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Effects of 8-bromo cyclic GMP and verapamil on depolarization-evoked Ca^{2+} signal and contraction in rat aorta

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1 The pharmacological action of NO donors is usually attributed to a cellular rise in guanosine 3':5'-cyclic monophosphate (cyclic GMP), but this hypothesis is based only on indirect evidence. Therefore, we have studied the effects of cyclic GMP on Ca^{2+} movements and contraction in rat isolated endothelium-denuded aorta stimulated by KCl depolarizing solution using the permeant analogue 8-bromo cyclic GMP (BrcGMP). Isometric contraction and fura-2 Ca^{2+} signals were measured simultaneously in preparations treated with BrcGMP and with verapamil. The activation of calcium channels was estimated by measuring the quenching rate of the intracellular fura-2 signal by Mn^{2+} and by the depolarization-dependent influx of $^{45}Ca^{2+}$.

2 Stimulation with 67 mM KCl-solution evoked an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and a contractile response which were inhibited by pretreatment with verapamil (0.1 μM) or BrcGMP (0.1–1 mM). However, the inhibition of the fura-2 Ca^{2+} signal was significantly higher with verapamil than with BrcGMP, whereas the contraction was inhibited to a similar extent.

3 When preparations were exposed to K^+-depolarizing solution in which the calcium concentration was cumulatively increased, the related increase in fura-2 Ca^{2+} signal was barely affected by BrcGMP, whereas the contractile tension was strongly and significantly inhibited.

4 Cellular Ca^{2+} changes were also estimated with $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ influx in resting preparations was significantly reduced by BrcGMP (0.1 mM) but not by verapamil (0.1 μM); $^{45}Ca^{2+}$ influx in KCl-depolarized preparations was reduced by verapamil but was unaffected by BrcGMP.

5 Measurements of Mn^{2+}-induced quenching of the intracellular fura-2 signal showed that BrcGMP did not affect divalent cation entry in K^+-stimulated preparations, whereas verapamil concentration-dependently inhibited Mn^{2+} entry stimulated by K^+-depolarization.

6 The present results indicate that BrcGMP did not affect voltage-dependent Ca^{2+} channel gating in the rat aorta. For a given fura-2 Ca^{2+} signal, the contraction was lower in preparations exposed to BrcGMP than in the untreated ones, suggesting that the activation of cyclic GMP-dependent kinases reduced the contractile efficacy of calcium. Furthermore, the reduction of depolarization-dependent $^{45}Ca^{2+}$ uptake reported with sodium nitroprusside, a NO donor, was not observed with biologically active concentrations of BrcGMP, suggesting that this drug could have additional mechanisms of action, unrelated to activation of protein G-kinase.

Keywords: Rat aorta; cyclic GMP; fura-2; verapamil; $^{45}Ca^{2+}$ influx; Mn^{2+}; L-type calcium channel

Introduction

Guanosine 3':5'-cyclic monophosphate (cyclic GMP) is involved in the signal transduction mechanism activated by several hormones and drugs. Recently, the recognition of the activation of the soluble guanylate cyclase by nitric oxide (NO) has extended the importance of the role played by cyclic GMP in many cells, particularly vascular smooth muscle cells. It is established that endothelium-dependent vasorelaxations are largely mediated by NO or a closely related compound (Palmer *et al.*, 1987; Ignarro, 1989) and that endothelium-derived NO stimulates the soluble guanylate cyclase in smooth muscle cells, leading to an increase in cyclic GMP (Rapoport & Murad, 1983). However, the mechanism by which cyclic GMP induces vasorelaxation is not known. It has been suggested that vasorelaxation is related to a lowering of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$, for review see Schmidt *et al.*, 1993) but other studies have demonstrated that cyclic GMP (Pfitzer *et al.*, 1986) or endothelium-derived NO (Dietrich *et al.*, 1994) regulate vascular tone by a $[Ca^{2+}]_{cyt}$-independent mechanism. The effect of cyclic GMP also depends on the nature of the stimulation applied to the artery: in the rat aorta, removal of the endothelium enhances the contractile response and the Ca^{2+} influx evoked by α-adrenergic agonists, whereas it does not affect the contraction or the $^{45}Ca^{2+}$ influx stimulated by high K^+ solution (Godfraind, 1986).

Nitrocompounds are known to release NO and to increase cellular cyclic GMP levels (Feelisch & Kelm, 1991). The latter property is usually considered to be responsible for the cellular actions of these compounds so that they are often used to study the action of cyclic GMP in vascular smooth cells. For example, it has been reported that sodium nitroprusside decreases the cytosolic Ca^{2+} concentration in vascular smooth muscle and the sensitivity of contractile elements to Ca^{2+} (Karaki *et al.*, 1988), and that it uncouples the development of active stress from myosin phosphorylation (McDaniel *et al.*, 1992). These effects were attributed to the increase in cyclic GMP levels. However, not all studies support a role for cyclic GMP in mediating the vasorelaxant effects of nitrocompounds. Some effects of sodium nitroprusside could be due to the generation of cyanide (McDaniel *et al.*, 1992). Moreover, some of the effects of NO itself are independent of cyclic GMP production and could be related either to the marked reactivity of the nitric oxide free radical

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(Braughler *et al.*, 1979) or to a direct effect of NO (Gupta *et al.*, 1994; Bolotina *et al.*, 1994).

