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INVOLVEMENT OF PROTEIN KINASE C IN Ca^{2+}-INDEPENDENT CONTRACTION OF RAT UTERINE SMOOTH MUSCLE

Hideji Karibe, Kazuhiko Oishi[1] and Masaatsu K. Uchida

Department of Molecular Pharmacology, Meiji College of Pharmacy,
1-35-23 Nozawa, Setagaya-ku, Tokyo 154, Japan

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SUMMARY: The contribution of protein kinase C to the contraction by oxytocin of rat uterine longitudinal smooth muscle in Ca^{2+}-free solution was investigated. Immunological analysis revealed that type II (β) and III (α) protein kinase C subspecies were present in rat uterine smooth muscle. The pretreatment of a diacylglycerol kinase inhibitor R59022 to accumulate diacylglycerol potentiated the Ca^{2+}-independent contraction. The contractile activity was diminished with the depletion of protein kinase C, when the contraction was evoked repeatedly by oxytocin during the prolonged exposure to a tumor-promoting phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate. These results suggested the involvement of protein kinase C in oxytocin-induced contraction in Ca^{2+}-free solution.

Oxytocin induces sustained contraction in Ca^{2+}-free solution containing 0.2 mM EGTA after exposing to Ca^{2+}-free solution containing 3 mM EGTA for 1 hr (Ca-free contraction) (1,2). This Ca-free contraction is observed repeatedly in Ca^{2+}-free solution without exposing the muscle to Ca^{2+}-containing solution for over 10 hr (3). Such Ca^{2+}-independent contraction is induced by various drugs besides oxytocin and observed in various smooth muscles such as rat stomach, vas deferens, thoracic aorta and guinea pig stomach, trachea (3,4). We reported previously that calcium antagonists such as D-600 and nicardipine and intracellular Ca^{2+} chelator quin-2 had no effect on Ca-free contraction (2,5). Furthermore, the study with fura-2 indicates that cytosolic free Ca^{2+} level ($[Ca^{2+}]_i$) does not rise during Ca-free contraction (5). These results suggest that this contraction is rather Ca^{2+}-independent, that is, it is not triggered by the usual increase in $[Ca^{2+}]_i$. Our recent study has shown that oxytocin-induced Ca-free contraction was not associated with any phosphorylation of myosin light chain (6). These findings clearly indicate that Ca^{2+}/calmodulin/myosin light chain kinase system is not involved in Ca-free contraction. On the contrary, protein kinase inhibitors such as staurosporine and H-7 are found to be effective inhibitors of this contraction (7). These results suggest the involvement of protein kinases, but not myosin light chain kinase, in the contraction.

Protein kinase C is a ubiquitous serine/threonine protein kinase that is thought to play a major role in signal transduction in a variety of cells and tissues (8). The activation of protein kinase C is believed to

[1] To whom all correspondence should be addressed.

Abbreviations used are: EGTA, ethyleneglycol-bis-(2-aminoethylester)-N,N,N',N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; IP_3, inositol 1,4,5-trisphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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be linked to receptor-coupled activation of phospholipase C that generates two intracellular second messengers, IP_3 and diacylglycerol (8,9). IP_3 releases Ca^{2+} from intracellular stores whereas diacylglycerol, in a concerted manner with membrane phospholipids and Ca^{2+}, promotes translocation of protein kinase C to the plasma membrane with a resulting activation of the enzyme.

The involvement of protein kinase C in cellular function has been investigated by stimulating or inhibiting the enzyme activity. The use of inhibitors for protein kinase C is, however, limited because of their poor selectivity (10,11). Tumor-promoting phorbol esters such as TPA has been widely used for the activation of protein kinase C. Although phorbol esters and diacylglycerol are often thought to exert their function via the common mechanism of protein kinase C activation, differences in their modes of activation of protein kinase C has been recently pointed out (12). Therefore, two further approaches have been chosen to clarify the contribution of protein kinase C to cellular function in the present study. One approach is the use of diacylglycerol kinase inhibitor R59022 to accumulate diacylglycerol, an endogenous activator of protein kinase C (13). Alternatively, the long-term treatment of cells with a phorbol ester for a depletion of protein kinase C and a concomitant loss of cell responsiveness was employed (14).

