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Myosin light chain phosphorylation and Mn^{2+}-dependent norepinephrine-induced contractions in guinea-pig vas deferens

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Abstract

We have reported that norepinephrine but not K^+ induced a sustained and dose-dependent contraction without extracellular Ca^{2+} and Mn^{2+} in Ca^{2+}-depleted Mn^{2+}-loaded vas deferens from the guinea-pig. In the present study, we determined the phosphorylation of the 20-kDa myosin light chain and examined the effects of inhibitors of calmodulin and myosin light chain kinase on the Mn^{2+}-dependent norepinephrine-induced contraction in order to evaluate the contribution of phosphorylation to this contraction. W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide], ML-9 [1-(5-chloronaphthalene-1-sulfonyl)-homopiperazine] and wortmannin inhibited this contraction. However, the Mn^{2+}-dependent norepinephrine-induced contraction developed without a significant increase in the phosphorylation of the 20-kDa myosin light chain. In β-escin-permeabilized preparations, Mn^{2+} induced contractions that were inhibited by ML-9. These results suggest that the activation of myosin light chain kinase is essential for the development of Mn^{2+}-dependent norepinephrine-induced contractions and that the phosphorylation of myosin light chain may act as a trigger for these contractions. © 1998 Elsevier Science B.V.

Keywords: Vas deferens; (Guinea pig); Myosin light chain phosphorylation; Norepinephrine; Mn^{2+}

1. Introduction

Ca^{2+}-dependent 20-kDa myosin light chain phosphorylation has been shown to be a dominant pathway for the regulation of contractions in smooth muscle (Kamm and Stull, 1989). The rise in cytoplasmic Ca^{2+} and the consequent activation of myosin light chain kinase triggers both phosphorylation and contractions. Various agonists shift the activity balance of myosin light chain kinase to phosphatases (Gong et al., 1992; Somlyo et al., 1995) and consequently change the relationships between intracellular Ca^{2+} concentration and either phosphorylation level or force (Defeo and Morgan, 1985; Sakata et al., 1989). In addition to such regulatory systems, a myosin light chain phosphorylation-independent mechanism activated by certain agonists has been proposed (Karaki, 1995). Hoar and Kerrick (1988) also reported that Mn^{2+} induced contractions independently of the phosphorylation of 20-kDa myosin light chain in chicken gizzard skinned preparations. They speculated that the development of such contractions was due to oxidization of contractile proteins by Mn^{2+} and/or direct activation of actomyosin ATPase.

We found that contractions induced by norepinephrine and acetylcholine, but not K^+, in Ca^{2+}-depleted preparations of the guinea-pig vas deferens were restored by intracellular accumulation of Mn^{2+} (Mn^{2+} loading, Tsunobuchi and Gomi, 1990; Tsunobuchi-Ushijima and Gomi, 1996a). These agonist-induced contractions were of the sustained type, and their magnitudes were comparable to those of the tonic component of contractions induced by the agonists in preparations without Ca^{2+} depletion and Mn^{2+} loading. It had been shown that the buffering or extrusion processes for Ca^{2+} are not completely inhibited by intracellular Mn^{2+} in Mn^{2+}-loaded preparations (Tsunobuchi-Ushijima and Gomi, 1996a). If the contractions induced in the preparations after Ca^{2+} depletion and Mn^{2+} loading (Mn^{2+}-loaded preparations) were due to contaminating Ca^{2+} remaining after Ca^{2+} depletion, the contractions would not be reproducible. However, the norepinephrine-induced contractions of the preparations were repeatedly induced without extracellular Mn^{2+} and Ca^{2+} even after removal of sarcoplasmic reticulum functions with cyclopiazonic acid, ryanodine or A23187 (Tsunobuchi-Ushijima et al., 1996b). Thus, we concluded that

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Mn^{2+} but not Ca^{2+} supported these contractions (Mn^{2+}-dependent norepinephrine-induced contractions).

In this study, we evaluated the phosphorylation of the 20-kDa myosin light chain in order to evaluate the contribution of phosphorylation to the Mn^{2+}-dependent norepinephrine-induced contractions. Further, we examined the effects of inhibitors of calmodulin and myosin light chain kinase on the Mn^{2+}-dependent norepinephrine-induced contractions in intact preparations and the Mn^{2+}-induced contractions in β-escin-permeabilized preparations of the guinea-pig vas deferens.