The purpose of the present study was to characterize the effect of cyclic GMP on the $[Ca^{2+}]_{cyt}$ and the Ca^{2+}-tension relationship in rat aorta contracted by high KCl. We used 8-bromo cyclic GMP (BrcGMP), a lipophilic and non-hydrolysable analogue of cyclic GMP, which enters smooth muscle cells and activates cyclic GMP-dependent kinases (Sekhar *et al.*, 1992). Experiments were carried out in the presence of N^{ω}-nitro-L-arginine (L-NOARG), an inhibitor of NO synthase (Moore *et al.*, 1990; Mulsch & Busse, 1990), in order to avoid interference by nitric oxide. Isometric contraction has been measured simultaneously with front-surface fluorometry of fura-2 loaded rings of aorta. Furthermore, the activation of calcium channels was estimated by measuring the rate of quenching of the intracellular fura-2 signal by Mn^{2+} simultaneously with muscle tone. In separate experiments, $^{45}Ca^{2+}$ influx through voltage-dependent Ca^{2+} channels was estimated by measuring the K^+ depolarization-evoked fast change in the specific activity of cellular calcium resistant to lanthanum (Godfraind, 1976).

The results show that BrcGMP decreases fura-2 Ca^{2+} signals in the rat aorta depolarized by KCl without affecting voltage-dependent Ca^{2+} influx, indicating that this could be due to increased rate of Ca^{2+} sequestration or efflux. Moreover, the inhibition of contractile tension by BrcGMP cannot be entirely related to the lowering of $[Ca^{2+}]_{cyt}$, but also to a modification of the relationship between $[Ca^{2+}]_{cyt}$ and contraction. Furthermore, since biologically active concentrations of BrcGMP did not modify depolarization-dependent $^{45}Ca^{2+}$ influx, reduction of this influx by sodium nitroprusside, as reported by Karaki *et al.* (1984), is likely to occur by a mechanism that is independent of the rise in cyclic GMP evoked by this drug.

Methods

Measurement of contractile tension and $[Ca^{2+}]_{cyt}$

Aortic rings (mean length 3.28 ± 0.02 mm) were inverted and lightly rubbed to remove the endothelium. They were incubated for 3–5 h at room temperature in a physiological solution (composition, mM: NaCl 122, KCl 5.9, $NaHCO_3$ 15, $MgCl_2$ 1.25, $CaCl_2$ 1.25, glucose 11) containing 5 μM fura-2 acetoxymethyl ester (fura-2 AM) and 0.05% Cremophor EL, and further incubated for 30 min with 0.1 mM L-NOARG and with or without BrcGMP and/or verapamil. L-NOARG was used in order to avoid interference by nitric oxide which could possibly be produced by the inducible NO synthase activity of smooth muscle; this enzymatic activity could be induced by bacterial contamination during the long lasting fura-2 loading period. Rings were mounted between two hooks under a tension of 2 g in a 5 ml organ bath filled with physiological solution at 37°C gassed with 95% O_2 and 5% CO_2. This bath was part of a fluorimeter (CAF 110, JASCO, Tokyo) which allows us to estimate the calcium signal. The muscle tone was measured by an isometric lever connected to an isometric force transducer. The luminal face of the aortic ring was alternatively illuminated (128 Hz) with two excitation wavelengths (340 ± 10 nm and 380 ± 10 nm) obtained from a xenon high pressure lamp (75 W) coupled to two monochromators. The emitted light from the muscle was collected by a photomultiplier through a 500 ± 12 nm filter. The time constant for the optical channel was 0.26 s. The ratio of the fluorescence due to excitation at 340 nm (F340) to that at 380 nm (F380) was calculated from successive illumination periods. The fluorescence signals, F340, F380 and the ratio F340/F380 were measured simultaneously with contractile tension and recorded on a computer, by using data acquisition hardware (MacLab) and data recording software (Chart v3.2, AD Instruments Pty Ltd., Castle Hill, Australia). At the end of the experiment, the fura-2-Ca^{2+} signal was calibrated. The maximal ratio ($R_{max} = 1.497 \pm 0.038$, $n = 50$) was measured in calcium saturating medium by adding ionomycin (14 μM) in high KCl solution (KCl, 128 mM), while the minimal ratio ($R_{min} = 0.318 \pm 0.03$, $n = 50$) was obtained in calcium-free medium in the presence of ethylene glycol-*bis* (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA, 8 mM). The autofluorescence was finally measured at 340 nm and 380 nm by quenching the fura-2 fluorescence with $MnCl_2$ (10 mM) and it was subtracted from the experimental values for the calculation of the cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$). R_{max}, R_{min} and autofluorescence were determined in each preparation. $[Ca^{2+}]_{cyt}$ was calculated by the application of the following equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_{cyt} = \frac{R - R_{min}}{R_{max} - R} \quad \beta \times K_D$$

where K_D is the apparent dissociation constant of the fura-2/Ca^{2+} complex and is assumed to be equal to 224 nM; β (1.510 ± 0.038, $n = 50$) is a correcting factor equal to the ratio of the fluorescence intensities of the free dye (measured with EGTA) and the calcium saturated dye (measured with ionomycin), at 380 nm; the β value was calculated for each preparation according to the fluorescence intensities of the free dye and of the calcium-saturated dye which were measured in the same preparation. R is the ratio of the fluorescence intensities emitted at 500 nm at the two excitation wavelengths 340 nm and 380 nm for each experimental value.