EXPERIMENTAL PROCEDURES

Preparation of rat uterine longitudinal smooth muscle; Rat uterine longitudinal smooth muscle was obtained as described by Karibe *et al.* (7). Briefly, female Wister rats (160-200 g) were ovariectomized. After a recovery period of over 5 days, they were given estradiol benzoate (0.1 mg/kg, s.c.) once a day for 4 days, put them unconscious by a blow on the neck and exsanguinated and uterine horns were dissected.

Mechanical recording of muscle contraction; The muscle strips were mounted in a 10 ml organ bath containing normal Locke-Ringer solution at 30 °C and aerated with 5% CO_2 in O_2. After equilibration in normal Locke-Ringer solution for 1 hr at a resting tension of 0.5 g, the strips were incubated in Ca^{2+}-free solution containing 3 mM EGTA for 1 hr at a resting tension of 0.2 g ('EGTA treatment') to remove Ca^{2+} bound to the muscle tissues. After EGTA treatment, the medium was replaced by Ca^{2+}-free solution and incubated further for 10 min. Ca-free contraction was induced by the addition of oxytocin and allowed to reach a plateau. At 20 min after the addition of oxytocin ($3x10^{-3}$ unit/ml), the strips were washed with Ca^{2+}-free solution for 40 min. Next contraction was then induced by the addition of oxytocin in the presence and absence of R59022 (10^{-6} M) or TPA (10^{-7} M). After 20 min, the strips were washed with Ca^{2+}-free solution with or without drug for 40 min. The contractions were likewise repeated several times in the presence and absence of drug. Normal Locke-Ringer solution had the following composition (mM): NaCl 154, KCl 5.63, $CaCl_2$ 2.16, $MgCl_2$ 2.10, $NaHCO_3$ 5.95, and glucose 5.55. Ca^{2+}-free solution had the same composition except that $CaCl_2$ was replaced by 0.2 mM EGTA.

Electrophoresis and Western blotting analysis; SDS-PAGE was performed in 10% polyacrylamide gels according to the procedure of Laemmli (15). The proteins separated in the gel was subject to electrophoretical transfer on nitrocellulose sheet. Immunoreactive proteins to protein kinase C subspecies specific monoclonal antibodies MC-1a, MC-2a, and MC-3a which recognize respectively three types of protein kinase C subspecies α, β, and γ were stained by using Amersham detection kit.

Other methods; Protein was determined by the method of Lowry *et al.* (16) by using bovine serum albumin as a standard. Protein kinase C was partially purified from rat brain according to the method of Oishi *et al.* (17).

Materials; Protein kinase C subspecies specific monoclonal antibodies MC-1a, MC-2a, and MC-3a were purchased from Seikagaku Kogyo Co. (Tokyo). R59022 was from Funakoshi Yakuhin Co. (Tokyo). TPA and 4α-phorbol didecanoate were from Sigma Chemical Co. (St. Louis, MO). Estradiol Benzoate and oxytocin were from Teikoku Hormone Manufacturing Co. (Tokyo).

RESULTS

It is reported that protein kinase C is present in various tissues and cell types including smooth muscles (8,18,19), and also shown the diversity of tissue-distribution of protein kinase C subspecies

