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Different regulation of myofilament Ca^{2+} sensitivity in β-escin-skinned cardiac and vascular smooth muscles

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Abstract

We compared the effects of guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS, an activator of G-protein), phorbol 12,13-dibutylate (PDB, an activator of protein kinase C) and pimobendan (an inotropic agent with Ca^{2+}-sensitizing action) on the Ca^{2+} sensitivity of the contractile proteins in β-escin-skinned muscle preparations obtained from rabbit left ventricles and mesenteric arteries. After the skinning procedure, when GTPγS (100 μM) or PDB (1 μM) was added to the Ca^{2+} solutions, pCa_{50} were significantly increased in preparations obtained from vascular smooth muscle, but not from cardiac muscle, indicating that G-protein- and protein kinase C-mediated direct Ca^{2+} sensitization may occur only in smooth muscle, but not in cardiac muscle. In contrast, pimobendan (50 μM) increased the Ca^{2+} responsiveness only in cardiac muscle. Therefore, we conclude that, in addition to the common regulatory factors affecting Ca^{2+} sensitivity such as intracellular pH and phosphorylation by protein kinase A, there are other means of regulation of Ca^{2+} sensitivity working differently in cardiac and in vascular smooth muscles.

Keywords: Ca^{2+} sensitivity; G-protein; Protein kinase C; Pimobendan; β-Escin; Cardiac muscle; Smooth muscle, vascular

1. Introduction

Although it is evident that the contractility of muscle, whether smooth or striated, can be modulated by mechanisms that cause changes in the rise of intracellular Ca^{2+}, chemical modifications of the contractile proteins are additionally involved in the physiological regulation of muscle contraction. Phosphorylation of contractile proteins by cyclic AMP (cAMP)-dependent protein kinase (protein kinase A) decreases Ca^{2+} sensitivity similarly in both cardiac and smooth muscles. Changes in intracellular pH also affect Ca^{2+} sensitivity in both muscle types. However, there may be factors which regulate Ca^{2+} sensitivity differently in the two muscle types. In vascular smooth muscle, recent studies have shown that α_1-adrenoceptor agonists and endothelin-1 with guanosine 5′-triphosphate (GTP) (Kitazawa et al., 1989; Nishimura et al., 1992; Satoh et al., 1994), or guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) alone (Fujiwara et al., 1989; Kitazawa et al., 1991; Kubota et al., 1992) directly increase Ca^{2+} sensitivity in chemically skinned muscles, suggesting that there is a G-protein-mediated direct Ca^{2+} sensitization of contractile proteins in vascular smooth muscle. However, in cardiac muscle, although these agonists are known to increase Ca^{2+} sensitivity in intact preparations (Endoh and Blinks, 1988; Wang et al., 1991), it is still uncertain whether activation of G-proteins directly modulates the myofilament responsiveness to Ca^{2+} in skinned preparations. Therefore, the purpose of this study was to test the hypothesis that activation of G-proteins directly regulates the Ca^{2+} sensitivity of the contractile proteins in cardiac muscle. We also compared the effects of phorbol 12,13-dibutylate (PDB, an activator of protein kinase C) and pimobendan (an inotropic agent with Ca^{2+}-sensitizing action) on the Ca^{2+} sensitivity of cardiac and of vascular smooth muscles. These agents increase the Ca^{2+} sensitivity of contractile proteins via different mechanisms (Fujino et al., 1988b; Krämer et al., 1991). Experiments were conducted with chemically (β-escin) skinned muscle preparations obtained from rabbit left ventricles and mesenteric arteries.

