple picture is complicated by observations that both drugs completely block the cGMP-dependent currents in excised patches of plasma membrane but not always (benzamil) or completely (diltiazem) block the light-sensitive current in whole rods (Stern et al., 1986; Nicol et al., 1987). Furthermore, a kinetic analysis of the cGMP-dependent Ca^{2+} flux across bovine disk membranes reveals two kinetic components (Koch & Kaupp, 1985), whereas only a single component is obvious in frog disk membranes (Schnetkamp & Bownds, 1987). The present study describes experiments that enable the two different components of cGMP-dependent Ca^{2+} release in bovine ROS disks to be distinguished.

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Experimental Procedures

Preparation of Rod Outer Segment Disks. Bovine eyes were purchased from a local abbatoir and collected in a light-tight box. ROS with a sealed plasma membrane and enriched in Ca^{2+} were isolated according to procedures described before (Schnetkamp et al., 1979; Schnetkamp, 1986). The isolation medium contained 600 mM sucrose, 5% w/v Ficoll 400, 20 mM Tris-HCl (pH 7.4), 10 mM glucose, 1 mM $CaCl_2$, and 0.1 mM EDTA. After purification and washing, intact ROS were resuspended in a medium containing 600 mM sucrose, 5% w/v Ficoll 400, and 20 mM Hepes (adjusted to pH 7.4 with arginine). The suspension of intact ROS (overall rhodopsin concentration between 150 and 250 μM) was placed overnight at −20 °C. After thawing, the ROS suspension was hypotonically shocked by dilution with 20 volumes of 10 mM Hepes (adjusted to pH 7.4 with arginine). After 2–3 min 25 mM tetramethylammonium chloride was added from a 2.5 M stock solution. ROS disks were sedimented for 10 min at 10000 rpm in a fixed-angle rotor (Beckman J-20). The supernatant was discarded and the pellet resuspended in 300 mM sucrose, 2.5% w/v Ficoll 400, and 20 mM Hepes (adjusted to pH 7.4 with arginine). The overall rhodopsin concentration in the disk suspension was 150–200 μM. Experiments were performed within 3 h. Aliquots of the disk suspension were diluted in the desired medium immediately before use. The isolation procedure and all experimental procedures were carried out under dim red light illumination.

Ca^{2+} Measurements. cGMP-induced Ca^{2+} release was measured with the Ca^{2+}-indicating dye Arsenazo III in an SLM-Aminco DW2C spectrophotometer. The cuvette house

[1] Abbreviations: ROS, rod outer segment(s); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TMA, tetramethylammonium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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was equipped with a magnetic stirrer. In all measurements the dual-wavelength mode was used with the wavelength pair of 650 and 750 nm and a bandwidth of 6 nm. The calibration of Ca^{2+}-indicating signals was obtained by adding known amounts of Ca^{2+} to the cuvette containing the ROS suspension. Validation of the experimental procedures and further details have been documented elsewhere (Kaupp & Koch, 1985; Schnetkamp, 1986).

An aliquot of the concentrated disk suspension was diluted in a medium containing 150 mM KCl, 20 mM Hepes (adjusted to pH 7.4 with arginine), and 200 μM Arsenazo III. In all experiments Arsenazo III was occupied by Ca^{2+} at levels of 10–20%. The overall rhodopsin concentration in the cuvette ranged between 7 and 16 μM. The cuvette was cooled to 5 °C by a circulating water bath. Lowering the temperature reduced the Ca^{2+} leakage from ROS disks in the absence of cGMP. At 5 °C Ca^{2+} leakage was unaffected by the presence of tetracaine or l-cis diltiazem, whereas at room temperature addition of the drugs sometimes increased the leakage rate of Ca^{2+}. A second advantage of the lowered temperature was that the difference between the two kinetic components of cGMP-induced Ca^{2+} release became more conspicuous on the time scale of our recordings. Experiments carried out at 25 °C gave similar results. The amount of cGMP-induced Ca^{2+} release was the same at both temperatures, with the rate differing by a factor of about 2.2. Na^+ selectively eliminated the fast component while leaving the slow component unaffected, despite the fact that Na^+ removed more Ca^{2+} at the high temperature [cf. Schnetkamp (1986)].