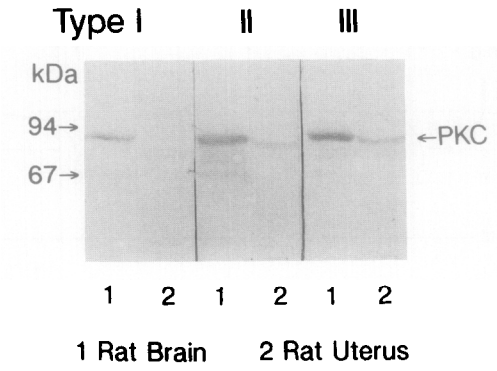

Fig. 1. Immunoblot showing identification of protein kinase C subspecies in rat uterine longitudinal smooth muscles. Rat uterine longitudinal smooth muscles were homogenized at 4 °C in the solution containing 20 mM Tris/HCl (pH 7.5), 1 mM dithiothreitol, 5 mM EGTA, 1 mM EDTA, 10% (v/v) glycerol, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and 0.5% (v/v) Nonidet P-40. The homogenate was left for 1 hr and then centrifuged at 10,000 xg for 1 hr at 4 °C. The supernatant (10 μg protein/lane) was subject to SDS-PAGE followed by Western blotting as described in "Methods". Partially purified protein kinase C from rat brain was used as a control.

for example in bovine aorta (20). In the present study, immunological analysis revealed that type II (β) and III (α) protein kinase C subspecies were present in rat uterine smooth muscle, whereas all three subspecies were in the purified rat brain protein kinase C preparation coinciding with previous observations by others (Fig. 1) (21).

Fig. 2 shows the effect of R59022 on oxytocin-induced Ca-free contraction. Ca-free contraction could be repeatedly evoked by oxytocin ($3x10^{-3}$ unit/ml) in the similar contractile activity. In the presence of R59022, although first contraction after the addition of R59022 was not changed

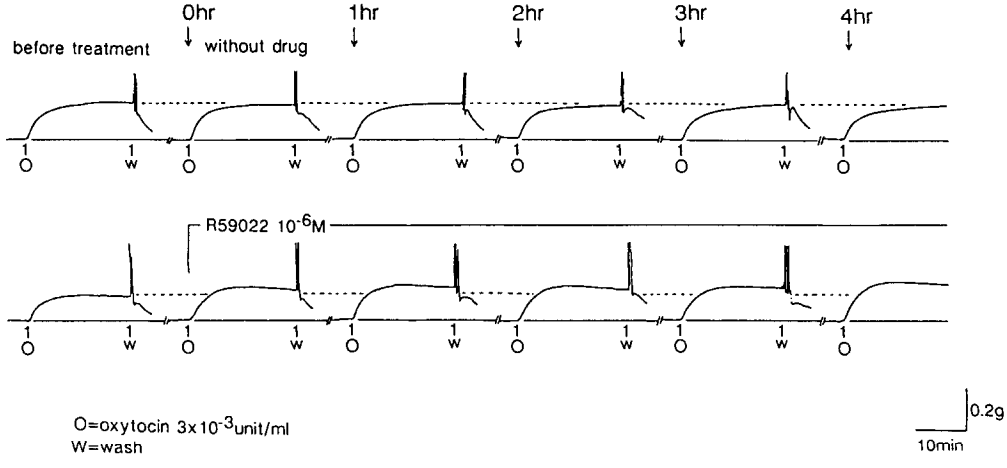

Fig. 2. Typical traces showing the effect of R59022 on oxytocin-induced Ca-free contraction. Ca-free contraction was induced by the addition of oxytocin and allowed to reach a plateau. At 20 min after the addition of oxytocin ($3x10^{-3}$ unit/ml), the strips were washed with Ca^{2+}-free solution for 40 min. Next contraction was then induced by the addition of oxytocin in the presence and absence of R59022 (10^{-6} M). After 20 min, the strips were washed with Ca^{2+}-free solution with or without drug for 40 min. The contractions were likewise repeated several times in the presence (lower trace) and absence (upper trace) of R59022.