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2. Materials and methods

2.1. Preparations

New Zealand White rabbits weighing 1.8–2.2 kg were used for this study. The animals were anesthetized with pentobarbital sodium (40 mg/kg i.v.). The heart and then the mesenteric artery (about 1 mm in diameter) were removed and placed in oxygenated physiological salt solution of the following composition (in mM): NaCl 130, KCl 4.0, $CaCl_2$ 1.5, $MgCl_2$ 1.0, glucose 10, and Tris-HCl 10 (pH 7.4) at room temperature (22–25°C). In the case of the mesenteric artery, connective tissues and endothelial cells were carefully removed under a dissection microscope. A small bundle of muscle fibers (0.1–0.2 mm wide and 0.5–0.8 mm long) was tied with monofilament silk to the fine tips of two tungsten needles, one of which was connected to a force transducer, and mounted in one of the wells (0.5 ml volume each) engraved in a plastic plate. Solutions were rapidly changed by sliding the plate to an adjacent well. After a 10-min equilibration period, resting tension was adjusted in steps by means of a micromanipulator to produce maximal development during exposure to high-K^+ solution (K^+ contracture); the high-K^+ solution had the same composition as the physiological salt solution except that it contained 134 mM KCl and no NaCl. The preparations were then treated with β-escin (50 μM) for 30 min in a relaxing solution whose composition was (in mM): K^+ methanesulphonate 87, piperazine-*N*-*N*′-bis-(2-ethanesulphonic acid) (PIPES) 20, $Mg(methanesulphonate)_2$ 5.1, ATP 5.2, phosphocreatine 10, creatine phosphokinase 0.5 mg/ml, and EGTA 10. Various Ca^{2+} concentrations were prepared by adding appropriate amounts of $Ca(methanesulphonate)_2$ to the relaxing solution. The pH of the solution was adjusted to 7.0 with KOH and the ionic strength was standardized at 0.2 M by changing the amount of K(methanesulphonate) added.

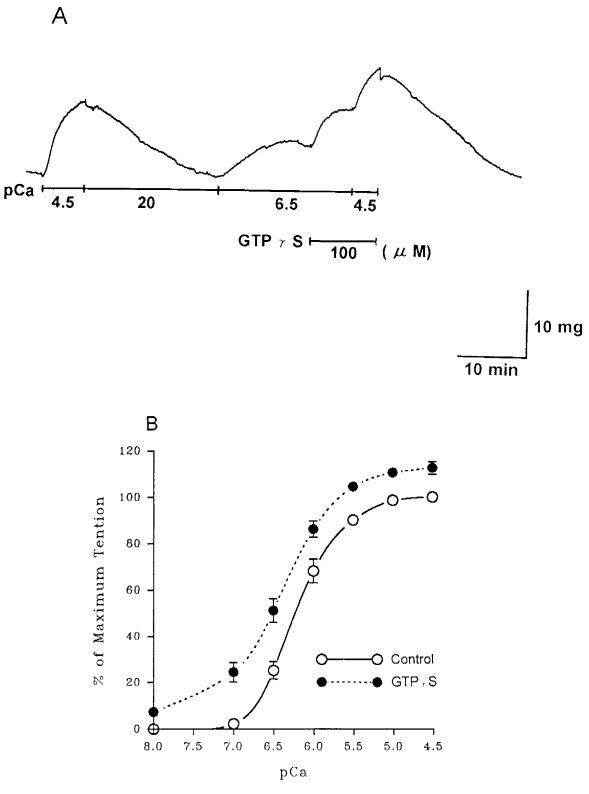

Fig. 1. Effect of 100 μM GTPγS on Ca^{2+}-induced contraction in β-escin-skinned vascular smooth muscle. A: Representative recording which shows that application of 100 μM GTPγS increased 3×10^{-7} M Ca^{2+}-induced contraction. B: pCa-force relationship in the absence (open circles) and presence (filled circles) of 100 μM GTPγS in Ca^{2+} solutions. Points are means ± S.E.M. for 6 fibers in each Ca^{2+} solution. Tension is expressed as percentage of the maximal tension obtained at 3×10^{-5} M Ca^{2+} without GTPγS.