cGMP-induced Ca^{2+} release was initiated by addition of cGMP from a concentrated stock solution (200 mM) under continuous stirring with a magnetic stirrer. This addition caused a small decrease in the difference in absorption ($A_{650nm} - A_{750nm}$) due to dilution (0.001–0.005 absorbance unit as compared with a change of 0.1 absorbance unit due to cGMP-induced Ca^{2+} release). All traces are corrected for this small dilution artifact. This correction was obtained from the change in absorption resulting from the addition of a second portion of 500 μM cGMP at the end of the experiment that did not cause any further Ca^{2+} release. Three further controls were done. First, addition of cGMP to the ROS disk suspension in the absence of Arsenazo III did not give rise to any absorption changes. Second, in the presence of the Ca^{2+} ionophore A23187 ROS disks discharge all their internal Ca^{2+} under our experimental conditions, and subsequent addition of cGMP resulted only in the dilution artifact. Third, addition of cGMP to the suspension medium without ROS disks illustrated the dilution artifact.

Results and Discussion

ROS disks subjected to strong osmotic shocks consistently gave rise to a larger release of Ca^{2+} upon addition of cGMP as compared with stacked disks with a leaky plasma membrane [cf. Koch and Kaupp (1985)]. In particular, the osmotic shock exposed a very rapid component of cGMP-induced Ca^{2+} release, which was completed within the mixing time of the addition of cyclic GMP (1–2 s) and which was less obvious in stacked disks with a leaky plasma membrane. A typical time course of cGMP-induced Ca^{2+} release from ROS disks at 5 °C initiated by a saturating cGMP concentration of 500 μM is shown in Figure 1. A time-unresolved release (fast component; half-rise time <1 s) of 0.075 ± 0.026 mol of Ca^{2+}/mol of rhodopsin (average ± SD of 15 preparations) is followed by a much slower release (slow component; half-rise time of 30–50 s), bringing the total Ca^{2+} release to 0.28 ± 0.05 mol of Ca^{2+}/mol of rhodopsin (average ± SD of 16 preparations). The total Ca^{2+} content of the disks as judged from Ca^{2+} release induced by the Ca^{2+} ionophore A23187 amounted to 1.04 ± 0.18 mol of Ca^{2+}/mol of rhodopsin (average ± SD of seven preparations). The initial rate of Ca^{2+} release by the slow component was estimated to be 1.12 ± 0.26 × 10^5 Ca^{2+} (outer segment)$^{-1}$ s^{-1} (average ± SD of 14 preparations). It appears unlikely that resealed inside-out vesicles of ROS plasma membrane contributed to the observed cGMP-induced Ca^{2+} release; the medium employed for the hypotonic shock did not contain Ca^{2+}, and such vesicles would therefore not be expected to contain Ca^{2+}.

