Effect of Phorbol Ester, 12-Deoxyphorbol 13-Isobutylate (DPB), on Muscle Tension and Cytosolic Ca²⁺ in Rat Anococcygeus Muscle

Takeharu Kaneda, Kazumasa Shimizu, Shinjiro Nakajyo and Norimoto Urakawa

Division of Veterinary Pharmacology, Nippon Veterinary and Animal Science University, 7-1 Kyonan-cho 1-chome, Musashino, Tokyo 180, Japan

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Abstract—Effects of phorbol ester, 12-deoxyphorbol 13-isobutyrate (DPB), on muscle tension and cytosolic Ca²⁺ ([Ca²⁺]i) level was investigated in rat anococcygeus muscle in comparison with other smooth muscles. 1) DPB (10⁻⁶ M) induced a large contraction and an elevation of [Ca²⁺]i level in rat aorta and small and rhythmic changes in tension and [Ca²⁺]i level in guinea pig ileum. However, DPB did not change either of the parameters in rat anococcygeus muscle. 2) DPB caused tension development without changing the [Ca²⁺]i level elevated by high K⁺, ionomycin or ß-escin in the anococcygeus muscle. 3) In the ß-escin permeabilized muscles of guinea pig ileum and urinary bladder, rabbit mesenteric artery and rat anococcygeus muscle, DPB enhanced the Ca²⁺-developed tension. Moreover, the enhancement was inhibited by H-7 (3 x 10⁻⁵ M). 4) DPB did not cause muscle tension to develop in the muscle of rat aorta, guinea pig ileum and rat anococcygeus muscle, pretreated with phorbol 12-myristate 13-acetate for 24 hr. In conclusion, DPB showed different contractile effects on the aorta, ileum and anococcygeus muscle, respectively. The initiation of muscle tension by DPB probably requires [Ca²⁺]i and the DPB-induced enhancement may be due to a Ca²⁺ sensitization of contractile elements in the anococcygeus muscle. Therefore, the difference between the DPB-induced response of the anococcygeus muscle and those of the other muscles seems to be due to a different Ca²⁺ movement caused by DPB. Moreover, it is suggested that DPB develops muscle tension by increasing [Ca²⁺]i and enhances it through the mediation of protein kinase C in the anococcygeus muscle as well as the other smooth muscles.

Keywords: 12-Deoxyphorbol 13-isobutyrate, Anococcygeus muscle, Cytosolic Ca²⁺, Ca²⁺ sensitization, Protein kinase C

It was reported that phorbol esters activated protein kinase C (1) and induced a contraction in vascular smooth muscle (2–4) and tracheal one (5, 6). The esters induced a larger contraction than high K⁺ did for a given increase in cytosolic Ca²⁺ ([Ca²⁺]i) in vascular smooth muscle (7). In the intestinal smooth muscle, phorbol esters enhanced the contraction induced by high K⁺ (8–11) or histamine (12). In contrast, phorbol esters inhibited the contraction induced by histamine (12, 13) or carbachol (8, 9) in the intestinal smooth muscle. Although it has been reported that phorbol esters diversely changed contractile activities on various smooth muscles, it is difficult to find a paper that compared the effect of phorbol esters on various smooth muscles in detail.

On the other hand, it is known that rat anococcygeus muscle does not show a muscle tonus and spontaneous contractions but show contractions induced by adrenergic and cholinergic agonists (14–16). That is, the pharmacological characteristics of the anococcygeus muscle is quite different from that of rat aorta that is not affected by cholinergic agonists. To clarify the effects of phorbol esters in rat anococcygeus muscle in comparison with rat aorta and other smooth muscles, we investigated the effects of 12-deoxyphorbol 13-isobutyrate (DPB) on muscle tension and [Ca²⁺]i level in intact smooth muscles, on muscle tension development by Ca²⁺ in ß-escin-permeabilized muscles and on phorbol 12-myristate 13-acetate (TPA)-pretreated ones.

