ROLE OF THE CALCIUM-CALMODULIN SYSTEM IN THE ADENYLAZE CYCLASE ACTIVITY OF VASCULAR SMOOTH MUSCLE

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In order to evaluate the relationship between the calcium-calmodulin system and the adenylate cyclase activity of vascular smooth muscle, we examined the effects of several calcium effectors on the basal and stimulated adenylate cyclase activity.

Thoracic aortae were removed from Wistar rats and the tissues were homogenated with cold homogenizing buffer containing 1 mM EDTA. Membrane protein fraction of the smooth muscle was prepared by centrifugation at 37,000g. In this procedure, endogenous guanine nucleotides and contractile proteins remained. The protein fraction was incubated with 2 mM EGTA, 50 μM trifluoperazine, 0.1 μM A23187 or 25 μM calmodulin under basal and stimulated (50 μM isoproterenol, 100 μM GTP and 50 μM forskolin) conditions. The adenylate cyclase activity was determined by a method modified in our laboratory using double isotope counting.

Trifluoperazine reduced the basal adenylate cyclase activity significantly (p < 0.01) as well as the stimulated enzyme activities. A23187 did not affect the basal enzyme activity, but elevated the isoproterenol stimulated enzyme activity significantly (p < 0.05). EGTA did not affect the basal and stimulated adenylate cyclase activities. Calmodulin elevated the basal enzyme activity significantly (p < 0.02), but did not affect the stimulated enzyme activities.

These results suggest that the calcium-calmodulin system is necessary for maintenance of the adenylate cyclase activity of vascular smooth muscle cells. The calmodulin acting site is considered to be the catalytic subunit, and stimulation of the enzyme is accelerated by calcium ion.

THE adenylate cyclase-cyclic AMP system is considered to be an important factor in the smooth muscle relaxation induced by β-adrenergic agonists. Several lines of evidence to support this have been reported by many investigators: β-agonist increases the cyclic AMP in blood vessels. β-agonist stimulates adenylate cyclase activity in broken cells from blood vessels; a derivative of cyclic AMP, dibutyryl cyclic AMP, causes relaxation of blood vessels when added to the bath and other agents which elevate cyclic AMP in blood vessels (e.g., phosphodiesterase inhibitors) cause such relaxation.

It has been reported that the β-adrenergic smooth muscle relaxation is mediated by protein phosphorylations catalyzed by protein kinase A. While most of the evidence has suggested that

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this relaxation results from a decrease in calcium availability\cite{5,7} it has been further suggested that the protein phosphorylated by this mechanism either or indirectly controls the calcium distribution\cite{8} Scheid et al\cite{9} reported that $\beta$-adrenergic agents stimulate cyclic AMP dependent phosphorylation which enhances sodium-potassium transport and induces the relaxation, and they suggested that this stimulation induces relaxation through enhanced sodium-calcium exchange. Webb and Bohr\cite{3} observed that the relaxation of blood vessels induced by isoproterenol or dibutyryl cyclic AMP was inhibited by ouabain, suggesting that cyclic AMP-elicited relaxation is mediated in part by stimulation of the sodium pump. The $\beta$-adrenergic relaxation of vascular smooth muscle is therefore considered to arise through the adenylate cyclase-cyclic AMP system which acts to amplify the Na-K ATPase activity within the cell.

On the other hand, it has been reported recently that the adenylate cyclase activity of vascular smooth muscle is regulated by the intracellular calcium ion concentration\cite{10,11,12} Piascik et al\cite{13} observed that intracellular calcium ion, at physiological concentrations, can stimulate as well as inhibit the adenylate cyclase activity of smooth muscle, and that the stimulation of the enzyme activity is mediated by calmodulin.

Thus, to investigate the relationship between the adenylate cyclase activity and the calcium-calmodulin system, both of which are important in the control of vascular smooth muscle tone, we incubated various calcium effectors with basal and stimulated aortic membrane protein fraction, and measured the adenylate cyclase activity of vascular smooth muscle.

**MATERIALS AND METHODS**

**Materials** $^{14}$C-labeled adenosine triphosphate ($^{14}$C-ATP, specific activity, 50 mCi/mmole), $^3$H-labeled adenosine $^3$', 5' monophosphate ($^3$H-cyclic AMP, specific activity, 40 mCi/mmole) and $^{14}$C-labeled adenosine 5'-monophosphate ($^{14}$C-5'AMP, specific activity, 40 mCi/mmole) were purchased from ICN Radiochemical, USA. A23187 was from Hoechst Calbiochem, USA. Isoproterenol, GTP, forskolin, ATP, cyclic AMP and calmodulin were from Sigma Chemical USA.