Measurement of $^{45}Ca^{2+}$ influx

Calcium influx was estimated by measuring changes in the specific activity of the calcium fraction resistant to displacement by lanthanum (Godfraind, 1976). After an equilibration period of 60 min in physiological solution maintained at 37°C and aerated with a gas mixture of 95% O_2 and 5% CO_2, aortic segments were further incubated, for 30 min, in the same solution in the presence of 0.1 mM L-NOARG, and in the absence or presence of BrcGMP (0.1 mM–1 mM) and/or verapamil (0.1 μM). Thereafter, the aortic segments were transferred, for 2 min, either in a physiological solution, or in a 100 mM KCl depolarizing solution, both containing $^{45}Ca^{2+}$ (4 μCi ml^{-1}). After this 2 min-incubation, they were washed for 5 min in 500 ml of La^{3+} solution (composition, mM: NaCl 122, KCl 5.9, $MgCl_2$ 1.25, $LaCl_3$ 50, glucose, 11 and Tris-maleate buffer, 15; pH 6.8) to remove extracellular Ca^{2+}. The aortic segments were then dried on filter paper, weighed and dissolved in 0.1 ml of a mixture of perchloric acid: H_2O_2 (1:1). The radioactivity bound to the tissue was counted in 4 ml of Pico-fluor 30 by liquid scintillation.

Measurement of contractile tension and quenching rate of fura-2 evoked by Mn^{2+}

The Mn^{2+}-induced quenching of fura-2 fluorescence was estimated at 363 ± 10 nm excitation wavelength, which represented the fura-2 isosbestic wavelength in our system. This was determined by recording fura-2 excitation spectra at different Ca^{2+} concentrations, according to Grynkiewicz *et al.* (1985). The fluorescence signal F363 and contractile tension were measured simultaneously, at 2 s intervals. Mechanically endothelium-denuded aortae were loaded with fura-2 AM and preincubated with 0.1 mM L-NOARG which was present in all bathing solutions during the experiment. Rings were mounted between two hooks under a tension of 2 g in a 5 ml organ bath filled with a zero Ca^{2+} physiological solution (containing 0.1 mM EGTA) at 37°C, gassed with 95% O_2 and 5% CO_2. After 5 min incubation, 0.1 mM $MnCl_2$ was added to the bath and the fluorescence, as well as the muscle tone were measured. After 3 min the rings were

stimulated by a 67 mM KCl solution which produced an increase in the quenching rate and a contractile response. After 15 min, 10 mM $MnCl_2$ was added to the organ bath to quench rapidly the remaining fluorescence. The minimum value measured in the presence of 10 mM $MnCl_2$ was considered as background (autofluorescence) and subtracted from the values recorded during the experiment. Experimental values were normalized by dividing all fluorescence values by the fluorescence measured before the addition of 0.1 mM $MnCl_2$, such that the maximum value was 1 and the minimum value was 0. The quenching rate was estimated from the slope of the change in the normalized fluorescence (fluorescence units s^{-1}). It was calculated by linear regression of the data obtained during the 60 s preceding (Mn^{2+} influx in resting preparation) and the 60 s following (Mn^{2+} influx in depolarized preparation) the change to the 67 mM KCl solution.

Drugs

Fura-2 acetoxymethyl ester (Fura-2 AM) and ionomycin were obtained from Boehringer Mannheim. Verapamil was obtained from Sigma, a millimolar stock solution was prepared in water. N^{ω}-nitro-L-arginine and 8-bromo-cyclic GMP were from Sigma, they were dissolved directly in physiological or depolarizing solutions.

Statistical analysis

Results of the experiments are expressed as means ± standard error (s.e.mean). Tests of significance have been made using Student's paired or unpaired *t* test, or by analysis of variance (ANOVA), when comparisons involved more than two groups. *P* values smaller than 0.05 were considered significant.

Results

Effects of verapamil and BrcGMP on contractile tension and $[Ca^{2+}]_{cyt}$

The $[Ca^{2+}]_{cyt}$ of fura-2 loaded rat aortae was measured in rings without endothelium (mechanically rubbed), in the presence of L-NOARG. The preparations were mounted in the organ chamber, in physiological solution. After 3 min they were stimulated with a 67 mM KCl solution for 3 min. The physiological solution was then readmitted in the bath and, after 7 min, the fluorescence was measured; the $[Ca^{2+}]_{cyt}$ estimated from this fluorescence value was considered as resting $[Ca^{2+}]_{cyt}$. The rings were then further stimulated by the 67 mM KCl solution and fluorescence as well as contractile tension were measured up to 5 min after the beginning of this second stimulation. Matched preparations had been pretreated for 30 min at the end of the fura-2 loading period with verapamil (0.1 μM) and/or BrcGMP (0.1 mM–1 mM).

The resting $[Ca^{2+}]_{cyt}$ measured in physiological solution after the first KCl-stimulation of the muscle was not significantly different between untreated rings and rings treated either with verapamil (0.1 μM), BrcGMP (0.1 and 1 mM) or both (Table 1). Stimulation of the artery rings with the 67 mM KCl solution evoked an increase in $[Ca^{2+}]_{cyt}$ ($\Delta[Ca^{2+}]_{cyt}$) and in contractile tension. As illustrated in Figure 1, the increase in $[Ca^{2+}]_{cyt}$ reached a maximum value after 46 ± 4 s ($n = 50$). The development of muscle tone typically showed a fast and a slow component. In order to quantify the action of the various treatments, Ca^{2+} signals and muscle tone were measured at two time points: (1) the time of the first peak of the Ca^{2+} signal, usually occurring between 20 and 70 s after exposure to the high K^+ solution; (2) 5 min after exposure to the high K^+ solution, a time needed to reach the steady state of the contractile response. As shown in Figure 1, verapamil (0.1 μM) similarly inhibited the increase in $[Ca^{2+}]_{cyt}$ and in tension evoked by the KCl-solution. BrcGMP (0.1 mM–1 mM) produced a marked inhibition of the contractile response evoked by the high KCl solution and only a slight depression of the Ca^{2+} signal.