Materials and Methods

Muscle preparations
Male guinea pigs (Hartley strain, 300–400 g; Funabashi Farm, Funabashi) and rats of either sex (Wistar strain, 250–300 g; Imamichi Institute for Animal Reproduction, Ibaragi) were killed by a blow on
the head and bled to death. After exsanguination, the thorax and the abdomen were opened, then trachea, ileum, taenia coli and urinary bladder were removed from guinea pigs. The thoracic aorta and anococcygeus muscle were removed from male rats. The uterus was removed from female rats in the stage of the estrus cycle, which was determined by microscopic examination of a vaginal smear. Male New Zealand white rabbits (3-3.5 kg, Funabashi Farm) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and they were killed by rapidly injecting 5 ml of air into the ear vein. Then the abdomen was opened and the mesenteric artery was removed. The strips of aorta and mesenteric artery were prepared by removing the endothelium.

Muscle tension measurement

One end of each strip was bound to a glass holder and the other end was connected to a strain-gauge transducer (Nihon Kohden, Tokyo), with silk thread in an organ bath. The muscle tension was isometrically recorded. The physiological salt solution (PSS) employed was a modified Tyrode's solution of the following composition: 136.8 mM NaCl, 5.4 mM KCl, 1.5 mM CaCl₂, 1.0 mM MgCl₂, 11.9 mM NaHCO₃ and 5.5 mM glucose. Ca²⁺-free PSS was made by adding 1 mM ethyleneglycol bis-(β-aminoethylether)-N,N',N',N'-tetraacetic acid (EGTA) instead of 1.5 mM CaCl₂. These solutions were aerated with a 95% O₂, 5% CO₂ gas mixture at 37°C (pH 7.2). A hyperosmotically added 65 mM K⁺ (H-65K⁺) solution was made by increasing the KCl concentration in the PSS and an isosmotically added high K⁺ (Iso-K⁺) solution was made by substituting an equimolar amount of K⁺ for Na⁺ in the PSS.

Simultaneous measurement of muscle tension and [Ca²⁺], level

[Ca²⁺] level was measured simultaneously with muscle tension as described previously (17). Muscle strips were incubated with fura2/AM (5 μM) for 3 to 4 hr at room temperature. The noncytoxic detergent cremophor EL (0.02%) was also added 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 the PSS, and the other end of the muscle was attached to the transducer with silk thread. The muscle strip was kept horizontally in the organ bath. The muscle strip was alternately excited with light at 340 nm and 380 nm through the rotating filter wheel, and the 500 nm emission was measured with a fluorimeter (CAF-100; Japan Spectroscopic Co., Ltd., Tokyo).

Measurement of muscle tension in a permeabilized smooth muscle

A thin muscle strip (5.0 mm in length and 0.2 mm in width) was prepared from the isolated anococcygeus muscle, mesenteric artery, ileum or urinary bladder, respectively, as described above. We used guinea pig urinary bladder instead of rat aorta which belongs to type A (see Results), because it was extremely difficult to prepare a permeabilized muscle of the aorta with β-escin. The muscle strip was held horizontally in a 1-ml organ bath. One end of each strip was fixed and the other end was connected to a strain-gauge transducer. The muscle tension was isometrically recorded. The permeabilized muscle was made by treating the isolated tissue with a relaxing solution (RS) containing β-escin (4 × 10⁻⁵ M) for 20 to 30 min at room temperature. RS contained: 130 mM propanionic acid potassium salt, 4 mM MgCl₂, 4 mM ATP, 20 mM tris-maleate 2 mM creatine phosphate, 10 U/ml creatine phosphokinase and 2 mM EGTA. Each solution was adjusted to pH 6.8 at 24°C with KOH (0.1 N). Then, permeabilization was started after exchanging RS with β-escin for the normal RS. A RS containing drug was entirely exchange for the normal RS in the organ bath.

