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EFFECT OF EXTRACELLULAR CALCIUM ON VASCULAR CONTRACTION INDUCED BY PHORBOL ESTER

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In rat thoracic aorta, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) caused a slowly onset, sustained vascular contraction. The contraction was markedly reduced in the absence of extracellular Ca^{2+}, although small tension development was still observed. The tension developed by TPA in the presence of Ca^{2+} was decreased by serial addition of a Ca^{2+}-channel blocker, verapamil in a concentration-dependent manner. TPA could cause vascular contraction to almost maximum level at lower concentration of extracellular Ca^{2+}, compared with KCl- or norepinephrine-induced contraction. These results suggest that extracellular Ca^{2+} which influxes through Ca^{2+}-channels into cytoplasm is necessary for full tension development by TPA, and that TPA increases sensitivity of contractile mechanisms coupling with Ca^{2+}.

It is well known that Ca^{2+} plays an important role in vascular smooth muscle cell contraction. NE as well as other vasoactive agents causes PI turnover through the receptor on the cell surface (1). PI turnover produces inositol trisphosphate which makes microsome to release Ca^{2+} to the cytoplasm (2). At the same time Ca^{2+}-channels on the cell surface are also opened to influx Ca^{2+} from extracellular space. These mechanisms raise the concentration of cytoplasmic free Ca^{2+} from 10^{-7} M to 10^{-6} M which in turn combines with calmodulin to activate MLCK. The phosphorylated MLC produces tension of the smooth muscle through actin-activated Mg-ATPase (3).

Recently, phorbol esters, such as TPA, were reported to cause the sustained contraction of vascular smooth muscle (4-6). TPA is known to have various biological effects in addition to carcinogenesis (7), and furthermore, some of these effects have been found to be mediated through an ubiquitous protein kinase, protein kinase C (8, 9).

Abbreviations used are; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; NE, norepinephrine; PI, phosphatidylinositol; MLC, myosin light chain; MLCK, MLC kinase; PDBu, phorbol dibutylate; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; TMB-8, 8-(N,N'-Diethylamino)-octyl-3,4,5-trimethoxybenzoate; OAG, 1-oleoyl-2-acetyl-glycerol.

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Protein kinase C acts with Ca^{2+} synergistically in cells to make physiological effects (9). In fact, some of the effects of TPA are increased by calcium ionophore A23187. In the secretion of insulin from β-cells by glucose (10) and in the release of aldosterone from adrenal glomerulosa cells by angiotensin II (11), two phases of response are observed and the initial phase is caused by A23187 and the late phase by TPA. Moreover, the natural responses are mimicked in combination with the two.

Concerning the relation of phorbol ester-induced vascular contraction and the extracellular Ca^{2+}, there is an inconsistency on reports. Rasmussen et al. (4) and Danthulri et al. (5) reported that TPA-induced vascular contraction entirely depended upon extracellular Ca^{2+}. On the contrary, Sybertz et al. (6) reported that in rabbit aorta PDBu-induced contraction was independent of the extracellular Ca^{2+} and that the contraction was caused after depletion of the intracellular Ca^{2+} pool by NE.

In this report, detail studies on the role of extracellular Ca^{2+} on the vascular contraction induced by TPA were carried out.

MATERIALS AND METHODS

Materials: TPA, verapamil and nifedipine were purchased from Sigma Chemical. Diltiazem was kindly gifted by Tanabe Seiyaku Co., Ltd.. Other materials and chemicals were obtained from commercial sources. TPA was dissolved in dimethylsulfoxide to give a stock solution of 1 mM and was diluted with water to appropriate concentration. Krebs' bicarbonate solution contained (mM) NaCl, 118; KCl, 4.7; $CaCl_2$, 2.2; KH_2PO_4, 1.18; $MgSO_4$, 1.2; glucose, 5.6; $NaHCO_3$, 25, unless otherwise indicated.

Tissue preparations: Inbred Wistar-Kyoto male rats at three to five months old were decapitated, the thoracic aortae were quickly removed and placed in Krebs' bicarbonate solution aerated with a gas mixture of 95 % O_2 and 5 % CO_2. After cleaning of all loose connective tissues the vessels were cut into herical strips.

