Effects of rolipram on U46619-induced contraction and cyclic nucleotide content in the porcine coronary artery

Takeharu KANEDA¹, Takashi KUBOTA¹, Kenji FUJIMOTO¹,
Norimoto URAKAWA¹, Shinjiro NAKAJYO¹ and Kazumasa SHIMIZU¹

¹Laboratory of Veterinary Pharmacology, Department of Veterinary Medicine,
Veterinary and Life Science University, Japan

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Abstract

The effects of various selective phosphodiesterase (PDE) inhibitors on muscle contractility and cyclic nucleotide content in the porcine coronary artery were investigated. Various selective PDE inhibitors, vinpocetine (type 1), erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA, type 2), milrinone (type 3), rolipram (type 4), Ro20-1724 (type 4), and zaprinast (type 5), inhibited U46619-induced contractions in a concentration-dependent manner. The rank order of potency for the porcine coronary artery was rolipram > Ro20-1724 > milrinone > vinpocetine > zaprinast > EHNA, which was different from that of both the porcine carotid artery and aorta. Rolipram inhibited the U46619-induced muscle tension with a decreased $[Ca^{2+}]_i$ level, but inhibited the high K^+ -induced contraction without a change in $[Ca^{2+}]_i$ level. Rolipram increased cAMP but not cGMP content. Iberitoxin restored the inhibition of muscle tension and the $[Ca^{2+}]_i$ levels induced by rolipram. U46619 and caffeine induced a transient increase in the $[Ca^{2+}]_i$ levels in a Ca^{2+} -free solution, but rolipram only inhibited the U46619-induced Ca^{2+} transient. In conclusion, rolipram is the most potent inhibitor in the porcine coronary artery, but not in the carotid artery and aorta. Moreover it is suggested that the mechanism by which rolipram causes relaxation is due to a decrease in the $[Ca^{2+}]_i$ levels and of the Ca^{2+} sensitivity of the contractile elements to cAMP.

Key words: rolipram, coronary artery, cAMP, phosphodiesterase, intracellular Ca^{2+}

Introduction

Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are important second messengers, and are associated with smooth muscle relaxation (Diamond, 1978). These cyclic nucleotides are synthesized by adenylyl cyclase or guanylyl cyclase, and are degraded by phosphodiesterases (PDEs). Currently, PDEs are classified into 11 families (Beavo, 1955; Fawcett *et al.*, 2000; Yuasa *et al.*, 2000), and selective PDE inhibitors have been

Correspondence to: Dr. Takeharu Kaneda, Laboratory of Veterinary Pharmacology, Department of Veterinary Medicine, Veterinary and Life Science University, 7-1 Kyonan-cho 1-chome, Musashino, Tokyo 180-8602, Japan
Phone: +81-422-31-4457 Fax: +81-422-31-4457 e-mail: t-kaneda@nvlu.ac.jp

found (Beavo and Reifsnnyder, 1990). In the case of the cardiovascular system, PDE3 is one of the major PDE isozymes. Actually, selective PDE3 inhibitors such as milrinone and enoximone are used in the management of acute heart failure. It has been shown that the treatment effects of these PDE3 inhibitors are their positive inotropic action and the vasodilation due to increases in cAMP (Kikura and Levy, 1995; Shipley *et al.*, 1996).

Vascular smooth muscle cells (VSMCs) can exist as 2 distinct phenotypes, contractile and synthetic (Yoshida and Owens, 2005; Owens *et al.*, 2004). In healthy blood vessels, contractile VSMCs contract and dilate in response to numerous hormonal or biophysical demands. In vascular abnormalities such as atherosclerosis, growth factors and cytokines induce a phenotypic change in VSMCs from the contractile state to the synthetic state. Recently, it has been shown that PDE4 activity surpassed PDE3 activity in synthetic VSMCs (Houslay *et al.*, 2007). However, there are few papers that shown the effects of PDE4 inhibitors on muscle contraction in normal vascular vessels (Lindgren *et al.*, 1991; Noguera *et al.*, 2001).

In the present study, we have demonstrated the effects of selective PDE (type 1–5) inhibitors on contractility of the porcine coronary artery, and compared that with those of both the porcine carotid artery and aorta. In addition, we also investigated the relaxing mechanisms of rolipram in the coronary artery.