A TPA-pretreated muscle

The muscle strip was treated by the method of Rodriguez-Pena and Rozengurt (18), which was employed to down-regulate the C kinase activity. Muscle strips were incubated in the Dulbecco's modified Eagle medium (containing 10% fetal calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin) for 24 hr at 37°C in a CO₂ incubator in the presence of 3 × 10⁻⁶ M TPA. As a control, other strips were incubated in medium containing only 0.1% DMSO without TPA. These muscle contractions were isometrically recorded.

Chemicals

Chemicals used were 12-deoxyphorbol 13-isobutyrate (DPB), phorbol 12-myristate 13-acetate (TPA) (Funakoshi, Tokyo), 1-(5-isoquinolinesulfonyl)-2-methyl piperazine di-hydrochloride (H-7) (Seikagaku Kogyo, Tokyo), chelerythrine chloride, calphostin C, protein kinase C (19-31) (Cosmo Bio Co., Ltd., Tokyo), phenylephrine, carbachol, ionomycin, β-escin, etyleneglycol bis-(β-aminoethylether)-N,N',N',N'-tetraacetic acid (EGTA), verapamil (Sigma Chemical Co., St. Louis, MO, USA), fura2/AM (Dojindo Laboratories, Kumamoto) and cremophor EL (Nacalai Tesque, Kyoto).

Statistics

Values are expressed as means±S.E.M., and statistical analyses were performed by Student's t-test.
RESULTS

Effects of DPB on the muscle tension of various smooth muscles

DPB (10^{-6} M) induced a contraction that was the same size as or larger than a H-65K^{+} induced-contraction of guinea pig urinary bladder or rat aorta in normal PSS (2.5 mM Ca^{2+}). DPB induced a small contraction in guinea pig trachea, rabbit mesenteric artery and enhanced rhythmic contractions in guinea pig ileum or taenia coli. However, DPB did not affect the rat anococcygeus muscle and uterus (Fig. 1). Data on the rabbit mesenteric artery were not shown in the figure. A lower concentration (10^{-7} M) of DPB induced a contraction in the urinary bladder and aorta, but it had no effect on other muscles. The application of a higher concentration (3 \times 10^{-6} M) of DPB had no effect on the anococcygeus muscle and uterus. From these results, the responses to DPB in the various smooth muscles were classified into three groups: A) DPB induced a large contraction (guinea pig urinary bladder and rat aorta), B) DPB induced a small contraction (guinea pig trachea, ileum, taenia coli and rabbit mesenteric artery) and C) DPB had no effect (rat anococcygeus muscle and uterus).

Effect of DPB on muscle tension and [Ca^{2+}] level of the smooth muscles

Rat aorta as type A, guinea pig ileum as type B and rat anococcygeus muscle as type C were selected as representatives of the three groups, respectively; and the effects of DPB on these muscles were examined by simultaneous measurement of muscle tension and [Ca^{2+}] level using the fluorescent Ca^{2+} indicator fura2. DPB (10^{-6} M) developed a muscle tension and elevated [Ca^{2+}] level in the aorta. The DPB-induced contraction was greater than that by high K^{+}, while the increase in [Ca^{2+}] by DPB was less than that by high K^{+} (Fig. 2). Verapamil (10^{-5} M) partially inhibited the contraction to 79.9\pm4.0\% of the DPB-induced muscle tension and suppressed the elevated [Ca^{2+}] level to the resting one. A sequential addition of EGTA (4 mM) further decreased the [Ca^{2+}] level under the resting one to -20.8\pm5.0\% of DPB-induced

Fig. 1. Effects of DPB (10^{-6} M) on muscle tensions of various smooth muscles of guinea pig and rat. H-65K^{+}, Hyperosmotically added 65.4 mM KCl; DPB, 12-deoxyphorbol 13-isobutyrate. Traced from a result typical of 6 experiments.
[Ca$^{2+}$], while the contraction still remained. On the other hand, DPB induced small rhythmic changes in contraction and [Ca$^{2+}$], level in the ileum (Fig. 2), and the addition of verapamil abolished them. However, DPB did not affect the muscle tension and [Ca$^{2+}$], level in the anococcygeus muscle (Fig. 2). Data on verapamil or EGTA were not shown in the figure.