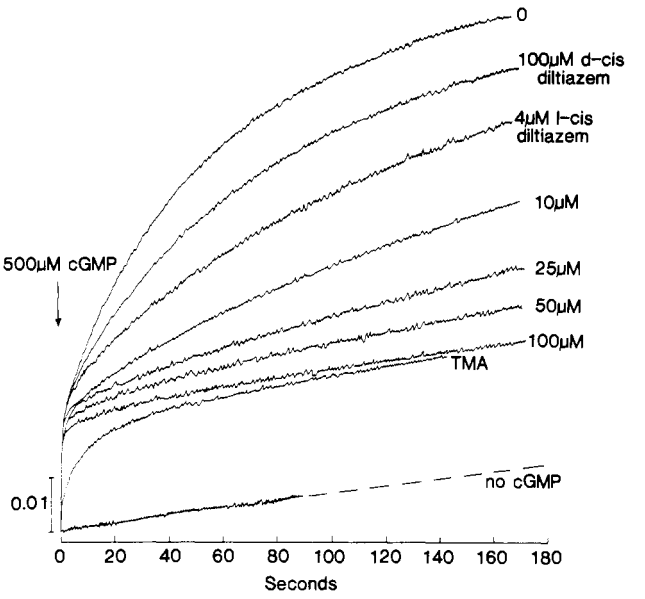

FIGURE 1: cGMP-induced Ca^{2+} release from bovine ROS disks at 5 °C. Hypotonically shocked disks were suspended in 150 mM KCl (tetramethylammonium chloride replaced KCl in the trace labeled TMA), 20 mM Hepes (adjusted to pH 7.4 with arginine), 200 μM Arsenazo III, and 1 μM FCCP. The rhodopsin concentration was 11 μM. At time zero 500 μM cGMP was added from a 200 mM stock solution. The disk suspension was preincubated for 5 min in the presence of the indicated concentrations of l-cis and d-cis diltiazem. Ca^{2+} leakage in the absence of cGMP was identical in all traces. d-Cis diltiazem was added at 100 μM, while l-cis diltiazem was present at the indicated concentrations. Ca^{2+} release was measured in the dual-wavelength mode by an increase in the absorption difference ($A_{650nm} - A_{750nm}$). The calibration bar of 0.01 absorbance unit represented a Ca^{2+} release of 0.039 mol of Ca^{2+}/mol of rhodopsin. The traces shown in Figure 1 and in all other figures were copied by hand from the original recordings. The noise in all traces was caused by the spin bar used to mix the suspension.

Koch and Kaupp (1985) reported that l-cis diltiazem was a potent blocker of the cGMP-induced Ca^{2+} release from bovine disks, whereas d-cis diltiazem was much less effective. This stereospecific block was confirmed with hypotonically shocked disks for the slow component of cGMP-induced Ca^{2+} release, but the fast component was not affected [Figure 1; see also Cook et al. (1987)]. At cGMP concentrations <50 μM the fast component predominated and could be resolved kinetically; l-cis diltiazem (100 μM) produced a blockage of <25%, whereas Ca^{2+} release induced by a subsequent addition of 500 μM cGMP was completely blocked (Figure 2C). Both slow and fast components were observed in media in which KCl was substituted by LiCl or CsCl. The amplitude of cGMP-induced Ca^{2+} release was greatly reduced when KCl was replaced by tetramethylammonium (TMA) chloride [cf. Koch & Kaupp (1985)]. In the TMACl medium the total cGMP-induced Ca^{2+} release was of similar magnitude as that observed in KCl medium with 100 μM l-cis diltiazem present (Figure 1). This suggests that the fast component does not require the presence of alkali cations but can be observed in

