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8-Bromoguanosine 3′:5′-cyclic monophosphate decreases intracellular free calcium concentrations in cultured vascular smooth muscle cells from rat aorta

Hisashi Kai, Hideo Kanaide, Takahiro Matsumoto and Motoomi Nakamura

Research Institute of Angiocardiology and Cardiovascular Clinic, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

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The effects of 8-bromoguanosine 3′:5′-cyclic monophosphate (8-Br cGMP) on intracellular free calcium concentrations ($[Ca^{2+}]_i$) in cultured rat aortic vascular smooth muscle cells (VSMCs) loaded with fura-2 were recorded microfluorometrically. Irrespective of whether VSMCs were at rest (in 5 mM K^+ PSS), under Ca^{2+} depletion (in Ca^{2+}-free medium for 10 min) and K^+ depolarization (in high K^+ PSS), $[Ca^{2+}]_i$ was actively reduced and reached a new and lower steady-state level with the application of 8-Br cGMP. This may be the first and direct evidence that cGMP, a putative mediator of various vasodilators, actively reduces $[Ca^{2+}]_i$ in VSMCs.

8-Bromo cyclic GMP; Fura-2; (Vascular smooth muscle cell)

1. INTRODUCTION

Although the relaxation of VSMCs induced by vasodilators such as nitrocompounds, atrial natriuretic peptide and endothelium-dependent vasodilators is accompanied by an increase in cGMP concentrations [1–4], the underlying mechanism mediating this vasorelaxant effect has not been clearly defined. Possible mechanisms proposed for the VSMCs relaxation by cGMP are as follows: (i) cGMP may decrease the availability of Ca^{2+} for the contractile process. It has been proposed that cGMP reduces $[Ca^{2+}]_i$ by acceleration of Ca^{2+} extrusion through the sarcolemma [4–6], inhibition of Ca^{2+} release from intracellular Ca^{2+}-storage sites [7–9] or inhibition of Ca^{2+} influx into the cells [7–9]. (ii) It has been shown that cGMP induces phosphorylation of cGMP-dependent protein kinase and dephosphorylation of myosin light chain, a process which may lead to a reduction in the sensitivity of myofilaments for Ca^{2+} [1,2].

Direct evidence to show the effect of cGMP on $[Ca^{2+}]_i$ in VSMCs has apparently never been reported. Using a new technique of microfluorometry of the Ca^{2+}-sensitive dye fura-2, we report here the successful recording of $[Ca^{2+}]_i$ transients in VSMCs in primary culture, as induced by a membrane-permeable analogue 8-Br cGMP. cGMP decreased the $[Ca^{2+}]_i$ levels, regardless of whether VSMCs were at rest, at K^+ depolarization or at Ca^{2+} depletion.

Correspondence address: H. Kai, Research Institute of Angiocardiology and Cardiovascular Clinic, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

Abbreviations: 8-Br cGMP; 8-bromoguanosine 3′:5′-cyclic monophosphate; db-cAMP, dibutyryl adenosine 3′:5′-cyclic monophosphate; $[Ca^{2+}]_i$, intracellular free calcium concentration; PSS, physiological saline solution; VSMCs, vascular smooth muscle cells

2. MATERIALS AND METHODS

Fura-2/AM was purchased from Dotite (Japan),

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8-Br cGMP and db-cAMP from Sigma (USA) and ionomycin from Calbiochem (USA).

2.1. *Loading cells with fura-2*

Rat aortic medial VSMCs were cultured as described [10] and the primary cell culture was used for all experiments. On days 5–6, just before reaching confluence, the cultured cells on Lux chamber slides were incubated with growth medium containing 5 μM fura-2, as the acetoxy methyl ester (fura-2/AM) [11], for 60 min at 37°C. The measurements were performed in PSS at 25°C. The millimolar composition of the normal PSS (pH 7.4 at 25°C) was: 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5.5 glucose, 10 Hepes. High K^+ solutions were prepared by replacing NaCl with KCl, iso-osmotically. The composition of Ca^{2+}-free PSS was the same as for normal PSS, except that it contained 2 mM EGTA instead of 1 mM $CaCl_2$.

