Increased Inhibitory Effect of Phorbol Ester on Cytosolic Ca^{2+} Level and Contraction in Rat Myometrium after Gestation

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ABSTRACT—Activation of voltage-dependent Ca^{2+} channels by high K^{+} (40 mM) increased the cytosolic Ca^{2+} level ($[\text{Ca}^{2+}]_i$) (estimated by fura-PE3 fluorescence ratio) and force in myometrium isolated from pregnant (21 days after gestation) and non-pregnant (estrus) rats. 12-Deoxyphorbol 13-isobutyrate (DPB, 1 mM) decreased the high K^{+} -stimulated $[\text{Ca}^{2+}]_i$ and force in a concentration-dependent manner. The inhibitory effect was stronger in the pregnant myometrium than in the non-pregnant myometrium. In the pregnant myometrium, the increase in Ca^{2+} permeability by ionomycin (1 μM) greatly increased $[\text{Ca}^{2+}]_i$ and force, which were only partially inhibited by verapamil (10 μM). DPB (1 μM) inhibited the verapamil-insensitive component of the increases in $[\text{Ca}^{2+}]_i$ and muscle tension. Oxytocin (100 nM) and thapsigargin (1 μM) also induced a verapamil-insensitive increase in $[\text{Ca}^{2+}]_i$ and force, and DPB (1 μM) inhibited these increments. Ca^{2+} sensitivity of contractile elements, estimated from the relationships between Ca^{2+} and muscle force in intact and α -toxin permeabilized muscle, was not significantly changed by DPB (1 μM). In summary, DPB inhibits the increase in $[\text{Ca}^{2+}]_i$ more strongly in myometrium isolated from pregnant rats than that from non-pregnant rats without any change in the $[\text{Ca}^{2+}]_i$ /tension relationship. Since DPB decreased $[\text{Ca}^{2+}]_i$ -rise induced by three different mechanisms, DPB may activate Ca^{2+} extrusion, rather than to inhibit a specific influx pathway, to decrease $[\text{Ca}^{2+}]_i$.

Keywords: Uterine smooth muscle, Phorbol ester, Relaxation, Cytosolic Ca^{2+} level, Pregnancy

It is well-known that uterotonic agonists, such as oxytocin, carbachol and prostaglandins, increase intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and contraction (1–3). These receptor agonists stimulate phosphoinositide turnover in the uterine smooth muscle (4, 5). In various types of smooth muscle, inositol 1,4,5-trisphosphate, one of the phosphoinositide hydrolysis products, releases Ca^{2+} from the intracellular Ca^{2+} store and induces transient contraction (6). Another phosphoinositide product, diacylglycerol, stimulates protein kinase C that phosphorylates various proteins involved in cellular function (7). It has been reported that protein kinase C activation with phorbol ester inhibits smooth muscle contraction by inhibiting Ca^{2+} channel activity or inositol phosphate production (8, 9), while it induces contraction by activa-

tion of contractile proteins (10–12). In cultured cells from rat portal vein, phorbol esters increase Ca^{2+} channel activity (13). In myometrium, it has been reported that phorbol ester induces stimulatory and inhibitory effects on contraction (14–16). However, few attempts have been made to compare the role of protein kinase C before and after pregnancy. In the present studies, we compared the effect of protein kinase C activation by phorbol esters on $[\text{Ca}^{2+}]_i$ and muscle contraction in isolated uterine smooth muscle from non-pregnant and pregnant rats.

MATERIALS AND METHODS

Female Wistar rats (200–250 g; Shiraishi Laboratory Animals, Tokyo) were used for this study. Vaginal smears were taken, and the pro-oestrus rats were mated with male rats overnight. The day of gestation when sperm were observed in the vaginal lavage was defined as day 0 of gestation. The normal length of gestation in the colony

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of rats was 21 days; and in most cases, delivery was observed in the afternoon at day 21. Uteri of pregnant rats were removed at day 21, 9:00–12:00 after the gestation. Myometrium, isolated from rats in oestrus, was used as the non-pregnant myometrium. Rats were stunned and bled, and a strip of longitudinal uterine muscle (1–2 mm wide and 7–8 mm length) was isolated from the middle of each horn.