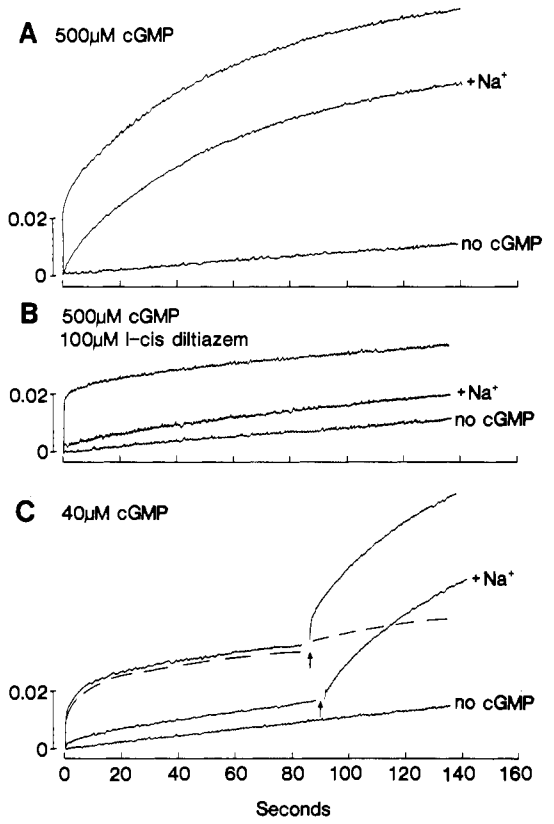

FIGURE 2: Na^+ eliminates the fast component of cGMP-induced Ca^{2+} release. Hypotonically shocked disks were preincubated for 10 min at 5 °C in 150 mM KCl, 20 mM Hepes (adjusted to pH 7.4 with arginine), 200 μM Arsenazo III, and 1 μM FCCP; 50 mM NaCl was added 10 min before addition of cGMP to all traces labeled Na^+. The rhodopsin concentration was 16 μM. The Ca^{2+} leakage immediately before addition of cGMP was the same in all traces, and a representative trace is illustrated (labeled no cGMP). At time zero cGMP was added to the indicated final concentration. The calibration bar of 0.02 absorbance unit represented a Ca^{2+} release of 0.055 mol of Ca^{2+}/mol of rhodopsin. (A) At time zero 500 μM cGMP was added. There were no further additions. (B) At time zero 500 μM cGMP was added; 100 μM 1-cis diltiazem was added to all traces 1 min before addition of cGMP. (C) At time zero 40 μM cGMP was added. In one trace 100 μM 1-cis diltiazem was added 1 min before addition of cGMP (broken line). About 90 s after the addition of 40 μM cGMP, a further addition of 500 μM cGMP was made in all traces except that labeled no cGMP (indicated by the arrows).

the TMACl medium albeit slower. In further support, 1-cis diltiazem (100 μM) had little effect on cGMP-induced Ca^{2+} release in TMACl medium (not shown). The remainder of this paper describes experiments on the effect of Na^+ on cGMP-induced Ca^{2+} release from bovine ROS disks. The results suggest that the two components of cGMP-induced Ca^{2+} release represent distinct Ca^{2+} pools.

Treatment with Na^+ Selectively Eliminates the Fast Component of cGMP-Induced Ca^{2+} Release. In addition to a cGMP-dependent pathway for Ca^{2+} transport, ROS disk membranes also contain a Na–Ca exchanger (Schnetkamp et al., 1977; Caretta, 1985; Schnetkamp, 1986; Schnetkamp & Bownds, 1987). Not surprisingly, it has been reported that the presence of Na^+ reduces the Ca^{2+} content of disks and decreases the amount of cGMP-induced Ca^{2+} release in bovine disks, although the reported effects are quantitatively quite different (Koch & Kaupp, 1985; Caretta, 1985). In frog disks, Na^+ and cGMP appear to have access to different Ca^{2+} pools since Na^+ does not preempt the cGMP-dependent pool (Schnetkamp & Bownds, 1987).

The experiment illustrated in Figure 2A shows the effect of preincubating bovine ROS disks in KCl medium to which