2.2. *Microfluorometry of fura-2*

We used a fluorescence microscope (model Standard 18, Zeiss) equipped with a photon-counting system (Zeiss), a water immersion objective system (Plan-Neofluor 63, Zeiss), and appropriate combinations of filters (Zeiss and Toshiba), in which cells were excited at 380 nm and analysed at wavelengths between 470 and 560 nm. The fluorescence intensity in a spot (<1 μm^2) of the cytosol 3 μm distant from the nucleus was measured using our method [12,13]. Each cell was exposed to excitation light, only once, for no longer than 2 s to avoid the photobleaching effect on the dye. In figs.2 and 3, there was a decrease in fluorescence intensity, as deduced from the upward deflection of the ordinate, because it corresponds to an increase in $[Ca^{2+}]_i$ when dye in the VSMCs was excited at 380 nm [11]. Estimation of $[Ca^{2+}]_i$ was made according to Grynkiewicz et al. [11]. The maximum and minimum levels of fluorescence intensity were obtained by permeabilizing cells, loaded with fura-2/AM as described, with 5×10^{-8} M ionomycin in the presence of excess Ca^{2+} (10^{-3} M) and 10 mM EGTA (approx. 10^{-9} M Ca^{2+}), respectively.

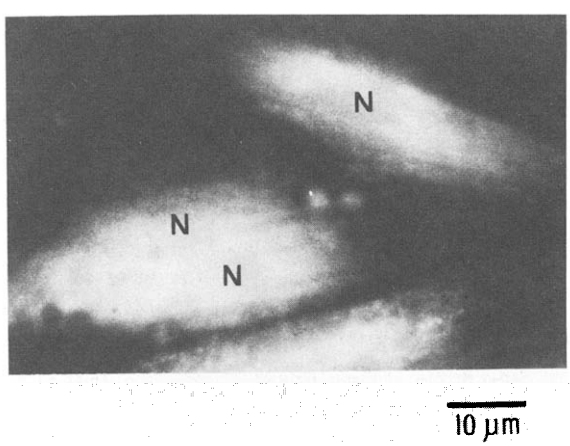

Fig.1. Fluorescence photograph of VSMCs loaded with fura-2 in normal PSS. Excitation wavelength, 380 nm; emission wavelength, 470–560 nm. N, nucleus.

3. RESULTS AND DISCUSSION

Fluorescence photographs of VSMCs loaded with fura-2 in normal PSS showed that the specific greenish-blue fluorescence of the fura-2·Ca^{2+} complex appeared almost exclusively in the cytosol (fig.1). The nucleus was also stained, but with a more greenish tint. Stress fibers and the extracellular space stained negatively.

When 10^{-4} M 8-Br cGMP was applied to VSMCs in normal PSS (5 mM K^+), $[Ca^{2+}]_i$ gradually decreased, and reached a lower steady-state level within 12 min, and was maintained as long as 8-Br cGMP was present (fig.2A). $[Ca^{2+}]_i$ recovered to the pre-exposure level at 6 min after the removal of 8-Br cGMP from the bathing PSS. A typical time course of the effect of the application of 8-Br cGMP on $[Ca^{2+}]_i$ of VSMCs under Ca^{2+} depletion is shown in fig.2B. When VSMCs were exposed to Ca^{2+}-free PSS containing 2 mM EGTA, $[Ca^{2+}]_i$ was reduced and reached a low steady-state level within 10 min. When these Ca^{2+}-depleted VSMCs were subsequently exposed to 10^{-4} M 8-Br cGMP, a further decrease in $[Ca^{2+}]_i$ was observed and $[Ca^{2+}]_i$ reached a new lower steady-state level. The time course of these events was similar to that observed in the presence of extracellular Ca^{2+}. This steady-state level remained unchanged for as long as 8-Br cGMP was present.