When preparations were treated with both 0.1 mM BrcGMP and 0.1 μM verapamil, the contractile tension was almost completely abolished while the $\Delta[Ca^{2+}]_{cyt}$ was not significantly different from the response measured in the presence of verapamil alone (Table 1).

These observations suggested that BrcGMP decreased the contractile response without markedly affecting the increase in $[Ca^{2+}]_{cyt}$, and thus could change the $[Ca^{2+}]_{cyt}$/tension relationship. We further tested this hypothesis by measuring the contractile tension and the $[Ca^{2+}]_{cyt}$ in preparations exposed to increasing concentrations of extracellular Ca^{2+} in 100 mM KCl solution, which maintains the membrane potential at a constant depolarized level. Figure 2 illustrates the relationship between $[Ca^{2+}]_o$ and either the contractile tension or $[Ca^{2+}]_{cyt}$. In 0.1 mM BrcGMP-treated rings, the $[Ca^{2+}]_o$-contraction curve was significantly shifted to the right compared to untreated rings. The threshold of $[Ca^{2+}]_o$ needed to obtain a contraction was increased by a factor of ten and 3 mM $CaCl_2$ in the bath produced a contraction equal to only 25% of the response recorded in the absence of BrcGMP. In contrast, the increase in $[Ca^{2+}]_{cyt}$ evoked by increasing $[Ca^{2+}]_o$ was hardly affected by BrcGMP. As a result, the $[Ca^{2+}]_{cyt}$-tension relation was clearly depressed by BrcGMP (0.1 mM), as illustrated in Figure 3.

Effects of verapamil and BrcGMP on $^{45}Ca^{2+}$ influx

Aortic segments were incubated for 30 min in physiological solution in the presence of 0.1 mM L-NOARG, and in the absence or presence of verapamil and/or BrcGMP. Thereafter, they were transferred, for 2 min, either in a physiological solution, or in a 100 mM KCl depolarizing solution, both containing $^{45}Ca^{2+}$. Figure 4 shows the effects of 0.1 μM verapamil and 0.1 mM BrcGMP on $^{45}Ca^{2+}$ influx. In aortic rings bathed in physiological solution, $^{45}Ca^{2+}$ influx

Table 1 Effects of BrcGMP and verapamil on resting $[Ca^{2+}]_{cyt}$, on $\Delta[Ca^{2+}]_{cyt}$ and contraction evoked by 67 mM KCl-solution

	Resting $[Ca^{2+}]_{cyt}$ (nM)	*$\Delta[Ca^{2+}]_{cyt}$* (nM)		*Contraction* (mN)	
		Peak	*5 min*	*Peak*	*5 min*
Untreated ($n = 16$)	98.0 ± 11.6	110.2 ± 7.4	100.9 ± 5.0	12.2 ± 1.1	19.9 ± 1.3
BrcGMP 0.1 mM ($n = 8$)	85.5 ± 9.6	81.5 ± 9.7*	88.0 ± 10.2	5.8 ± 1.1**	7.8 ± 1.1**
BrcGMP 1 mM ($n = 10$)	84.2 ± 8.1	85.4 ± 8.5*‡‡	82.5 ± 8.8*‡	4.0 ± 0.6**	4.5 ± 0.6**
Verapamil 0.1 μM ($n = 8$)	92.0 ± 12.0	53.2 ± 5.3**†	54.6 ± 4.4**††	3.1 ± 0.5**	7.1 ± 1.2**
Verapamil 0.1 μM + BrcGMP 0.1 mM ($n = 8$)	95.3 ± 14.1	37.2 ± 5.9**††	45.8 ± 5.8**††	0.3 ± 0.2**††	0.7 ± 0.3**††‡‡

$\Delta[Ca^{2+}]_{cyt}$ and contraction were measured simultaneously at the peak time of $[Ca^{2+}]_{cyt}$ (46 ± 4 s, see also text) and at 5 min after the stimulation with the KCl-solution. The number of preparations for each group is given in parentheses.
*$P<0.05$, **$P<0.01$, vs. untreated; †$P<0.05$, ††$P<0.01$, vs. BrcGMP 0.1 mM; ‡$P<0.05$, ‡‡$P<0.01$, vs. verapamil 0.1 μM; ANOVA.