Each strip was attached to a holder under a resting tension of 10 mN. After equilibration for 20 min in a physiological salt solution, each strip was repeatedly exposed to 40 mM KCl solution until responses became stable. The physiological salt solution contained: 136.9 mM NaCl, 5.4 mM KCl, 1.5 mM CaCl_2 , 1.0 mM MgCl_2 , 23.8 mM NaHCO_3 , 5.5 mM glucose and 0.01 mM ethylenediaminetetraacetic acid (EDTA). The high K^+ solution was prepared by replacing NaCl with equimolar KCl. These solutions were saturated with a 95% O_2 and 5% CO_2 mixture at 37°C and pH 7.4. Muscle contraction was recorded isometrically with a force-displacement transducer (Model TB611T; Nihon Kohden, Tokyo) connected to a Model 3134 strain amplifier and Model 3056 ink-writing recorder (Yokogawa, Tokyo). We employed the contraction induced by 40 mM K^+ as a reference response, because this concentration of K^+ induced maximum contraction in the myometrium.

$[\text{Ca}^{2+}]_i$ was measured as reported by Ozaki et al. (17) and Sato et al. (18) with a fluorescent Ca^{2+} indicator, fura-PE3. The use of fura-PE3 enabled us to measure $[\text{Ca}^{2+}]_i$ for several hours without a significant decline of fluorescence (19). Muscle strips were treated with the acetoxymethyl ester of fura-PE3 (fura-PE3/AM, 5 μM) for 4–5 hr at room temperature. A non-cytotoxic detergent, cremophor EL (0.02%), was added to increase the solubility of fura-PE3/AM. After loading, the muscle strip was washed with physiological salt solution at 37°C for 20 min to remove uncleaved fura-PE3/AM and was held horizontally in a temperature-controlled, 7-ml organ bath. One end of the muscle strip was connected to a force-displacement transducer to monitor the muscle contraction. The muscle strip was illuminated alternately (48 Hz) at two excitation wavelengths (340 and 380 nm). The intensity of 500 nm fluorescence (F340 and F380) was measured by using a fluorimeter (CAF100; Jasco, Tokyo). The ratio of F340 to F380 (R340/380) was calculated as an indicator of $[\text{Ca}^{2+}]_i$. The absolute Ca^{2+} concentration was not calculated in this experiment because the dissociation constant of the fluorescent indicator for Ca^{2+} in the cytosol may be different from that obtained in vitro (20). Therefore, the ratio obtained in resting and each stimulant-stimulated muscle was taken as 0 and 100%, respectively.

Permeabilized muscle strips were prepared by treat-

ment of *Staphylococcal* α -toxin as described previously (21, 22). Small muscle strips, 0.1–0.2 mm in diameter and 1–2 mm in length, were made. Permeabilization was accomplished by incubating the muscle strips for 30 min with 80 $\mu\text{g}/\text{ml}$ of α -toxin in a relaxing solution containing: 130.0 mM potassium propionate, 4.0 mM MgCl_2 , 4.0 mM Na_2ATP , 20.0 mM tris-maleate, 2.0 mM creatine phosphate, 10 unit/ml creatine phosphokinase, 1 mM carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazide, 1 mM E-64 and 2 mM EGTA (pH 6.8) and the indicated concentrations of free Ca^{2+} . All of the solutions were treated with 10 μM ionomycin to remove the effects of releasing Ca^{2+} from the sarcoplasmic reticulum. The apparent binding constant of EGTA for Ca^{2+} was considered to be 10^6 M^{-1} at pH 6.8. Experiments were prepared isometrically at room temperature (22–24°C).