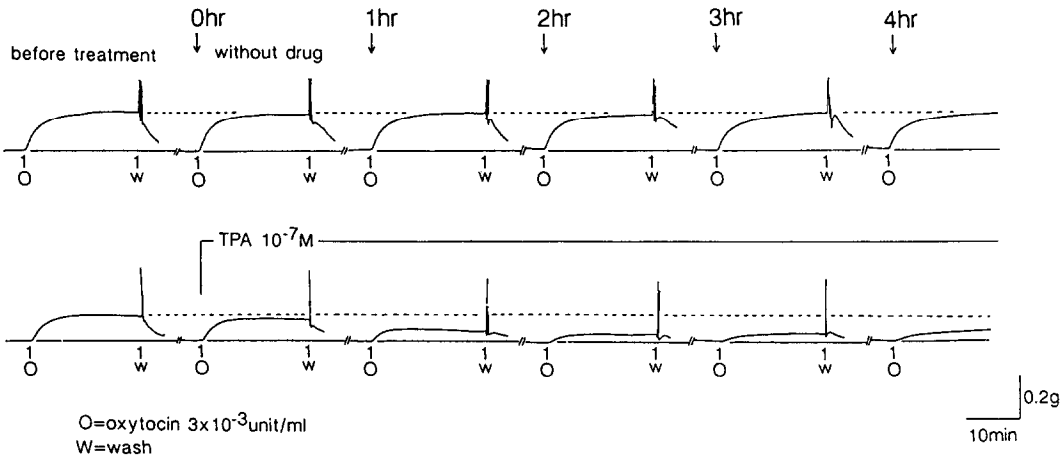

Fig. 3. Typical traces showing the effect of TPA on oxytocin-induced Ca-free contraction. Ca-free contraction was induced by the addition of oxytocin and allowed to reach a plateau. At 20 min after the addition of oxytocin ($3x10^{-3}$ unit/ml), the strips were washed with Ca^{2+}-free solution for 40 min. Next contraction was then induced by the addition of oxytocin in the presence and absence of TPA (10^{-7} M). After 20 min, the strips were washed with Ca^{2+}-free solution with or without drug for 40 min. The contractions were likewise repeated several times in the presence (lower trace) and absence (upper trace) of TPA.

compared to the contraction before that, Ca-free contraction was potentiated with repeating the contraction in the presence of R59022. Fifth contraction after the addition of R59022 (4 hr of drug treatment) was potentiated to 130% compared to the previous contraction (Fig. 4).

Next, the effect of the long-term treatment of TPA on oxytocin-induced Ca-free contraction was examined (Fig. 3). Ca-free contraction was decreased with repeating the contraction in the presence of TPA (10^{-7} M). Fifth contraction after the addition of TPA (4 hr of drug treatment) was inhibited to 24% by TPA compared to the previous contraction (Fig. 4). The Ca-free contraction was not affected

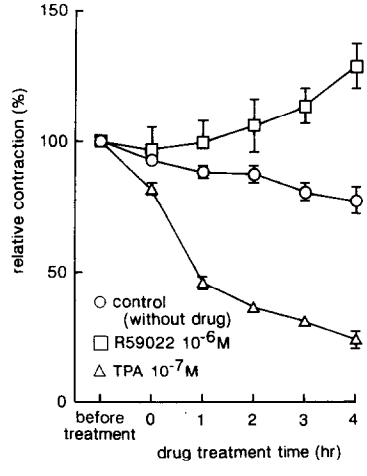

Fig. 4. The effects of R59022 and TPA on Ca-free contraction. The plots were based upon the data shown in Fig. 2 and 3. The contraction before any treatment was plotted as 100%. The contraction without drug (circle), with 10^{-6} M R59022 (square), and with 10^{-7} M TPA (triangle).