2. Materials and methods

2.1. Normal and Mn^{2+}-loaded preparations of isolated vas deferens from guinea-pigs

Male Hartley guinea-pigs of 350–550 g were stunned and killed by cervical dislocation. The studies reported in this manuscript were carried out in accordance with the Guide for the Care and Use of Laboratory Animals in the Takara-machi Campus of Kanazawa University which was defined on the basis of Notification No. 6 by the Prime Minister's Office, Standards Relating to the Care and Management etc. of Experimental Animals.

The vasa deferentia were excised and the serous membrane was stripped away. A strip from 8 to 10 mm in length was prepared from the epididymal portion of the isolated vas deferens. Each strip was incubated in aerated Hepes–Locke–Ringer solution (normal medium) of the following composition (in mM): NaCl, 154; KCl, 5.6; $MgCl_2$, 2.1; $CaCl_2$, 2.2; glucose, 2.8; Hepes, 4.4, adjusted to pH 7.4 at 30°C with 1 M NaOH. For Ca^{2+}-free medium, $CaCl_2$ was omitted from the normal medium and Na_2EGTA was added at a final concentration of 0.2 mM. After a 1 h equilibration, contraction was induced by 10 μM norepinephrine in the normal medium. Subsequently the strips were incubated in Ca^{2+}-free medium to deplete Ca^{2+} and then Mn^{2+} was loaded as previously described (designated as Mn^{2+}-loaded preparations, Tsunobuchi-Ushijima and Gomi, 1996a). Briefly, after the disappearance of norepinephrine-induced contractions in Ca^{2+}-free medium, the preparations were exposed to Mn^{2+} (2.1 mM) for 90 min. During this period, K^+ (100 mM) was applied for 5 min every 15 min. Preparations without Ca^{2+} depletion and Mn^{2+} loading were designated as normal preparations.

2.2. Determination of 20-kDa myosin light chain phosphorylation

Normal and Mn^{2+}-loaded preparations were frozen by immersion in an acetone-dry ice slurry containing 10% trichloroacetic acid and 10 mM dithiothreitol at 0, 0.5 and 5 min after the application of norepinephrine or K^+. Myosin light chain phosphorylation was determined as described by Seto et al. (1990). Briefly, myosin was extracted from the strips by 14 h incubation in sample buffer containing 20 mM Tris base, 23 mM glycine, 10 mM dithiothreitol, 8 M urea and 0.1% bromophenol blue, pH 8.6. The phosphorylated and unphosphorylated myosin light chains were separated by urea–glycerol polyacrylamide gel electrophoresis (Persechini et al., 1986), blotted to Clear Blot Membrane P® (ATTO Co., Japan) and labeled with mouse anti-myosin light chain immunoglobulin M (IgM). The separated myosin light chains were visualized using biotinylated goat anti-mouse immunoglobulin G (IgG) and streptavidin–alkaline phosphatase. The area of each myosin light chain band was determined with a scanning densitometer. The extent of myosin light chain phosphorylation was expressed as a percentage of total myosin light chain.

2.3. Measurement of mechanical responses in intact preparations

Isotonic contractions induced by norepinephrine in normal and Mn^{2+}-loaded preparations were recorded at a 12.5-fold magnification using an isotonic transducer (TD-112S, Nihon Kohden, Japan). Unless otherwise stated, each inhibitor was applied 30 min before the application of norepinephrine.

2.4. Measurement of mechanical responses in β-escin-permeabilized preparations

A small strip (100–150 μm wide and 1 mm long) of longitudinal muscle from guinea-pig vas deferens was treated for 30 min with 100 μM β-escin and 10 μM A23187 in a relaxing solution containing (in mM): K^+-methanesulfonate, 89.9; Mg^{2+}-methanesulfonate, 6.94; Na_2ATP, 4.92; EGTA, 2.0; creatine phosphate, 10; PIPES, 30 (pH 6.8, 25°C). In activating solution, the concentration of EGTA was increased to 4 mM and appropriate amounts of Ca^{2+}-methanesulfonate or $MnCl_2$ were added. The apparent binding constants of EGTA for Ca^{2+} and Mn^{2+} were considered to be 1.0×10^6 and 4×10^7 M^{-1} at pH 6.8, respectively (Itoh et al., 1982). To prevent deterioration of the cation-induced contractions, calmodulin (0.5 μM) was added to the medium throughout the experiments. Contractile force was recorded isometrically with a strain gauge (UL-2GR, Minebea, Japan).