**Animals** Male Wistar rats, weighing 250–300g at 8 weeks of age, were used. They were housed and fed with tap water and normal rat chow containing 0.26g sodium/100g and 0.75g potassium/100g ad libitum for 2 weeks before the experiments.

**Preparation of aortic smooth muscle** The thoracic aortae were removed from rats under pentobarbital anesthesia. The materials were cleaned of loose connective tissue and adherent fat. The aortic tissues were washed twice with cold homogenizing buffer (20 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 1 mM EDTA, 1 mM MgCl$_2$ and 1 mM dithiothreitol) and then homogenized in 6–8 vol of the cold buffer using a polytron PT10 (Nihonseiki Co., Ltd., Tokyo). The homogenate was filtered through a nylon mesh and centrifuged at 9,000g for 10 min. The pellet obtained was rehomogenized and recentrifuged. The two supernatants were combined and centrifuged at 37,000g for 30 min to yield membrane protein fraction. The pellet was finally dispersed in cyclase assay buffer consisting of 10 mM Tris-HCl, 1 mM dithiothreitol and 1 mM MgCl$_2$, pH 7.4, at a protein concentration of 4 mg/ml, and stored at −80°C until analysis.

**Adenylate cyclase assay** Adenylate cyclase activity was determined by a method modified from Krishna’s method\cite{14} The incubation medium for the adenylate cyclase assay contained 0.4 mM Tris-HCl, pH 7.3, 3.3 mM MgSO$_4$, 0.1 mM NaF, 0.1 mM theophylline, 2 mM ATP, $^{14}$C-AMP, and 0.1 ml of the membrane protein. To the incubation medium, 50 $\mu$M isoproterenol, 100 $\mu$M GTP or 50 $\mu$M forskolin was applied as a stimulator. Under such stimulation, 2 mM EGTA, 50 $\mu$M trifluoperazine, 0.1 $\mu$M A23187 or 25 $\mu$M calmodulin was further added as a calcium effector (final volume, 0.7 ml). The incubations were carried out at 30°C for 20 min and were stopped by the addition of 0.1 mg/0.3 ml cyclic AMP containing $^3$H-cyclic AMP (≥ 20,000 dpm) as recovery followed by boiling for 3 min. The tubes were centrifuged and the supernatant fluid was chromatographed on 1 ml Dowex 50-H$^+$ (50W-X4, 200–400 mesh, BioRad Laboratory, USA) columns (0.4 x 4.5 cm). The column was eluted with water and 1 ml fractions were collected. Approximately 80% of the cyclic AMP was recovered in the 3rd and 4th fractions. The 1st and 2nd fractions contained most of the ATP which was eluted before the cyclic AMP fraction (Fig. 1). To the 2 ml of cyclic AMP fraction, 0.2 ml each of Ba(OH)$_2$ (0.25M) and ZnSO$_4$ (0.25M) solutions were added, and the mixture was centrifuged at 9,000g.
Fig. 1. Chromatographic separation of AMP, cyclic AMP and 5′AMP on a Dowex 50 ion-exchanged column. $^{14}$C-AMP ($\approx 30,000$ dpm), $^3$H-cyclic AMP ($\approx 25,000$ dpm) and $^{14}$C-5′AMP ($\approx 15,000$ dpm) were added to a 1.0 ml Dowex 50-H⁺ (50WX-4, 200–400 mesh) column. The column was eluted with water and 1 ml fractions were collected. The radioactivities of $^{14}$C and $^3$H were measured with a liquid scintillation spectrometer.

Fig. 2. Effects of 0.25 M Ba (OH)$_2$ with ZnSO$_4$ (0.4 ml) on radiolabeled $^{14}$C-ATP ($\approx 40,000$ dpm), $^3$H-cyclic AMP (cAMP) ($\approx 65,000$ dpm) and $^{14}$C-5′AMP ($\approx 18,000$ dpm) dissolved in 2.0 ml of water, respectively.

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Fig.3. Adenylate cyclase activities of rat aortic smooth muscle observed by various concentrations of isoproterenol in the presence of 10 (●) and 100 (○) μM of GTP.