Materials and Methods

Muscle preparations and tension measurement

The heart, carotid arteries and thoracic aorta were removed from adult pigs of either sex at the local abattoir. In each case, the medial layer of the left coronary artery was isolated from the hearts and cut into spiral strips. The carotid artery and thoracic aorta were prepared by removing the adventitia and cutting rings into circular strips. All strips were 2- to 3-mm wide and 8- to 10-mm long. The endothelium was removed by gentle rubbing of the inner surface of the strips with absorbent cotton. The muscle strips were incubated with physiological salt solution (PSS) containing (in mM) 136.8 NaCl, 5.4 KCl, 1.5 CaCl₂, 1.0 MgCl₂, 11.9 NaHCO₃, and 5.6 glucose. PSS was aerated with 95% O₂ and 5% CO₂ to adjust pH to 7.2 at 37°C. Muscle tension was recorded isometrically. One end of each strip was tied to a glass holder and the other end was connected by a silk thread to a strain-gauge transducer (TB-611T; Nihon Kohden, Tokyo) in an organ bath containing PSS with a resting tension of 1 g for both the coronary and carotid artery, and 10 g for the thoracic aorta. The muscle strips were equilibrated for 30 min to obtain a stable contractility induced by isosmotically added 40 mM KCl. When the contractile response induced by 1 μ M U46619 reached a steady level about 15–20 min after addition to the preparations, either vinpocetine (type 1), EHNA (type 2), milrinone (type 3), rolipram (type 4), Ro20-1724 (type 4) or zaprinast (type 5) were added cumulatively.

Simultaneous measurement of a muscle contraction and [Ca²⁺]_i level

The [Ca²⁺]_i level was measured simultaneously with each muscle contraction as reported previously (Ozaki *et al.*, 1987). Muscle strips were incubated overnight at 4°C with PSS containing 5 μ M fura2/AM and 0.02% cremophor EL (a non-cytotoxic detergent) to increase the solubility of

fura2/AM. One end of the muscle was pinned to the bottom of the organ bath which was filled with 8 ml of PSS, and the other end attached to the transducer with silk thread. The muscle strip was held horizontally in the organ bath. The muscle strip was alternately excited with light at either 340 nm or 380 nm using a rotating filter wheel, while the 500 nm emission was measured through a band-pass filter with a fluorometer (CAF-100; Japan Spectroscopic Co., Ltd., Tokyo). The fluorescence emission ratio (F340/F380) was recorded as an indicator of $[Ca^{2+}]_i$. The fluorescence ratio was expressed as a percentage, by assigning the values at rest in normal PSS to be 0% and those at a steady level of $[Ca^{2+}]_i$ in 1 μ M U46619 or 40 mM KCl to be 100%.

Assay of cGMP or cAMP content

The cGMP and cAMP content of the muscle strips was measured by enzyme immunoassay, as reported previously (Kaneda *et al.*, 2004). After incubation of the strips with each of the PDE inhibitors for 10 min in the presence of U46619 (1 μ M), the strips were rapidly frozen in liquid nitrogen, and stored at -80°C until homogenized in 6% trichloroacetic acid (0.4 ml). The homogenate was centrifuged at $3,000 \times g$ for 15 min, and the supernatant washed four times with 1.5 ml of water-saturated diethyl ether. The cGMP and cAMP content of the strips was assayed with an enzyme immunoassay kit (GE Healthcare, UK). The cGMP and cAMP content was expressed as picomoles per gram wet weight of tissue.

Chemicals

The chemicals used were milrinone, zaprinast (Sigma-Aldrich, St. Louis, MO, USA), vinpocetine, erythro-9-(2-hydroxy-3-nonyl) adenine $\cdot$ HCl (EHNA), rolipram, U46619 (BIOMOL Research Laboratories, USA) and Ro20-1724 (LC Laboratories, New Boston, USA). The concentration of vinpocetine was held below 30 μ M in the present experiments as it will crystallize if its concentration exceeds 30 μ M in PSS.

Statistics

Values are expressed as the mean $\pm$ SEM, and the IC_{50} values (the concentration producing 50% relaxation) were determined by linear regression analysis. Statistical analyses were performed by using the Student's *t*-test, and probabilities less than 5% were considered significant ($P < 0.05$).

