Cyclic AMP Modulation of Adrenoreceptor-mediated Arterial Smooth Muscle Contraction

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ABSTRACT We examined the effects of cyclic AMP (cAMP) on the intracellular Ca^{2+} release in both the intact and skinned arterial smooth muscle. The amount of Ca^{2+} in the sarcoplasmic reticulum (SR) was estimated indirectly by caffeine-induced contraction of the skinned preparation and directly by caffeine-stimulated ^{45}Ca efflux from the previously labeled skinned preparation. The norepinephrine-induced release contraction was markedly enhanced by dibutyryl cAMP (dbcAMP) and reduced by propranolol. The stimulatory effect of dbcAMP was best observed when the muscle was exposed to 10^{-5} M dbcAMP and 2×10^{-6} M norepinephrine was used to induce the release contraction. 10^{-5} M cAMP had no effect on the Ca^{2+}-induced contraction or on the pCa-tension relationship in the skinned preparation. This concentration of cAMP increased Ca^{2+} uptake into the SR of the skinned preparation when the Ca^{2+} in the SR was first depleted. 10^{-5} M cAMP stimulated Ca^{2+}-induced Ca^{2+} release from the SR after optimal Ca^{2+} accumulation by the SR. The results indicate that the stimulatory effect of cAMP on the norepinephrine-induced release contraction could be due to enhancement of the Ca^{2+}-induced Ca^{2+} release from the SR in arterial smooth muscle.

INTRODUCTION

Free calcium ions (Ca^{2+}) and cyclic AMP (cAMP) are ubiquitous intracellular messengers. In most cell types, rapid changes in the intracellular Ca^{2+} concentration exert direct control over cellular functions, while slower fluctuations in cAMP levels modulate the Ca^{2+} control system. In smooth muscle, cAMP modulation has been postulated to involve stimulation of Ca^{2+} and Na^{+} pumps (Scheid et al., 1979; Bulbring and den Hertog, 1980), partial inhibition of potential-operated Ca^{2+} channels (Meisheri and van Breemen, 1982), and a decrease in the affinity of myosin light chain kinase for the Ca-calmodulin complex (Adelstein et al., 1978, 1982). All of the above mechanisms act toward a reduction in active tension. It is therefore not clear why activation of arterial

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smooth muscle by norepinephrine (NE) should be accompanied by an increase of both the intracellular Ca^{2+} and cAMP.

An important step in arterial constriction induced by neurotransmitters and certain autocoids is the release of intracellular Ca^{2+} (van Breemen et al., 1972; Saida and van Breemen, 1984). In the present study, we investigate a possible role of cAMP in facilitating these processes of agonist-mediated intracellular Ca^{2+} release.

METHODS

The preparation of rabbit mesenteric artery and the measurement of isometric tension have been described previously (Saida and van Breemen, 1983*a*). A thin bundle of muscle (50 μm in width, 8 μm in thickness, and 300 μm in length) was prepared in physiological salt solution (PSS) of the following composition: 140 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES (brought to pH 7.2 with NaOH) saturated with 100% O_2. Removal of extracellular Ca^{2+} was accomplished by preincubating the preparation in Ca^{2+}-free PSS with 2 mM EGTA (Ca^{2+}-free medium) before applying the agonists. Maximal tension development, which was induced by 10^{-5} M NE in PSS or by 5×10^{-5} M Ca^{2+} in the intact and skinned preparation, respectively, was ~1.6 kg/cm^2. In order to directly compare the responses of intact and skinned muscle in the same preparation, all experiments were carried out at room temperature.

TABLE I

Solution Composition for an Experiment Using a Skinned Preparation

Solution	KCl	K propionate	Tris-maleate	$MgCl_2$	ATP	EGTA	Millimolar
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	
Relaxing	65	65	20	5	3.3	4	
Ca^{2+}	65	65	20	5	3.3	5	$\chi CaCl_2$
Ca^{2+} loading (10^{-6} M Ca^{2+})	65	65	20	5	3.3	0.5	0.3 $CaCl_2$
Ca^{2+} releasing	65	65	20	5	3.3	0.1	25 caffeine

Skinned Preparation

Skinned smooth muscle was prepared and studied by the methods described in our previous papers (Saida and Nonomura, 1978; Saida, 1982). After contractions were measured on the intact preparation, the muscle was treated with saponin (120–150 μg/ml for 20 min) in a relaxing solution (the composition is shown in Table I). In each preparation, the concentration of saponin was adjusted to ensure that a reproducible contraction could be generated during the duration of the experiment. 10^{-5} M Ca^{2+} was used to induce an initial and a terminal contraction to examine whether the skinned preparation showed deterioration of the contraction.