Effects of DPB on muscle tension and [Ca$^{2+}$], level in the anococcygeus muscle pretreated with high K$^+$, ionomycin or β-escin

The anococcygeus muscle, in which DPB did not affect...
the muscle tension and [Ca\(^{2+}\)] level, was pretreated with high K\(^{+}\), ionomycin or β-escin to elevate the [Ca\(^{2+}\)] level. After that, we examined the effects of DPB on the developed tension and elevated [Ca\(^{2+}\)] level in the muscle. Iso-38K\(^{+}\) developed muscle tension and elevated the [Ca\(^{2+}\)] level in the muscle. Although the addition of DPB (10\(^{-6}\) M) remarkably enhanced the developed tension by Iso-38K\(^{+}\), it did not affect the [Ca\(^{2+}\)] level.

Iso-38K\(^{-}\) solution containing 0.1 mM Ca\(^{2+}\) induced a muscle tension that was 4.2 ± 1.5% of the one induced by Iso-77K\(^{+}\) solution containing 2.5 mM Ca\(^{2+}\), and the elevation of [Ca\(^{2+}\)] was 42.6% of the Iso-77K\(^{+}\)-elevated one. The DPB-enhanced muscle tension in 0.1 mM Ca\(^{2+}\) was 20.7 ± 4.2% of the Iso-77K\(^{+}\)-induced one. An Iso-38K\(^{-}\) solution containing 2.5 mM Ca\(^{2+}\) induced a muscle tension that was 34.2 ± 6.5% of the one induced by Iso-77K\(^{+}\) solution containing 2.5 mM Ca\(^{2+}\) and elevated the [Ca\(^{2+}\)] level to 84.3 ± 2.1% of the Iso-77K\(^{+}\)-elevated one. The DPB-enhanced muscle tension in 2.5 mM Ca\(^{2+}\) was 84.1 ± 9.9% of the Iso-77K\(^{-}\)-induced one.

Ionomycin (10\(^{-6}\) M) induced an increase in [Ca\(^{2+}\)] level, but it had no effect on muscle tension. DPB developed a small tension but did not change the elevated [Ca\(^{2+}\)] level in the ionomycin-pretreated muscle. The addition of β-escin (4 × 10\(^{-5}\) M) to an intact muscle in the normal medium induced a slow increase in tension and a large and successive increase in [Ca\(^{2+}\)] level in the muscle within 10 min. DPB further increased the tension but not the [Ca\(^{2+}\)] level (Fig. 3). From these results, it seems likely that the elevation of [Ca\(^{2+}\)] level above the resting level is essential for DPB to initiate or enhance the muscle tension and DPB induces Ca\(^{2+}\) sensitization in the tension development of the anococcygeus muscle.

Effects of DPB on a Ca\(^{2+}\)-developed tension in permeabilized muscles

To clarify the Ca\(^{2+}\) sensitizing effect of DPB, Ca\(^{2+}\)-induced contractions in permeabilized smooth muscles were examined. Permeabilized muscles were prepared in guinea pig urinary bladder (type A); guinea pig ileum and