One of the most typical time courses of the effect of the application of 10^{-4} M 8-Br cGMP on

$[Ca^{2+}]_i$ in K^+-depolarized VSMCs is shown in fig.3A. When VSMCs were exposed to 135 mM K^+ PSS, $[Ca^{2+}]_i$ rapidly increased to a steady state within 4 min, and remained at this level for the duration of the exposure period. The subsequent application of 8-Br cGMP induced a gradual decrease in $[Ca^{2+}]_i$, and then $[Ca^{2+}]_i$ reached a new steady level, within 12 min. When 8-Br cGMP was washed out in the presence of 135 mM K^+, $[Ca^{2+}]_i$ rapidly rose to a higher level, which was not significantly lower than the value observed prior to the application of 8-Br cGMP, in the presence of 135 mM K^+. Reductions of $[Ca^{2+}]_i$ by 8-Br cGMP in VSMCs at rest (in 5 mM K^+ PSS) and under depolarization (30 mM K^+ and 135 mM K^+ PSS) were dose-dependent (fig.3B).

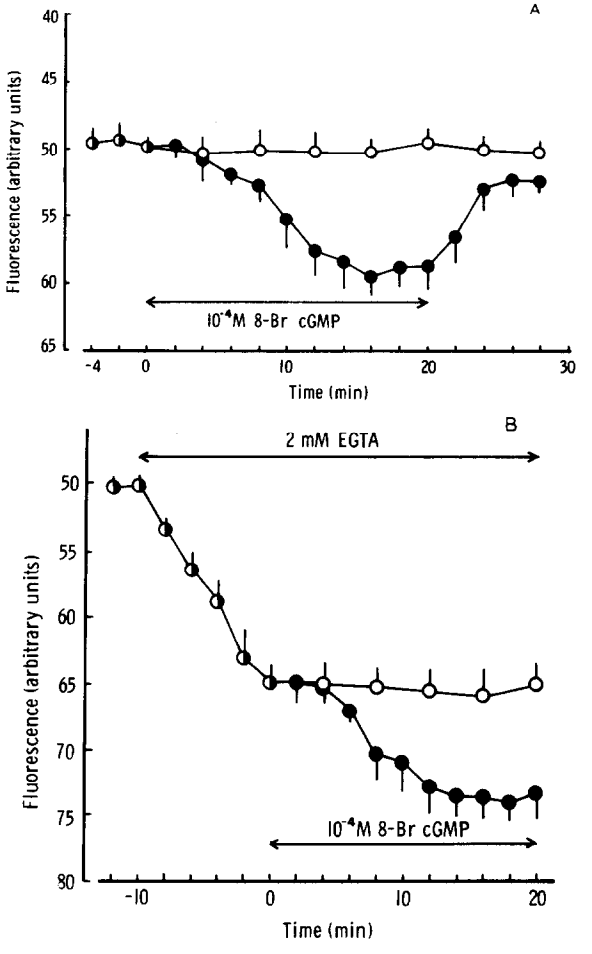

Fig.2. (A) A typical time course of the cytosolic fluorescence of VSMCs induced by the application of 10^{-4} M 8-Br cGMP in normal PSS (●) (○, control VSMCs, no application of 8-Br cGMP). (B) A typical time course of the cytosolic fluorescence of VSMCs induced by the application of 10^{-4} M 8-Br cGMP to VSMCs in Ca^{2+}-free media (●) (○, control VSMCs, no application of 8-Br cGMP in Ca^{2+}-free media). Data are means ± SD of 5 experiments, and 8 cells were examined in each experiment.

