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cGMP-kinase mediates cGMP- and cAMP-induced Ca^{2+} desensitization of skinned rat artery

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Abstract

(Rp)-8-Bromo-guanosine 3′,5′-cyclic monophosphorothioate (Rp-8-Br-cGMPS) inhibited competitively both isozymes of type Iα and Iβ cGMP-dependent protein kinase (cGMP-kinase) purified from porcine aorta with apparent K_i values (μM) of 3.7 for Iα and 1.8 for Iβ. The compound also inhibited bovine heart type II cAMP-dependent protein kinase (cAMP-kinase), but with a K_i of 25 μM. Thus, it is a selective inhibitor of cGMP-kinase. In α-toxin-skinned smooth muscle preparations from rat mesenteric artery, 8-Br-cGMP (10^{-7} M) and 8-Br-cAMP (10^{-6} M) produced a rightward shift of the concentration-contraction curves for Ca^{2+}, denoting a decrease in Ca^{2+} sensitivity of the contractile elements. The shift by 8-Br-cAMP as well as by 8-Br-cGMP was completely reversed by Rp-8-Br-cGMPS, while a selective inhibitor of activation of cAMP-kinase, (Rp)-adenosine-3′,5′-cyclic monophosphorothioate (Rp-cAMPS), was without effects on the shift produced by these two compounds. These findings indicate the pivotal role that the activation of cGMP-kinase plays in the production of a decrease in Ca^{2+} sensitivity of contractile elements.

Keywords: cGMP-dependent protein kinase; cAMP-dependent protein kinase; (Rp)-8-Bromo-guanosine-3′,5′-cyclic monophosphorothioate; (Rp)-Adenosine-3′,5′-cyclic monophosphorothioate; Ca^{2+} sensitivity; Smooth muscle, skinned, vascular

1. Introduction

It is now generally accepted that nitric oxide-generating vasodilators such as nitroglycerin and sodium nitroprusside induce relaxation of vascular smooth muscle via cyclic GMP (cGMP) which these compounds produce within the cell through activation of soluble guanylate cyclase (Schults et al., 1977; Murad, 1986; Kawada et al., 1994). However, the molecular events that link the increase in cGMP with the relaxation are not well understood. A line of evidence has indicated that the effects of cGMP are likely to be mediated by activation of cGMP-dependent protein kinase (cGMP-kinase), and that activation of this kinase brings about a decrease in intracellular concentration of Ca^{2+}, an effect leading to relaxation of vascular smooth muscle (Cornwell and Lincoln, 1989; Lincoln and Cornwell, 1991).

However, using aequorin as a Ca^{2+} indicator, DeFeo and Morgan (1985) demonstrated that changes in mechanical tension of vascular smooth muscle produced by vasoconstrictors were not always connected with changes in intracellular Ca^{2+} concentration. The relaxation of vascular smooth muscle by nitroglycerin (Yanagisawa et al., 1989) or nitroprusside (Karaki et al., 1988) was also shown not to be related with the reduction of the cytosolic Ca^{2+} level measured with a Ca^{2+} indicator, fura-2. These findings strongly suggest a decrease in sensitivity of contractile elements to Ca^{2+} as a possible mechanism by which cGMP relaxes vascular smooth muscle.

Using rat mesenteric artery skinned with α-toxin, Nishimura and Van Breemen (1989) demonstrated that cGMP could produce a decrease in Ca^{2+} sensitivity of contractile elements. However, whether or not the decrease is mediated through activation of cGMP-kinase has not been determined yet. Likewise, skinned vascular smooth muscle was used to show that cAMP produces a decrease in Ca^{2+} sensitivity of contractile elements (Pfitzer et al., 1984; Nishimura and Van Breemen, 1989). Again, it has not been tested whether or not the activation of protein kinase is involved in the observed decrease in Ca^{2+} sensitivity.

Phosphorothioate stereoisomers of cGMP and cAMP,

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(Rp)-guanosine-3′,5′-cyclic monophosphorothioate (Rp-cGMPS) and (Rp)-adenosine-3′,5′-cyclic monophosphorothioate (Rp-cAMPS), have been reported to inhibit the activation of cGMP- and cAMP-kinases (Butt et al., 1990; Hofmann et al., 1985; Van Haastert et al., 1984). Rp-cAMPS was shown to be about 10 times more selective as inhibitor of cAMP-kinase than of cGMP-kinase, while Rp-cGMPS was an inhibitor of moderate potency with no selectivity for these two types of kinases. Neither of these compounds is thought to be metabolized by mammalian cyclic nucleotide phosphodiesterases (Van Haastert et al., 1983; Zimmerman et al., 1985).