Results

Effects of various PDE inhibitors on high K^+ and U46619-induced contractions

High K^+ (40 mM) and U46619 induced sustained contractions in the coronary artery. Vinpocetine (type 1), EHNA (type 2), milrinone (type 3), rolipram (type 4), Ro20-1724 (type 4) and zaprinast (type 5) inhibited these high K^+ and U46619-induced contractions in a concentration-dependent manner in the coronary artery (Fig. 1). The concentrations of these agents producing 50% relaxation (IC_{50}) of both the high K^+ - and U46619-induced contractions is presented in Table 1. In the present experiment, these PDE inhibitors inhibited U46619-induced contractions more potently than those of high K^+ - induced contractions. The rank order

Fig. 1. The effects of vinpocetine ($\circ$), EHNA ($\bullet$), milrinone ($\triangle$), Ro20-1724 ($\blacktriangle$), rolipram ($\square$) and zaprinast ($\blacksquare$) on contractions induced by 1 μ M U46619 (A) and 40 mM KCl (B) in preparations of the porcine coronary artery. The contractions induced by 1 μ M U46619 or 40 mM KCl just before the application of these PDE inhibitors were taken as 100%. Each point represents the mean of 4–7 preparations. Vertical bars indicate the SEM.

for the relaxant effect was rolipram > Ro20-1724 > milrinone > vinpocetine > zaprinast > EHNA for the U46619-induced contractions.

Effects of various PDE inhibitors on cGMP and cAMP content

Milrinone (100 μ M), rolipram (100 μ M) and Ro20-1724 (100 μ M) caused a significant increase in the cAMP content (Fig. 2A). On the other hand, zaprinast (100 μ M) only caused a significant increase in the cGMP content (Fig. 2B).

Table 1. IC₅₀ of various selective PDE inhibitors in coronary artery

Agents	IC ₅₀ (μ M)	
	40 K ⁺	U46619 (1 μ M)
Vinpocetine	>100	20 (16.8–24.9)
EHNA	>100	>100
Milrinone	>100	7.9 (6.6–9.3)
Ro20-1724	>100	4.0 (2.8–5.9)
Rolipram	>100	3.4 (2.4–4.9)
Zaprinast	>100	40 (39.5–47.0)

Numbers in parentheses indicate 95% confidence limits. n=4–7.

Fig. 2. The effects of vinpocetine (30 μ M, Vin), EHNA (100 μ M), milrinone (30 μ M, Mil 30, 100 μ M, Mil 100), rolipram (30 μ M, Roli 30; 100 μ M, Roli 100), Ro20-1724 (100 μ M, Ro 20) and zaprinast (Zap) on the cAMP (A) and cGMP (B) contents of the porcine coronary artery. Preparations were precontracted with 1 μ M U46619, and were treated with these agents for 10 min. The control was treated with vehicle instead of these PDE inhibitors. Each point represents the mean of 4 experiments. Vertical bars indicate the SEM. **, significant difference from control.

Fig. 3. Effects of rolipram on the U46619-induced (A) or 40 mM KCl-induced (40K⁺, B) increase in [Ca²⁺]_i levels (F340/F380, upper trace) and muscle tension (lower trace). The increase in [Ca²⁺]_i levels induced by U46619 or 40 mM K⁺ just before an application of rolipram was taken as 100%. Each trace was obtained from typical results of 4–5 experiments.

Effects of rolipram on high K⁺ and U46619-induced increases in both [Ca²⁺]_i levels and muscle tension

The effects of rolipram on [Ca²⁺]_i levels were measured simultaneously with changes in muscle tension, using a fluorescent Ca²⁺ indicator, fura2. Either 40 mM K⁺ or U46619 induced muscle contraction and elevated [Ca²⁺]_i levels as indicated by the ratio of F340/F380. Rolipram (30 or 100 μM) decreased both muscle tension and the increases of [Ca²⁺]_i levels induced by U466189 (35.1 ± 2.2% for tension and 26.5 ± 1.7% for [Ca²⁺]_i level at 30 μM, n=4, data not shown; 2.8 ± 2.8% for tension and 1.7 ± 1.7% for [Ca²⁺]_i level at 100 μM, n=5, Fig. 3A). The resting

tension and $[Ca^{2+}]_i$ levels were not affected by the BK channel blocker iberiotoxin (30 nM; IbTX). In the presence of IbTX (30 nM), inhibition of the U466189 (30 μ M)-induced muscle tension and the increase in $[Ca^{2+}]_i$ levels caused by rolipram (30 μ M) were partially reversed (from $35.1 \pm 2.2\%$ to $58.7 \pm 3.5\%$ for tension, $n=4$, $P<0.01$, from $26.5 \pm 1.7\%$ to $46.3 \pm 4.2\%$ for $[Ca^{2+}]_i$ level, $n=4$, $P<0.01$, data not shown).