![Fig. 4. Effects of DPB (10\(^{-6}\) M) on Ca\(^{2+}\)-tension curves in permeabilized muscle of guinea pig urinary bladder (a), guinea pig ileum (b), rabbit mesenteric artery (c) and rat anococcygeus muscle (d). 100% represents the muscle tension induced by Ca\(^{2+}\) (urinary bladder, 10\(^{-5}\) M; ileum, 10\(^{-5}\) M; mesenteric artery, 3 × 10\(^{-5}\) M; anococcygeus muscle, 3 × 10\(^{-5}\) M). Control (○), 10\(^{-6}\) M DPB (●). Each point represents the mean of 5–6 experiments and S.E.M. is shown by a vertical bar. Significant difference from the control (*P < 0.05, **P < 0.01).](image-url)
Fig. 5. Effects of DPB (10⁻⁶ M) and H-7 (3×10⁻⁵ M) on the Ca²⁺-developed tension in β-escin permeabilized muscle of guinea pig urinary bladder (a), guinea pig ileum (b), rabbit mesenteric artery (c) or rat anococcygeus muscle (d). The upper trace shows a typical contraction. Lower one: 100% represents the muscle tension induced by a single application of 10⁻⁶ M Ca²⁺. Each point is the mean value derived from 5—6 experiments and vertical bars represent the S.E.M. Significant difference between the group (*P<0.05, **P<0.01).
rabbit mesenteric artery (type B); and rat anococcygeus muscle (type C). Ca\(^{2+}\) was added cumulatively in the absence or presence of DPB. The application of Ca\(^{2+}\) (10\(^{-7}\)–3 \times 10\(^{-5}\) M) developed tension dose-dependently in all the muscles; The maximum response was elicited by 10\(^{-5}\) M Ca\(^{2+}\) in the urinary bladder and ileum and by 3 \times 10\(^{-5}\) M Ca\(^{2+}\) in the mesenteric artery and anococcygeus muscle (Fig. 4). DPB (10\(^{-6}\) M) shifted the Ca\(^{2+}\)-tension curves to the left along the Ca\(^{2+}\) concentration axis, indicating an increase in the Ca\(^{2+}\) sensitivity of tension development (Fig. 4). The enhancement by DPB in the mesenteric artery or anococcygeus muscle was found at Ca\(^{2+}\) concentrations from 3 \times 10\(^{-7}\)–3 \times 10\(^{-6}\) M, but that in the ileum at Ca\(^{2+}\) concentrations from 3 \times 10\(^{-7}\) and 10\(^{-6}\) M. The urinary bladder showed the enhancement only at 3 \times 10\(^{-7}\) M. It can be said that the enhancement by DPB in the mesenteric artery or anococcygeus muscle was revealed in a wider range of Ca\(^{2+}\) concentration than that in the ileum, especially that in the urinary bladder. Moreover, the DPB-enhanced tension was inhibited by H-7, an inhibitor of protein kinase C activity, in all the muscles (Fig. 5). In contrast, 3 \times 10\(^{-5}\) M H-7 did not inhibit the muscle tension developed by 10\(^{-6}\) M Ca\(^{2+}\) in the muscles. Although other inhibitors of protein kinase C activity, chelerythrine, calphostine C and PKC(19–31), inhibit the DPB-enhanced tension in rabbit mesenteric artery, they did not inhibit the DPB-enhanced tension in guinea pig urinary bladder and ileum and rat anococcygeus muscle. Furthermore, chelerythrine, calphostine C and PKC(19–31) remarkably enhanced the Ca\(^{2+}\) (10\(^{-6}\) M)-developed tension.

Effects of TPA-pretreatment on the DPB-induced enhancement

Muscle strips of the aorta, ileum and anococcygeus muscle were incubated in Dulbecco’s modified Eagle medium with TPA (3 \times 10\(^{-6}\) M) for 24 hr at 37\(^\circ\)C, which has been reported as conditions to cause the down-regulation of protein kinase C. To confirm the contractility of the TPA-pretreated muscle, Iso-77K\(^+\) was added before the experiment. After that, the addition of DPB (10\(^{-6}\) M) did not induce a contraction in the TPA-pretreated aorta, while DPB caused a large contraction in the aorta without the TPA-pretreatment (Fig. 6). The muscle tension developed by Iso-77K\(^+\) in the TPA-pretreated aorta was 0.25±0.05 g and that in the TPA-untreated one was 0.42±0.09 g, and there was no difference in the Iso-77K\(^+\) induced contractions between a TPA-pretreated and TPA-untreated aorta. Although DPB did not affect the Iso-38K\(^-\)-induced contraction in the TPA-pretreated ileum, DPB enhanced the Iso-38K\(^-\)-induced one in the ileum without the TPA-pretreatment. The Iso-77K\(^+\) developed muscle tension in the TPA-pretreated ileum was
DISCUSSION