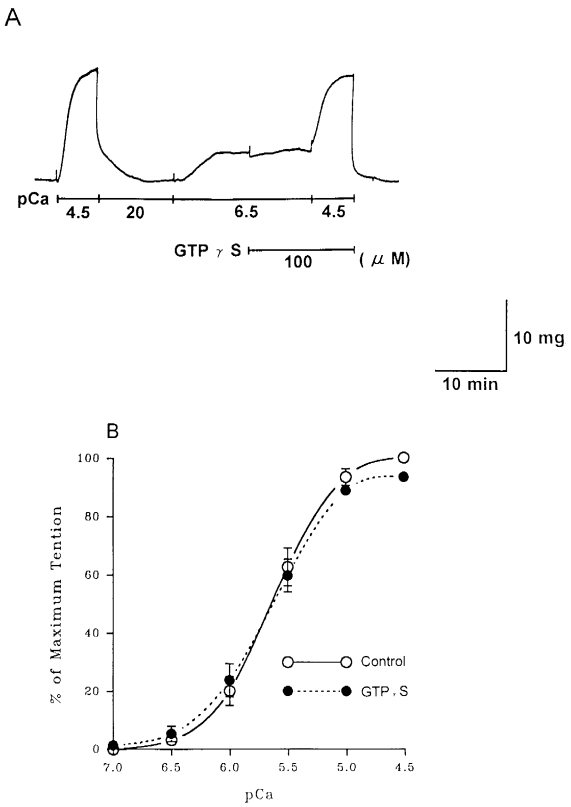

Fig. 2. Effect of 100 μM GTPγS on Ca^{2+}-induced contraction in β-escin-skinned cardiac muscle. A: Representative recording which shows that application of 100 μM GTPγS had no effect on 3×10^{-7} M Ca^{2+}-induced contraction. B: pCa-force relationship in the absence (open circles) and presence (filled circles) of 100 μM GTPγS in Ca^{2+} solutions. Points are means ± S.E.M. for 4 fibers in each Ca^{2+} solution. Tension is expressed as percentage of the maximal tension obtained at 3×10^{-5} M Ca^{2+} without GTPγS.

Pimobendan (UDCG 115-BS, kindly donated by Dr. K. Thomae, Biberach an der Riss, Germany) was dissolved in dimethyl sulfoxide and added to the Ca^{2+} solution. The final concentration (< 0.5%) of dimethyl sulfoxide in the Ca^{2+} solution had no significant effect on muscle contraction and relaxation.

2.2. pCa-force relationship

After the skinning procedure, the preparations were relaxed by exposure to the relaxing solution without β-

escin. Various concentrations of Ca^{2+} were applied cumulatively from low to high concentration. The amplitude of contraction induced by each of the various concentrations of Ca^{2+} was normalized with respect to that induced by 3×10^{-5} M Ca^{2+} in the same preparation. The data for the normalized pCa-force relationship were fitted to the Hill equation:

$$F/F_{max} = [Ca^{2+}]^{n_H} / \left([EC_{50}]^{n_H} + [Ca^{2+}]^{n_H} \right)$$

where F_{max} is the maximally activated force at 3×10^{-5} M Ca^{2+}, F is the developed force at the actual Ca^{2+} concentration, EC_{50} is the Ca^{2+} concentration giving half-maximal activation, n_H is the Hill coefficient that is an index of cooperativity. All experiments were carried out at room temperature (22–25°C).

2.3. Statistical analysis

All data are presented as means ± S.E.M. Statistical significance was evaluated with Student's paired *t*-test. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. Effects of GTPγS and PDB on the Ca^{2+} sensitivity of vascular smooth and cardiac muscles

First, we examined the effects of activation of G-protein by guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) on the Ca^{2+} sensitivity of vascular smooth and cardiac muscles.

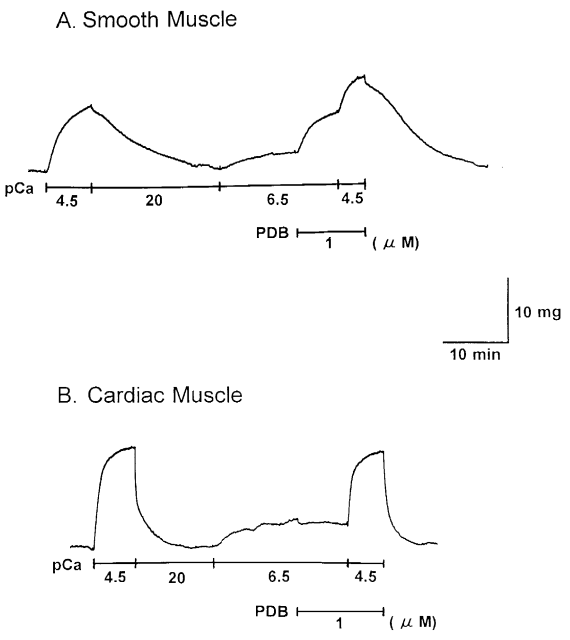

Fig. 3. Effect of 1 μM PDB on Ca^{2+}-induced contraction in β-escin-skinned muscles. Representative recordings which show that application of 1 μM PDB increased 3×10^{-7} M Ca^{2+}-induced contraction in vascular smooth muscle (A), but had no effect in cardiac muscle (B).