After permeabilization, 32 μM Ca^{2+}, the concentration at which Ca^{2+} induced the maximal contraction in the preparations, was applied for 2 min to induce a 'standard contraction'. Then, either 1 μM Ca^{2+} or 32 μM Mn^{2+} was applied. During a sustained phase of the cation-induced contraction, 10 μM GTP and 100 μM norepinephrine were applied cumulatively. The magnitude of the additional contraction induced by norepinephrine was measured 10 min after the application of norepinephrine. In

some experiments, 10 μM GTPγS was applied instead of GTP and norepinephrine. ML-9 was added to the bathing medium 30 min before applying the divalent cations. The interval between contractions was 40 min.

2.5. Other

The results are expressed as mean values ± S.E.M. Student's paired and unpaired *t*-tests were used for statistical analysis. $P < 0.05$ was considered significant.

The drugs applied were ML-9 [1-(5-chloronaphthalene-1-sulfonyl)-homopiperazine] (Biomol Research Lab., USA), biotinylated goat anti-mouse IgG (Biosource, USA), A23187 (Calbiochem, USA), EGTA (Dojindo Lab., Japan), manganese chloride (Nacalai Tesque, Japan), streptavidin–alkaline phosphatase (Oncogene Science, USA), W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide] (Research Biochemicals International, USA), calmodulin, β-escin, GTP, GTPγS, mouse anti-myosin light chain IgM, (−)-norepinephrine bitartrate and wortmannin (Sigma Chemical Co., USA), creatine phosphate and Na_2ATP (Wako Pure Chemical Industries, Japan). All other chemicals were of the highest reagent grade available. ML-9 and wortmannin were dissolved to a concentration of 10 mM in 50 and 100% ethanol, respectively. The final concentration of ethanol in the bathing medium was less than 0.2%. A23187 and β-escin were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in the medium was less than 0.1%. At these concentrations, the vehicles did not affect the contractions. All other agents were dissolved in deionized and distilled water.

3. Results

3.1. The effects of inhibitors of calmodulin and myosin light chain kinase on norepinephrine-induced contractions in normal and Mn^{2+}-loaded preparations

Norepinephrine (10 μM) induced a small transient contraction followed by a large transient and a sustained contraction in normal preparations, whereas it induced only a sustained contraction in Mn^{2+}-loaded preparations. The magnitude of the sustained phase of the Mn^{2+}-dependent norepinephrine-induced contraction reached about 80% of that of the contraction in normal preparations (Fig. 1A). W-7 inhibited the sustained phases of norepinephrine (10 μM)-induced contractions of both normal and Mn^{2+}-loaded preparations in a dose-dependent manner and abolished them at 100 μM (Fig. 2).

ML-9 (10, 30 μM) and wortmannin (1 μM) also inhibited these contractions (Figs. 3 and 4). The inhibitory effect of ML-9 on the contractions induced by norepinephrine in Mn^{2+}-loaded preparations was significantly greater than that on normal preparations. Wortmannin applied 30

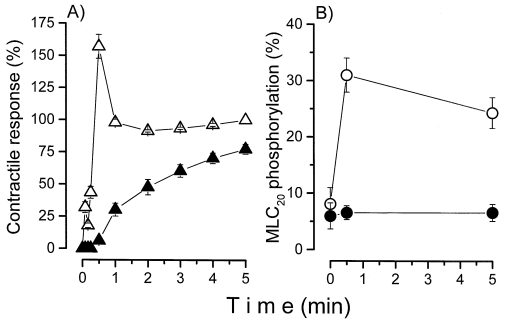

Fig. 1. Time course for the contractions and for the phosphorylation of 20-kDa myosin light chain (MLC_{20}) induced by 10 μM norepinephrine in normal and Mn^{2+}-loaded preparations from guinea-pig vas deferens. (A) Contractions induced by norepinephrine in normal (△) and Mn^{2+}-loaded preparations (▲). These contractions were recorded in the same preparations, i.e. before and after Mn^{2+} loading. Each point is expressed as a percentage of the sustained phase (at 5 min) of the norepinephrine-induced contraction in normal preparations. Vertical bars show S.E.M. ($n = 8$). (B) The phosphorylation of 20-kDa myosin light chain in normal (○) and Mn^{2+}-loaded preparations (●). Each point is a percentage of the total amount of 20-kDa myosin light chain. Vertical bars show S.E.M. ($n = 6$–7).