Isometric contraction: To record isometric contraction, preparations were equilibrated for 90 min. in aerated Krebs' solution changing every 20 min. under 1 g resting tension at 37°C. After equilibration, each strip was stimulated with 3×10^{-6} M NE, at which concentration, the preparation showed almost maximal contraction for NE in the preliminary experiment. Following wash out of NE, another equilibration for 80 min. was repeated before the tests described below.

a) Response for TPA and medium Ca^{2+}. Each strip was stimulated with 3×10^{-7} M TPA in Krebs' solution. After sustained contraction reached the plateau, the medium solution was replaced two times with Krebs' solution. Then, the medium was replaced with Ca^{2+}-free Krebs' solution with 0.5 mM EGTA. When the strips relaxed maximally, the medium was replaced again with Krebs' solution containing 2.2 mM Ca^{2+}.

b) TPA concentration-response experiment. Preparations were preincubated in the Krebs' solution with 1 mM Ca^{2+} or in the Ca^{2+}-free Krebs' solution with 0.5 mM EGTA for 30 min., changing the solution every 10 min.. Then, small volume of TPA solution was added cumulatively. The data obtained in this experiment were expressed as the percentage of the initial NE response. As shown later, the response in 1 mM Ca^{2+} Krebs' solution was not different from the response in 2.2 mM Ca^{2+} Krebs' solution.

c) Relaxation by verapamil. Preparations were preincubated in the Krebs' solution containing 2.2 mM Ca^{2+} for 30 min. as described above, then contracted with 3×10^{-8} M TPA or 30 mM KCl. After the tension developed maximally small volume of verapamil solution was added cumulatively. The data obtained during this experiment were expressed as the percentage of the response caused by TPA or KCl.
d) Ca^{2+} concentration-response experiment. Preparations were preincubated for 30 min. in Krebs' solution without Ca^{2+} or EGTA in this experiment except that the Ca^{2+} concentration was 0 mM. Then various drugs were added. To obtain the response at 0 mM Ca^{2+}, preincubation and stimulation were carried out in Ca^{2+}-free Krebs' solution with 0.5 mM EGTA. The data obtained in this experiment were expressed as the percentage of the response of each drug at 2.2 mM Ca^{2+}.

Statistical analysis: Data are presented as means ± S.E.M.. Student's t-test was used to determine statistical difference between groups.

RESULTS AND DISCUSSION

a) Response for TPA and medium Ca^{2+}. TPA caused slow onset, sustained contraction in rat thoracic aorta (Fig. 1). The contraction began at 5 min., developed slowly, reaching maximum at 40 to 60 min.. Once contracted, vascular smooth muscle was not relaxed by replacing the medium with TPA-free Krebs' solution. However, it relaxed by replacing the solution with Ca^{2+}-free Krebs' solution with EGTA, suggesting the role of extracellular Ca^{2+} for full tension development. Small tension development (26.5 ± 4.9 %, n=4) was still observed in Ca^{2+}-free Krebs' solution with EGTA. When Krebs' solution was replaced again, considerable contraction was observed without another addition of TPA (72.3 ± 15.4 %, n=4). These results were almost consistent with the report described previously by Danthuluri et al. (5).

b) TPA concentration-response experiment. Next set of experiments showed that TPA induced vascular contraction in a dose-dependent manner over a concentration range of 3×10^{-9} M to 3×10^{-7} M in the presence of 1 mM Ca^{2+} (Fig. 2). On the other hand, in Ca^{2+}-free Krebs' solution with EGTA, the contraction induced by cumulative addition of TPA was reduced signifi-

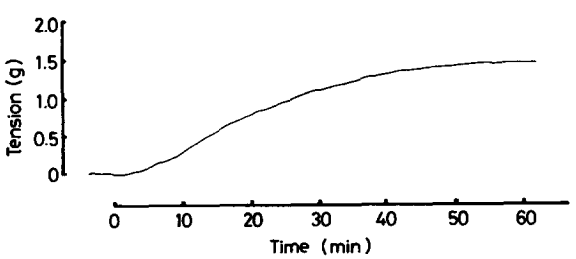

Fig. 1. A chart record showing TPA-induced contraction of rat thoracic aortic strips. 3×10^{-7} M TPA was added at time 0 and the tension developed was recorded isometrically.

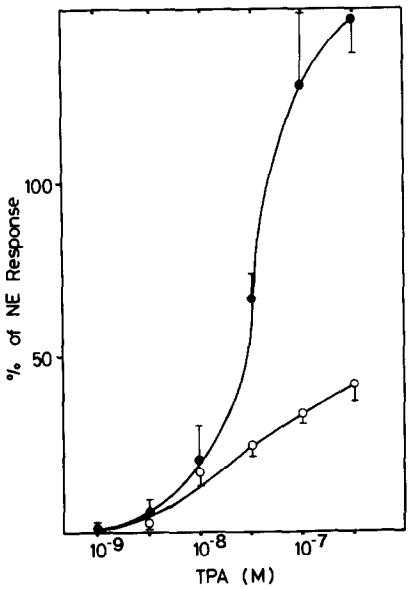

Fig. 2. TPA-induced concentration-response curves in the presence (●—●) or absence (○—○) of 1 mM Ca^{2+}. Each aortic strip was initially stimulated with 3 x 10^{-6} M NE, and the tension developed by cumulative addition of TPA was shown as % of the initial NE response. Mean values in the presence of Ca^{2+} differ statistically from those in the absence of Ca^{2+} ($p<0.02$, 3 x 10^{-8} M; $p<0.05$, 1 x 10^{-7} M; $p<0.01$, 3 x 10^{-7} M). (Mean ± S.E.M., n=4)