Chemicals used were oxytocin, thapsigargin, *p*-trifluoromethoxy-phenylhydrazide, creatine phosphokinase, creatine phosphate (Sigma Chemicals, St. Louis, MO, USA), EDTA, EGTA (Dojindo Laboratories, Kumamoto), fura-PE3/AM (Tafelabs, Austin, TX, USA), 12-deoxyphorbol 13-isobutyrate (DPB), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Funakoshi, Tokyo), clemophor EL (Nacalai Tesque, Kyoto), E-64 (Peptide Institute, Osaka) and ionomycin (Hoechst Japan, Tokyo). *Staphylococcus aureus* α -toxin was donated by Dr. Iwao Kato (Chiba University, Chiba). E-64 and ionomycin were dissolved in ethanol. Thapsigargin, DPB, TPA and fura-PE3/AM were dissolved in DMSO.

Results of the experiments are expressed as means $\pm$ S.E.M. Student's *t*-test was used for statistical analysis of the results, and $P < 0.05$ was considered to be significant.

RESULTS

The effects of DPB on high K^+ -induced increases in $[\text{Ca}^{2+}]_i$ and contraction

Figure 1A shows typical traces of the effect of 1 μM DPB on $[\text{Ca}^{2+}]_i$ and tension in non-pregnant myometrium stimulated with 40 mM KCl. DPB (1 μM), added during the 40 mM KCl-induced sustained increases in $[\text{Ca}^{2+}]_i$ and muscle tension, transiently increased and then decreased the $[\text{Ca}^{2+}]_i$ and muscle tension to approximately 70% and 50%, respectively, of the levels before the addition of DPB. In pregnant myometrium stimulated with 40 mM KCl, DPB (1 μM) inhibited $[\text{Ca}^{2+}]_i$ to approximately 30% and muscle tension to the baseline level (Fig. 1B). DPB also induced small and transient increase in $[\text{Ca}^{2+}]_i$ and muscle tension. The magnitude of transient contractions induced by 1 μM DPB was $25.2 \pm 1.9\%$ ($n=5$) in non-pregnant myometrium and $4.2 \pm 1.3\%$ ($n=7$) in pregnant myometrium.

Fig. 1. Effects of DPB on $[Ca^{2+}]_i$ (indicated by R340/380) and muscle tension in myometria isolated from non-pregnant and pregnant rat stimulated with 40 mM KCl. A: Typical recording of the effects of 1 μ M DPB in non-pregnant myometrium. B: Typical recording of the effects of 1 μ M DPB in pregnant myometrium. C and D: Concentration-response relationships for the inhibitory effect of DPB (10 nM–10 μ M) on $[Ca^{2+}]_i$ and muscle tension, respectively ($n=4$ each) (○, non-pregnant; ●, pregnant)

When various concentrations of DPB (10 nM–10 μ M) were applied after the high K^+ -induced responses reached a plateau, both $[Ca^{2+}]_i$ and muscle tension decreased in a concentration-dependent manner. The inhibitory effects of DPB on $[Ca^{2+}]_i$ and tension induced by high K^+ were greater in the pregnant myometrium than in the non-pregnant myometrium (Fig. 1: C and D). The relationship between $[Ca^{2+}]_i$ and muscle tension in the absence and presence of DPB in the non-pregnant and pregnant myometria are shown in Fig. 3, A and B, respectively. It is shown that DPB decreased $[Ca^{2+}]_i$ and contraction without changing the $[Ca^{2+}]_i$ /tension relationship in non-pregnant and pregnant myometria.

Another phorbol ester, 12-*O*-tetradecanoyl-phorbol-13-acetate (1 μ M), also inhibited the high K^+ -induced increases in $[Ca^{2+}]_i$ and contraction in non-pregnant and pregnant myometria in a similar manner to DPB (data not shown).