(Rp)-8-bromo-guanosine-3′,5′-cyclic monophosphorothioate (Rp-8-Br-cGMPS) is a derivative of Rp-cGMPS, and was shown in this laboratory to inhibit the relaxation of rabbit aortic smooth muscle elicited by nitroglycerin and 8-Br-cGMP, but not that elicited by 8-Br-cAMP (Nakazawa and Imai, 1994). We tested the effects of this compound on two isozymes of type I cGMP-kinase isolated from porcine aorta and type II cAMP-kinase purified from bovine heart. As the compound was found to be around 10 times more effective as an inhibitor of cGMP-kinase than of cAMP-kinase, the present study was undertaken with Rp-8-Br-cGMPS as a selective inhibitor of activation of cGMP-kinase, and Rp-cAMPS as a selective inhibitor of activation of cAMP-kinase. This was done in order to elucidate the part played by the activation of cGMP- and cAMP-kinases in the changes in Ca^{2+} sensitivity of contractile elements produced by cGMP and cAMP.

2. Materials and methods

2.1. Purification of cGMP-kinases Iα and Iβ

Two isozymes of cGMP kinase, Iα and Iβ, were purified from porcine aorta using the method developed by Wolfe et al. (1989) for bovine aortic enzymes. The tunica media layers dissected from fresh porcine aortas were cut into small pieces and reduced to a fine powder with stainless-steel percussion mortar at liquid N_2 temperature. Using the powder as a starting material, purification was conducted in the following way. The soluble extract from 3 kg of the frozen powder was sequentially chromatographed with diethylaminoethyl (DEAE)-cellulose (Whatman, DE52), 8-(6-aminohexyl)amino-cAMP-agarose (Pharmacia, AGcAMP type 3), and DEAE-Sephacel (Pharmacia) columns. The elution profile from the final DEAE-Sephacel chromatography was essentially similar to that reported for bovine aorta (Wolfe et al., 1989); monomeric Iβ, Iα, $Iα^+$ (Iα with bound cyclic nucleotide), and Iβ were eluted in this order. Fractions containing highly purified Iα or Iβ isozyme were separately pooled, dialyzed against TEM buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 25 mM 2-mercaptoethanol), and stored in TEM buffer containing 50% (w/v) glycerol at −20°C. The purified enzymes were apparently homogeneous as judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis under denaturing conditions followed by silver staining. The molecular mass of subunit of type Iα (76 kDa), was less than that of type Iβ (80 kDa; data not shown).

2.2. Protein kinase assay

cGMP-kinase activity was assayed by the method of Lincoln (1983) with minor modifications. Briefly, the purified cGMP-kinase (0.5 μg/ml) was incubated at 30°C for 3 min in a reaction mixture (100 μl) containing 20 mM Tris-HCl, pH 7.5, 100 mM $Mg(CH_3COO)_2$, 0.2 mg/ml histone II-A, 100 μM [γ-^{32}P]ATP (150–300 cpm/pmol) and various concentrations of cGMP and/or a test compound. The reaction was initiated by the addition of [γ-^{32}P]ATP and terminated by the addition of 2 ml of 10% trichloroacetic acid. The mixture was kept on ice for 10 min and filtered under reduced pressure through a Whatman GF/C filter using an automated cell harvester (Labomash LH-101, Labo Science, Tokyo, Japan). The filters were washed twice with 2 ml of 5% trichloroacetic acid, and ^{32}P radioactivity remaining on the filters was counted. cAMP-kinase activity was assayed as described previously (Kawada et al., 1989) using type II cAMP-kinase purified from bovine heart (Sigma, P5511) with calf thymus histone (Worthington, HLY) as a substrate. Protein concentration was determined by a modified Lowry method (Paterson, 1977) with bovine serum albumin as a standard.