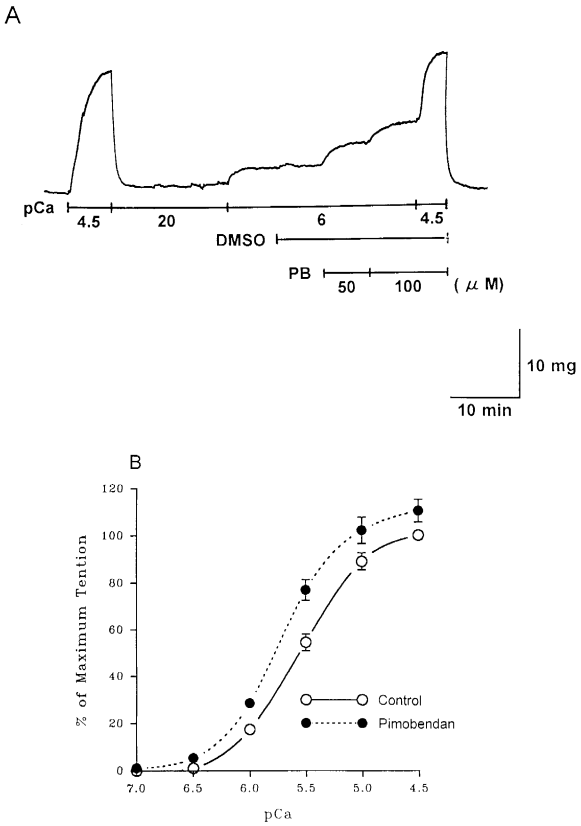

Fig. 4. Effect of 50 μM pimobendan on Ca^{2+}-induced contraction in β-escin-skinned cardiac muscle. A: Representative recording which shows that application of 50 μM pimobendan increased 3×10^{-7} M Ca^{2+}-induced contraction. B: pCa-force relationship in the absence (open circles) and presence (filled circles) of 50 μM pimobendan in Ca^{2+} solutions. Points are means ± S.E.M. for 6 fibers in each Ca^{2+} solution. Tension is expressed as percentage of the maximal tension obtained at 3×10^{-5} M Ca^{2+} without pimobendan. PB, pimobendan; DMSO, dimethyl sulfoxide.

Fig. 1A shows a representative recording indicating that GTPγS enhanced the Ca^{2+}-induced contraction in vascular smooth muscle. When 100 μM GTPγS was applied to the contraction induced by 3×10^{-7} M Ca^{2+}, the amplitude was enhanced, and on rinsing, the muscle was relaxed to the resting level. Fig. 1B summarizes the effect of 100 μM GTPγS on the contractions induced by various concentrations of Ca^{2+} (10^{-8}–3×10^{-5} M). GTPγS shifted the pCa-force relation curve to the left with a slight increase in the maximum amplitude of contraction induced by 3×10^{-5} M Ca^{2+}. In the presence of GTPγS, the pCa_{50} ($-\log[EC_{50}]$) value was 6.47 ± 0.07, a value which was significantly greater than the control value (6.20 ± 0.06, $n = 6$, $P < 0.01$). The Hill coefficients were 1.73 ± 0.10 in the control and 1.36 ± 0.11 in the presence of GTPγS ($P < 0.05$).

In cardiac muscle, as shown in Fig. 2A, the addition of 100 μM GTPγS had no effect on the Ca^{2+}-induced contraction. Fig. 2B shows the pCa-force relation curves indicating that neither Ca^{2+} sensitivity nor maximum Ca^{2+}-activated force was affected by 100 μM GTPγS, and there was no significant change in either the pCa_{50}

value (control: 6.15 ± 0.02, GTPγS: 6.23 ± 0.05, $n = 4$) or the Hill coefficient (control: 1.68 ± 0.21, GTPγS: 1.54 ± 0.19).