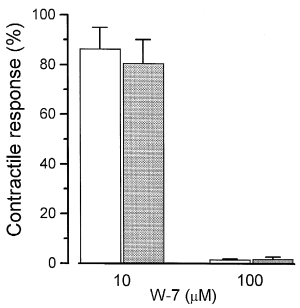

Fig. 2. The effects of W-7 (10, 100 μM) on the sustained phase of contractions induced by norepinephrine (10 μM) in normal (open column) and Mn^{2+}-loaded (shaded column) preparations. W-7 was applied 30 min before the application of norepinephrine. Norepinephrine-induced contractions were recorded for 5 min. Each result was expressed as a percentage of the sustained phase of the control contraction induced by norepinephrine (10 μM) in normal and Mn^{2+}-loaded preparations before application of W-7, respectively. Vertical bars show S.E.M. ($n = 3$–6).

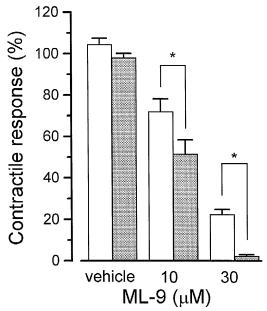

Fig. 3. The effects of ML-9 (10, 30 μM) on the sustained phase of contractions induced by norepinephrine (10 μM) in normal (open columns) and Mn^{2+}-loaded (shaded columns) preparations. ML-9 was applied 30 min before the application of norepinephrine. The vehicle was 0.15% ethanol. Each result was expressed as a percentage of the sustained phase of the control contractions induced by norepinephrine (10 μM) in normal and Mn^{2+}-loaded preparations before application of the inhibitor, respectively. Vertical bars indicate S.E.M. ($n = 6$–10).

min before the application of norepinephrine inhibited the sustained phases of the contractions, 5 min after the application of norepinephrine, to 10.2 ± 1.7% ($n = 5$) and 19.9 ± 5.5% ($n = 4$), respectively, in normal and Mn^{2+}-loaded preparations (Fig. 4A). When the inhibitor was applied 5 min after norepinephrine, the sustained phases of the contractions were slowly decreased and were inhibited to 37.7 ± 3.4% ($n = 5$) and 78.6 ± 5.6% ($n = 4$) of the initial values 30 min after the application of wortmannin in normal and Mn^{2+}-loaded preparations, respectively (Fig. 4B). Thus, wortmannin had a greater inhibitory effect when applied before contractions than when applied during the sustained phases of the contractions, in particular, in Mn^{2+}-loaded preparations (Fig. 4).

3.2. Relation between 20-kDa myosin light chain phosphorylation and contractions induced by norepinephrine in normal and Mn^{2+}-loaded preparations

The level of 20-kDa myosin light chain phosphorylation did not increase during Mn^{2+}-dependent norepinephrine-induced contractions, whereas the level of phosphorylation increased significantly within 30 s after the application of norepinephrine in normal preparations (Fig. 1B). The increase was slightly reduced 5 min after the application of norepinephrine.

There was a correlation between the phosphorylation level of 20-kDa myosin light chain and sustained contractions induced by norepinephrine (3.2–100 μM) in normal preparations, when determined in the sustained phase of contractions (5 min after the application of norepinephrine). In contrast, there was no correlation between these factors in Mn^{2+}-loaded preparations. Norepinephrine induced Mn^{2+}-dependent contractions without a significant increase in phosphorylation (Fig. 5).