Fig.4. Effects of several calcium effectors on the basal and isoproterenol, GTP and forskolin (FSK) stimulated adenylate cyclase activities of rat aortic smooth muscle.
NO: no addition, EGTA: 2 mM of EGTA, TFP: 50 μM of trifluoperazine, A23187: 0.1 μM of A23187. n: number of experiments
Vertical bars indicate the mean values ± SE.

for 10 min to remove the remaining ATP. The effects of the Ba(OH)$_2$ and ZnSO$_4$ treatment on the radiolabeled ATP, cyclic AMP and 5'-AMP are illustrated in Fig. 2. The supernatant solution was transferred into a scintillation vial containing scintillation cocktail (2, 5-diphenyloxazole, 3.5 g; 2, 2'-p-phenylenebis, 0.15 g; naphthalene, 50 g; in 500 ml dioxane). The $^{14}$C and $^3$H radioactivities were measured with a liquid scintillation spectrometer (LSC 1000, Aloka Co., Ltd., Japan).

Adenylate cyclase activity was expressed as pmol cyclic AMP/min/mg protein. Protein concentration was estimated according to Lowry et al$^{15}$ using bovine serum albumin as standard.

Statistics Statistical significance was assessed by Student's t-test for unpaired data, and the data were expressed as the mean ± SE.

RESULTS

The adenylate cyclase activities of vascular smooth muscle stimulated by various concentrations of isoproterenol in the presence of 10 or 100 μM of GTP are shown in Fig. 3. A dose dependency of the adenylate cyclase activity with respect to isoproterenol and GTP was demonstrated, and the maximum activity was recognized at 10 μM of isoproterenol (Fig. 3).

The effects of EGTA, trifluoperazine or A23187 on the adenylate cyclase activity of vascular smooth muscle, when nonstimulated (basal) and when stimulated by isoproterenol, GTP and forskolin, are illustrated in Fig. 4. Trifluoperazine reduced the basal adenylate cyclase activity significantly ($p < 0.01$). Also under stimulated conditions, a similar reducing tendency by trifluoperazine was observed, although the changes were not significant. A23187 did not affect the basal adenylate cyclase activity, but elevated the isoproterenol stimulated adenylate cyclase activity significantly ($p < 0.05$). EGTA did not affect the basal and stimulated adenylate cyclase activities appreciably (Fig. 4).

The effect of calmodulin on the adenylate cyclase activity of vascular smooth muscle under nonstimulated (basal) and the stimulated conditions are illustrated in Fig. 5. Calmodulin elevated the basal adenylate cyclase activity significantly ($p < 0.02$). While, under the stimulated conditions calmodulin did not affect the adenylate cyclase activity (Fig. 5).

DISCUSSION

It has been established that adenylate cyclase is a complex enzyme protein composed of three components, a receptor, GTP binding and catalytic subunit$^{16,17}$ and is present in the surface membranes of most cells$^{18}$ The adenylate cyclase activity is known to be regulated by the intracellular free calcium concentration$^{11,12}$ as well as
calcium-calmodulin complex\textsuperscript{13,19}

In the present experiments, homogenation of rat aorta to prepare membrane protein was carried out in the presence of 1 mM EDTA. Concerning the effect of EDTA on the elimination of calcium ion, it has been reported that pretreatment with EDTA chelates extracellular calcium ion and stabilizes the intracellular calcium ion at about $10^{-8}$–$10^{-7}$ mole\textsuperscript{20,21} which may be adjusted in these experimental conditions.

The membrane preparation from vascular smooth muscle was carried out by a modified version of the method of Sharma et al\textsuperscript{22} Their resultant membrane protein fraction was washed with 0.6M KCl to remove intrinsic guanine nucleotides and contractile protein. However, we did not wash the membrane with KCl, in order to preserve the membrane nucleotides and protein. It is considered therefore that endogenous guanine nucleotides and contractile protein, such as GTP and calmodulin, remained in the membrane protein fraction prepared for the present experiments.

Trifluoperazine, a calmodulin inhibitor, was found to reduce the basal adenylate cyclase activity significantly. Under stimulated conditions with isoproterenol, GTP and forskolin, similar reductions of the enzyme activities were also observed, suggesting that calmodulin is necessary for maintaining the basal and stimulated adenylate cyclase activity of vascular smooth muscle.