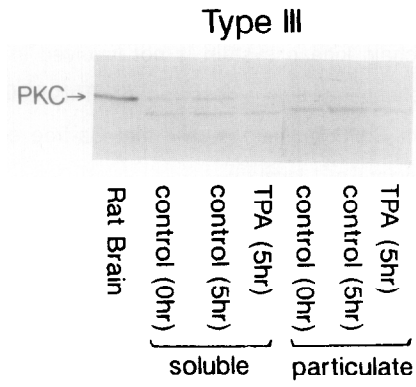

Fig. 5. Western blot showing TPA-induced down-regulation of type III protein kinase C subspecies in rat uterine smooth muscles. After the muscles were incubated with 10^{-7} M TPA for 5 hr at 30 °C, the muscles were homogenized at 4 °C in the solution containing 20 mM Tris/HCl (pH 7.5), 1 mM dithiothreitol, 5 mM EGTA, 1 mM EDTA, 10% (v/v) glycerol, 20 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 100,000 xg for 1 hr at 4 °C. The supernatant was referred to the soluble fraction. The pellet was resuspended in the above solution with 0.5% (v/v) Nonidet P-40, left for 1 hr, and then centrifuged at 100,000 xg for 1 hr at 4 °C. This supernatant was referred to the particulate fraction. The soluble and particulate fractions (10 μg protein/lane) were subject to SDS-PAGE followed by Western blotting as described in "Methods".

by long-term treatment with TPA unless the contraction was repeated. These results indicate that the repetition of contraction and long-term treatment of TPA cooperatively inhibit Ca-free contraction possibly in the result of down-regulation of protein kinase C.

It has been reported that long-term treatment with phorbol esters results in down-regulation of protein kinase C as assessed by immunoreactivity and enzyme activity (14). As shown in Fig. 5, type III protein kinase C subspecies in the soluble and particulate fractions of uterine smooth muscle was almost depleted when the muscle was incubated with TPA for 5 hr. A similar result was obtained in type II protein kinase C subspecies (data not shown). A band just under the protein kinase C band was a non-specific band because this band was also observed when normal mouse serum was used in the place of protein kinase C monoclonal antibodies or the monoclonal antibodies were omitted (data not shown). It is, therefore, suggested that the inhibition of Ca-free contraction observed in Fig. 3 is a result of the down-regulation of protein kinase C.

DISCUSSION

It is well established in various types of smooth muscle that contractile activity is determined by the increased level of intracellular free Ca^{2+} (22). Stimulation of a wide variety of cell surface receptors leads to an increase in intracellular levels of Ca^{2+} (9,22). Ca^{2+} mobilization from intracellular sources has been described as a result of the activation of such receptors which are coupled to the hydrolysis of inositol phospholipids by phospholipase C (9). The degradation of phosphatidylinositol 4,5-bisphosphate leads to the production of two second messengers IP_3 and diacylglycerol. Oxytocin-induced inositol phospholipid hydrolysis has been observed in rat uterus (23). IP_3 has been shown to release Ca^{2+} from intracellular Ca^{2+} storage sites in various types of smooth muscles (24,25) as well as from isolated uterine sarcoplasmic reticulum (26). An increased intracellular Ca^{2+} level induces activation of myosin light chain kinase followed by phosphorylation of myosin light chain. As a result,

the formation of crossbridges between actin and phosphorylated myosin initiates contraction. But this Ca^{2+}/calmodulin/myosin light chain kinase system is not involved in oxytocin-induced Ca-free contraction because no myosin phosphorylation is observed (6). This finding is also supported by the observation by using fluoresent Ca^{2+} indicator fura-2 that Ca-free contraction of rat uterus is independent of the rise in intracellular Ca^{2+} level (5).

The receptor-stimulated transient increase in diacylglycerol is important for signal-transducing system since diacylglycerol generated serves as an endogenous activator of protein kinase C (8,9) One of the main sources for the generation of diacylglycerol is thought to be the hydrolysis of phosphatidylinositol 4,5-bisphosphate as described above. It is now generally accepted that diacylglycerol generated by agonist-stimulation is quickly phosphorylated into phosphatidic acid by diacylglycerol kinase. A diacylglycerol kinase inhibitor R59022 has been shown to result in the accumulation of diacylglycerol and concomitantly stimulate the protein kinase C activity in intact platelets (13). R59022 was, therefore, employed for the present study. R59022 potentiated Ca-free contraction (Fig. 2 and 4), indicating that diacylglycerol accumulated as a result of the inhibition of diacylglycerol kinase increased the contractile activity possibly by the potentiation of protein kinase C activity. The repitition of contraction was seemed to be not required for the potentiation of Ca-free contraction, since the incubation of the muscle with R59022 for 3 hr without repeating the contraction potentiated the contractile activity (data not shown).