2.3. Experiments with skinned vascular smooth muscle preparations from rat mesenteric artery

Mesenteric arteries excised from male Wistar rats (250–350 g) under ether anaesthesia were placed in aerated (100% O_2) physiological salt solution (PSS) of the following composition (mM): NaCl, 140; KCl, 5; $CaCl_2$, 1.5; $MgCl_2$, 1; glucose, 10; HEPES, 5, pH 7.4. Small branches (diameter: 200–300 μm) were dissected out under the microscope and cleaned under the microscope of adhering fat and connective tissue. Small rings (300–400 μm long) were prepared and transferred to an experimental chamber (volume: about 0.7 ml). Isometric tension was recorded with a force-displacement transducer (U gauge, UL-2GR, Minebea, Tokyo, Japan). After recording of the maximal contractile tension induced by 145 mM K^+ PSS (NaCl was substituted for KCl in PSS), the solution was changed to a Ca^{2+}-free cytoplasmic substitution solution (CSS) of the following composition (mM) with 30 μg/ml staphylococcal α-toxin and 10 μM A23187: EGTA, 10; K propionate, 130; $MgCl_2$, 4; Na_2ATP, 3.75; creatine phosphate, 10; Tris maleate, 20, pH 6.84 to skin the preparations. After 30 min the preparations were washed with Ca^{2+}-free CSS without α-toxin and A23187. Contraction was then induced with CSS containing 10^{-5} M Ca^{2+}. If the contrac-

tion was greater than the maximal contraction induced by 145 mM K^+ solution in intact preparations, it was judged that most of the cells had been successfully permeabilized. After another 20 min, the preparations were exposed to various concentrations of Ca^{2+} (10^{-7}–10^{-5} M) and concentration-contraction curves for Ca^{2+} were made. The contraction produced by each concentration of Ca^{2+} was expressed as percentage of the contraction induced by 10^{-5} M Ca^{2+} in the control group. To examine the effects of cyclic nucleotides and/or inhibitors, a concentration-contraction curve for Ca^{2+} was made, using the same procedure as mentioned above about 20 min after administration of these agents. All experiments were carried out at room temperature (20–24°C).

2.4. Analysis of concentration-contraction curves

The concentration-contraction curves obtained were fitted to the following logistic equation described by Waud and Parker (1971):

$$Y = a/\left[1 + (b/X)^c\right]$$

where Y is the magnitude of contraction, a is the maximum contraction, b is the EC_{50} value, X is the concentration of Ca^{2+} and c is the slope factor. b and c were calculated with an IBM personal computer by means of software utilizing a least-square procedure based on the Marquardt-Levenberg algorithm (SigmaPlot, version 5.0; Jandel Scientific, CA, USA). Apparent pA_2 values were calculated according to the method of Van Rossum (1963).

2.5. Statistical methods

All values are presented as means ± S.E. Statistical evaluation of differences among groups was made by one-way analysis of variance followed by Tukey's method. Differences were considered significant at a probability value of less than 0.01.

2.6. Chemicals

The sources of chemicals used in this study were as follows: cGMP (sodium salt) and cAMP (sodium salt) from Yamasa Shouyu (Choushi, Japan); 8-bromo-guanosine 3′,5′-cyclic monophosphate (8-Br-cGMP), 8-bromo-adenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) and histone type II-A (calf thymus) from Sigma (St. Louis, MO, USA); (Rp)-8-bromo-guanosine 3′,5′-monophosphorothioate (Rp-8-Br-cGMPS), (Rp)-adenosine 3′,5′-monophosphorothioate (Rp-cAMPS) and (Rp)-8-bromo-adenosine 3′,5′-monophosphorothioate (Rp-8-Br-cAMPS) from Biolog (Bremen, Germany); [γ-^{32}P]ATP tetra(triethylammonium) salt (30 Ci/mmol) from Du Pont-New England Nuclear (Boston, MA, USA); staphylococcal α-toxin from Gibco BRL (Gaithersburg, MD, USA); A23187 from Hoechst Calbiochem (San Diego, CA, USA). All other chemicals were obtained from Wako (Osaka, Japan) and were of the highest purity grade commercially available.

3. Results

3.1. Effects of Rp-8-Br-cGMPS on cGMP- and cAMP-kinases

cGMP and 8-Br-cGMP were potent activators of type Iα and Iβ isozymes of cGMP-kinase isolated from porcine aorta (Table 1). As reported for isozymes purified from bovine aorta (Wolfe et al., 1989) these compounds were 4–6-fold more potent activators of isozyme Iα than of isozyme Iβ.

