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OXYTOCIN CONTRACTS RAT UTERINE SMOOTH MUSCLE IN Ca^{2+}-FREE MEDIUM WITHOUT ANY PHOSPHORYLATION OF MYOSIN LIGHT CHAIN

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SUMMARY: Contraction of rat uterine smooth muscle related to phosphorylation state of myosin light chain under various conditions was investigated. In the Ca^{2+}-containing medium, both high K^+ and oxytocin induced marked contraction of the muscle accompanied by pronounced phosphorylation of myosin light chain. In the Ca^{2+}-free medium, although both vanadate and oxytocin induced slight contraction, phosphorylation of myosin light chain was only evident for vanadate but not for oxytocin. It was suggested that another mechanism distinct from myosin light chain phosphorylation might be involved in Ca^{2+}-independent contraction of uterine smooth muscle elicted by oxytocin.

Oxytocin induces sustained contraction of rat uterine smooth muscle in the solution without Ca^{2+} after the muscle is incubated with 3 mM EGTA for 1 h (Ca-free contraction) (1,2). This oxytocin-induced contraction in Ca^{2+}-free medium gives 5-15% of the maximal contraction caused by oxytocin in the presence of Ca^{2+} (normal contraction). We have reported that calcium antagonists such as D-600 and nicardipine and intracellular Ca^{2+} chelator quin-2 have no effect on Ca-free contraction (2,3). Furthermore, the study with fura-2 indicates that cytosolic free Ca^{2+} level ($[Ca^{2+}]_i$) does not rise during this Ca-free contraction (3). Therefore, it is predicted that this contraction is rather Ca^{2+}-independent, that is, it is not triggered by the usual increase in $[Ca^{2+}]_i$.

It is generally accepted that phosphorylation of myosin light chain by MLCK is the most probable mechanism to initiate smooth muscle contraction (4). In fact, MLCK inhibitors, ML-9 and KT-5926 (5) effectively inhibit normal contraction of rat uterine smooth

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Abbreviations used are: EGTA, ethylene glycol bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid; MLCK, myosin light chain kinase; PPi, pyrophosphate; PAGE, polyacrylamide gel electrophoresis; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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muscle. But they have no effect on Ca-free contraction (6), indicating a possibility that myosin phosphorylation by MLCK is not involved in this process. Therefore, the present study was performed to clarify the phosphorylation state of myosin in an early stage of this sustained contraction by means of two different electrophoresis systems, namely, PPi PAGE (7,8) and urea-glycerol PAGE (9). We reported here that Ca-free contraction was not associated with any phosphorylation of myosin light chain.

EXPERIMENTAL PROCEDURES

The rat uterine longitudinal smooth muscle was obtained as described by Matsuo and Uchida (10). The muscle strips were mounted in a 10 ml organ bath filled with normal Locke-Ringer solution aerated with 5% carbon dioxide in oxygen at 30°C. After equilibration in normal Locke-Ringer solution for 1 h at a resting tension of 0.5g, the stripes were incubated in Ca^{2+}-free solution containing 3 mM EGTA for 1 h at a resting tension of 0.2g (EGTA treatment). The medium was then replaced by Ca^{2+}-free solution containing 0.2 mM EGTA. After further incubation for 10 min, contraction was induced by the addition of 10^{-2} unit/ml oxytocin (Ca-free contraction). Isometric tension was monitored with a force displacement transducer. At indicated times, the organ bath was removed without influencing tonic tension and strips were rapidly frozen by submersion in dry ice-acetone. 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 substituted by 3 or 0.2 mM EGTA.