Like that of GTPγS, the addition of 1 μM PDB to the Ca^{2+} solution enhanced the 3×10^{-7} M Ca^{2+}-induced contraction in smooth muscle (from 26.2% to 81.0% of 3×10^{-5} M Ca^{2+}-induced contraction without PDB; Fig. 3A), but had no effect in cardiac muscle (from 29.0% to 27.4% of 3×10^{-5} M Ca^{2+}-induced contraction without PDB; Fig. 3B).

3.2. Effects of pimobendan on the Ca^{2+} sensitivity of cardiac and vascular smooth muscles

We also examined the effects of pimobendan on the Ca^{2+} sensitivity of cardiac and vascular smooth muscles. In contrast with GTPγS and PDB, pimobendan augmented the Ca^{2+}-induced contraction only in cardiac muscle. Fig. 4A shows a representative recording indicating that the addition of 50 μM pimobendan to the 3×10^{-7} M Ca^{2+} solution enhanced the Ca^{2+}-induced contraction in cardiac muscle. As shown in Fig. 4B, 50 μM pimobendan shifted the pCa-force relation curve to the left with a slight increase in the maximum amplitude of contraction induced by 3×10^{-5} M Ca^{2+}. In the presence of pimobendan, the pCa_{50} value was 5.62 ± 0.03, a value which was significantly greater than the control value (5.46 ± 0.04, $n = 6$, $P < 0.001$). The Hill coefficients were 2.07 ± 0.16 in the control and 1.80 ± 0.12 in the presence of pimobendan ($P > 0.05$). However, pimobendan had no effects on the Ca^{2+}-induced contraction and the pCa-force relation curve in vascular smooth muscle (Fig. 5A and B). The pCa_{50} values were 6.15 ± 0.02 and 6.23 ± 0.05, and the Hill coefficients were 1.68 ± 0.21 and 1.54 ± 0.19 in the absence and presence of pimobendan, respectively ($n = 4$, $P > 0.05$).

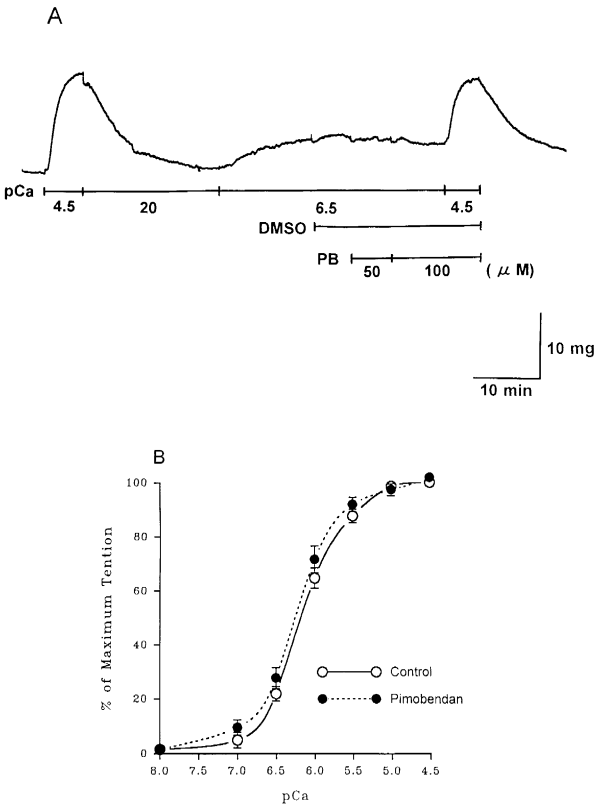

Fig. 5. Effect of 50 μM pimobendan on Ca^{2+}-induced contraction in β-escin-skinned vascular smooth muscle. A: Representative recording which shows that 50 μM pimobendan had no effect on 3×10^{-7} M Ca^{2+}-induced contraction. B: pCa-force relationship in the absence (open circles) and presence (filled circles) of 50 μM pimobendan in Ca^{2+} solutions. Points are means ± S.E.M. for 4 fibers in each Ca^{2+} solution. Tension is expressed as percentage of the maximal tension obtained at 3×10^{-5} M Ca^{2+} without pimobendan.

4. Discussion

The present study clearly demonstrated the existence of a different regulation of the Ca^{2+} sensitivity of the contractile proteins in cardiac and in vascular smooth muscles. These differences are as follows: (1) GTPγS and PDB enhanced myofilament Ca^{2+} sensitivity in β-escin-skinned preparations obtained from vascular smooth muscle, but not from cardiac muscle; in contrast, (2) pimobendan enhanced the Ca^{2+} sensitivity only in cardiac muscle.