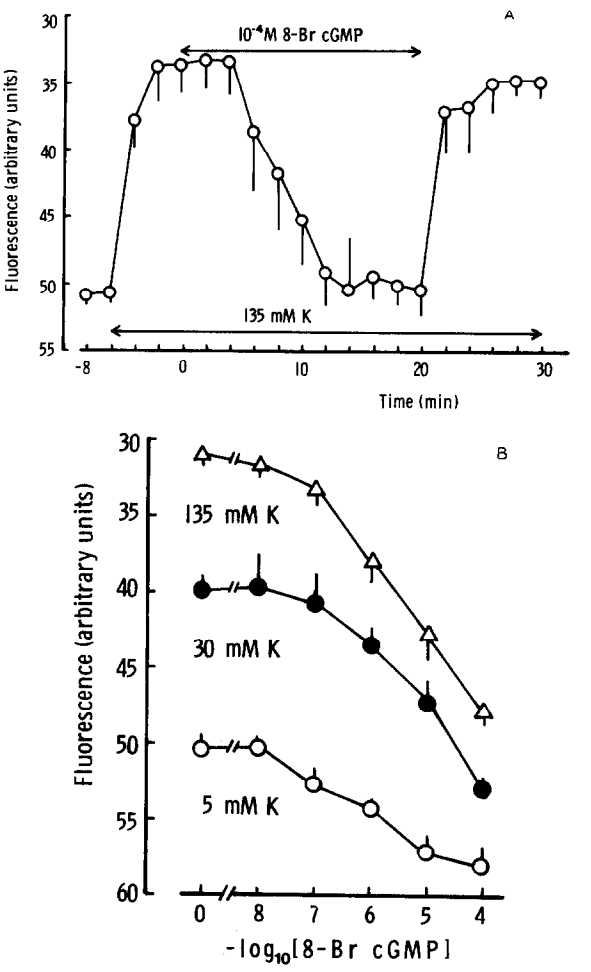

Fig.3. (A) A typical time course of the cytosolic fluorescence induced by the application of 10^{-4} M 8-Br cGMP to VSMCs in 135 mM K^+ PSS. (B) Dose-dependent effect of 8-Br cGMP on the increased $[Ca^{2+}]_i$ induced by various concentrations of extracellular K^+ (○, 5 mM K^+; ●, 30 mm K^+; △, 135 mM K^+). Fluorometry was carried out 20 min after the application of 8-Br cGMP to cells incubated for 6 min in PSS containing various concentrations of K^+. Data are means ± SD of 5 experiments.

Table 1 lists the levels of $[Ca^{2+}]_i$ in VSMCs before and 20 min after (at a new and lower steady state) the application of 8-Br cGMP, estimated as described. The levels of $[Ca^{2+}]_i$ at rest and in the case of K^+ depolarization were similar to those noted in studies using either fura-2 or quin-2 [14,15].

The present study demonstrates that 8-Br cGMP, a membrane-permeable analogue of cGMP, actively decreases $[Ca^{2+}]_i$ of VSMCs in primary culture, regardless of whether VSMCs are Ca^{2+}-depleted, at rest or in a state of K^+ depolarization. Thus, 8-Br cGMP decreases $[Ca^{2+}]_i$ in both Ca^{2+}-depleted and Ca^{2+}-overloaded VSMCs. The finding that 8-Br cGMP actively reduces $[Ca^{2+}]_i$ in Ca^{2+}-depleted VSMCs in the absence of extracellular Ca^{2+} indicates that there is an acceleration of Ca^{2+} extrusion through the sarcolemma. This observation provides evidence to support the hypotheses that acceleration of Ca^{2+} extrusion may induce a decrease in $[Ca^{2+}]_i$ in VSMCs [4–6].

As shown in table 1, the higher the $[Ca^{2+}]_i$ before application of 8-Br cGMP, the greater was the extent of the decrease in $[Ca^{2+}]_i$ in the VSMCs.

Table 1

Effects of 10^{-4} M 8-Br cGMP on $[Ca^{2+}]_i$ of VSMCs

State of VSMCs	Estimated value of $[Ca^{2+}]_i$ (nM)		
	Before application (*a*)	20 min after application (*b*)	Extent of the $[Ca^{2+}]_i$ decrease (*a*)–(*b*)
Ca^{2+} depletion (in Ca^{2+}-free media)	56 ± 7	31 ± 6	25
At rest (in 5 mM K^+ PSS)	148 ± 12	91 ± 10	57
K^+ depolarization (in 30 mM K^+ PSS)	292 ± 35	128 ± 11	164
K^+ depolarization (in 135 mM K^+ PSS)	553 ± 83	176 ± 22	377