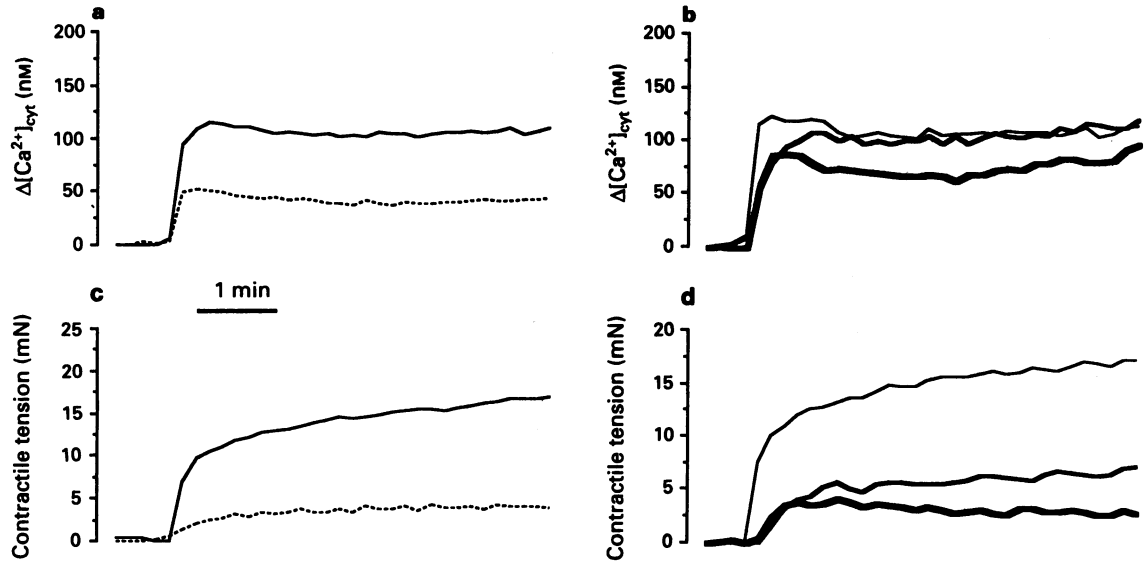

Figure 1 Effects of verapamil (0.1 μM) and BrcGMP (0.1–1 mM) on 67 mM KCl-stimulated calcium signals ($\Delta[Ca^{2+}]_{cyt}$; a, b) and contractile tension (c, d). (a, c) Solid line: untreated preparation; dashed line: 0.1 μM verapamil-treated preparation. (b, d) Thin line: untreated preparation; thicker line: 0.1 mM BrcGMP-treated preparation; thickest line: 1mM BrcGMP-treated preparation. Corresponding averaged values (from 8–16 different preparations) are shown in Table 1.

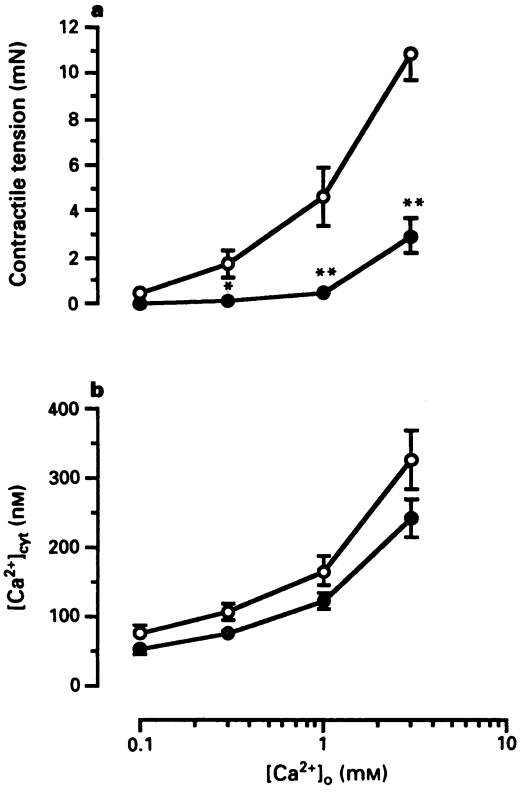

Figure 2 Effects of cumulative increases of extracellular calcium concentrations ($[Ca^{2+}]_o$) on contractile tension (a) and $[Ca^{2+}]_{cyt}$ (b), in untreated (○) and 0.1 mM BrcGMP-treated (●) rings. The preparations were incubated in Ca^{2+}-free solution for 15 min at room temperature, followed by 10 min at 37°C in a Ca^{2+}-free 100 mM KCl solution, before increasing the extracellular Ca^{2+} concentration. Values are expressed as mean ± s.e.mean from 7 rings. In some cases s.e.mean did not exceed the size of the symbol. $*P<0.05$, $**P<0.01$; Student's unpaired t test.

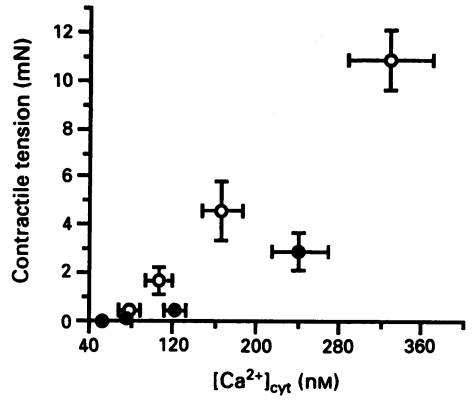

Figure 3 $[Ca^{2+}]_{cyt}$-tension relationship in untreated (○) and 0.1 mM BrcGMP-treated (●) rings, from data of Figure 2.

was slightly but significantly reduced in the presence of BrcGMP to 77.7 ± 6.4% of its value in untreated rings ($P<0.01$, ANOVA). $^{45}Ca^{2+}$ influx in physiological solution was unaffected by 0.1 μM verapamil (89.5 ± 7.7% of the value in untreated preparations; $P = 0.28$). The combination of verapamil and BrcGMP reduced $^{45}Ca^{2+}$ influx to 74.9 ± 11.9% of its value in untreated rings ($P<0.05$). Incubation of aortic rings in 100 mM KCl-solution markedly increased the $^{45}Ca^{2+}$ influx. In KCl-depolarized aortic rings, verapamil significantly inhibited the total $^{45}Ca^{2+}$ influx to 76.6 ± 4.4% of the value measured in untreated preparations ($P<0.01$), which corresponds to an inhibition of 44.6% of the KCl-evoked stimulation of $^{45}Ca^{2+}$ influx, obtained after subtraction of the basal $^{45}Ca^{2+}$ influx measured in physiological solution. BrcGMP did not affect $^{45}Ca^{2+}$ influx in the KCl-solution, in the presence or in the absence of verapamil. Increasing the concentration of BrcGMP to 1 mM did not induce an inhibition of the K^+-stimulated $^{45}Ca^{2+}$ influx which was equal to 103.1 ± 8.7% ($n = 5$) of the value obtained in the absence of BrcGMP.