Although it is evident that Ca^{2+} is the major physiological regulator of both cardiac and smooth muscle contraction, a growing body of recent evidence suggests that Ca^{2+} sensitivity of the contractile proteins, which is another important regulator of muscle contractions, can be modified by physiological mechanisms. Studies of intact muscles done with Ca^{2+} indicators have shown that endothelin-1 (Sakata et al., 1989; Wang et al., 1991) and an α_1-adrenoceptor agonist (Endoh and Blinks, 1988; Karaki et al., 1988) increase contractions with a much smaller increase in intracellular Ca^{2+} concentration in both cardiac and smooth muscles, indicating agonist-induced sensitization of contractility to Ca^{2+}. In vascular smooth muscle, several studies using α-toxin- or β-escin-permeabilized skinned preparations, which retain a receptor-coupled signal transduction system, have demonstrated that, together with GTP, an α_1-adrenoceptor agonist (Kitazawa et al., 1989; Satoh et al., 1994) and endothelin-1 (Nishimura et al., 1992) can markedly increase the contractile response to a given submaximal level of Ca^{2+}. These studies also showed that such potentiated contractions are mimicked with GTPγS and inhibited by GDPβS, indicating that the Ca^{2+}-sensitizing effect of these agonists is mediated by G-proteins. In addition, activation of protein kinase C has also been reported to enhance Ca^{2+} sensitivity in smooth muscle (Fujiwara et al., 1988; Itoh et al., 1994; Masuo et al., 1994).

Myosin light chain phosphorylation is one of the major regulatory mechanisms of contraction of smooth muscle,

and both G-protein- and protein kinase C-dependent enhancement of force is accompanied by an increase in myosin light chain phosphorylation (Kitazawa et al., 1991; Kubota et al., 1992; Masuo et al., 1994). α_1-Adrenoceptors and endothelin receptors couple the same G-protein (G_q) which stimulates phospholipase C. GTPγS activates several G-proteins including G_q. Phospholipase C increases production of diacylglycerol, which, in turn, activates protein kinase C. However, Hori et al. (1993) suggested that there are two pathways for Ca^{2+} sensitization in rat aorta: a protein kinase C-dependent pathway which is activated by phorbol esters, and a protein kinase C-independent pathway which is activated by receptor agonists. Furthermore, results of several recent studies indicated that protein kinase C is little involved in the GTPγS-induced Ca^{2+} sensitization in smooth muscle, but tyrosine kinase appears to play a role via activation of small G-protein, rho, in this process (Di Salvo et al., 1993; Fujita et al., 1995; Hirata et al., 1992; Itoh et al., 1994).

Endoh and Blinks (1988) and Wang et al. (1991), using the bioluminescent indicator, aequorin, demonstrated that endothelin-1 and an α_1-adrenoceptor agonist increase Ca^{2+} sensitivity in intact cardiac muscles. However, the results concerning effects of these agonists on Ca^{2+} sensitivity in skinned cardiac muscle preparations are controversial. Puceat et al. (1990) reported that single rat ventricular cells pretreated with an α_1-adrenoceptor agonist and then skinned had an increased Ca^{2+} sensitivity. Paik et al. (1994) demonstrated that ferret papillary muscle pretreated with endothelin-1 showed no change in Ca^{2+} sensitivity after the skinning process. We also found that these agonists failed to affect Ca^{2+} sensitivity in α-toxin-skinned rabbit cardiac muscle preparations (unpublished observation).

Although both α_1-adrenoceptors and endothelin receptors couple G-proteins and activate protein kinase C in cardiac muscle as well as in smooth muscle, it is still unclear whether activation of G-proteins and protein kinase C directly affects the Ca^{2+} sensitivity of the contractile proteins in cardiac muscle. There have been no reports regarding G-protein-mediated regulation of the Ca^{2+} sensitivity in cardiac muscle. The present data represent the first published evidence that activation of G-protein fails to change the Ca^{2+} sensitivity. GTPγS activates several G-proteins, including stimulatory G-protein (G_s). G_s stimulates protein kinase A through the adenylate cyclase-cAMP pathway. Protein kinase A phosphorylates troponin I, which decreases the Ca^{2+} sensitivity and may overcome other G-protein-mediated enhancement of Ca^{2+} sensitivity. However, application of protein kinase A inhibitor together with GTPγS did not change the Ca^{2+}-induced contraction (data not shown).