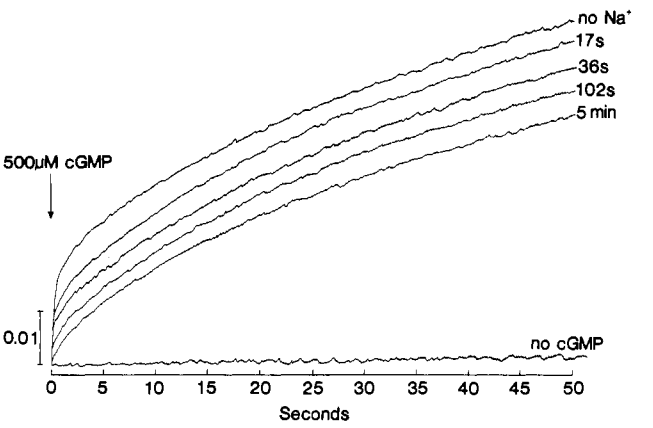

FIGURE 3: Na–Ca exchange removes Ca^{2+} to be released by cGMP in the fast component. Hypotonically shocked disks were preincubated for 5 min at 5 °C in 150 mM KCl, 20 mM Hepes (adjusted to pH 7.4 with arginine), 200 μM Arsenazo III, and 1 μM FCCP. The rhodopsin concentration was 14 μM; 50 mM NaCl was added in the four traces labeled with the time elapsed between the NaCl addition and the subsequent addition of cGMP. At time zero 500 μM cGMP was added except for the trace labeled no cGMP. The calibration bar of 0.01 absorbance unit represented a Ca^{2+} release of 0.031 mol of Ca^{2+}/mol of rhodopsin.

50 mM NaCl was added. The resulting traces suggest that Na^+ selectively eliminates the fast component of cGMP-induced Ca^{2+} efflux while leaving the slow component unaffected (the trace labeled "Na^+" can be superimposed on the control trace by moving it up by 0.025 absorbance unit). To corroborate this interpretation, two further experiments were done. First, since 1-cis diltiazem blocks the slow component of cGMP-induced Ca^{2+} release (Figure 1), and since Na^+ "blocks" the fast component, the presence of both Na^+ and 1-cis diltiazem should completely block cGMP-induced Ca^{2+} release (cf. Figure 2A,B). Second, Koch and Kaupp (1985) reported that only the fast component of cGMP-induced Ca^{2+} release is observed at cGMP concentrations under 50 μM, which are too low to activate the slow component. This observation suggests another way to demonstrate that Na^+ selectively eliminates the fast component of cGMP-induced Ca^{2+} release from bovine ROS disks. Addition of 40 μM cGMP activated only the fast component of Ca^{2+} release as shown by the lack of inhibition by 1-cis diltiazem (Figure 2C, broken line). If Na^+ selectively eliminates the fast component, pretreatment with Na^+ should abolish Ca^{2+} release at low cGMP concentrations as is observed (Figure 2C). To these cuvettes was added a high concentration of cGMP (500 μM) at the second arrow about 90 s after addition of low cGMP (40 μM). Since most of the fast component was already released by the addition of 40 μM cGMP, the second addition of cGMP is expected to activate predominantly the slow component of Ca^{2+} efflux. This means that the Ca^{2+} release caused by the second cGMP addition should be blocked by 1-cis diltiazem but should not be affected by Na^+ (Figure 2C).

The results of the experiments illustrated in Figure 1 and 2 show that cGMP-induced Ca^{2+} release from bovine ROS disks has two distinct components: one is blocked by 1-cis diltiazem, while the other is "blocked" by Na^+. It is interesting to note that cGMP-induced Ca^{2+} release from frog ROS disks shows only a slow component not affected by Na^+ (Schnetkamp & Bownds, 1987). The "blocking" action of Na^+ could be attributed to at least two mechanisms: either Na^+ is a true blocker of the transport mechanism, or Na^+ serves to deplete intradiskal Ca^{2+} by Na–Ca exchange so that no Ca^{2+} is left in the compartment accessible to the fast component of the cGMP-dependent transporter. This possibility was tested in