Values are means ± SD ($n = 8$)

Thus, it was suggested that the extent of the $[Ca^{2+}]_i$ decrease induced by 8-Br cGMP depended on the level of $[Ca^{2+}]_i$ on application of this nucleotide. This is consistent with reports that sarcolemmal Ca^{2+}-ATPase of VSMCs was stimulated in a dose-dependent manner by free concentrations of Ca^{2+} and that the Ca^{2+}-ATPase was accelerated, dose-dependently, by nitroglycerin through an increase in cGMP levels and activation of cGMP-dependent protein kinase [5,6]. Thus, acceleration of the sarcolemmal Ca^{2+}-ATPase activity may be involved in the $[Ca^{2+}]_i$ reduction seen with cGMP. We proposed that nitroglycerin accelerates the extrusion of Ca^{2+} through the sarcolemma [12].

The possibility has been discussed that cAMP-dependent protein kinase can be activated by cGMP, at high concentrations [16]. Since the concentrations of 8-Br cGMP used here are relatively high, it is possible that the cAMP-dependent mechanism may be involved in the reduction in $[Ca^{2+}]_i$ by 8-Br cGMP in VSMCs [17]. Fig.4 demonstrates a typical time course observed when 10^{-4} M db-cAMP was applied to VSMCs in 135 mM K^+ PSS. $[Ca^{2+}]_i$ elevated by K^+ depolarization was gradually decreased and reached a lower steady-state level, within 16 min. It is apparent that the extent of $[Ca^{2+}]_i$ reduction induced by 10^{-4} M db-cAMP is much less than that induced by 10^{-4} M 8-Br cGMP (fig.3A,B). Thus, it was indicated that the effects of 8-Br cGMP on the reduction in

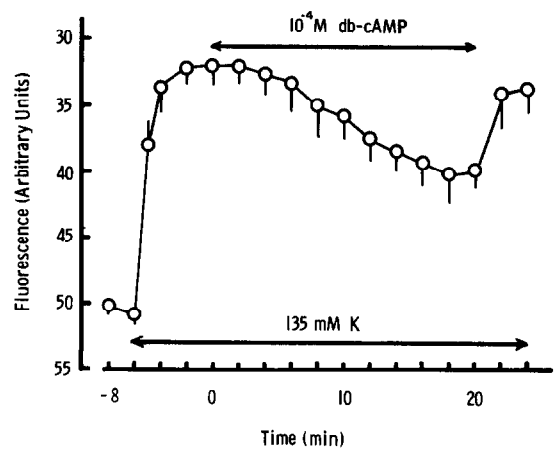

Fig.4. A typical time course of the cytosolic fluorescence observed when 10^{-4} M db-cAMP was applied to VSMCs in 135 mM K^+ PSS. Data are means ± SD of 4 experiments.

$[Ca^{2+}]_i$ may be much more potent than those of db-cAMP. Furthermore, it was reported that cGMP was less than 1/200 as effective as cAMP, as an apparent competitor of cAMP-dependent protein kinase [18], and the molar concentration of 8-Br cGMP required to induce a reducation in $[Ca^{2+}]_i$ is markedly low ($< 10^{-6}$ M; $p < 0.01$, Student's t-test). Thus, it was suggested that the mechanism via a cAMP-dependent system may have a less important role in the effects of 8-Br cGMP on $[Ca^{2+}]_i$ of VSMCs, in the present study.

The present report may be the first to show that an increase in intracellular cGMP levels actively reduces $[Ca^{2+}]_i$ of VSMCs at rest, under conditions of K^+ depolarization or Ca^{2+} depletion. The reduction in $[Ca^{2+}]_i$ through activation of the Ca^{2+} pump is probably one of the major mechanisms involved in cGMP-mediated vasodilatation. The effect of cGMP on the Ca^{2+} influx through the sarcolemma was not evaluated and remained to be elucidated in the present study.

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