Protein kinase C is present in various tissue and cell types including smooth muscles (8,18,19,20). It has recently become apparent that more than seven subspecies of protein kinase C exist (27). Immunological and biochemical studies have demonstrated the different tissue and subcellular distribution of types I, II, and III (γ, β, and α, respectively) protein kinase C subspecies. Western blotting analysis by using protein kinase C subspecies specific monoclonal antibodies MC-1a, MC-2a, and MC-3a revealed that rat uterine longitudinal smooth muscle has type II (β) and type III (α) subspecies (Fig. 1). The differential susceptibility of the protein kinase C subspecies to proteolysis and to phorbol ester-induced down regulation has been demonstrated in purified preparations of the enzymes (28,29) and in several cell types including basophilic leukemia cells (28) and KM3 cells (30). The present findings demonstrated that type III protein kinase C subspecies in the soluble and particulate fractions was down-regulated when the uterine smooth muscle was treated with TPA for 5 hr (Fig. 5). Similar result was obtained in type II protein kinase C subspecies (data not shown). In the present study, the differential susceptibility of the protein kinase C subspecies to phorbol ester-induced down regulation was not observed. In an another approach, we examined oxytocin-induced Ca-free contraction under the condition where protein kinase C has been down-regulated by long-term treatment of the muscle with TPA. The long-term treatment with TPA results in a significant decrease of contractile activity correlated with a down-regulation of protein kinase C (Fig. 4). An inactive form of phorbol ester 4α-phorbol didecanoate had no effect on Ca-free contraction (data not shown). From these results, we concluded that protein kinase C was involved in oxytocin-induced Ca-free contraction.

The intracellular Ca^{2+} level during Ca-free contraction is so low that myosin light chain kinase is not activated during Ca-free contraction (6). Since the intracellular Ca^{2+} concentrations of uterine and vascular smooth muscle cells in the medium containing Ca^{2+} are reported to be 1.4×10^{-7} M (31) and 2.3×10^{-7} M (32), respectively, the intracellular Ca^{2+} concentration during Ca-free contraction is

estimated to be less than 10^{-7} M. A major question is whether protein kinase C is activated under the condition. Protein kinase C is originally reported to be dependent on Ca^{2+} in the presence of diacylglycerol and phospholipid, but now it became certain that the susceptibility to Ca^{2+} varied with protein kinase C subspecies (27). Type II subspecies, which is observed to be in rat uterine smooth muscle (Fig. 1), does not depend on Ca^{2+} and responds partially to diacylglycerol and phospholipid even in the presence of 0.5 mM EGTA (33). The novel protein kinase C subspecies such as δ, ε, and ζ are also known as diacylglycerol and phospholipid-dependent and Ca^{2+}-independent protein kinases (34,35), although the presence of these novel protein kinase C subspecies in rat uterine smooth muscle is not clear at present. It is, therefore, conceivable that type II subspecies (and novel protein kinase C, if present in the uterus) is predominantly involved in Ca-free contraction.

The susceptibility of the enzyme to Ca^{2+} also varied with substrate proteins (17,36). Protamine is one of the typical substrate for protein kinase C which dose not require Ca^{2+} for activation (17). At present, the endogenous substrate proteins which are involved in Ca-free contraction are not known yet. The substrate proteins for protein kinase C with a characteristic of low requirement of Ca^{2+} for phosphorylation could be the good candidates. There are several reports indicating the possible contribution of phosphorylation of actin-interacting proteins such as calponin (37) and caldesmon (38,39) and intermediate filaments (40) to smooth muscle contractions. We reported previously that cytochalasins as well as protein kinase inhibitors are good inhibitor of Ca-free contraction and that simultaneous addition of cytochalasin D and staurosporine synergistically inhibits Ca-free contraction (7). One possibility is that the protein related to the actin filaments is one of the candidates for protein kinase C substrate during Ca-free contraction.

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