The effects of verapamil on high K^+ -induced increase in $[Ca^{2+}]_i$ and contraction

To determine if the effect of another relaxant is also affected by pregnancy, we examined the effects of a Ca^{2+} channel blocker, verapamil. Different concentrations of verapamil (1 nM–10 μ M) were added during the sustained contraction induced by 40 mM KCl, and changes in $[Ca^{2+}]_i$ and contraction were measured. Verapamil decreased both $[Ca^{2+}]_i$ and muscle tension in a concentration-dependent manner (Fig. 2). Results are plotted as the $[Ca^{2+}]_i$ /tension relationships in Fig. 3, A and B. These figures indicate that verapamil decreases both $[Ca^{2+}]_i$ and contraction in the high K^+ -stimulated muscle and, as compared with the effects of DPB, the inhibitory effects were not greatly modified by pregnancy. These figures also indicate that the $[Ca^{2+}]_i$ /tension relationships in the presence of DPB and verapamil are similar because DPB and verapamil decreased $[Ca^{2+}]_i$ and tension in a similar manner.

Fig. 2. Concentration-response relationships for the inhibitory effect of verapamil (1 nM–10 μ M) on $[Ca^{2+}]_i$ (A) and muscle tension (B) in non-pregnant ($\circ$ or $\square$) and pregnant ($\bullet$ or $\blacksquare$) myometria stimulated with 40 mM KCl ($n=4$ each).

The effects of DPB on ionomycin-, oxytocin- and thapsigargin-induced increases in $[Ca^{2+}]_i$ and contraction

To further characterize the inhibitory effects of DPB, we examined the effects of DPB on the $[Ca^{2+}]_i$ and muscle tension stimulated by a Ca^{2+} ionophore, ionomycin, in the pregnant myometrium. Ionomycin (1 μ M) increased $[Ca^{2+}]_i$ and muscle tension in the pregnant myometrium, and verapamil (10 μ M) inhibited the stimulated $[Ca^{2+}]_i$ and muscle tension to $65.4 \pm 5.6\%$ and $73.3 \pm 6.8\%$ ($n=4$) (% of control response without verapamil), respectively (Fig. 4A). The same concentration of verapamil almost completely inhibited the high K^+ -induced increase in $[Ca^{2+}]_i$ and tension in pregnant myometrium (see Fig. 2). Additional application of DPB (1 μ M) decreased $[Ca^{2+}]_i$ and muscle tension to $42.8 \pm 5.3\%$ and $38.8 \pm 9.2\%$ ($n=4$) (% of control response without DPB and verapamil), respectively. La^{3+} (30 μ M) further decreased $[Ca^{2+}]_i$ below the resting level and muscle tension to the resting level ($n=2$).

Oxytocin (100 nM) induced sustained increases in $[Ca^{2+}]_i$ and tension in the pregnant myometrium (Fig. 4B). Application of verapamil (10 μ M) only partially

A. Non-pregnant

B. Pregnant

Fig. 3. $[Ca^{2+}]_i$ /tension relationship in high K^+ stimulated myometria at various concentrations of verapamil or DPB in non-pregnant (A) and pregnant (B) myometria. After the observation of the control response to 40 mM K^+ , DPB ($\bullet$) (a, 0; b, 10 nM; c, 100 nM; d, 1 μ M; e, 10 μ M) or verapamil ($\circ$) (a', 0; b', 10 nM; c', 100 nM; d', 1 μ M; e', 10 μ M) was cumulatively added ($n=4$).

inhibited $[Ca^{2+}]_i$ and muscle tension. The verapamil-resistant components of $[Ca^{2+}]_i$ and tension were inhibited by 1 μ M DPB. La^{3+} (30 μ M) inhibited the remaining $[Ca^{2+}]_i$ to the resting level ($n=3$).

Thapsigargin (1 μ M), an inhibitor of the Ca^{2+} pump in sarcoplasmic reticulum, rapidly increased $[Ca^{2+}]_i$ with a slow increase in muscle tension (Fig. 4C). Verapamil (10 μ M) only partially inhibited the thapsigargin-induced increases in $[Ca^{2+}]_i$ and muscle tension. The verapamil-resistant portion of $[Ca^{2+}]_i$ and muscle tension were inhibited by 1 μ M DPB ($n=4$).