For the Ca^{2+} release experiments, the muscle was treated with lower concentrations of saponin (50–80 μg/ml for 20 min) in order to preserve the functionality of the SR as described in a previous paper (Saida, 1982). The following procedures were used for estimation of the amount of Ca^{2+} released from the SR: (*a*) the Ca^{2+} in the SR was depleted by 25 mM caffeine in the presence of 4 mM EGTA; (*b*) the SR was loaded with 10^{-6} M Ca^{2+} by bathing the skinned preparation in a Ca^{2+} solution weakly buffered with

0.5 mM EGTA for 3 min; (*c*) the skinned preparation was washed with relaxing solution containing 0.5 mM EGTA; (*d*) the skinned preparation was exposed to the "test" solution; and (*e*) 25 mM caffeine was applied to the preparation in the presence of 0.1 mM EGTA. The amount of Ca^{2+} released was estimated by comparing the caffeine-induced contraction with that of the control experiment in which the same procedures were followed, except for the application of the Ca^{2+}-releasing stimulus. The control experiments were carried out before and after the test experiment to examine whether the skinned preparation showed deterioration of the contraction.

The concentration of EGTA was altered when necessary as shown in Table I. In buffered solutions, Ca^{2+} concentrations were calculated by assuming an apparent binding constant of the Ca-EGTA complex to be 10^6 M^{-1} at pH 6.8, 20°C (Saida and Nonomura, 1978). The value of 4×10^5 M^{-1} was used as the binding constant of ATP for Mg^{2+} at pH 6.8 (Martell and Schwarzenbach, 1956).

In both experiments for the Ca^{2+}-induced contraction and the Ca^{2+} release from the SR, preparations showing deterioration of the contraction were discarded.

Ca^{2+} Efflux

The skinned preparations were loaded with 10^{-6} M Ca^{2+} labeled with ^{45}Ca for 10 min, followed by exposure to 10^{-5} M Ca^{2+} labeled with ^{45}Ca at the same specific activity in the absence or presence of 10^{-5} M cAMP for 2 min. The preparations were then passed through a series of test tubes, each containing the relaxing solution with 0.1 mM EGTA. The preparations were left for 1 min in each tube and, at 9 min, 25 mM caffeine was applied to the muscle.

Chemicals

NE bitartrate, propranolol hydrochloride, dibutyryl cAMP (dbcAMP), and cAMP were obtained from Sigma Chemical Co., St. Louis, MO. Saponin was from Merck & Co., Inc. ^{45}Ca (specific activity 24.5 mC/mg) was obtained from New England Nuclear, Boston, MA.

RESULTS

Effect of dbcAMP on NE-induced Contraction

Dibutyryl cAMP had an inhibitory effect on the tonic phase of the NE-induced contraction. This inhibition increased monotonically over the dbcAMP concentration range of 10^{-6}–10^{-3} M (Fig. 1, closed circles). On the other hand, dbcAMP had a biphasic effect on the initial rapid component of the NE-induced contraction, with a small stimulation at 10^{-5} M followed by an equally slight inhibition at 10^{-3} M (Fig. 1, open circles). The NE concentration used in these experiments was 2×10^{-6} M, which was half-maximally effective.

Since the initial rapid contractile phase is due largely to intracellular Ca^{2+} release, we decided to study the stimulating effect of dbcAMP in the absence of Ca^{2+} entry from the extracellular space. Fig. 2 shows that the transient NE contraction obtained after a 10-min treatment with EGTA (NE-induced release contraction) is markedly enhanced by dbcAMP and reduced by the β-adrenergic blocker propranolol. The dependences of dbcAMP stimulation of the NE-induced release contraction on the concentrations of dbcAMP and NE are given in Fig. 3. The results show that the stimulation reaches a maximum at 10^{-5} M dbcAMP and is most pronounced at submaximal NE concentration.