Rp-8-Br-cGMPS inhibited the activation by cGMP of these two isozymes of type I cGMP-kinase. As is evident from the Dixon plot depicted in Fig. 1, the inhibition was of a competitive type; apparent K_i values were 3.7 μM for isozyme Iα and 1.8 μM for isozyme Iβ (Fig. 1 and Table 1). The compound was also a competitive inhibitor of type II cAMP-kinase. However, the apparent K_i value

Table 1
Activation and inhibition constants of porcine aorta cGMP kinase types Iα and Iβ, and bovine heart cAMP kinase type II

Compound	cGMP kinase				cAMP kinase	
	K_a (μM)		K_i (μM)		K_a (μM)	K_i (μM)
	Type Iα	Type Iβ	Type Iα	Type Iβ	Type II	
cGMP	0.047	0.26			4.7	
8-Br-cGMP	0.018	0.071			6.3	
cAMP	5.0	2.0			0.044	
8-Br-cAMP	0.72	0.52			0.007	
Rp-8-Br-cGMPS			3.7	1.8		25
Rp-8-Br-cAMPS			3.8	3.5	N.D. [a]	N.I. [b]

The apparent inhibition constants (K_i) and the apparent activation constants (K_a, concentration required for half-maximal activation) are shown. Each value represents the mean of two experiments, each performed in triplicate.

[a] N.D., not determined because activation was variable depending on the Rp-8-Br-cAMPS preparations.

[b] N.I., weak or no inhibition.

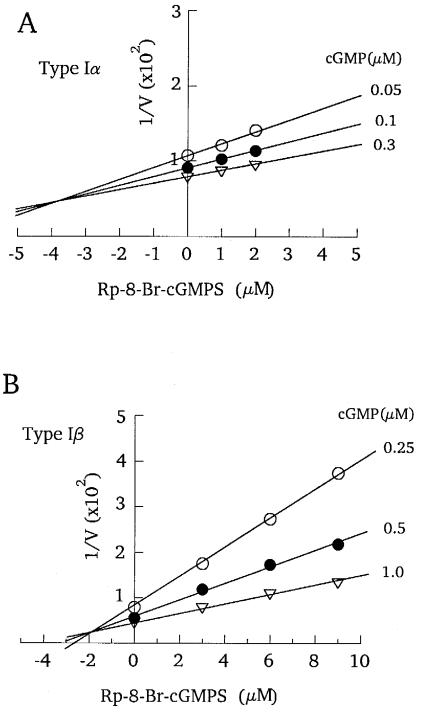

Fig. 1. Inhibition of cGMP-kinases Iα and Iβ by Rp-8-Br-cGMPS. Dixon plot analyses of the inhibition of cGMP-kinase Iα (A) and cGMP-kinase Iβ (B) by Rp-8-Br-cGMPS were shown. $1/V$ is the reciprocal of protein kinase activity (μmol/min per mg). Each value represents the mean of two experiments, each performed in triplicate.

of 25 μM for cAMP-kinase indicates that the compound is around 10-times more effective as inhibitor of cGMP-kinase than of cAMP-kinase.

For comparison, effects of Rp-8-Br-cAMPS, a putative, selective inhibitor of cAMP-kinase, were also studied. The compound was also a potent, competitive inhibitor of both types of isozymes of cGMP-kinase. The K_i values of 3.8 μM for isozyme Iα and 3.5 μM for isozyme Iβ were comparable to those of Rp-8-Br-cGMPS (Table 1). Rp-8-Br-cAMPS was found to activate, but not to inhibit, type II cAMP-kinase as shown by Gjersten et al. (1995). We, therefore, did not use the compound in the present study.

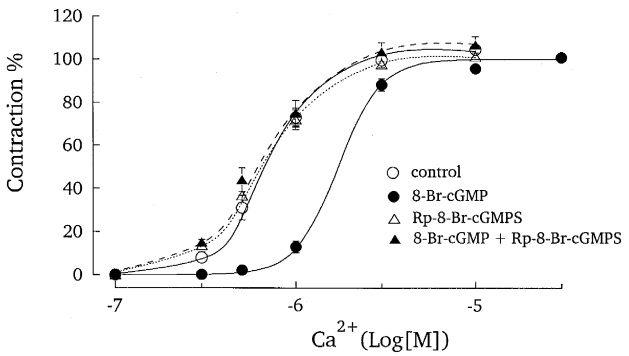

Fig. 2. Effects of 8-Br-cGMP on the concentration-contraction curve for Ca^{2+} and antagonistic effects of Rp-8-Br-cGMPS thereupon. Values are expressed as percentages of contraction induced by 10^{-5} M Ca^{2+}. All data represent means ± S.E. of six experiments. (○) control preparations; (●) 8-Br-cGMP (10^{-7} M); (△) Rp-8-Br-cGMPS (10^{-5} M); (▲) 8-Br-cGMP (10^{-7} M) + Rp-8-Br-cGMPS (10^{-5} M).