cantly. However, small but considerable contraction was observed. The tensions induced by 3 x 10^{-7} M TPA were 147 ± 10.3 % (n=4) and 42.3 ± 5.5 % (n=4) of the initial NE response in the presence and absence of Ca^{2+}, respectively ($p<0.01$). Using rat aortic ring preparation in HEPES-saline buffer (1.5 mM $CaCl_2$), Danthuluri et al. (5) reported that 10^{-5} M TPA caused contraction to the extent at 63 % of maximum phenylephrine response. Thus, larger and more sensitive response to TPA was observed in the present preparation. Present results indicated that the contraction of rat aorta induced by TPA was partially independent of extracellular Ca^{2+}. The contraction caused by TPA may be divided into two parts; one is dependent on extracellular Ca^{2+} and the other independent. These agreed with the results described by Gleason et al. (12). However, in this respect, Rasmussen et al. showed that the TPA-induced vascular contraction depended entirely on extracellular Ca^{2+} (4). In their preparation, 3 x 10^{-9} M to 3 x 10^{-7} M TPA induced dose-dependent contraction of perfused rabbit ear artery in 2.5 mM Ca^{2+}, but did not in 1.5 mM Ca^{2+}. Danthuluri et al. (5) also reported the dependency of the contraction on the availability of extracellular Ca^{2+}, as the contraction of their rat aortic ring preparation induced by TPA was almost relaxed in Ca-free EGTA buffer. On the contrary, Sybertz et al. reported that in rabbit aorta PDBu-induced contraction was not dependent

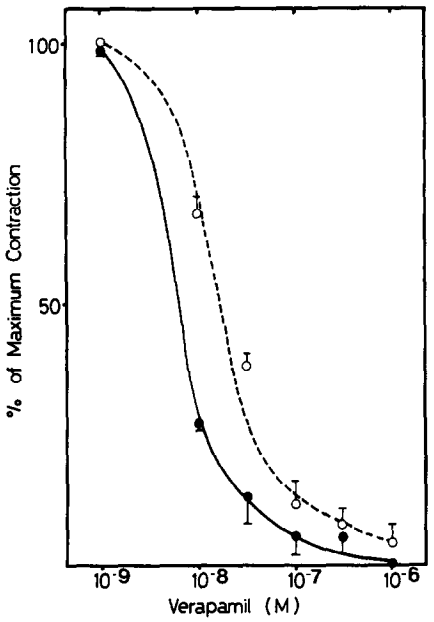

Fig. 3. Vasodilatory effect of verapamil on the aortic strips contracted by TPA and KCl. Aortic strips were contracted with 3×10^{-8} M TPA (●—●) or 30 mM KCl (O----O) in the presence of 2.2 mM Ca^{2+} (100 %), and verapamil was added cumulatively. (Mean ± S.E.M., n=5)

on extracellular Ca^{2+} at all (6). These inconsistencies led us to the further investigation on the role of extracellular Ca^{2+} in vascular contraction induced by TPA.

c) Relaxation by verapamil. As shown in Fig. 3, verapamil showed vasodilation on the strips contracted by 3×10^{-8} M TPA as well as by 30 mM KCl in the presence of 2.2 mM Ca^{2+} in a concentration-dependent fashion. The ID_{50} was 4.9×10^{-9} M and 2.0×10^{-8} M for TPA and KCl, respectively. Same results were obtained with diltiazem and nifedipine (data not shown). These results suggested that at least for full tension development by TPA, extracellular Ca^{2+} influx into cytoplasm through Ca^{2+}-channel was necessary. There have been discussed two possibilities on the role of TPA on vascular contraction. First, TPA opens the Ca^{2+}-channels to increase the Ca^{2+} influx, or inhibits the Ca^{2+} efflux in turn elevated the intracellular Ca^{2+} concentration. Second, TPA does not change the Ca^{2+} influx for its response, but stimulates contractile mechanisms coupling with intracellular Ca^{2+}. Gleason et al. reported that in rabbit aorta extracellular Ca^{2+}-dependent contraction by PDBu was due to increasing Ca^{2+} influx from extracellular space (12), which was consistent with the first possibility. Our result also seems to support this possibility. On the contrary, Sybertz et al. showed that PDBu had no effect on ^{45}Ca influx as well as on ^{45}Ca efflux