As shown in Fig. 5, DPB ($1 \mu\text{M}$ DPB) changed neither $[\text{Ca}^{2+}]_i$ nor muscle tension in the resting myometria isolated from pregnant and also from non-pregnant rats.

We also confirmed that DPB (100 nM and $1 \mu\text{M}$) did not change muscle tension in the non-pregnant and pregnant rat myometria.

Fig. 4. Effects of DPB on $[\text{Ca}^{2+}]_i$ and muscle tension in myometrium isolated from pregnant rat stimulated by ionomycin (A), oxytocin (B) or thapsigargin (C). One micromolar ionomycin, 100 nM oxytocin or $1 \mu\text{M}$ thapsigargin was added after the observation of the control response to 40 mM K^+ . After the $[\text{Ca}^{2+}]_i$ and tension reached maximum, $10 \mu\text{M}$ verapamil, $1 \mu\text{M}$ DPB and $30 \mu\text{M}$ La^{3+} (except C) were sequentially added.

Fig. 5. Effects of DPB on resting $[Ca^{2+}]_i$ and muscle tension in myometrium isolated from pregnant rat. After the observation of the control response to 40 mM K^+ , 1 μ M DPB and 4 mM EGTA were sequentially added ($n=4$).

The effects of DPB on permeabilized muscle

In the α -toxin permeabilized myometrium isolated from pregnant rat, addition of Ca^{2+} (10 μ M) induced sustained contractions that reached the maximum in 10 min. Washing the muscle strips with relaxing solution ($Ca^{2+} < 0.01 \mu$ M) decreased muscle tension to the resting level. Ca^{2+} (0.1–10 μ M) was then cumulatively added in the absence or presence of 1 μ M DPB (pretreated for 30 min). DPB did not alter the contractions induced by

Ca^{2+} (Fig. 6).

DISCUSSION

In the myometrium isolated from rat uterus, it has been reported that phorbol ester inhibits contraction induced by several agonists such as oxytocin and high K^+ (14–16). We confirmed this and further revealed that phorbol esters more strongly inhibited the uterine smooth

Fig. 6. Ca^{2+} /tension relationship in the absence or presence of 1 μ M DPB in pregnant myometrium permeabilized with α -toxin. After the observation of the control response to 10 μ M Ca^{2+} , 0.1–10 μ M Ca^{2+} was cumulatively added to induce graded contractions. ($n=5-7$). ○, control; ●, DPB.

muscle contraction in the pregnant myometrium (21 day after gestation) than that in non-pregnant myometrium. We also found that phorbol esters slightly increased the high K^+ induced contraction before inducing relaxation, confirming the previous findings (15). In contrast to the relaxing action, the contractile effect of phorbol ester was greater in the non-pregnant myometrium than in the pregnant myometrium. The initial contraction produced by DPB was associated with the increase in $[Ca^{2+}]_i$. This contraction may be attributable to the enhancement of Ca^{2+} influx through high K^+ -activated Ca^{2+} channels, because it has been demonstrated that the activation of protein kinase C by phorbol ester increases the Ca^{2+} current in the myometrial cells isolated from pregnant rat (23).

The phorbol ester-induced relaxation was accompanied by a decrease in $[Ca^{2+}]_i$, and the amount of relaxation was closely associated with the decrease in $[Ca^{2+}]_i$ in the muscle stimulated by high K^+ . Verapamil showed similar effects to DPB. Although DPB and verapamil decreased $[Ca^{2+}]_i$ by approximately 70%, muscle tension decreased to the resting level. Similar results have been obtained in which verapamil decreased the platelet activating factor-induced increase in $[Ca^{2+}]_i$ to approximately 50% and decreased the contraction to or near the resting level (19). These results suggest that the threshold $[Ca^{2+}]_i$ for contraction in the rat myometrium is much higher than resting $[Ca^{2+}]_i$.