Table 2
Effects of Rp-8-Br-cGMPS and Rp-cAMPS on the rightward shift of concentration-contraction curves for Ca^{2+} induced by 8-Br-cGMP and 8-Br-cAMP as expressed by pD_2 values

Drug	pD_2
Group 1	
Control	6.15 ± 0.04
8-Br-cGMP	5.76 ± 0.02 [a]
Rp-8-Br-cGMPS	6.18 ± 0.03 [b]
8-Br-cGMP + Rp-8-Br-cGMPS	6.18 ± 0.04 [b]
Group 2	
Control	6.16 ± 0.01
8-Br-cGMP	5.85 ± 0.03 [a]
Rp-cAMPS	6.21 ± 0.02 [b]
8-Br-cGMP + Rp-cAMPS	5.88 ± 0.05 [a,c]
Group 3	
Control	6.17 ± 0.01
8-Br-cAMP	5.89 ± 0.02 [a]
Rp-cAMPS	6.20 ± 0.03 [b]
8-Br-cAMP + Rp-cAMPS	5.92 ± 0.04 [a,c]
Group 4	
Control	6.07 ± 0.03
8-Br-cAMP	5.81 ± 0.03 [a]
Rp-8-Br-cGMPS	6.07 ± 0.05 [b]
8-Br-cAMP + Rp-8-Br-cGMPS	6.02 ± 0.03 [b]

Concentration of drugs used: 8-Br-cGMP, 10^{-7} M; 8-Br-cAMP, 10^{-6} M; Rp-8-Br-cGMPS, 10^{-5} M; Rp-cAMPS, 10^{-5} M. All data represent means ± S.E. of five or six experiments. pD_2 values ($-\log EC_{50}$) were obtained by logistic curve analysis. Alphabetical symbols indicate significant difference ($P < 0.01$) among treatments within a group: [a] different from control; [b] different from 8-Br-cGMP or 8-Br-cAMP treatment; [c] different from Rp-8-Br-cGMPS or Rp-cAMPS.

3.2. Ca^{2+} sensitivity of contractile elements of α-toxin-skinned mesenteric artery

3.2.1. 8-Br-cGMP on Ca^{2+} sensitivity of contractile elements and the effects of Rp-8-Br-cGMPS

As shown in Fig. 2, 8-Br-cGMP (10^{-7} M) produced a clear rightward shift of the concentration-contraction curve for Ca^{2+} in skinned smooth muscle preparations from rat mesenteric arteries. After treatment with 8-Br-cGMP the pD_2 values for Ca^{2+} were significantly changed from 6.15 ± 0.04 to 5.76 ± 0.02 ($P < 0.01$) (Table 2, group 1). The apparent pA_2 value for 8-Br-cGMP calculated on the basis of the change was 7.15.

Rp-8-Br-cGMPS (10^{-5} M) itself did not produce any effect on the concentration-contraction curve for Ca^{2+} (pD_2 value in the presence of Rp-8-Br-cGMPS was 6.18 ± 0.03), but completely counteracted the rightward shift of the concentration-contraction curve for Ca^{2+} produced by 8-Br-cGMP (pD_2 in the presence of 8-Br-cGMP and Rp-8-Br-cGMPS was 6.18 ± 0.04).

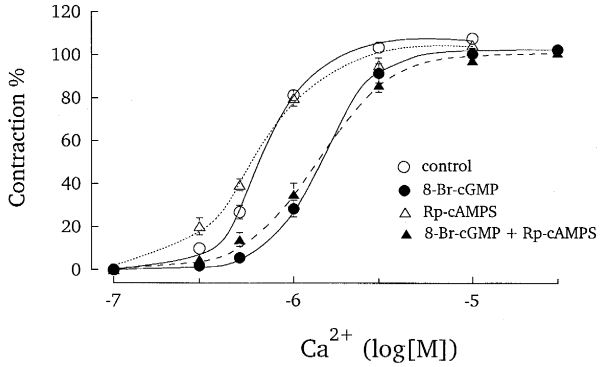

Fig. 3. Effects of 8-Br-cGMP on the concentration-contraction curve for Ca^{2+} and antagonistic effects of Rp-cAMPS thereupon. Values are expressed as percentages of contraction induced by 10^{-5} M Ca^{2+}. All data represent means ± S.E. of five experiments. (○) control preparations; (●) 8-Br-cGMP (10^{-7} M); (△) Rp-cAMPS (10^{-5} M); (▲) 8-Br-cGMP (10^{-7} M) + Rp-cAMPS (10^{-5} M).