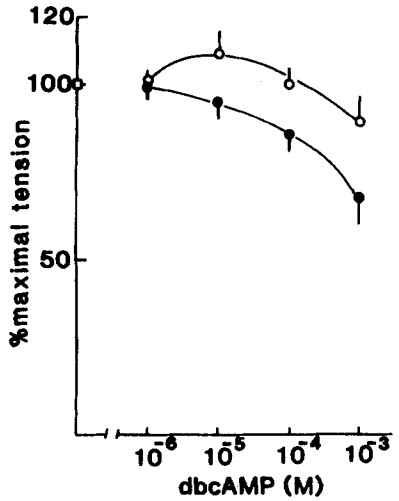

FIGURE 1. Dose-response curve of the effect of dbcAMP on 2×10^{-6} M NE-induced contraction in PSS. Open circles: effect of dbcAMP on the phasic component of NE contraction. Closed circles: effect of dbcAMP on the tonic component of NE contraction. The square represents the maximal tension development induced with 2×10^{-6} M NE. Points are means ± SEM ($N = 6$).

Effect of cAMP on the Contractile Proteins

To elucidate the effects of cAMP on myofilament interaction, we performed the following experiments using the skinned preparation.

To avoid participation of the SR, Ca^{2+} solutions were strongly buffered with 5 mM EGTA. As shown in Fig. 4*A*, the saponin-treated skinned preparation demonstrated Ca^{2+}-induced contractions in the presence of 2 mM Mg^{2+} and 3

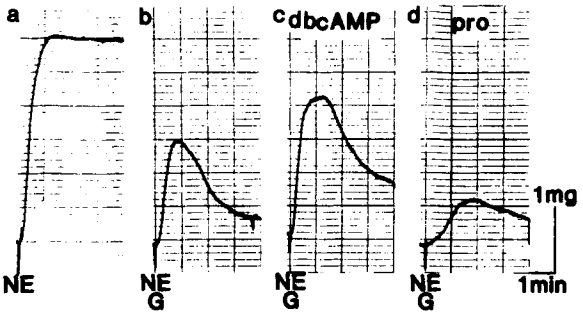

FIGURE 2. Effect of dbcAMP on NE-induced release contraction. (*a*) 2×10^{-6} M NE induced a sustained contraction in PSS. (*b*) NE induced a release contraction in Ca^{2+}-free medium (G) after bathing the muscle in Ca^{2+}-free medium for 10 min. (*c*) Effect of 10^{-5} M dbcAMP on the NE-induced release contraction. (*d*) Effect of 10^{-5} M propranolol (pro) on the NE-induced release contraction. The times required for relaxation (*b–d*) were 7, 9, and 6 min, respectively.

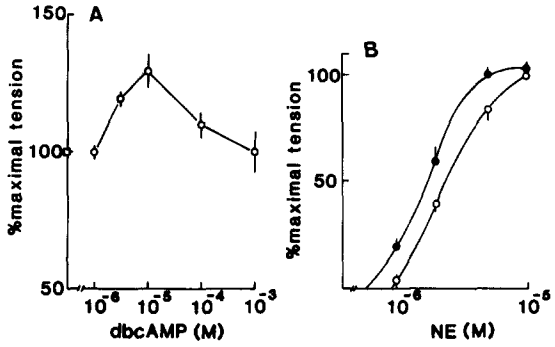

FIGURE 3. Effect of dbcAMP on NE-induced release contraction in Ca^{2+}-free medium. (*A*) Dose-response curve of the effect of dbcAMP on 2×10^{-6} M NE-induced release contraction in Ca^{2+}-free medium. The square represents the maximal tension development induced with 2×10^{-6} M NE. (*B*) Effect of 10^{-5} M dbcAMP on the dose-response curve for NE in Ca^{2+}-free medium. Points are means ± SEM ($N = 6$).

mM Mg ATP. Under these conditions, treatment of the skinned preparation with 10^{-5} M cAMP had no effect on the contraction induced with 10^{-6} M Ca^{2+} (Fig. 4*A*).