The frozen strips were crushed in a frozen stainless steel mill at -170°C. The frozen powder was extracted in 60 volumes of PPi sample buffer containing 100 mM $Na_4P_2O_7$, 5 mM EGTA, 2.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 0.6 M KI and 10% glycerol (pH 8.8), which was slightly modified from the original composition of Persechini *et al.* (11). After centrifugation of the extract, glycerol and sucrose were added to give final concentration of 30 and 20%, respectively. The samples were subjected to PPi PAGE as described previously by Takano-Ohmuro and Kohama (7,8) for 16 h to examine the phosphorylation state of myosin by separating phosphorylated myosin from unphosphorylated one. The pattern of protein on PPi PAGE disk gel was visualized by staining with 0.1% Coomassie brilliant blue R-250. In order to analyze phosphorylation state of myosin light chain by urea-glycerol PAGE, PPi PAGE of the muscle extract was carried out for 3 h . After staining briefly with Coomassie brilliant blue R-250, a major protein band which contained unseparated myosin and filamine was excised from the gel and treated with the urea solution containing 9 M urea, 100 mM Tris-HCl (pH 6.8) and 5% 2-mercaptoethanol for 10 min. Urea-glycerol PAGE of the excised gel was then performed as described previously (9) to examine the phosphorylation state of myosin light chain by separating phosphorylated myosin light chain from unphosphorylated one in order of increased mobility. The pattern of protein on urea-glycerol PAGE slab gel was visualized by means of a slightly modified procedure (12) of the highly sensitive silver stain of Oakley *et al.* (13), which is approximately 20-fold sensitive than the Coomassie brilliant blue staining. Identification of filamin on PPi PAGE gel was carried out as described previously (7).

RESULTS

Exposure of the muscle strips to 45.6 mM K^+ medium in the presence of Ca^{2+} caused a sustained contraction which gave a sub-maximal tension of about 2g after 60 sec, as shown in Fig.1. At time zero, one myosin band (designated by M) which comigrated with the unphosphorylated gizzard myosin was observed just under the filamin band on PPi PAGE. At 15 sec after the muscle was exposed to the high K^+ medium, additional two bands which were migrated faster than the unphosphorylated myosin band appeared on PPi PAGE. The upper