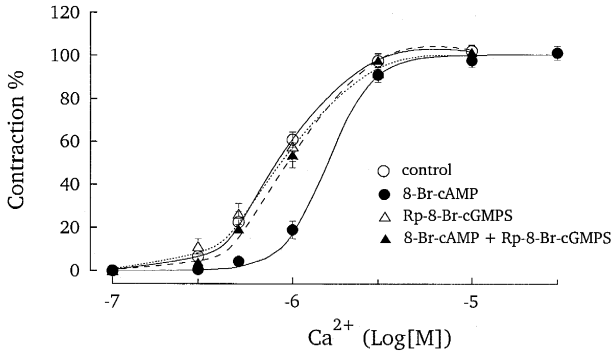

Fig. 5. Effects of 8-Br-cAMP on the concentration-contraction curve for Ca^{2+} and antagonistic effects of Rp-8-Br-cGMPS thereupon. Values are expressed as percentages of contraction induced by 10^{-5} M Ca^{2+}. All data represent means ± S.E. of six experiments. (○) control preparations; (●) 8-Br-cAMP (10^{-6} M); (△) Rp-8-Br-cGMPS (10^{-5} M); (▲) 8-Br-cAMP (10^{-6} M) + Rp-8-Br-cGMPS (10^{-5} M).

3.2.2. 8-Br-cGMP on Ca^{2+} sensitivity of contractile elements and the effects of Rp-cAMPS

As shown in Fig. 3, Rp-cAMPS (10^{-5} M) itself did not significantly affect the concentration-contraction curve for Ca^{2+} (pD_2 value was 6.21 ± 0.02) (Table 2, group 2). It also did not reverse the rightward sift of the concentration-contraction curve for Ca^{2+} induced by 8-Br-cGMP. The value of pD_2 in the presence of 8-Br-cGMP and Rp-cAMPS (5.88 ± 0.05) was not significantly different from the pD_2 value in the presence of 8-Br-cGMP alone (5.85 ± 0.03).

3.2.3. 8-Br-cAMP on Ca^{2+} sensitivity of contractile elements and the effects of Rp-cAMPS

As shown in Fig. 4, 8-Br-cAMP (10^{-6} M) produced a clear rightward shift of the concentration-contraction curve for Ca^{2+}. The values of pD_2 for Ca^{2+} were significantly changed from 6.17 ± 0.01 to 5.89 ± 0.02 after treatment with 8-Br-cAMP (Table 2, group 3). The apparent pA_2 value for 8-Br-cAMP calculated on the basis of the changes was 5.97. A lower concentration of 8-Br-cAMP (10^{-7} M) did not affect the concentration-contraction curve for Ca^{2+} (data not shown).

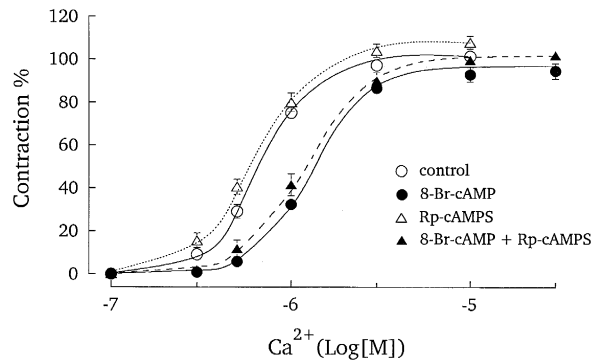

Fig. 4. Effects of 8-Br-cAMP on the concentration-contraction curve for Ca^{2+} and antagonistic effects of Rp-cAMPS thereupon. Values are expressed as percentages of contraction induced by 10^{-5} M Ca^{2+}. All data represent means ± S.E. of six experiments. (○) control preparations; (●) 8-Br-cAMP (10^{-6} M); (△) Rp-cAMPS (10^{-5} M); (▲) 8-Br-cAMP (10^{-6} M) + Rp-cAMPS (10^{-5} M).

Rp-cAMPS (10^{-5} M) failed to reverse the rightward shift of the concentration-contraction curve for Ca^{2+} induced by 8-Br-cAMP. The pD_2 value of 5.92 ± 0.04 in the presence of 8-Br-cAMP and Rp-cAMPS was not significantly different from that of 5.89 ± 0.02 in the presence of 8-Br-cAMP alone.