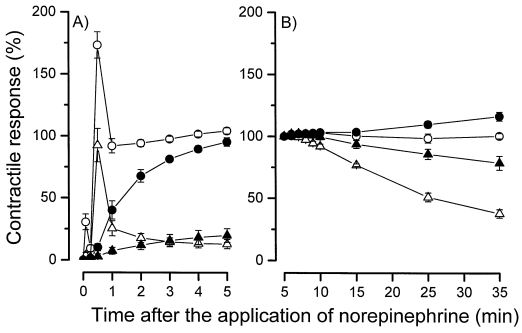

Fig. 4. The effects of wortmannin (1 μM) on the contractions induced by norepinephrine (10 μM) in normal (open symbols) and Mn^{2+}-loaded (solid symbols) preparations. (A) Wortmannin (triangles) or vehicle (circles) was applied 30 min before the application of norepinephrine. Each result was expressed as a percentage of the sustained phase of the control contractions induced by norepinephrine (10 μM) in normal and Mn^{2+}-loaded preparations respectively before application of the inhibitor. (B) Wortmannin (triangles) or vehicle (circles) was applied 5 min after the application of norepinephrine (during the sustained phase). Each result is expressed as a percentage of the sustained phase just before the application of the inhibitor. The vehicle was 0.1% ethanol. The abscissa shows the time after the application of norepinephrine. Vertical bars indicate S.E.M. ($n = 4$–6).

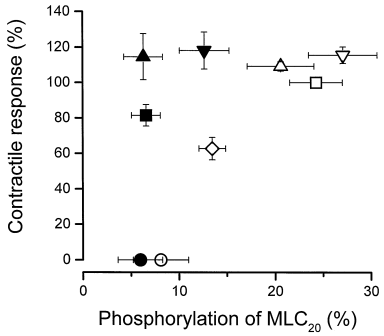

Fig. 5. The relation between the phosphorylation of 20-kDa myosin light chain (MLC_{20}) and the contraction induced by norepinephrine in normal and Mn^{2+}-loaded preparations from guinea-pig vas deferens. The magnitudes of phosphorylation and contractions were determined during the sustained phase of contractions, or 5 min after the application of norepinephrine. The phosphorylation levels were expressed as percentages of the total amount of 20-kDa myosin light chain. The contraction magnitudes were expressed as percentages of the sustained phase (at 5 min) of the control contraction induced by 10 μM norepinephrine in normal medium before Ca^{2+} depletion. Open and solid symbols show the results in normal and Mn^{2+}-loaded preparations, respectively (○●, without norepinephrine; ◇, 3.2 μM; □ ■, 10 μM; △ ▲, 32 μM; ▽ ▼, 100 μM norepinephrine). Horizontal and vertical bars show S.E.M. ($n = 6$–12).

3.3. Enhancement of Ca^{2+}- and Mn^{2+}-induced contractions by GTP, norepinephrine and GTPγS in β-escin-permeabilized smooth muscle of guinea-pig vas deferens

Both 1 μM Ca^{2+} and 32 μM Mn^{2+} induced sustained contractions with magnitudes 31.9 ± 3.5% ($n = 7$) and 33.9 ± 4.7% ($n = 11$) of the standard contraction induced by 32 μM Ca^{2+}, respectively. The development rate of Mn^{2+}-induced contractions was slower than that of the contractions induced by Ca^{2+}. GTP (10 μM) increased the Ca^{2+}- and Mn^{2+}-induced contractions by 56.6 ± 7.1% ($n = 7$) and 71.1 ± 6.4% ($n = 11$) of each primary cation-induced contraction, respectively. Subsequently added norepinephrine (100 μM) induced a further sustained contraction. The magnitudes of the norepinephrine-induced additional contractions were 70.4 ± 16.5% ($n = 7$) and 88.5 ± 6.6% ($n = 11$) of the primary Ca^{2+}- and Mn^{2+}-induced contractions. Neither GTP nor norepinephrine could induce contractions when the concentration of Mn^{2+} was lower than its own threshold for induction of a contraction.

GTPγS (10 μM) also increased the Ca^{2+}- and Mn^{2+}-induced contractions by 43.9 ± 7.0% ($n = 10$) and 93.3 ± 7.7% ($n = 10$), respectively.

3.4. The effect of ML-9 on the contractions induced by cations and agonists in β-escin-permeabilized preparations

ML-9 (100 μM) inhibited both Ca^{2+}- and Mn^{2+}-induced contractions by about 70% of the initial level. The