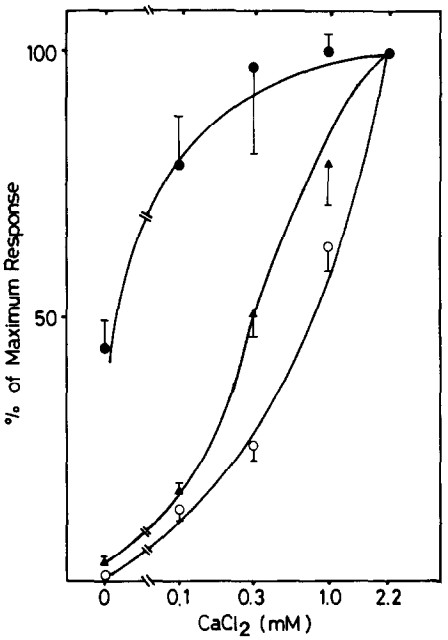

Fig. 4. Effects of extracellular Ca^{2+} on vascular contraction induced by TPA, KCl and NE. Preparations were pretreated and stimulated in Ca^{2+}-free Krebs' solution with EGTA at 0 mM. In other cases strips were pretreated and added various drugs in Ca^{2+}-free Krebs' solution without EGTA, and Ca^{2+} was added cumulatively. 1 x 10^{-7} M TPA (●——●); 3 x 10^{-6} M NE (▲——▲); 70 mM KCl (○——○). Data are expressed as % of the response at the Ca^{2+} concentration of 2.2 mM for each strip. Mean value for TPA at 0.1 mM Ca^{2+} differs statistically from those for NE and KCl ($p<0.01$). (Mean ± S.E.M., n=4)

in rabbit aorta (6). Itoh et al. observed that in porcine coronary artery TPA had no effect on the Ca^{2+} transient but did enhance the contraction induced by KCl (14). In the second possibility, Ca^{2+} still turns over and extracellular Ca^{2+} may influx through a small number of Ca^{2+} channels that open in the presence or absence of TPA. Verapamyl might show its effect by inhibiting the small amount of Ca^{2+} influx. Miller et al. reported an evidence consistent with this possibility (14).

d) Ca^{2+} concentration-response experiment. Figure 4 shows the sensitivity to extracellular Ca^{2+} of the contracton induced by TPA compared with NE and KCl. The tension produced by TPA (10^{-7} M), NE (3 x 10^{-6} M) and KCl (70 mM) in the presence of 2.2 mM Ca^{2+} were about 150 %, 107 % and 50 % of the one by the initial addition of NE, respectively. TPA caused significant contraction at low concentration of Ca^{2+} in comparison with NE and KCl. This result was consistent with that described by Miller et al. in which in skinned vessels TPA increased the contraction in the presence of 3 x 10^{-7} M Ca^{2+} (14). Thus, the main effect of TPA may be to increase the sensitivity of contractile mechanisms coupling with Ca^{2+}.

Concerning the small contraction induced by TPA in the Ca^{2+}-free solution with EGTA, Ca^{2+} pooled in the cell may be used for the contraction. However, Sybertz et al. (6) reported that in rabbit aorta PDBu-induced contraction did not depend on NE sensitive Ca^{2+}-pool in the cells. In our preliminary experiment, TMB-8, an inhibitor of intracellular Ca^{2+} mobilization, could not cancel the response induced by TPA (10^{-7} M) in rat aortic strips (unpublished observation). These may suggest that the contraction independent of extracellular Ca^{2+} is not caused by mobilization of Ca^{2+} from intracellular store. Further studies are necessary to clarify this point.

Various effects of TPA are known to be mediated through protein kinase C (8-11). Some investigators showed the possible role of protein kinase C on vascular contraction induced by phorbol esters. Miller et al. observed that polymyxin B, one of the inhibitors of protein kinase C, inhibited TPA-induced contraction (14). Phosphatidylserine, on which the activity of protein kinase C is dependent, potentiated TPA-induced contraction in skinned vessels (13, 15). Moreover, some phorbol esters which could not activate protein kinase C did not show vascular contracting effect (14, 15). However, OAG (up to 60 μg/ml), which is another activator of protein kinase C, did not produce vascular contraction in our experiment (data not shown), although Miller et al. reported the contractile effect of OAG in skinned vessels (14). Thus, further studies are required to decide the role of protein kinase C in vascular contraction induced by phorbol esters.

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