Additional experiments done in the presence of various concentrations of Ca^{2+} showed that 10^{-5} M cAMP did not modify the relationship between tension

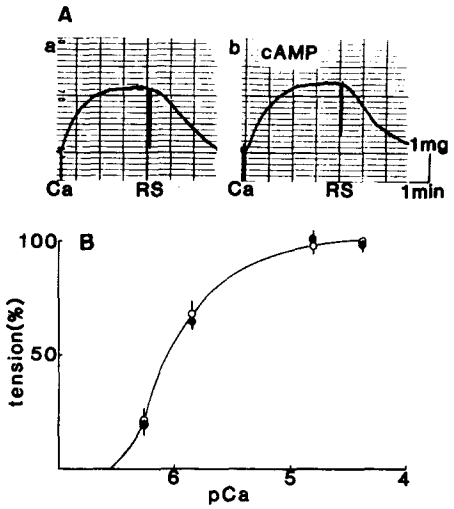

FIGURE 4. Effect of cAMP on Ca^{2+}-induced contraction in a skinned preparation. (*A*) 10^{-6} M Ca^{2+}-induced contraction in the absence (*a*) or presence (*b*) of 10^{-5} M cAMP. The total concentration of EGTA in Ca-EGTA buffered solution was 5 mM. Rs, relaxing solution. (*B*) Tension curve as a function of pCa in Ca-EGTA–buffered solutions in the absence (open circles) or presence (closed circles) of 10^{-5} M cAMP. Points are means ± SEM ($N = 5$).

development of the skinned preparation and the pCa in the heavily Ca-EGTA–buffered solution (Fig. 4*B*).

Effect of cAMP on Ca^{2+} Uptake by the SR and on Ca^{2+} Loss from the SR

Since 10^{-5} M cAMP had no effect on the contractile proteins, as shown above, we examined the effect of 10^{-5} M cAMP on the SR. The amount of Ca^{2+} refilled in the SR after bathing the skinned preparation in a Ca^{2+} solution was estimated by the 25 mM caffeine-induced contraction in the relaxing solution.

The time course of Ca^{2+} uptake by the SR from the solution containing 10^{-6} M Ca^{2+} is shown in Fig. 5*A*. The effects of 10^{-5} M cAMP on this time course appear to be complex because of an early stimulation and a late inhibition.

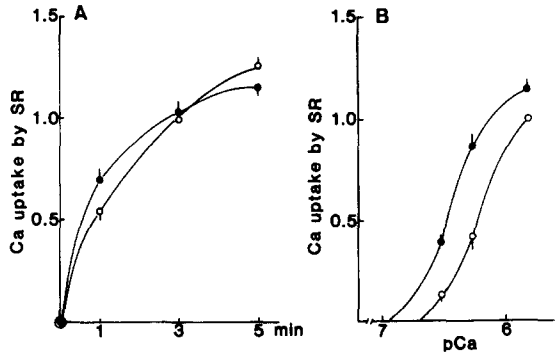

FIGURE 5. Effect of cAMP on Ca^{2+} uptake by the SR of the skinned preparation. The amount of Ca^{2+} present in the SR was estimated from the 25 mM caffeine-induced contraction. (*A*) Time course of Ca^{2+} uptake by the SR in the absence (open circles) or presence (closed circles) of 10^{-5} M cAMP. The skinned preparation was loaded with 10^{-6} M Ca^{2+} for various periods of time. 1.0 (ordinate) corresponds to the area under the caffeine contraction after loading with 10^{-6} M Ca^{2+} for 3 min. (*B*) The skinned preparation was loaded with various concentrations of Ca^{2+} (pCa) for 1 min in the absence (open circles) or presence (closed circles) of 10^{-5} M cAMP. 1.0 (ordinate) corresponds to the area under the caffeine contraction after loading with 10^{-6} M Ca^{2+} for 1 min. Points are means ± SEM ($N = 5$).

However, 10^{-5} M cAMP consistently increased the Ca^{2+} uptake by the SR if the concentration of Ca^{2+} was below 10^{-6} M and the time of uptake was limited to 1 min (Fig. 5*B*).

To test for a possible effect of cAMP on the rate of loss of SR Ca^{2+}, the SR was filled with 10^{-6} M Ca^{2+} for 3 min and then exposed to the relaxing solution containing 0.5 mM EGTA. Fig. 6 shows that 10^{-5} M cAMP did not affect the decay of SR Ca^{2+} under these conditions.