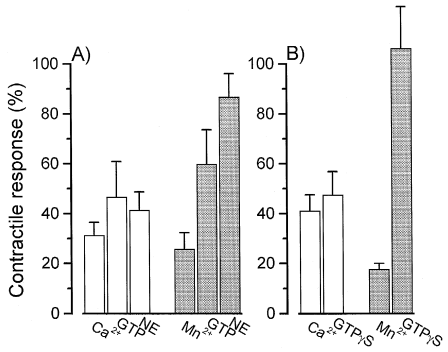

Fig. 6. The effects of ML-9 (100 μM) on contractions in β-escin-permeabilized preparations from guinea-pig vas deferens. After recording the control contraction induced by cumulative application of cations and agonists, ML-9 was applied for 30 min, and then contractions were elicited again in the presence of ML-9. The magnitude of the tension change induced by each stimulant applied cumulatively was measured at the plateau level (10 min after the application of stimulants), and was expressed as a percentage of the corresponding increase in tension of the control contraction. (A) GTP (10 μM) and norepinephrine (NE, 100 μM) were applied cumulatively during a plateau of the cation-induced contraction. (B) GTPγS (10 μM) was applied during a plateau of the cation-induced contraction. Open columns, contractions induced by 1 μM Ca^{2+}; shaded columns, contractions induced by 32 μM Mn^{2+}. Vertical bars show S.E.M. (A: $n = 6$, B: $n = 5$).

increases in the cation-induced contractions caused by GTP, norepinephrine and GTPγS were also attenuated by ML-9. The inhibitory effect of ML-9 on the additional contractions induced by these agonists with Ca^{2+} was similar to that on the primary Ca^{2+}-induced contraction. However, the inhibition of the additional contractions induced by the agonists with Mn^{2+} was significantly weaker than that of the primary contraction (Fig. 6). The sustained components of the additional Mn^{2+}-induced contractions by norepinephrine and GTPγS were resistant to ML-9.

4. Discussion

W-7, ML-9 and wortmannin inhibited Mn^{2+}-dependent norepinephrine-induced contractions as well as norepinephrine-induced contractions in normal preparations. These results suggest that activation of myosin light chain kinase through calmodulin may be involved in the Mn^{2+}-dependent norepinephrine-induced contractions.

However, there was no correlation between the phosphorylation level of the 20-kDa myosin light chain and the magnitude of the contractions in Mn^{2+}-loaded preparations. The Mn^{2+}-dependent norepinephrine-induced contractions developed without a significant increase in phosphorylation in guinea-pig vas deferens, whereas norepinephrine-induced contractions in normal preparations were accompanied by a significant increase in the phosphorylation of 20-kDa myosin light chain. Although the highest concentration of norepinephrine (100 μM) used significantly increased the phosphorylation level in Mn^{2+}-loaded preparations, the magnitude of the stimulated Mn^{2+}-dependent contraction was double that of norepinephrine-induced contraction at a similar level of phosphorylation in normal preparations.

The absence of an increase in phosphorylation during Mn^{2+} dependent norepinephrine-induced contraction conflicted with the observation that the inhibitors of calmodulin and myosin light chain kinase suppressed the contraction. Mn^{2+} activates the catalytic subunit of protein phosphatase 1 (Chu et al., 1996). However, it is unlikely that dephosphorylation progressed during the measurement of phosphorylation, because immediate immersion of the preparation into the acetone-dry ice slurry containing trichloroacetic acid stopped the phosphorylation and dephosphorylation processes. Alternatively, it is likely that the phosphatase activated by Mn^{2+} might rapidly dephosphorylate the 20-kDa myosin light chain during contractions. A significant increase in phosphorylation could not be detected 30 s after the application of norepinephrine to Mn^{2+}-loaded preparations. At that time the magnitude of the Mn^{2+}-dependent norepinephrine-induced contraction was less than 10% and the contractions that developed subsequently were also not associated with phosphorylation. However, these contractions were sensitive to the suppression of myosin light chain kinase activation. These results indicate that the role of phosphorylation may be as an activation trigger for these contractions, or as an initiator for the formation of attached cross-bridges.

This mechanism may be explained by means of the latch bridge model proposed by Murphy (1994) or the positive cooperative model proposed by Vyas et al. (1992). According to the former model, the slow dissociation rate of an attached cross-bridge or a latch bridge, which is formed by dephosphorylation of an activated cross-bridge, contributes to maintaining the sustained phase of contractions. According to the latter model, although a dephosphorylated cycling cross-bridge is formed without previous phosphorylation of the 20-kDa myosin light chain, the phosphorylated myosin light chain regulates this formation. Thus, myosin light chain kinase directly and indirectly regulates both phosphorylated and dephosphorylated cross-bridge cycling and contractions can be developed at a low phosphorylation level. Both models propose that the low level of myosin light chain phosphorylation can maintain sustained contractions, although the activation of myosin light chain kinase is necessary to initiate contractions. In the present study, because wortmannin applied during the sustained phase of contractions significantly inhibited the contractions, myosin light chain must have been phosphorylated during the sustained phase. However, the inhibitory effects were much less than those of the inhibitor that was applied before initiation of the contractions, in particular, in Mn^{2+}-loaded preparations. These results suggest a contribution of dephosphorylated and/or