There have been many reports on the effects of phorbol esters on contractility in smooth muscle. Phorbol esters induced a contraction in vascular smooth muscles (2–4, 7) or tracheal smooth muscles (5, 6) and enhanced the contractions induced by high K+ (8–11) or by histamine (12) in intestinal smooth muscle. On the other hand, phorbol esters inhibited the contractions induced by histamine (12, 13) or carbachol (8, 9) in intestinal smooth muscles. Thus, phorbol esters showed diverse responses in various kinds of smooth muscles or in muscles pretreated with various agonists. In this study, the responses to DPB in the smooth muscles of guinea pig, rat and rabbit were classified into three groups: A) DPB induced a large contraction (guinea pig urinary bladder and rat aorta), B) DPB induced a small contraction (guinea pig trachea, ileum taenia coli and rabbit mesenteric artery) and C) DPB had no effect (rat anococcygeus muscle and uterus).

DPB (10^{-6} M) induced an increase of [Ca^{2+}], and a large contraction in the aorta, being a type A muscle. In the ileum, as type B, DPB elicited a small rhythmic contraction and only a small change in [Ca^{2+}] level. On the other hand, DPB had no effect on either of the parameters in a type C muscle like anococcygeus muscle. Since the increases of [Ca^{2+}] were completely inhibited by an application of verapamil after the treatment with DPB in the aorta and ileum, it seems likely that DPB stimulates a verapamil-sensitive Ca^{2+} channel (L-type voltage-dependent Ca^{2+} channel) and enters Ca^{2+} into cells of the two muscles to induce a contraction. This assumption is consistent with those in some papers (19–21). Although verapamil seems to block the Ca^{2+} channel stimulated by DPB in the aorta, verapamil partially inhibited the DPB-induced contraction. The result confirms the observation that DPB increased the Ca^{2+} sensitivity of contractile elements of rat aorta (22). Thus, there is a possibility that the different DPB-induced responses are implicated in different mechanisms of Ca^{2+} influx and Ca^{2+} sensitivity in the three muscles.

The reason why DPB has no effect in the anococcygeus muscle is probably because DPB does not elevate the [Ca^{2+}] level above the resting one, which is caused by Ca^{2+} influx to the cytoplasm and/or Ca^{2+} release from the sarcoplasmic reticulum. Then, we examined if DPB develops muscle tension under an elevated [Ca^{2+}] level, i.e., the pretreatment with high K+, ionomycin or β-escin. While DPB did not show any effect on the intact muscle, DPB initiated or enhanced the muscle tension in the pretreated muscle that had an increased [Ca^{2+}] level. Furthermore, the magnitude of the enhancement was dependent on the degree of increase in the [Ca^{2+}] level under the high K+-pretreated muscle. The data showed that the DPB-induced enhancement was exhibited only at a certain [Ca^{2+}] level over the resting one in the anococcygeus muscle.

The effect of DPB on the relationship between tension development and [Ca^{2+}] was examined in the β-escin-permeabilized muscles, the urinary bladder (type A), the mesenteric artery and the ileum (type B) and the anococcygeus muscle (type C). Ca^{2+} developed a muscle tension dose-dependently, and post-treatment with DPB enhanced the Ca^{2+}-developed tension in these permeabilized muscles. While the post-treatment with DPB shifted the Ca^{2+}-tension curves to the left in these muscles, the degrees of enhancement of tension development were diverse in these muscles. These results suggest that [Ca^{2+}] is necessary for the DPB-induced enhancement in the permeabilized anococcygeus muscle as well as the other muscle preparations and that DPB enhanced Ca^{2+} sensitization of tension development in the lower Ca^{2+} concentration (3 \times 10^{-7}–3 \times 10^{-6} M).