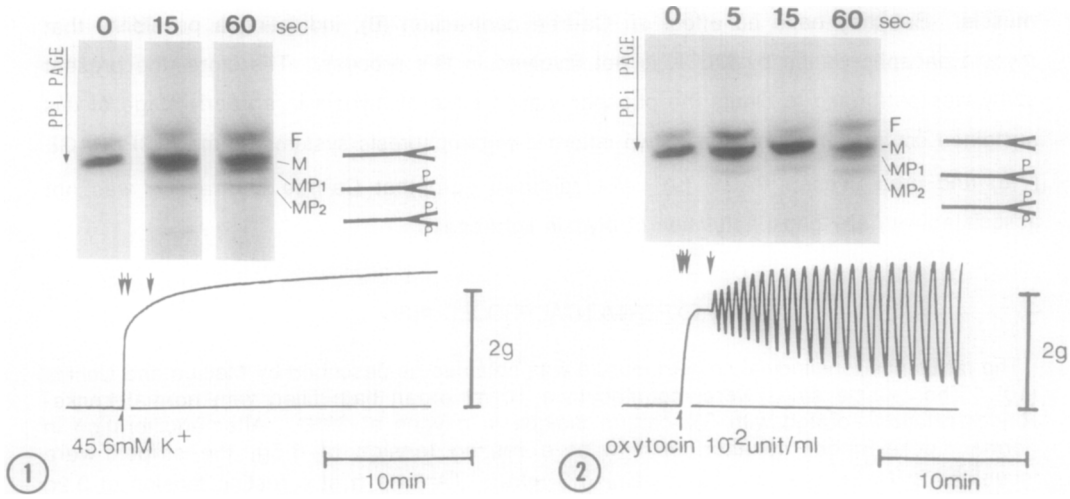

Fig.1. Analysis of the phosphorylation state of myosin by PPi PAGE during 45.6 mM K^+-induced contraction in the medium containing Ca^{2+}. After equilibration of uterine smooth muscle in normal Locke-Ringer solution for 1 h at a resting tension of 0.5g, contraction was induced by changing the medium to K^+ rich solution containing 45.6 mM K^+. Isometric tension was monitored with a force displacement transducer. Myosin phosphorylation was determined for individual strips frozen after indicated times of stimulation (indicated with the arrows). Unphosphorylated myosin (M), the myosin with one mono-phosphorylated 20 kDa light chain (MP1) and the myosin with two mono-phosphorylated 20 kDa light chains (MP2) were separated in the order of increasing mobility on PPi PAGE. The pattern of protein on the PPi PAGE disk gel was visualized by staining with 0.1% Coomassie brilliant blue R-250. The band represented by "F" was filamin. To the right of M, MP1 and MP2 is schematically shown how myosin is phosphorylated at 20 kDa light chain, which was deduced from the results of urea-glycerol PAGE analysis of each myosin band separated on the PPi PAGE (figures not shown).

Fig.2. Analysis of the phosphorylation state of myosin by PPi PAGE during oxytocin-induced contraction in the medium containing Ca^{2+}. After 1 h at a resting tension of 0.5g, contraction was induced by the addition of 10^{-2} unit/ml oxytocin. See"Fig.1 legend" for details.

band (MP1) was the myosin with one unphosphorylated 20 kDa light chain and the lower band (MP2) was the myosin with two mono-phosphorylated 20 kDa light chains, deduced from the results of subsequent analysis of excised band from PPi PAGE disk gel by urea-glycerol PAGE (data not shown). After 60 sec the phosphorylation state of these two bands increased. Approximately 40% of myosin were the phosphorylated form at this time as expressed by the percent of total myosin.

In the presence of Ca^{2+}, oxytocin (10^{-2} unit/ml) induced a tonic contraction at early stage followed by a pendular contraction as shown in Fig.2. Phosphorylation of myosin was detected as early as 5 sec after the addition of oxytocin and a time-dependent increase of MP1 and MP2 bands was observed up to 60 sec. These results indicate the involvement of myosin phosphorylation in the contractions induced by high K^+ (Fig.1) and oxytocin (Fig.2) in the presence of Ca^{2+} and also confirm the general agreement that the initiation of smooth muscle contraction requires myosin light chain phosphorylation (4).

Phosphorylation of myosin during oxytocin-induced Ca-free contraction was then examined by PPi PAGE. Oxytocin induced sustained contraction with a maximal tension of about 0.2g which was about one-tenth of normal contraction (Fig.3). At any indicated times of 5, 15,

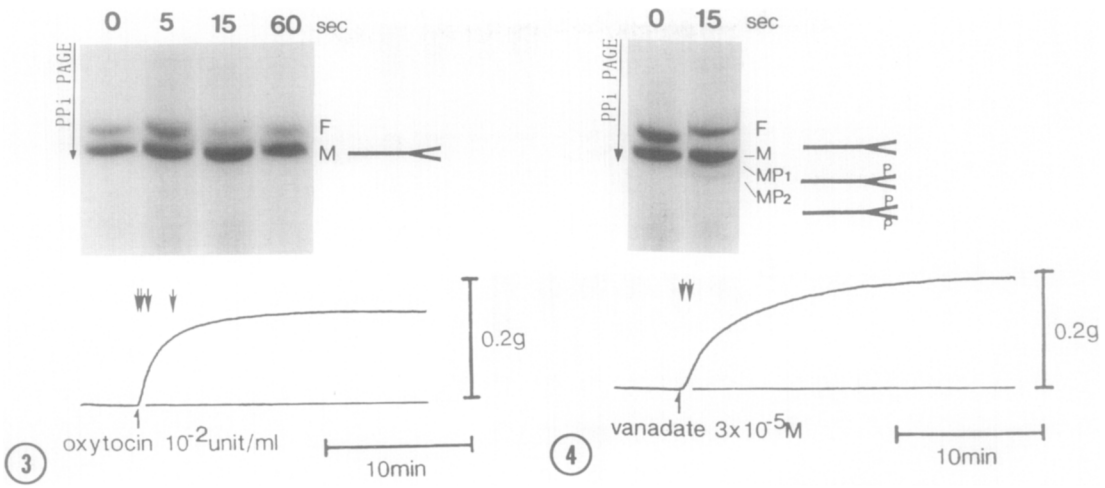

Fig.3. Analysis of the phosphorylation states of myosin by PPi PAGE during oxytocin-induced contraction in the Ca^{2+}-free medium. After "EGTA treatment" contraction was induced by the addition of 10^{-2} unit/ml oxytocin. See "Fig.1 legend" for details.