Effects of verapamil and BrcGMP on the quenching rate of fura-2 and the contractile response evoked by Mn^{2+}

Fura-2 loaded aortic rings were first incubated for 5 min in a zero Ca^{2+} physiological solution (containing 0.1 mM EGTA). The addition of 0.1 mM $MnCl_2$ to the bath slightly enhanced the quenching rate of fura-2 fluorescence. When the K^+ concentration in the bath was increased to 67 mM, the quen-

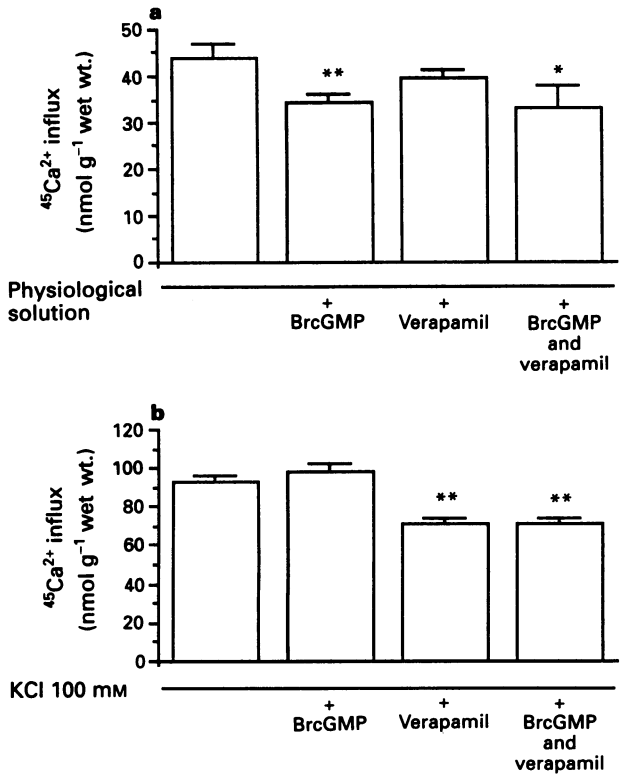

Figure 4 Effects of 0.1 mM BrcGMP and 0.1 μM verapamil on $^{45}Ca^{2+}$ influx in rat aorta. Aortic segments were incubated for 30 min in physiological solution in the presence of 0.1 mM N^{ω}-nitro-L-arginine (L-NOARG) and in the absence or in the presence of 0.1 mM BrcGMP and/or 0.1 μM verapamil. Thereafter, they were transferred, for 2 min, either in a physiological solution (a), or in a 100 mM KCl depolarizing solution (b), both containing $^{45}Ca^{2+}$. Each value is the mean with s.e.mean from 6–17 aortic segments. $^{*}P<0.05$, $^{**}P<0.01$ vs. untreated preparations; ANOVA.

ching rate of the fluorescence rapidly increased and a contractile response was recorded. As shown in Figure 5, BrcGMP (1 mM) did not affect the slope of the fluorescence decay in 67 mM KCl, whereas it strongly inhibited the contractile response. Verapamil concentration-dependently inhibited both the KCl-evoked increase in the rate of fluorescence decay and the contractile response evoked by $MnCl_2$ in KCl-solution (Figure 6). A high concentration of verapamil (10 μM) abolished both responses. Figure 7 illustrates the mean effect of BrcGMP and verapamil on the quenching rate and on the contractile response evoked by Mn^{2+} in several K^{+}-depolarized preparations. Those treatments did not significantly affect the quenching rate measured in physiological solution.

Discussion

The present results show that the inhibitory effect of BrcGMP on K^{+}-evoked contraction was stronger than its effect on the fura-2 Ca^{2+} signal; moreover, the lowering of $[Ca^{2+}]_{cyt}$ resulting from BrcGMP-treatment in K^{+}-depolarized preparations was neither associated with an inhibition of $^{45}Ca^{2+}$ influx nor with an inhibition of Mn^{2+} entry, while they were inhibited by verapamil, an inhibitor of voltage-dependent L-type calcium channels (Godfraind *et al.*, 1986), suggesting that BrcGMP does not affect voltage-dependent calcium channel gating. Various cyclic GMP-dependent mechanisms affecting $[Ca^{2+}]_{cyt}$ have been reported: (1) phosphorylation of phospholamban with consequent activation of the Ca^{2+}-ATPase of the endoplasmic reticulum (Cornwell *et al.*, 1991); (2) phosphorylation of the PI-kinase (Vrolix *et al.*, 1988) or of a 240 kDa protein (Yoshida *et al.*, 1991) with consequent activation of the Ca^{2+}-ATPase of plasma membrane; (3) activation of the Na^{+}/Ca^{2+} exchange (Furukawa *et al.*, 1991); (4) inhibition of voltage-dependent L-type calcium channels (Ishikawa *et al.*, 1993; Lorenz *et al.*, 1994). The measurement of the rate of fura-2 quenching by Mn^{2+} is a direct estimate of Mn^{2+} influx, which has been