Effect of cAMP on the Ca^{2+}-induced Ca^{2+} Release Mechanism of the SR

Fig. 7*A* shows the experimental protocol for testing the effect of cAMP on Ca^{2+}-induced Ca^{2+} release from the SR in the skinned preparation. The SR was loaded with 10^{-6} M Ca^{2+} by bathing the skinned preparation in a Ca^{2+} solution weakly buffered with 0.5 mM EGTA. This concentration of Ca^{2+} was demonstrated to

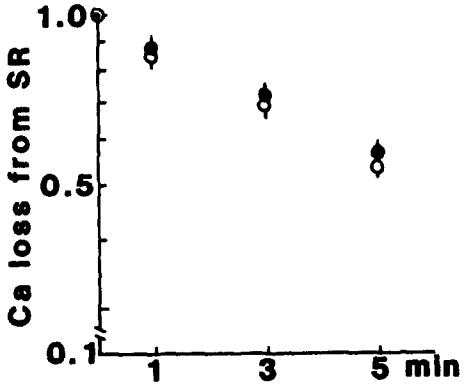

FIGURE 6. Effects of cAMP on Ca^{2+} loss from the SR. The skinned preparation was loaded with 10^{-6} M Ca^{2+} for 3 min and then exposed to relaxing solution containing 0.5 mM EGTA for various periods of time in the absence (open circles) or presence (closed circles) of 10^{-5} M cAMP. 1.0 (ordinate) corresponds to the area under caffeine contraction immediately after loading with 10^{-6} M Ca^{2+} for 3 min. Points are means ± SEM (N = 5).

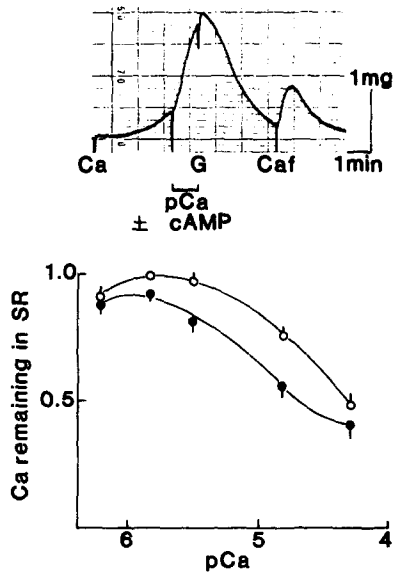

FIGURE 7. Effect of cAMP on Ca^{2+}-induced Ca^{2+} release from the SR. (*A*) Experimental procedure. The skinned preparation was loaded with 10^{-6} M Ca^{2+} for 3 min and then exposed to various concentrations of Ca (pCa) with or without 10^{-5} M cAMP for 1 min. The amount of Ca^{2+} remaining in the SR was estimated by the 25 mM caffeine contraction after washing with relaxing solution containing 0.5 mM EGTA (G). (*B*) Ca^{2+} remaining in the SR as a function of pCa. Ca^{2+} values were plotted relative to that in which the preparation was loaded with 10^{-6} M Ca^{2+}, with (closed circles) or without (open circles) 10^{-5} M cAMP. Points are means ± SEM (N = 5).

be optimal for loading the SR with Ca^{2+}. After the SR was fully loaded, it was exposed to various concentrations of Ca^{2+} for 1 min. To test for the effect of the change in Ca^{2+} concentration, the preparation was returned to the relaxing solution containing 0.5 mM EGTA for 3 min, and then the amount of Ca^{2+} remaining in the SR was estimated by the application of 25 mM caffeine. The amount of Ca^{2+} remaining in the SR decreased after treatment with Ca^{2+} above 3×10^{-6} M, which indicated activation of Ca^{2+}-induced Ca^{2+} release (Fig. 7*B*, open circles). As shown in Fig. 7*B*, 10^{-5} M cAMP facilitated the Ca^{2+}-induced Ca^{2+} release mechanism of the SR.

To confirm the above observation in a more direct manner, we measured the amount of Ca^{2+} present in the SR as ^{45}Ca efflux stimulated by 25 mM caffeine in the presence of 0.1 mM EGTA. For this purpose, we used the skinned preparations, which had been tested mechanically for their ability to support Ca^{2+}- and caffeine-induced contractions. The Ca^{2+} in the SR was first depleted by the application of 25 mM caffeine in the relaxing solution. Subsequently, the skinned preparation was loaded with 10^{-6} M Ca^{2+} labeled with ^{45}Ca for 10 min, and then exposed to 10^{-5} M Ca^{2+} labeled with ^{45}Ca at the same specific activity