Rolipram also decreased both muscle contraction and the increases in $[Ca^{2+}]_i$ levels induced by 40 mM K^+ , but the decrease in $[Ca^{2+}]_i$ levels was not significant ($81.3 \pm 6.0\%$, $n=4$; Fig. 3B).

Effects of rolipram on U466189 or caffeine induced increase in $[Ca^{2+}]_i$ levels and contraction in the absence of extracellular Ca^{2+}

In Ca^{2+} -free solution (using 2 mM EGTA), U46619 (1 μ M) induced a sustained increase in muscle tension and a transient increase in $[Ca^{2+}]_i$ levels ($71.5 \pm 4.1\%$ for tension, $40.0 \pm 3.3\%$ for $[Ca^{2+}]_i$ levels of the high K^+ -induced response, $n=6$). Rolipram (100 μ M) diminished the transient increase in both muscle tension and $[Ca^{2+}]_i$ levels elicited by U46619 to $37.6 \pm 7.5\%$ and $21.8 \pm 1.2\%$ (Fig. 4).

Caffeine (20 mM) also induced a transient increase in muscle tension and $[Ca^{2+}]_i$ levels in Ca^{2+} -free solution ($14.1 \pm 1.0\%$ for tension, $100.6 \pm 2.0\%$ for $[Ca^{2+}]_i$ level of high K^+ -induced response, $n=5$). Rolipram (100 μ M) had no significant effect on the caffeine-induced transient increase in both muscle tension and $[Ca^{2+}]_i$ levels ($19.4 \pm 1.8\%$ for tension, $102.1 \pm 4.3\%$ for $[Ca^{2+}]_i$ level, data not shown).

Effects of various selective PDE inhibitors on contractility in carotid artery and thoracic aorta preparations

Vinpocetine, EHNA, milrinone, rolipram, Ro20-1724, and zaprinast inhibited U46619-induced contractions in a concentration-dependent manner in both porcine carotid artery and thoracic aorta preparations (Fig. 5). The concentrations of IC_{50} are presented in Table 2. In both carotid artery and thoracic aorta preparations, milrinone only inhibited U46619-induced contractions to resting levels at their maximum concentration.

The rank orders for the relaxant effects were milrinone > vinpocetine, EHNA, Ro20-1724, rolipram, zaprinast for the carotid artery, and milrinone > zaprinast > vinpocetine, EHNA, Ro 20-1724, rolipram for the thoracic aorta.

Discussion

In the present study, the effects of the selective PDE (type 1–5) inhibitors on the contractility of preparations of the porcine coronary artery were compared with those of both the porcine carotid artery and aorta. Moreover, we also investigated the relaxing mechanism of rolipram in the porcine coronary artery. The major findings are as follows: (1) The rank order for the relaxant effect were rolipram > Ro20-1724 > milrinone > vinpocetine > zaprinast > EHNA for U46619-induced contraction. (2) In the presence of U46619, 100 μ M rolipram increased the content of cAMP significantly, but not that of cGMP. (3) As has been reported for many cyclic nucleotide-related agents, rolipram also inhibited U46619-induced muscle tension with a

Fig. 4. Representative recordings showing the increase in $[Ca^{2+}]_i$ levels (F340/F380, upper trace) and muscle tension (lower trace) induced by U46619 in Ca^{2+} -free PSS containing 2 mM EGTA, in the absence (A) and presence (B) of rolipram. Rolipram was applied 15 min prior to the application of U46619. The increase in $[Ca^{2+}]_i$ levels induced by 40 mM K^+ at 5 min in normal PSS was taken as 100%. Each trace, typical results from 4–5 experiments.

decrease in $[Ca^{2+}]_i$ levels. (4) In preparations of both the porcine carotid artery and aorta, the inhibitory effects of both rolipram and Ro20-1724 on the U46619-induced contraction were lower than those of other PDE inhibitors used in our study.