Fig.4. Analysis of the phosphorylation state of myosin by PPi PAGE during vanadate-induced contraction in the Ca^{2+}-free medium. After "EGTA treatment" contraction was induced by the addition of 10^{-5} M vanadate. See "Fig.1 legend" for details.

and 60 sec, we failed to detect phosphorylated myosin bands such as MP1 and MP2. These results indicate that Ca-free contraction occured without any phosphorylation of myosin light chain.

We next examined the phosphorylation of myosin during vanadate-induced contraction in Ca^{2+}-free medium. Vanadate ($3x10^{-5}$ M) induced sustained contraction in a similar manner (Fig.4). Although the mechanism of action of vanadate in contracting uterine smooth muscle is still obscure, the vanadate-induced contraction seems to be a result of the phosphorylation of myosin light chains by MLCK, because a MLCK inhibitor KT-5926 (5) inhibits the contraction induced by $3x10^{-5}$ M vanadate in Ca^{2+}-free medium).[2] In contrast to the case of oxytocin-induced Ca-free contraction, small but significant MP1 and MP2 bands were observed on PPi PAGE during $3x10^{-5}$ M vanadate-induced sustained contraction (Fig.4).

Finally, urea-glycerol PAGE was employed to analyze phosphorylation state of 20 kDa myosin light chain. The phosphorylation state of the myosin light chain was increased in a time-dependent manner in an early stage of contractions induced by oxytocin and high K^+ in the presence of Ca^{2+} (Fig.5A). The phosphorylated myosin light chain was also observed during vanadate-induced contraction in the absence of Ca^{2+} (Fig.5B, f). However, no phosphorylated 20 kDa myosin light chain was observed during oxytocin-induced Ca-free contraction (Fig.5B, b, c, and d). These results confirmed the above observations obtained by PPi PAGE analysis and also indicated that oxytosin induced Ca-free contraction without causing phosphorylation of 20 kDa myosin light chain.

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2 Karibe,H. and Uchida,M.K., unpublished observations.