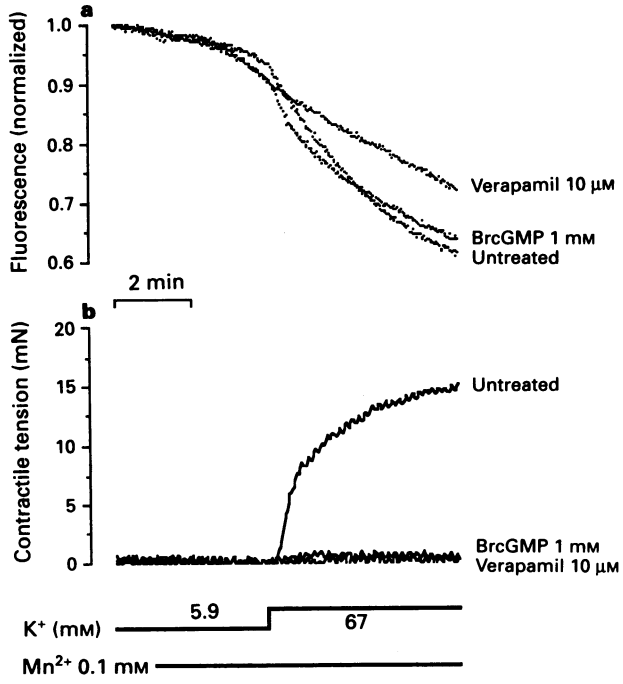

Figure 5 Effects of 1 mM BrcGMP and 10 μM verapamil on Mn^{2+}-induced quenching of 363 nm fura-2 fluorescence (a; ordinate scale: normalized fluorescence units) and Mn^{2+}-induced contraction (b) after 67 mM KCl-stimulation. Fluorescence was normalized by subtracting the autofluorescence from each measurement and dividing by the fluorescence value measured before the addition of $MnCl_2$.

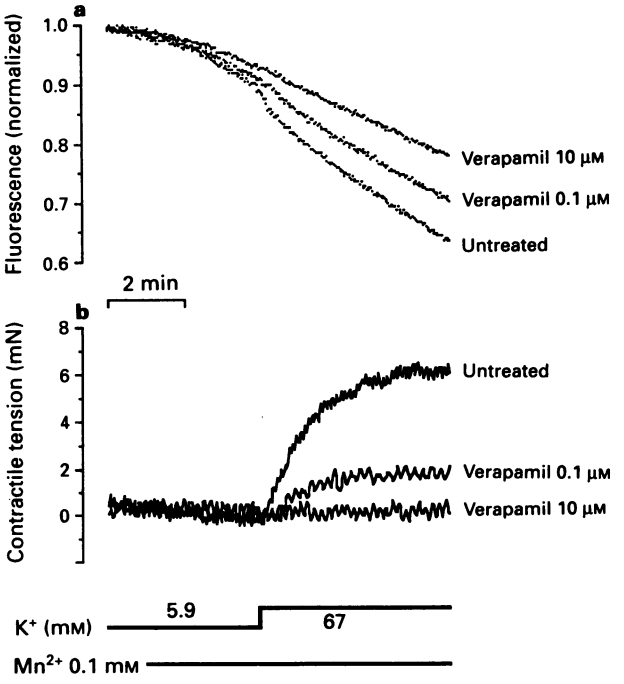

Figure 6 Effects of verapamil (0.1 μM and 10 μM) on Mn^{2+}-induced quenching of 363 nm fura-2 fluorescence (a; ordinate scale: normalized fluorescence units) and Mn^{2+}-induced contraction (b) after 67 mM KCl-stimulation. Fluorescence was normalized by subtracting the autofluorescence from each measurement and dividing by the fluorescence value measured before the addition of $MnCl_2$.

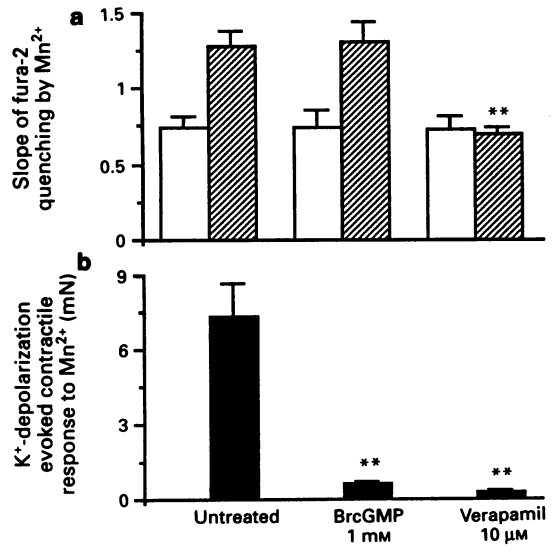

Figure 7 Effects of verapamil and BrcGMP on the quenching rate of fura-2 and the contractile response evoked by Mn^{2+}. Means ± s.e.mean (from 4–8 different preparations) of slopes of fura-2 quenching by Mn^{2+} (a; ordinate scale: 10^{-3} normalized fluorescence units/s) and of contractile responses to Mn^{2+} (b), in preparations depolarized by 67 mM KCl and treated or not with BrcGMP (1 mM) or verapamil (10 μM). After addition of 0.1 mM $MnCl_2$ to the bath, 363-nm fluorescence was measured at 2 s intervals; it was then normalized by subtracting from each measurement the autofluorescence and dividing by the fluorescence value measured before the addition of $MnCl_2$. Slope of change in normalized fluorescence was calculated by linear regression of data from the 60 s preceding (open columns: Mn^{2+} influx in resting preparation) and the 60 s following (hatched columns: Mn^{2+} influx in depolarized preparation) the change to the 67 mM KCl solution. $^{**}P<0.01$ vs. untreated preparations; ANOVA.

reported to occur through voltage-dependent Ca^{2+} channels (Chen & Van Breemen, 1993). Mn^{2+} influx was sensitive to verapamil but not to BrcGMP. Thus, the reduction of the fura-2 Ca^{2+} signal observed in BrcGMP-treated preparations depolarized by KCl cannot be ascribed to an inhibition of Ca^{2+} influx through voltage-dependent Ca^{2+} channels, but it is likely to result from a stimulation of Ca^{2+} sequestration into sarcoplasmic reticulum (SR) by cyclic GMP-dependent kinases activated processes, a view consistent with the absence of effect of BrcGMP on $^{45}Ca^{2+}$ uptake.