unphosphorylated attached cross-bridges in maintaining the contractions and the slow rate of the inhibition may reflect slow dissociation of the cross-bridges. The activation of phosphatase by Mn^{2+} may accelerate dephosphorylation of cross-bridges, resulting in the acceleration of latch bridge formation. Alternatively, Mn^{2+} may accelerate such a cooperative mechanism (Vyas et al., 1992).

Hoar and Kerrick (1988) reported that, in skinned preparations, Mn^{2+} induced two kinds of contractions independently of the phosphorylation of myosin light chain: a rigor contraction induced by the oxidation of contractile proteins and an active contraction induced by the direct activation of contractile proteins by Mn^{2+} in the presence of a reducing agent. In the present study, however, the Mn^{2+}-induced contractions of β-escin-permeabilized preparations were readily restored to the resting level in a relaxing solution without a reducing agent. This suggests that Mn^{2+} does not induce contractions by the oxidation of contractile proteins in the permeabilized preparations of guinea-pig vas deferens. The result, that the rate of development of Mn^{2+}-induced contractions was slower than that of Ca^{2+}-induced contractions was also different from their observations. Thus, the Mn^{2+}-induced contractions that they showed were different from ours. Further, ML-9 inhibited equally Ca^{2+}- and Mn^{2+}-induced contractions in this study. This result suggests that Mn^{2+} as well as Ca^{2+} induced a contraction through the phosphorylation of the 20-kDa myosin light chain in the present preparations.

In β-escin-permeabilized preparations, Mn^{2+} and Ca^{2+}, at the concentration used in this study, induced contractions of a similar magnitude. GTPγS, GTP and norepinephrine increased both Mn^{2+} and Ca^{2+} sensitivity and induced contractions additional to the primary cation-induced contractions. As mentioned above, ML-9 inhibited equally Ca^{2+}- and Mn^{2+}-induced contractions. However, it inhibited the additional Ca^{2+}-induced contractions more strongly than the additional Mn^{2+}-induced contractions. The magnitudes of the inhibitory effect of ML-9 on the additional Ca^{2+}-induced contractions were similar to those of the effect on the primary Ca^{2+}-induced contractions. These findings suggest that myosin light chain kinase plays important roles in these cation-induced contractions and Ca^{2+} sensitization, but is less involved in Mn^{2+} sensitization. Karaki (1995) described similar observations of Ca^{2+} sensitization induced by GTPγS in permeabilized preparations. He suggested that part of the Ca^{2+}-sensitizing effect was not due to myosin light chain phosphorylation and that GTP-binding protein is involved in this phosphorylation-independent mechanism. Mn^{2+} and agonists may activate such a mechanism under the conditions of the present experiments. However, this notion is inconsistent with the effectiveness of the inhibitors of myosin light chain kinase in intact preparations. Although the reason for this discrepancy is not clear, some factors regulating cation sensitivity may be lost from permeabilized preparations and it is likely that the high concentration of Mn^{2+} (32 μM) may activate an alternative mechanism. In β-escin-permeabilized preparations, norepinephrine-induced Mn^{2+} sensitization could not be observed when the concentration of Mn^{2+} was lower than its own threshold for inducing a contraction. Thus, there remains the possibility that the phosphorylation of myosin light chain is also essential for Mn^{2+} sensitization induced by GTP and norepinephrine in skinned preparations.

In summary, although inhibitors of calmodulin and myosin light chain kinase inhibited both types of contractions, Mn^{2+}-dependent norepinephrine-induced contractions developed without a significant increase in the phosphorylation of 20-kDa myosin light chain. These results suggest that the activation of myosin light chain kinase is essential for the development of Mn^{2+}-dependent norepinephrine-induced contractions and that the phosphorylation of myosin light chain may act as an activation trigger for these contractions, or as initiator for the formation of attached cross-bridges.

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