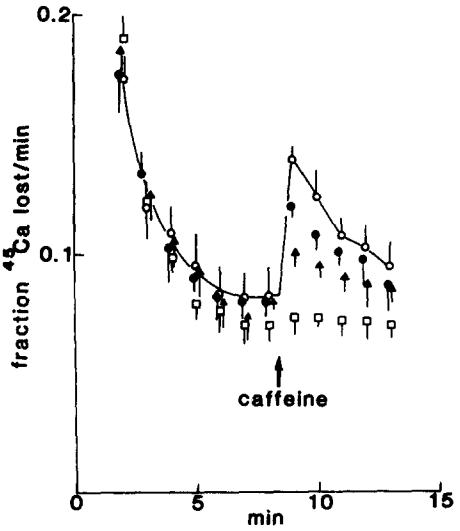

FIGURE 8. Effect of cAMP on 10^{-5} M Ca^{2+}-induced Ca^{2+} release from the SR. The skinned preparations were loaded with 10^{-6} M Ca^{2+} labeled with ^{45}Ca for 10 min, and then exposed to 10^{-5} M Ca^{2+} labeled with ^{45}Ca at the same specific activity in the absence (closed circles) or presence (triangles) of 10^{-5} M cAMP for 2 min. The label was then washed out by passing the tissue through a series of test tubes, each containing the relaxing solution with 0.1 mM EGTA. The preparations were left for 1 min in each tube and, at 9 min, 25 mM caffeine was applied to the muscle. Open circles represent the control experiment in which the preparation was loaded only with 10^{-6} M Ca^{2+} labeled with ^{45}Ca. Squares represent the experiment using the intact muscle under the same conditions as the control experiment using the skinned preparation. Points are means ± SEM ($N = 6$).

for 2 min. The preparation was washed out in the relaxing solution with 0.1 mM EGTA for 8 min. The amount of ^{45}Ca remaining in the SR (visualized as caffeine-stimulated ^{45}Ca efflux) decreased after treatment with 10^{-5} M labeled Ca^{2+} in comparison with the control (Fig. 8). This Ca^{2+}-induced Ca^{2+} loss was enhanced by the presence of 10^{-5} M cAMP during exposure to 10^{-5} M labeled Ca^{2+} (Fig. 8, triangles). Since the specific radioactivity was held constant in these experiments, the results are not due to ^{40}Ca-^{45}Ca exchange but entail a direct demonstration of Ca^{2+}-induced Ca^{2+} release from the SR. As a control, we used the intact preparation, in which Ca^{2+} in the SR had been depleted by high caffeine, to measure the ^{45}Ca efflux under the same conditions as the skinned preparation. As shown in Fig. 8 (squares), caffeine did not stimulate ^{45}Ca efflux, because the presence of the plasma membrane prevented filling of the SR from extracellular Ca^{2+} in the micromolar range.

DISCUSSION

The main findings reported in this paper are that cAMP stimulated the NE-induced release contraction and also the Ca^{2+}-induced Ca^{2+} release from arterial smooth muscle SR. The NE-induced release contraction may involve the Ca^{2+}-induced Ca^{2+} release mechanism, as described in a preceding paper (Saida and van Breemen, 1983*b*). The present results therefore confirm our hypothesis that α-adrenergic activation of arterial smooth muscle initiates Ca^{2+}-induced Ca^{2+} release from the SR (Saida and van Breemen, 1984).

Dibutyryl cAMP had an inhibitory effect on the tonic phase of NE-induced contraction. This inhibitory effect may be explained by the reduction of the intracellular Ca^{2+} concentration through stimulation of Ca^{2+} extrusion (Kroeger et al., 1975; Bulbring and Hertog, 1980) or Ca^{2+} sequestration (Casteels and Raeymaekers, 1979; Mueller and van Breemen, 1979). An important aspect of this study is the stimulating effect of dbcAMP on the NE-induced release contraction. This stimulatory effect was best observed when the muscle was exposed to 10^{-5} M dbcAMP and 2×10^{-6} M NE was used for the release contraction. The action of dbcAMP on the NE-induced release contraction could be due to stimulation of Ca^{2+}-induced Ca^{2+} release from the SR. Using skinned smooth muscle, we have previously demonstrated that Ca^{2+} itself triggers a Ca^{2+} release from the SR under physiological conditions (Saida, 1981; Saida and van Breemen, 1983*b*). This Ca^{2+}-induced Ca^{2+} release mechanism is inhibited by high concentrations of Mg^{2+} or procaine (Saida, 1982; Saida and van Breemen, 1984). In our previous work, the amount of Ca^{2+} present in the SR was indirectly estimated by the contractile response to caffeine on the skinned preparation. In the present work, in addition to the indirect method, we succeeded in directly measuring the amount of Ca^{2+} released as stimulation of ^{45}Ca efflux using a skinned preparation. Similar experiments have recently been reported by Stout and Diecke (1983). However, since they measured stimulation of ^{45}Ca efflux upon addition of ^{40}Ca, isotopic Ca exchange prevented the recording of a net Ca^{2+} loss from the SR. The protocol described in this paper circumvents this problem by the maintenance of constant specific radioactivity so that we were