3.2.4. 8-Br-cAMP on Ca^{2+} sensitivity of contractile elements and the effects of Rp-8-Br-cGMPS

As shown in Fig. 5, Rp-8-Br-cGMPS (10^{-5} M) counteracted the rightward shift of the concentration-contraction curve for Ca^{2+} produced by 8-Br-cAMP. The value of pD_2 of 6.02 ± 0.03 obtained in the presence of 8-Br-cAMP and Rp-8-Br-cGMPS was significantly different from that of 5.81 ± 0.03 obtained in the presence of 8-Br-cAMP alone. The value was almost the same as that of the control, 6.07 ± 0.03 (Table 2, group 4).

4. Discussion

Using rat mesenteric artery skinned with α-toxin, Nishimura and Van Breemen (1989) demonstrated a rightward shift of the concentration-contraction curve for Ca^{2+} with 3×10^{-5} M cGMP and cAMP. We confirmed their findings using cGMP (10^{-4} M) and cAMP (10^{-5} M) (data not shown).

In the present study, 8-Br-cGMP (10^{-7} M) and 8-Br-cAMP (10^{-6} M) were shown to produce a rightward shift of the concentration-contraction curve for Ca^{2+}. Apparent pA_2 values for 8-Br-cGMP (7.15) and for 8-Br-cAMP (5.97) indicate that 8-Br-cGMP is 12 times more potent than 8-Br-cAMP to decrease the Ca^{2+} sensitivity of contractile elements in skinned mesenteric artery of the rat.

Since 8-Br-cGMP and 8-Br-cAMP are substances that are known to be resistant to hydrolysis by mammalian cyclic nucleotide phosphodiesterases (Francis et al., 1988), it is conceivable that the observed changes in Ca^{2+} sensitivity were brought about by 8-Br-cGMP and 8-Br-cAMP themselves.

To elucidate the participation of cGMP-kinase and cAMP-kinase in 8-Br-cGMP- and 8-Br-cAMP-induced changes in Ca^{2+} sensitivity of contractile elements of vascular smooth muscle, we used Rp-8-Br-cGMPS as a selective inhibitor of cGMP-kinase and Rp-cAMPS as a selective inhibitor of cAMP-kinase (Hofmann et al., 1985; Van Haastert et al., 1984). Rp-cAMPS was reported to be a competitive inhibitor of cAMP- and cGMP-kinases with apparent K_i values of 3.7 and 53 μM, respectively. Rp-8-Br-cGMPS was found in the present study to inhibit both Iα and Iβ cGMP-kinase with K_i values of 3.7 μM and 1.8 μM, respectively, while it inhibited cAMP-kinase with a K_i value of 25 μM. Thus, unlike Rp-cGMPS, which was reported to inhibit the activation of both cGMP- and cAMP-kinases equally well with an apparent K_i value of 20 μM (Butt et al., 1990), Rp-8-Br-cGMPS is more potent as an inhibitor of cGMP-kinase than of cAMP-kinase.

Phosphorothioate stereoisomers of cGMP and cAMP (Rp derivatives) inhibit kinase activity by competing with activating cyclic nucleotides for cyclic nucleotide binding sites (De Wit et al., 1982, 1984; Butt et al., 1990). Thus, unlike most of the protein kinase inhibitors so far reported upon, the inhibitory actions of these compounds are not influenced by physiological concentration of ATP (De Wit et al., 1982), an obvious advantage in conducting experiments with inhibitors as pharmacological tools under physiological conditions.

Rp-8-Br-cGMPS produced a complete reversal of the rightward shift of the concentration-contraction curve for Ca^{2+} produced by 8-Br-cGMP, while Rp-cAMPS did not reverse this shift (Figs. 2 and 3), suggesting the participation of the activation of cGMP-kinase in the decrease in Ca^{2+} sensitivity of the contractile elements produced by 8-Br-cGMP. Interestingly, the rightward shift of the concentration-contraction curve for Ca^{2+} produced by 8-Br-cAMP was also counteracted, not by Rp-cAMPS, but by Rp-8-Br-cGMPS (Figs. 4 and 5). These results suggest that the decrease in Ca^{2+} sensitivity of the contractile elements produced by 8-Br-cAMP was mediated, not by activation of cAMP-kinase, but by activation of cGMP-kinase.