PDEs are involved in the regulation of signal transductions, and therefore various PDEs play important roles in mediating different cellular functions. Several studies have shown the functional role of PDE families in vascular smooth muscle. In the endothelium-denuded aorta,

Fig. 5. The effects of vinpocetine (○), EHNA (●), milrinone (△), Ro20-1724 (▲), rolipram (□) and zaprinast (■) on contractions induced by 1 μ M U46619 in preparations of the porcine carotid artery (A) and aorta (B). The contractions induced by 1 μ M U46619 just before the application of these PDE inhibitors were taken as 100%. Each point represents the mean of 4–7 preparations. Vertical bars indicate the SEM.

Table 2. IC_{50} and maximal inhibition (%) of various selective PDE inhibitors for contraction induced by U46619 in carotid artery and aorta

	Vinpocetine	EHNA	Milrinone	Ro20-1724	Rolipram	Zaprinast
Carotid artery						
IC_{50} (μ M)	>30	>100	1.1 (0.8–1.4)	>100	>100	83.1 (41.0–>100)
Maximal inhibition (%)	46.5 $\pm$ 5.5	49.9 $\pm$ 3.2	100	35.8 $\pm$ 3.3	46.7 $\pm$ 3.3	57.5 $\pm$ 5.9
n	6	6	6	5	6	6
Aorta						
IC_{50} (μ M)	>30	>100	11.1 (10.6–11.6)	>100	>100	46.3 (30.5–70.2)
Maximal inhibition (%)	40.7 $\pm$ 3.1	18.8 $\pm$ 1.7	100	18.4 $\pm$ 1.2	23.1 $\pm$ 2.7	61.5 $\pm$ 3.3
n	5	5	6	5	6	6

Numbers in parentheses indicate 95% confidence limits. The inhibition induced by PDE inhibitors which fully recover to the basal tension before the application of U46619 (1 μ M) was taken as 100% inhibition.

the relaxing effect of PDE3 inhibitors was the most potent of the selective PDE inhibitors (Komas *et al.*, 1991). On the other hand, the inhibitory effect of rolipram (type 4) on phenylephrine-induced contraction was more potent than that of milrinone (type 3) in the renal artery (Karsten *et al.*, 2003). In the present study, the selective PDE inhibitors inhibited the U46619-induced contraction and the rank order for the relaxant effect were rolipram > Ro20-1724 > milrinone > vinpocetine > zaprinast > EHNA in the porcine coronary artery. In contrast, the inhibitory effects of rolipram and Ro20-1724 on the U46619-induced contraction were less potent than those of milrinone and other PDE inhibitors in both the porcine carotid artery and aorta. These results indicate that PDE3 isozymes express in the smooth muscle of both elastic and muscular arteries, and regulate the smooth muscle contraction, however, PDE4 isozymes express only in muscular arteries. Moreover, the expressions of other PDE isozymes may be also different in various arteries.

In the present study, we could not detect an increase in cAMP in the mid-range concentrations of milrinone and rolipram or other PDE inhibitors used in the present study. Other researchers have shown that the PDE inhibitors, such as rolipram or cilostamide, increase the cAMP content at only very high concentrations (Eckly and Lugnier, 1994; Schoeffter *et al.*, 1987). These dissociations between vasodilation and increases in cAMP content have been shown in other cAMP elevating agents such as forskolin, isoproterenol and prostaglandin E₁ (Vegesna and Diamond, 1986). However, it has been reported that PDE3 and PDE4 inhibitors potentiate the relaxant effects and/or cAMP elevation produced by isoproterenol or forskolin in vascular smooth muscle (Tanaka *et al.*, 1988; Lindgren *et al.*, 1990; Maurice *et al.*, 1991). It has been proposed that cAMP signaling involves its sequestration into functional sub-cellular compartments and that significant local increases in cAMP in the cell are immeasurable (Vegesna and Diamond, 1986). On the other hand, it has been reported that the vasorelaxant action of amlinone, a PDE3 inhibitor, does not involve a cAMP-dependent pathway (van der Zyppe *et al.*, 2000). Changes in the local cyclic nucleotide concentration remain to be clarified.