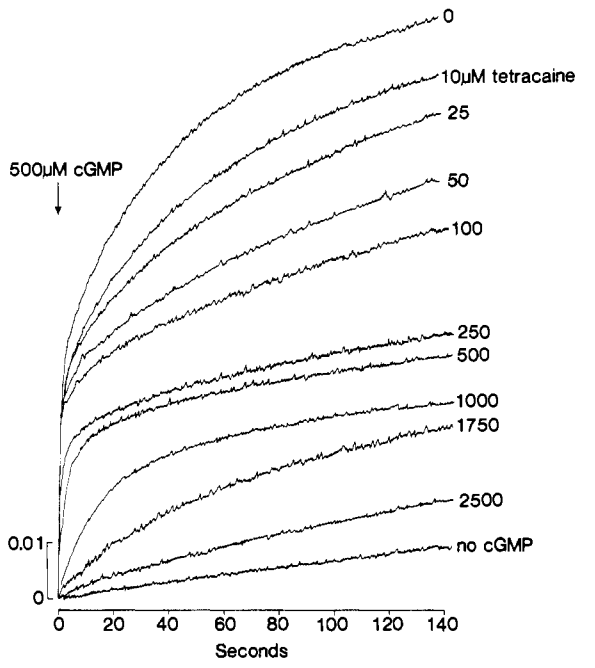

FIGURE 4: Tetracaine blocks both fast and slow components of cGMP-induced Ca^{2+} release. Hypotonically shocked disks were preincubated for 5 min at 5 °C in 150 mM KCl, 20 mM Hepes (adjusted to pH 7.4 with arginine), 200 μM Arsenazo III, and the indicated concentrations of tetracaine. The rhodopsin concentration was 15 μM. At time zero 500 μM cGMP was added except for the trace labeled no cGMP. Tetracaine did not alter the Ca^{2+} leakage prior to the addition of cGMP. The calibration bar of 0.01 absorbance unit represented a Ca^{2+} release of 0.029 mol of Ca^{2+}/mol of rhodopsin.

the experiment illustrated in Figure 3. Bovine disks were exposed to 50 mM Na^+ in the KCl medium for various lengths of time before addition of 500 μM cGMP. The addition of 50 mM NaCl caused a rapid burst of Ca^{2+} release of about 0.04 mol of Ca^{2+}/mol of rhodopsin completed in 5 s. The rapid burst of release was followed by a prolonged slow release, which totaled 0.17 mol of Ca^{2+}/mol of rhodopsin after 5 min (not shown). As more Ca^{2+} was removed by Na–Ca exchange, more of the fast component of the subsequent cGMP-induced Ca^{2+} release was eliminated (Figure 3). It should be emphasized that the fast component of cGMP-induced Ca^{2+} release amounted to only a small fraction of the total amount of Ca^{2+} present (0.075 mol of Ca^{2+}/mol of rhodopsin as compared with 1.14 mol of Ca^{2+}/mol of rhodopsin total Ca^{2+}). This suggests that the "slow" and "fast" cGMP-dependent Ca^{2+} transporters control distinct Ca^{2+} pools and perhaps reside in different disks. Thus, only a minor fraction of the disks would contain the fast cGMP-dependent transporter (and also a functional Na–Ca exchanger), and another fraction of the disks would contain the slow cGMP-dependent transporter (but no functional Na–Ca exchanger). This argument rests on the assumption that separate Ca^{2+} pools cannot be compartmentalized within a single disk; i.e., all Ca^{2+} within a single disk exchanges rapidly as has been demonstrated explicitly by the rapid release of all Ca^{2+} from disks upon addition the Ca^{2+} ionophore A23187 (Schnetkamp et al., 1977; Koch & Kaupp, 1985; Schnetkamp, 1986). In our experiments, the ionophore A23187 (2.5 μM) caused a complete Ca^{2+} release with a half-time of less than 2 s.

Tetracaine Blocks Both Components of cGMP-Induced Ca^{2+} Release. The local anesthetic tetracaine was found to block both the slow and the fast components of cGMP-induced Ca^{2+} release; blocking of the fast component required much higher concentrations than blocking of the slow component (Figure 4). Other local anesthetics such as lidocaine, procaine, and benzocaine (tested at 1 mM) were at most as effective as tetracaine at a concentration of 10 μM.