According to Francis et al. (1988), the K_a values for activation of cGMP- and cAMP-kinases by 8-Br-cGMP in pig coronary artery are 25 nM and 2.8 μM, respectively. The apparent pA_2 value of 7.15, found with 8-Br-cGMP in the present study, for the rightward shift of the concentration-contraction curve for Ca^{2+} is sufficient for activation of cGMP-kinase, but not for activation of cAMP-kinase. As regards 8-Br-cAMP, Francis et al. (1988) reported the K_a values for activation of cAMP- and cGMP-kinases to be 65 nM and 5.8 μM, respectively. The apparent pA_2 value found in the present study for the decrease in Ca^{2+} sensitivity produced by 8-Br-cAMP was 5.97, a value far higher than the value reported to be necessary for activation of cAMP-kinase, but equal to that necessary for activation of cGMP-kinase. These quantitative considerations also support the hypothesis that the decrease in Ca^{2+} sensitivity produced by 8-Br-cAMP was due to activation of cGMP-kinase.

In repetitively subcultured vascular smooth muscle cells in culture, the Ca^{2+}-lowering effects of cAMP as well as cGMP became lost despite the persisting presence of cAMP-kinase in these cells. As the Ca^{2+}-lowering effects could be restored after addition of cGMP-kinase, but not after addition of cAMP-kinase, Lincoln et al. (1990) concluded that not only cGMP but also cAMP exert Ca^{2+}-lowering effects through activation of cGMP-kinase. In support of this hypothesis, 'cross-activation' of cGMP-kinase by cAMP was reported by Jiang et al. (1992). Recently, cross-activation of cGMP-kinase by cAMP has been reported by Komalavilas and Lincoln (1996) in intact rat aorta with respect to phosphorylation of the inositol 1,4,5-trisphosphate receptor.

McDaniel et al. (1992) demonstrated that nitrovasodilators relax histamine-contracted porcine carotid artery by decreasing the intracellular Ca^{2+} concentration and uncoupling contraction from myosin phosphorylation. In contrast, Van Riper et al. (1995) reported that forskolin induced the relaxation of porcine carotid artery contracted with high K^+ by decreasing the Ca^{2+} sensitivity of myosin phosphorylation. These results suggest the involvement of different mechanisms in decreases in Ca^{2+} sensitivity of vascular smooth muscle produced by cGMP and cAMP: cGMP does not affect the phosphorylation of myosin but uncouples force generation from myosin phosphorylation, while cAMP decreases the Ca^{2+} sensitivity of contractile elements by lowering the Ca^{2+} sensitivity of myosin phosphorylation. However, in Triton X-100-skinned guinea-pig mesenteric arteriole, the addition of cGMP-kinase reduces myosin phosphorylation and contractile force proportionally despite a constant Ca^{2+} concentration, suggesting that cGMP primarily decreases the Ca^{2+} sensitivity of myosin phosphorylation (Pfizer and Boels, 1991). Likewise, Wu et al. (1996) have recently shown that 8-Br-cGMP decreases the Ca^{2+} sensitivity of α-toxin-skinned rabbit ileum by decreasing myosin phosphorylation at constant Ca^{2+}. Thus, cyclic nucleotides appear to produce the decrease in Ca^{2+} sensitivity of smooth muscle through multiple mechanisms depending on tissue, contraction stimulus, and possibly animal species and smooth muscle preparation.

cAMP-kinase is ubiquitous in mammalian tissues and in several tissues the concentration of this kinase is higher than that of cGMP-kinase (Lincoln and Corbin, 1983). For example, in porcine vascular smooth muscle the intracellular cAMP-kinase and cGMP-kinase concentrations are 0.17 μM and 0.13 μM, respectively (Francis et al., 1988).

It is, therefore, natural to suppose that the enzyme is also present in rat mesenteric artery. The reason why the enzyme did not participate in the decrease in Ca^{2+} sensitivity in the present study is not clear at present. The idea of a selective leak of cAMP-kinase may not be tenable in view of the striking similarities found between cGMP- and cAMP-kinases, e.g., in size, shape, affinity for cyclic nucleotide binding, K_m for ATP, and divalent metal ion requirement (Corbin and Lincoln, 1978). One possible explanation is the localization of cGMP-kinase, but not cAMP-kinase, in the vicinity of substrate protein(s), phosphorylation of which would lead to a decrease in Ca^{2+} sensitivity, as suggested by Cornwell et al. (1991) for the phosphorylation of phospholamban, one of cGMP-kinase substrates, in rat aortic smooth muscle cells.

In conclusion, the present study clearly demonstrated the pivotal role played by activation of cGMP-kinase in the production of decreases in Ca^{2+} sensitivity of contractile elements. Not only cGMP but also cAMP seem to produce the decrease in Ca^{2+} sensitivity through activation of cGMP-kinase.

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