It has been reported that cAMP-related agents inhibited U46619 muscle tension and increases in $[Ca^{2+}]_i$ levels, however, the inhibition of high K⁺-induced muscle tension with a slight decreases in $[Ca^{2+}]_i$ levels in the porcine coronary artery (Yamagishi *et al.*, 1994; Yamanaka *et al.*, 2003). In this study, rolipram inhibited the U46619-induced muscle tension with a decrease in $[Ca^{2+}]_i$ levels, but inhibited the high K⁺-induced contraction without changes in $[Ca^{2+}]_i$ levels. Thus, this suggests that the decrease in $[Ca^{2+}]_i$ levels caused by rolipram is due to a cAMP-dependent pathway.

In the present study, pretreatment with IbTX, a large-conductance Ca²⁺-activated K⁺ channel (BK channel) blocker, partially reversed the inhibition of the U46619-induced muscle tension and the increases in $[Ca^{2+}]_i$ levels by rolipram. In whole cell patch-clamp experiments, IbTX reduced isoproterenol (Iso)-enhanced K⁺ currents in smooth muscle cells isolated from the rat coronary artery (Li *et al.*, 2003). Thus, the decrease in $[Ca^{2+}]_i$ levels caused by rolipram may involve BK channels in the porcine coronary artery. Other mechanisms for the cAMP-induced decrease in $[Ca^{2+}]_i$ levels have been proposed, the stimulation of Ca²⁺ uptake into intracellular stores (Raeymaekers *et al.*, 1990), including and an increase in Ca²⁺ extrusion from the cell by

both the sarcolemmal Ca^{2+} pump (Bülbring and Tomita, 1987) and Na^+ - Ca^{2+} exchanger (NCX) (Yamanaka *et al.*, 2003). In the porcine coronary artery, U46619 and caffeine induced a transient increase in $[\text{Ca}^{2+}]_i$ levels in a Ca^{2+} -free solution (Yamagishi, 1994). They also showed that both Iso and IBMX significantly inhibited the U46619-induced Ca^{2+} transient, but not the caffeine-induced one. That is similar to our present data for rolipram. These results suggest that cAMP may decrease phosphatidylinositol turnover for the inhibition of the U46619-induced Ca^{2+} transient, but not stimulate Ca^{2+} uptake into intracellular stores, in the porcine coronary artery. The involvement of the sarcolemmal Ca^{2+} pump and the NCX in the cAMP-induced decrease in $[\text{Ca}^{2+}]_i$ levels of smooth muscle has been reported mainly in vascular smooth muscle (Ishida and Paul, 2005). Yamanaka *et al.* (2003) showed that pretreatment with low Na^+ physiological salt solution (PSS) partially reversed the inhibition of U46619-induced muscle tension and the increase in $[\text{Ca}^{2+}]_i$ levels by Iso, and Iso-induced decrease in tension during contraction by U46619 was greatly inhibited by 2',4'-dichlorobenzamil, a forward- and reverse-mode NCX inhibitor. Further study is needed to clarify the regulation of $[\text{Ca}^{2+}]_i$ levels in the smooth muscle of the coronary artery.

In the present study, rolipram reduced both muscle tension and the increase of $[\text{Ca}^{2+}]_i$ levels induced by high K^+ , but the decrease in $[\text{Ca}^{2+}]_i$ was only slight. In the α -toxin permeabilized rat mesenteric artery, cAMP decreased Ca^{2+} -induced contraction (Nishimura and van Breeman, 1989). Their data indicated that cAMP decreased the Ca^{2+} sensitivity of the contractile elements in vascular smooth muscle. Thus, it is suggested that the inhibition of high K^+ -induced contraction is related to the cAMP-dependent decrease of Ca^{2+} sensitivity of the contractile elements.

In summary, PDE4 inhibitors are the most potent inhibitors of the U46619-induced contraction amongst the other selective PDE inhibitors used in our study in the porcine coronary artery. But in the porcine carotid artery and aorta, the potency of PDE4 inhibitors is less than other PDE inhibitors. Moreover, it is suggested that the rolipram-induced relaxation is due to a decrease in $[\text{Ca}^{2+}]_i$ levels and the Ca^{2+} sensitivity of the contractile elements with relation to cAMP.

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