Conclusions

The results of this study show that cGMP-induced Ca^{2+} release in bovine ROS disks consists of two components that appear to reside in different disks. The fast component resides in disks that also contain a functional Na–Ca exchanger and is not blocked by 1-cis diltiazem. It is interesting to note that the cGMP-dependent flux mechanism purified from bovine ROS membranes was not blocked by 1-cis diltiazem (Cook et al., 1987). The slow component resides in disks that do not contain a functional Na–Ca exchanger and is blocked by 1-cis diltiazem. Both slow and fast components can be blocked by tetracaine, although higher concentrations of tetracaine are required for blocking the fast component. It is possible that the two kinetic components represent different proteins or different conformations of the same protein. In the latter case it is curious that conformational states of the cGMP-dependent mechanism are associated with the presence or absence of Na–Ca exchange.

Our results do not define a specific mechanism for cGMP-induced Ca^{2+} transport. The slow component requires the presence of alkali cations, and it has been suggested that the alkali cations electrically compensate for an electrogenic Ca^{2+} efflux (Caretta, 1985; Koch & Kaupp, 1985). However, electrical shunting of disk membranes by the electrogenic protonophore FCCP or by gramicidin [e.g., see Schnetkamp (1985)] does not result in the appearance of the slow component of cGMP-induced Ca^{2+} efflux in TMACl medium (Figure 1). The combination of FCCP and a cGMP-dependent Ca^{2+} channel is equivalent to the presence of the Ca–H exchanger A23187, which causes a complete release of all internal Ca^{2+} from disks (Schnetkamp et al., 1977; Caretta, 1985; Koch & Kaupp, 1985). Also, Mg^{2+} is believed to pass through the cGMP-dependent transporter in disks (Caretta & Cavaggioni, 1983), but addition of Mg^{2+} to the TMA medium did not change the cGMP-induced Ca^{2+} release (not shown). Therefore, the requirement for alkali cations appears to have some structural basis rather than compensation for an electrogenic Ca^{2+} efflux. Precedence for such a structural effect can be found in the electroneutral Ca–Ca exchange mode of the Na–Ca exchanger in heart sarcolemma. Ca–Ca exchange in this system is stimulated by alkali cations such as Li^+ or K^+, which are not themselves transported (Slaughter et al., 1983).

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References

Caretta, A. (1985) *Eur. J. Biochem. 148*, 599–606.

Caretta, A., & Cavaggioni, A. (1983) *Eur. J. Biochem. 132*, 1–8.

Caretta, A., Cavaggioni, A., & Sorbi, R. T. (1979) *J. Physiol. (London) 295*, 171–178.

Cook, N. J., Hanke, W., & Kaupp, U. B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 585–589.

Fesenko, E. E., Kolesnikov, S. S., & Lynbarsky, A. L. (1985) *Nature (London) 313*, 310–313.

Haynes, L. W., Kay, A. R., & Yau, K.-W. (1986) *Nature (London) 321*, 66–70.

Koch, K. W., & Kaupp, U. B. (1985) *J. Biol. Chem. 260*, 6788–6800.
Matthews, G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 299–302.
Nicol, G. D., Schnetkamp, P. P. M., Siami, Y., Cragoe, E., & Bownds, M. D. (1987) *Biophys. J. 51*, 272a.
Puckett, K. L., & Goldin, S. M. (1986) *Biochemistry 25*, 1739–1746.
Schnetkamp, P. P. M. (1985) *J. Membr. Biol. 88*, 263–275.
Schnetkamp, P. P. M. (1986) *J. Physiol. (London) 373*, 25–45.
Schnetkamp, P. P. M., & Bownds, M. D. (1987) *J. Gen. Physiol. 89*, 481–500.
Schnetkamp, P. P. M., Daemen, F. J. M., & Bonting, S. L. (1977) *Biochim. Biophys. Acta 468*, 259–270.
Schnetkamp, P. P. M., Klompmakers, A. A., & Daemen, F. J. M. (1979) *Biochim. Biophys. Acta 552*, 379–389.
Slaughter, R. S., Sutko, J. L., & Reeves, J. P. (1983) *J. Biol. Chem. 258*, 3183–3190.
Stern, J. H., Kaupp, U. B., & MacLeish, P. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1163–1167.
Yau, K.-W., & Nakatani, K. (1985) *Nature (London) 317*, 252–255.
Zimmerman, A. L., & Baylor, D. A. (1986) *Nature (London) 321*, 70–72.