The present observations confirm the results reported earlier by Godfraind (1986) and by Collins *et al.* (1988) showing that BrcGMP does not affect $^{45}Ca^{2+}$ influx stimulated by high K^+ solution in the rat aorta, but are at variance with the inhibition of the L-type Ca^{2+} current by BrcGMP observed in rabbit portal vein (Ishikawa *et al.*, 1993) and in A7r5 cells (Lorenz *et al.*, 1994; Blatter & Wier, 1994). This difference may be related to experimental conditions. For example, the enzymatic dispersion of cells has been shown to affect the properties of calcium channels (Feron *et al.*, 1992). Differences in the sensitivity to cyclic GMP could also arise from the existence of different subtypes of L-type Ca^{2+} channels, which are differentially expressed in dispersed and cultured cells and differentially regulated (Neveu *et al.*, 1994).

It has been reported (Karaki *et al.*, 1984) that sodium nitroprusside inhibits KCl-stimulated $^{45}Ca^{2+}$ influx in rat aorta; such an effect was not reproduced with BrcGMP in the present study, suggesting that it is unrelated to the stimulation of guanylate cyclase. This is consistent with the view that effects of NO and nitrocompounds (which are NO-donors) are only in part mediated by the increase of cellular cyclic GMP, and that some of them could be supported by direct interactions of NO with ionic channels (Bolotina *et al.*, 1994).

We observed that in resting tissues, deprived of functional endothelium, BrcGMP slightly reduced both the $^{45}Ca^{2+}$ influx and the $[Ca^{2+}]_{cyt}$ estimated by the fura-2 technique (with the latter technique, however, the effect of BrcGMP did not reach statistical significance). These effects could result from either a hyperpolarization (which decreases the probability of opening of voltage-dependent Ca^{2+} channels and so the $^{45}Ca^{2+}$ influx in resting preparations) or from an increase of Ca^{2+} sequestration and/or extrusion, by the mechanisms mentioned above. Krippeit-Drews *et al.* (1992) indeed showed that the spontaneous release of endothelium-derived relaxing factor (EDRF) hyperpolarizes rat aortic smooth muscle cells by 4–6 mV and that the blockade of the NO-synthase activity by L-NOARG, which lowers membrane potential in resting aortic rings, induces an increase in the basal $^{45}Ca^{2+}$ influx. The hyperpolarizing effect of NO could be mediated by a cyclic GMP-dependent protein kinase activation of Ca^{2+}-dependent K^+ channels (Robertson *et al.*, 1993; Taniguchi *et al.*, 1993). However, at variance with the effect of NO, a hyperpolarizing effect of BrcGMP has not been detected in rat aortic smooth muscle with intact functional endothelium (Vanheel *et al.*, 1994). Thus, the cause of the reduction of $^{45}Ca^{2+}$ influx in physiological solution by BrcGMP remains to be elucidated.

The major finding of the present study was that the effect of BrcGMP on the contraction evoked by high KCl solution was not related to its effect on $[Ca^{2+}]_{cyt}$ and that BrcGMP modified the relationship between $[Ca^{2+}]_{cyt}$ and contraction. In contrast, verapamil produced an inhibition of the KCl-induced contraction and a decrease in $[Ca^{2+}]_{cyt}$ that were of the same level. This can be explained by a cyclic GMP-dependent decrease in the sensitivity of the contractile elements to Ca^{2+}, a conclusion consistent with the report by Nishimura & Van Breemen (1989) in α-toxin permeabilized mesenteric artery. These findings are also consistent with the report by Karaki *et al.* (1988) who showed that sodium nitroprusside, a NO donor that stimulates cyclic GMP production, affects the $[Ca^{2+}]_{cyt}$-tension relationship for KCl-induced contractions in rat aorta.

It is noteworthy that, in depolarized preparations, BrcGMP inhibited Mn^{2+}-evoked contraction (in zero Ca^{2+}, 0.1 mM EGTA) as well as it did with Ca^{2+}-evoked contraction. The mechanism of Mn^{2+}-evoked contraction in smooth muscle has not been completely elucidated. It seems to involve the direct stimulation of actomyosin ATPase activity and acts independently of myosin light chain phosphorylation (Hoar & Kerrick, 1988). Thus, the inhibitory effect of BrcGMP on Mn^{2+}-evoked contraction could probably not be accounted for by an effect on myosin light chain phosphorylation. Whether or not such a conclusion could also be extended to Ca^{2+}-evoked contraction remains a matter of speculation.

In summary, the present results show that in contrast to verapamil, the inhibition of contractile tension by BrcGMP cannot be related to a calcium channel blocking mechanism, but to a modification of the relationship between $[Ca^{2+}]_{cyt}$ and contraction. Furthermore, because the reduction of K^+ depolarization-dependent $^{45}Ca^{2+}$ influx reported by Karaki *et al.* (1984) with sodium nitroprusside was not observed with BrcGMP, it is likely that this drug has additional mechanisms of action, unrelated to a rise in cyclic GMP.

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