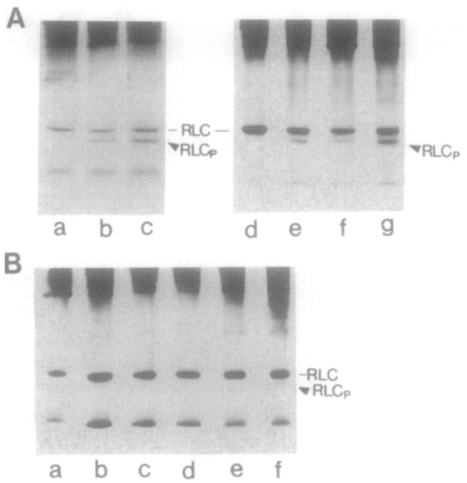

Fig.5. Analysis of the phosphorylation state of 20 kDa myosin light chain by urea-glycerol PAGE during contractions. The PPi PAGE samples prepared as described in "EXPERIMENTAL PROCEDURES" were subjected to PPi PAGE for 3 h to separate and recover whole myosin in the sample. The myosin excised from the PPi PAGE gel was treated with the urea solution. Unphosphorylated (RLC) and monophosphorylated (RLCp) forms of the light chain were separated as a function of increasing mobility in urea-glycerol PAGE. The pattern of urea-glycerol PAGE gel was visualized by the silver staining. (A) Urea-glycerol PAGE showing the state of 20 kDa myosin light chain phosphorylation during contractions in the presence of Ca^{2+}. Muscles were frozen at 0 sec (lane a), 15 sec (lane b), and 60 sec (lane c) after changing to K^+ rich (45.6 mM) solution and at 0 sec (lane d), 5 sec (lane e), 15 sec (lane f), and 60 sec (lane g) after the addition of 10^{-2} unit/ml oxytocin. (B) Urea-glycerol PAGE showing the state of 20 kDa myosin light chain phosphorylation during contractions in the absence of Ca^{2+}. Muscles were frozen at 0 sec (lane a), 5 sec (lane b), 15 sec (lane c), and 60 sec (lane d) after the addition of 10^{-2} unit/ml oxytocin and at 0 sec (lane e) and 15 sec (lane f) after the addition of 10^{-5} M vanadate.

DISCUSSION

PPi PAGE and urea-glycerol PAGE to analyze myosin phosphorylation states in contracting uterine smooth muscle were employed by following reasons. Firstly, small amount (1-2 μg) of myosin molecule can be analyzed by PPi PAGE from small tissue samples without any purification. (7,8). Secondly, neither phosphorylation nor dephosphorylation of regulatory myosin light chain occurs during PPi PAGE analysis (7,8). And lastly, the mobility of smooth muscle myosin on PPi PAGE increases only when the myosin is phosphorylated by MLCK (7,8,14,15), whereas the increased mobility of myosin light chain on urea-glycerol PAGE reflects increased negative charge as a result of phosphorylation. In this study, the phosphorylated myosin bands (MP1 and MP2) were evident on PPi PAGE during the contraction by oxytocin and high K^+ in the presence of Ca^{2+} and vanadate-induced contraction in the absence of Ca^{2+} (Figs.1, 2, and 4), indicating the involvement of phosphorylation by MLCK in these processes. Phosphorylation of myosin light chain during oxytocin-induced Ca-free contraction, however, was not detected in both PPi and urea-glycerol PAGE systems (Fig.3 and 5B). To our best knowledge, this is the first report showing that oxytocin can induce contraction without causing phosphorylation of myosin light chain.

In Ca^{2+}-induced contraction systems as shown in Figs.1, 2, and 5, it is obvious that increased $[Ca^{2+}]_i$ induces activation of MLCK followed by phosphorylation of myosin light chain. As a result, the formation of crossbridges between actin and phosphorylated myosin initiates muscle contraction. But in oxytocin-induced Ca-free contraction, this is not the case because no myosin phosphorylation is observed. This finding is also supported by the previous observation by using fluoresent Ca^{2+} indicator fura-2 that Ca-free contraction of rat uterus is independent of the rise in $[Ca^{2+}]_i$ (3). Sensitization of Ca^{2+}-induced contraction by agonist stimulation is a possibility that can account for the above phenomenon. This mechanism is probably mediated by GTP-binding protein in various smooth muscles (16,17) and uterine smooth muscle.[3] Such mechanism is likely to result in myosin phosphorylation, although small, to initiate contraction even with much smaller increase of $[Ca^{2+}]_i$ than its usual increase (18). However, this is also unlikely in oxytocin-induced Ca-free contraction since no phosphorylation of myosin light chain is observed.

Besides receptor agonist, a phorbol ester TPA, at a concentration to activate protein kinase C, clearly caused Ca-free contraction in rat thoracic aorta (19). It has been reported by others that TPA contracts some vascular smooth muscles even under Ca^{2+}-free conditions (20,21). Although no phosphorylation of myosin light chain was observed in this study, protein kinase C phosphorylates various functional proteins including contractile and cytoskeletal proteins (22). Indeed, Ca-free contraction was inhibited by protein kinase C and cytoskeletal inhibitors (6). Protein kinases (but not MLCK) and cytoskeletal elements might be actually involved in Ca-free contraction. Further study is need to be done.

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