able to obtain direct evidence for Ca^{2+}-induced Ca^{2+} release, as well as its potentiation by cAMP (Fig. 8).

Adelstein et al. (1978) first proposed that cAMP has a direct effect on actin-myosin interaction in smooth muscle. Their proposal has stimulated intensive research into the effects of cAMP on the Ca^{2+} regulation in smooth muscle. Several investigators (Kerrick and Hoar, 1981; Ruegg et al., 1981, 1983; Sparrow et al., 1981; Ruegg and Paul, 1982) have reported that cAMP inhibited Ca^{2+}-induced contraction of Triton X-100-treated, glycerinated smooth muscle. They concluded that this effect may be mediated through activation of cAMP-dependent protein kinase, producing phosphorylation of the myosin light chain kinase, which may result in an inhibition of actin-myosin interaction. It should be noted, however, that the inhibitory reffect of cAMP was demonstrated under special conditions using high concentrations of cAMP ($\geq 10^{-4}$ M) in the presence of fluoride or theophylline or by the use of protein kinase. In contrast, Itoh et al. (1982) reported that 3×10^{-6} M cAMP had no effect on 10^{-5} M Ca^{2+}-induced contraction of saponin-treated skinned smooth muscle in the presence of exogenous protein kinase. According to Ruegg et al. (1983), the inhibitory effect of cAMP was best observed when a low concentration of Ca^{2+} was used for the contraction. Nevertheless, the present observation shows that 10^{-5} M cAMP had no effect on the contraction induced with 10^{-6} M Ca^{2+} or with 10^{-5} M Ca^{2+} in the saponin-treated skinned preparation. The reason for this discrepancy is still unclear, although there is a difference in procedure for destroying the sarcolemma between the Triton X-100–treated, glycerinated preparation and the saponin-treated skinned preparation. Recently, Meisheri and Ruegg (1983) reported that the inhibitory effect of cAMP on the Ca^{2+}-induced contraction increased with decreasing the concentration of calmodulin in the solution bathing the Triton X-100-treated glycerinated preparation. In accord with their result, the discrepancy may be partially due to the difference in the concentration of calmodulin between the Triton X-100–treated, glycerinated preparation and the saponin-treated skinned preparation. Since this problem is very important for understanding the Ca^{2+} regulation in smooth muscle, further studies are required.

In their studies of smooth muscle relaxation by β-adrenergic agonists, Casteels and Raeymaekers (1979) and van Eldere et al. (1982) proposed that cAMP increased Ca^{2+} uptake in an agonist-releasable intracellular store. In an earlier paper, Deth and Casteels (1977) showed that the intracellular Ca^{2+} store was identical to the caffeine-sensitive Ca^{2+} store (most probably SR), which was separated from dinitrophenyl-sensitive mitochondria. Itoh et al. (1982) reported that application of cAMP and protein kinase increased the Ca^{2+} accumulation into SR of skinned arterial smooth muscle. In the present study using the skinned preparation, the stimulating effect of cAMP (10^{-5} M) on the Ca^{2+} uptake by the SR was clearly observed when the SR was loaded with Ca^{2+} below 10^{-6} M for 1 min. When the SR was loaded with 10^{-6} M Ca^{2+} for >3 min, cAMP reduced the Ca^{2+} uptake. Thus, it is assumed that cAMP predominantly stimulates the Ca^{2+}-induced Ca^{2+} release mechanism rather than the Ca^{2+} uptake mechanism after Ca^{2+} accumulation by the SR has reached a certain optimal level.

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