In the pregnant rat myometrium, oxytocin increased $[Ca^{2+}]_i$, part of which was insensitive to verapamil. This increase may be due to the activation of non-L type Ca^{2+} channels and/or inhibition of Ca^{2+} extrusion. DPB inhibited the verapamil-insensitive increase in $[Ca^{2+}]_i$. Thapsigargin also increased $[Ca^{2+}]_i$ and muscle tension, which may be attributable to the increase in Ca^{2+} influx through capacitative Ca^{2+} channels (24). The thapsigargin-induced increase in $[Ca^{2+}]_i$ was only partially inhibited by verapamil. DPB inhibited the verapamil-insensitive increase in $[Ca^{2+}]_i$. Furthermore, DPB decreased the increment of $[Ca^{2+}]_i$ mediated by the stimulation of passive Ca^{2+} influx due to the Ca^{2+} ionophore ionomycin. Since it has been shown that activation of protein kinase C by phorbol ester increases the activity of the membrane Ca^{2+} pump in vascular smooth muscle cells (25), this mechanism may at least partly be responsible for the decrease in $[Ca^{2+}]_i$ in the rat myometrium. Ca^{2+} pump in sarcoplasmic reticulum may not be involved in the DPB-induced decrease in $[Ca^{2+}]_i$, since DPB decreased $[Ca^{2+}]_i$ in the presence of an inhibitor of the Ca^{2+} pump in sarcoplasmic reticulum, thapsigargin.

In various types of smooth muscle, activation of protein kinase C by phorbol esters increases the Ca^{2+} sensitivity of contractile elements (11, 18, 22). In the rat myo-

metrium permeabilized with saponin, on the other hand, phorbol ester has dual actions; phorbol 12,13-dibutyrate enhanced the Ca^{2+} -induced contraction at a lower concentration (0.1 μM) but inhibited it at a higher concentration (1 μM) (15). However, we could not observe any effects of 1 μM DPB on the Ca^{2+} -induced contraction in the α -toxin permeabilized muscle. We also compared the relationship between $[Ca^{2+}]_i$ and muscle tension in high K^+ -stimulated myometria. However, there was no significant difference between the relationships in the presence of verapamil and that in the presence of DPB. It has been reported that endothelin-1, which greatly increases the Ca^{2+} sensitivity of contractile elements in vascular smooth muscle (26), did not alter the $[Ca^{2+}]_i$ /tension relationship in the rat myometrium (27). These results suggest that a change in Ca^{2+} -sensitivity of contractile elements is not involved in the protein kinase C-mediated relaxation.

In the muscle stimulated with ionomycin, it was observed that the contraction (200% of the high K^+ -induced contraction) was smaller than that predicted from the increase in $[Ca^{2+}]_i$ (470% of the high K^+ -stimulated $[Ca^{2+}]_i$). Such a relationship did not change in the presence of verapamil, DPB and La^{3+} . This result may indicate that ionomycin decreases the Ca^{2+} sensitivity of contractile elements. In the α -toxin-permeabilized muscle, however, this possibility was not confirmed (H. Ozaki et al., unpublished observation). Another possibility is that because ionomycin, a cation-proton exchanger, may inhibit mitochondrial activity (28), decreased ATP production might dissociate contraction from the increase in $[Ca^{2+}]_i$. In the muscle stimulated with thapsigargin, the increase in $[Ca^{2+}]_i$ at the initial phase was not accompanied by an increase in tension. Similar results were obtained in guinea pig ileum and rat aorta (our unpublished observations). The reason for this dissociation between $[Ca^{2+}]_i$ and tension is not known at present.

In conclusion, we have found that protein kinase C activation by phorbol ester decreases $[Ca^{2+}]_i$ and contraction in the rat myometrium without changing the $[Ca^{2+}]_i$ /tension relationship. The mechanism for the increased sensitivity to phorbol ester observed in pregnant myometrium needs further examination.

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