There are conflicting reports regarding the effect of protein kinase C activation on the Ca^{2+} sensitivity in cardiac muscle. Gwathmey and Hajjar (1990) showed that protein kinase C activation by phorbol ester, 12-deoxyphorbol 13-isobutyrate 20-acetate, decreased the Ca^{2+} sensitivity in skinned muscles from human myocardium. In contrast, Puceat et al. (1990) showed that protein kinase C activation before the skinning procedure increased the Ca^{2+} sensitivity in skinned rat single heart cells. In the present study, we found no effects of protein kinase C activation by PDB on the Ca^{2+} sensitivity in skinned preparations obtained from rabbit left ventricular muscles. The exact reason for these discrepancies is not clear but may be related to differences in experimental conditions and animal species.

Protein kinase C can phosphorylate specific intracellular substrates, including myosin light chain and the sarcolemmal bound Na^+-H^+ exchanger. Unlike that in smooth muscle, the physiological role of myosin light chain phosphorylation in the regulation of cardiac muscle contraction has not been unequivocally established. In contrast, alkalosis, induced by protein kinase C-mediated stimulation of the Na^+-H^+ exchanger, is a well-known sensitizer of myofilaments to Ca^{2+}. In skinned muscle preparations, the sarcolemma is disrupted so that the intracellular environment can be controlled. In the present study, the pH of the Ca^{2+} buffer solutions used for contraction and relaxation of the myofilaments was titrated to 7.0 and maintained via an effective buffering system. Thus, if the intracellular alkalinization mediated by the G-protein/protein kinase C pathway is a major mechanism of enhancement of Ca^{2+} sensitivity by agonists in intact cardiac muscle (Krämer et al., 1991), experiments using skinned muscle preparations would fail to show changes in the Ca^{2+} sensitivity induced by activation of G-protein or protein kinase C. Another explanation for the present results is that some soluble factors, which would be lost in the skinning procedure, may be part of the Ca^{2+}-sensitizing pathway in which G-protein and protein kinase C are involved. Further experiments are necessary to elucidate if there is direct G-protein- or protein kinase C-mediated regulation of Ca^{2+} sensitivity in cardiac muscle.

Pimobendan exhibits combined inotropic and peripheral vasodilator properties, which are attributable to selective inhibition of phosphodiesterase III (inotropic and vasodilator effects) and sensitization of cardiac myofilaments to intracellular Ca^{2+} (inotropic effect) (Fujino et al., 1988a,b; Lee et al., 1989). The Ca^{2+}-sensitizing action of pimobendan on the contractile protein is thought to be due to its increasing the Ca^{2+}-binding affinity of troponin C in the cardiac myofilament. Because smooth muscle lacks the troponin complex, pimobendan appears not to exert the Ca^{2+}-sensitizing action in smooth muscles (Fujimoto, 1994) as shown in the present results. Although phosphodiesterase III inhibition increases cAMP and may decrease Ca^{2+} sensitivity via activation of the cAMP/protein kinase A pathway, we did not find that pimobendan decreased the Ca^{2+} sensitivity in skinned preparations of vascular smooth muscle. This may have been because skinned preparations are devoid of a cell membrane and

the concentration of cAMP was too low after dilution with bath solution to affect the Ca^{2+} sensitivity.

It is now widely accepted that the main calcium regulatory mechanisms of muscle contraction are different in cardiac and in smooth muscles: an actin-linked mechanism in the former and a myosin-linked mechanism in the latter. However, little is known about differences in regulatory mechanisms of Ca^{2+} sensitivity between the two muscle types. The present results showed that activation of G-protein and protein kinase C, and pimobendan affect Ca^{2+} sensitivity differently in skinned preparations obtained from cardiac and from vascular smooth muscle. Thus, we conclude that, in addition to the common regulatory factors affecting the Ca^{2+} sensitivity of contractile proteins, such as intracellular pH and phosphorylation by protein kinase A, there are other means of regulation of Ca^{2+} sensitivity working differently in cardiac and in vascular smooth muscles.

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