Furthermore, we evaluated the effect of calmodulin on the adenylate cyclase activity of vascular smooth muscle. Calmodulin was found to elevate the basal adenylate cyclase activity significantly. However, calmodulin did not affect the stimulated adenylate cyclase activities. From these findings, it is suggested that calmodulin itself has stimulated potency on the basal adenylate cyclase activity of vascular smooth muscle.

It has been shown that calcium and calmodulin regulate the adenylate cyclase activity in the brain\textsuperscript{23} C6 astrocytoma cells\textsuperscript{24} and primary cultures of neonatal glial cells\textsuperscript{25} Brostrom et al\textsuperscript{26} observed that calcium-dependent enhancement of hormone secretion induced an increase in cyclic AMP in glial cells, and they suggested that calmodulin might play a physiological role in the regulation of adenylate cyclase activity. These findings are consistent with the results obtained in the present experiments.

Piasecki et al\textsuperscript{13} reported that in the absence of exogenous calmodulin, calcium ion concentrations of greater than 0.8 $\mu$M inhibited the adenylate cyclase activity of bovine aortic smooth muscle, but in the presence of 5 or 9 $\mu$M of calmodulin, calcium ion stimulated the enzyme activity. Furthermore, they observed that calmodulin stimulated the enzyme activity when between 0.1 and 0.8 $\mu$M of calcium was present. From these findings, they suggested that calcium ion, at physiological concentrations, can stimulate as well as inhibit the smooth muscle adenylate cyclase activity, and that stimulation of adenylate cyclase activity is mediated by calmodulin, the level of which associated with the adenylate cyclase may modulate the response of the enzyme to calcium.

Salter et al\textsuperscript{27} showed that calcium-calmodulin complex stimulated the activity of a partially purified preparation of the catalytic subunit of brain adenylate cyclase which was free of guanine nucleotide. It has thus been established that calmodulin or calcium-calmodulin complex apparently interacts directly with the catalytic subunit of adenylate cyclase\textsuperscript{28,29}.

In the present experiments, trifluoperazine reduced the isoproterenol, GTP and forskolin stimulated adenylate cyclase activity. It is considered therefore that the calmodulin acting site was also located on the catalytic subunit of the adenylate cyclase of the vascular smooth muscle used, since it has been established that isoproterenol, GTP and forskolin act to amplify adenylate cyclase activity through the receptor, GTP binding and catalytic subunit, respectively\textsuperscript{30}.

On the other hand, A23187, a calcium ionophore, did not affect the basal adenylate cyclase activity of the vascular smooth muscle, but did elevate the isoproterenol stimulated enzyme activity in the present experiments, suggesting that the stimulation of the adenylate cyclase activity of vascular smooth muscle is accelerated by calcium ion, in contrast to the basal adenylate cyclase activity. It has been reported that in the presence of calmodulin, a physiological concentration of calcium stimulates the adenylate cyclase activity of vascular smooth muscle\textsuperscript{10,13}. Calcium-calmodulin complex formed by calcium mobilization following the A23187 administration, is therefore assumed to have enhanced the stimulated adenylate cyclase activity in the present experiments.

Sulakhe\textsuperscript{31} investigated the regulation of the adenylate cyclase activity in washed membrane isolated from the rat cerebral cortex by divalent
cations and GTP. He reported that there were two classes of adenylyl cyclase, EGTA-sensitive and EGTA-insensitive, and observed that the EGTA-insensitive enzyme activity was increased by norepinephrine by a mechanism which depended on GTP.

In the present experiments, EGTA did not affect the basal and stimulated adenylyl cyclase activities of vascular smooth muscle. It is possible therefore that the adenylyl cyclase activity of the vascular smooth muscle was maintained by GTP, since the enzyme was insensitive to EGTA and endogenous contractile protein such as GTP remained after the present experimental procedures. Furthermore, already formed calcium-calmodulin complex is assumed to have preserved the adenylyl cyclase activity of the vascular smooth muscle, despite calcium chelation by the EGTA administration in the experiments.

In conclusion, the present results suggest that calmodulin is necessary to maintain the adenylyl cyclase activity of vascular smooth muscle, and the acting site is considered to be the catalytic subunit. Stimulation of the enzyme is accelerated by calcium ion, since A23187 increased the stimulated enzyme activity, whereas the adenylyl cyclase activity of the vascular smooth muscle may be preserved by GTP or calcium-calmodulin complex, since no effective calcium chelation by EGTA was observed under the basal and stimulated conditions in the present experiments.

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