Articles

Time-Dependent Binding of Paramagnetic and Fluorescent Hydrophobic Ions to the Acetylcholine Receptor from *Torpedo*†

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ABSTRACT: In receptor-rich vesicles isolated from *Torpedo*, paramagnetic or fluorescent phosphonium ions bind to both the acetylcholine receptor (AcChR) and the receptor membrane. When added to receptor vesicles, two to three phosphoniums undergo a slow time-dependent binding to the AcChR. The presence of agonist increases the rate but not the extent of binding of the alkylphosphonium nitroxides. Approximately one phosphonium per receptor can be displaced by the addition of saturating concentrations of the high-affinity histrionicotoxin derivative isodihydrohistrionicotoxin or by the addition of phencyclidine or quinacrine mustard. In addition, preincubation of the receptor with these channel blockers prevents approximately one phosphonium from binding to the receptor. When a series of alkyltriphenylphosphonium ions was studied, it was found that the rate of phosphonium binding to the receptor decreased with increasing probe hydrophobicity. This appears to be a function of the partitioning of the probe between membrane and aqueous phases. The phosphonium ions used here promote desensitization of the receptor, as judged by the binding rate of the fluorescent agonist NBDA-C_5-acylcholine or α-bungarotoxin. Preincubation of the receptor with isodihydrohistrionicotoxin virtually eliminates the phosphonium-mediated desensitization. The rates of the phosphonium-mediated desensitization also appear to be dependent upon the phase partitioning of the probe. These results strongly suggest that the binding sites for the phosphonium ion (and the high-affinity histrionicotoxin blocking site) are accessible only through the aqueous phase. The phosphonium binding and agonist-induced transitions observed here are not observed with a negative hydrophobic ion probe, or a negative surface amphiphile, indicating that modifications in membrane electrostatics do not contribute to the observed changes. These spin-labeled and fluorescent phosphonium derivatives provide a new set of probes to study channel blocking sites on the acetylcholine receptor.

Among the intrinsic ion channels present in excitable membranes, the chemically gated nicotinic acetylcholine receptor (AcChR)[1] represents a unique opportunity to examine both the structural and the electrical properties of a biological ion channel. The electric organs of certain eels and rays provide an abundant source of the AcChR, and this has greatly facilitated the biochemical and physical characterization of this important membrane protein [see Changeux et al. (1984) for a review]. In this receptor channel, there are a number of functionally and structurally distinct sites. In addition to the primary agonist binding site (for acetylcholine), there appear to be both high- and low-affinity sites for noncompetitive channel blockers (Heidmann et al., 1983; Oswald et al., 1983) and an additional regulatory binding site for agonist (Takeyasu et al., 1983, 1986). Compounds that bind to the high-affinity channel blocking site are particularly intriguing,

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[1] Abbreviations: AcChR, nicotinic acetylcholine receptor ion channel complex; PCP, phencyclidine; HTX, histrionicotoxin; isoHTX, isodihydrohistrionicotoxin; EbTX, erabutoxin b; Carb, carbamylcholine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; TEMPOL, 4-hydroxy-TEMPO; NBDA, (7-nitro-2,1,3-benzoxadiazol-4-yl)-amino; $TPMP^+$, triphenylmethylphosphonium; $TPDP^+$, triphenyldodecylphosphonium; EPR, electron paramagnetic resonance.

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