It has been suggested that phorbol esters have dual effects, increasing [Ca^{2+}], and Ca^{2+} sensitivity of contractile elements (22), and that [Ca^{2+}] entered, by the mediation of phorbol esters, into the smooth muscle cell activates myosin light chain (MLC) kinase and increases MLC phosphorylation, which may be a part of the contractile mechanism induced by phorbol ester (23). On the other hand, it was proposed that the increase in Ca^{2+} sensitivity induced by phorbol esters was not only due to an increase in Ca^{2+} sensitivity of the phosphorylation of MLC but also due to the activation of mechanism that was not dependent on the increase in MLC phosphorylation (22). The former may possibly have resulted from an altered balance between the activity of MLC kinase and MLC phosphatase (24–26). The latter may be partially due to a phosphorylation of calponin, actin-binding pro-
tein, by protein kinase C, which reduces the inhibitory regulation of calponin on the interaction of myosin and actin (27).

Protein kinase C is activated by phorbol esters or diacylglycerol that is endogenously generated by phosphatidyl-inositol turnover, and the enzyme requires Ca\(^{2+}\) and phospholipid. An activation of protein kinase C by phorbol esters in smooth muscle cells is known to be accompanied by a translocation of the enzyme from the cytosol to the membrane fraction (28, 29). On the other hand, it was shown that in TPA-incubated muscle cells, the activity of protein kinase C translocated and then decreased (30). This phenomenon has been called a down-regulation, and reported in smooth muscles (9, 11, 31). In the next series of experiments, we attempted to confirm that the activation of protein kinase C was responsible for the DPB-developed tension and enhanced Ca\(^{2+}\) sensitivity in the anococcygeus muscle; The relationship of DPB action to protein kinase C was examined by using H-7, an inhibitor of protein kinase C (32), and using preparations pretreated with TPA in which protein kinase C seems to be down-regulated. DPB elicited enhancements of Ca\(^{2+}\)-development tension in these permeabilized muscles, which were significantly inhibited by H-7. Moreover, DPB (10\(^{-6}\) M) did not develop tension in the three muscles that were incubated with TPA, but did in the untreated muscles. From these results, it is assumed that DPB activates the contractile mechanism and increases the Ca\(^{2+}\) sensitivity of the contractile elements, which are mediated by protein kinase C in the anococcygeus muscle like it is in other smooth muscles. Because the specificity of H-7 is not so high for inhibition of protein kinase C (33–35), it remains a possibility that H-7 inhibits other kinases. However, other protein kinase C inhibitors, chelerythrine, calphostine C and PKC\((19-31)\), inhibited the DPB-enhanced tension in only rabbit mesenteric artery and did not inhibit the DPB-enhanced tension in the other three muscles.

It has been reported that phorbol esters activate protein kinase C and induce an increase in [Ca\(^{2+}\)], in the aorta (7, 20, 36). However, DPB did not increase [Ca\(^{2+}\)], accompanying tension development in the anococcygeus muscle in the present study. Protein kinase C that is directly activated by phorbol ester (1) is classified into several isozymes according to the type of the original genes (37–39), and each isozone assembly is involved in a different response. Therefore, the different effect of phorbol esters may come from the difference in isozone in each type of smooth muscle.

In conclusion, DPB elicited contractile responses in various kinds of smooth muscles, but did not in rat anococcygeus muscle. However, DPB enhanced the developed tension in the anococcygeus muscle of which [Ca\(^{2+}\)] level had been elevated by some factors above its resting one. In the anococcygeus muscles, it is suggested that DPB requires an increase in [Ca\(^{2+}\)], and Ca\(^{2+}\)-sensitization of the contractile elements for an initiation and enhancement of muscle tension as in other smooth muscles. Accordingly, it seems likely that the difference of the tension development by DPB between the anococcygeus muscle and other smooth muscles is due to a difference in the Ca\(^{2+}\) movement of cells after an application of DPB to these muscles. Furthermore, the action of DPB is suggested to be involved in the contractile mechanism mediated by protein kinase C in the anococcygeus muscle as well as in the other smooth muscles.

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