Contraction of Smooth Muscle in Ca-free Solution

Faramarz ASHOORI, Hiroyoshi HIDAKA*, Akira TAKAI
and Tadao TOMITA

Department of Physiology, School of Medicine, Nagoya University,
Nagoya 466, and *Department of Pharmacology, School of
Medicine, Mie University, Tsu 514, Japan

Abstract

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— The tonic contractions which are extremely resistant to removal of the external Ca were investigated in the rat vas deferens and myometrium. Both the nora-drenaline response in the vas deferens and the oxytocin response in the myometrium could be repeatedly produced without appreciable diminution in Ca-free solution for more than 24 hrs. On the other hand, the tissue Ca content decreased exponentially after Ca-removal with a half time of 130-180 min. When Ca was readmitted, no indication of the early transient contraction was observed in the subsequent response in Ca-free solution, but the response was reduced compared with the response before Ca readmission. Verapamil suppressed the response in the presence of Ca, while it had very weak inhibitory effect even at 10 μ M. Calmodulin antagonists of phenothi-azine derivatives had a strong inhibitory effect on Ca-induced contractions, whereas they had very weak effects on the receptor-mediated contraction in Ca-free solution. Another calmodulin antagonist, W-7 suppressed both Ca-induced contraction and the contractions independent of external Ca. HA-1004, a vasodilator which has a struc-ture similar to W-7, reduced the receptor-mediated contraction in Ca-free solution without much effect on Ca-induced contractions. These results may suggest that the receptor-mediated contractions resistant to Ca-removal are caused by some process without a contribution of the Ca-calmodulin system.

Introduction

The contraction of muscles, including smooth muscle, is produced by interaction between actin and myosin filaments and this interaction is controlled by Ca ions. In most of smooth muscle, the contraction disappears when Ca^{2+} is removed from the external medium, suggesting an important role of Ca^{2+} influx. However, the rate of disappearance differs to some extent

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ファラマル アシュリー, 日高弘義*, 高井 章, 富田忠雄

名古屋大学医学部 第一生理学教室, 〒466 名古屋市昭和区鶴舞町 65

*三重大学医学部 薬理学教室, 〒514 津市江戸橋 2-174

in various types of smooth muscles. After removal of the external Ca, a transient contraction is often observed and this is interpreted to be due to release of Ca from the intracellular store (Brading & Sneddon, 1980; Casteels & Droogmans, 1981; Casteels & Raeymaekers, 1979; Deth & Van Breemen, 1977; Droogmans *et al.*, 1977; Hurwitz, 1975).

On the other hand, a small tonic contraction, which is extremely resistant to Ca-removal has recently been reported in some smooth muscles (vascular smooth muscle: Casteels *et al.*, 1981; Heaslip & Rahwan, 1982; myometrium: Sakai *et al.*, 1980, 1981, 1982; Mironneau *et al.*, 1984; Ashoori *et al.*, 1985; vas deferens: Ashoori & Tomita, 1983; stomach: Golenhofen *et al.*, 1982). These contractions are also generally explained by intracellular Ca release, but a possibility remains that they are produced by some Ca-independent process (Casteels *et al.*, 1981). In the present experiments, the underlying mechanism for the contraction observed in Ca-free solution was further analysed in the noradrenaline response of rat vas deferens and the oxytocin response of the non-pregnant rat myometrium.

Methods

Wister rats (200–250 g) were stunned and bled. Nonpregnant rats of metestrus stage were usually selected by examining the vaginal smear, because of large responses in Ca-free solution. After isolating the vasa deferentia and the uterus from male and female rats, respectively, longitudinal muscle strips about 1 mm wide and 7 mm long were dissected out. The preparation was mounted vertically in an organ bath of 1 ml capacity and isometric tension development was measured with a strain gauge and recorded on a potentiometric pen-recorder. The preparation was superfused at a constant rate of 2 ml/min. The normal solution had the following composition (mM): NaCl 136.9, KHCO₃ 5.9, CaCl₂ 2.4, MgCl₂ 1.2, glucose 11.8. When Ca was omitted, Ca was isosmotically replaced with NaCl and 2 mM EGTA was added. In order to produce a contraction by readmission of Ca, the excess K (40 mM) solution was used: NaCl 106.3, KCl 34, KHCO₃ 5.9, MgCl₂ 1.2, glucose 11.8 and EGTA 2. For readmission of a low concentration of Ca, EGTA was removed 10 min before the addition of Ca. When the pH of the solution was changed, 10 mM 3-(N-morpholino) propane sulphonic acid (MOPS) (Grover *et al.*, 1983 a) or a mixture of Tris, maleic acid and acetyl glycine, 2 mM each (Yatani & Goto, 1983) was used as the buffer.

The tissue Ca contents were measured by atomic absorption spectrophotometer (Hitachi 208), after digesting the tissues with HNO₃ and HClO₂ at 100°C for 3–6 hrs. For ⁴⁵Ca efflux experiments, the tissues (50–100 mg) were loaded with solution containing $1.5\text{--}5.1 \times 10^{-8}$ M ⁴⁵Ca for 22–24 hrs at room temperature (20–23°C). ⁴⁵Ca efflux was obtained by sequentially passing the preparations at 10–20 min intervals through a series of scintillation vials and the radioactivity was measured with the Aloka mini-scintillation counter. All experiments were carried out at 35°C.

Results

1. Mechanical response to noradrenaline in Ca-free solution in the rat vas deferens

The mechanical response to noradrenaline (NA) differed in the epididymal and prostatic sides of the vas deferens, as previously observed (Ashoori & Tomita, 1983). In the presence of

Fig. 1. Mechanical responses to noradrenaline in Ca-free solution in the rat vas deferens and effect of Ca readmission. a: control response to noradrenaline (NA, $100\mu\text{M}$); b-d: NA responses in Ca-free solution at 20 min intervals. e and g: NA responses 10 min after 0.5 and 1 mM Ca readmission, respectively; f and h: 20 min after removal of Ca. NA application (4 min) is indicated by horizontal bar below each record. Note different calibration for tension in (a) and in others (b-h).

Ca (2.4 mM), early large phasic contractions were followed by a sustained tonic contraction in the epididymal part, while a slow monophasic response with some twitches during an early phase was observed in the prostatic part. In Ca-free solution, the phasic contraction and twitches were completely abolished, but a tonic contraction was still produced by NA, particularly in the preparations taken from the epididymal part. The threshold concentration of NA to produce the response was similar ($0.5\mu\text{M}$) in the presence and absence of Ca. The response in Ca-free solution was essentially similar when the external K concentration was increased to 40 mM .

When NA ($100\mu\text{M}$) application for 4 min was repeated at 20 min intervals in Ca-free solution, the response gradually increased during first 1-2 hrs after Ca-removal and then reached to a more or less constant size (Fig. 1b-d). In lower records of Fig. 1, effects of Ca readmission are shown. In the presence of 0.5 mM Ca, NA produced small phasic contraction superimposed on the tonic response (e). The next response (f) obtained 20 min after Ca-removal was smaller than the 3rd response in Ca-free solution (d) and similar to the response observed 20 min after the first Ca-removal (b). Readmission of 1 mM Ca had roughly the same effect on the subsequent response in Ca-free solution (h).

When NA application was resumed after a prolonged interval, but still in Ca-free solution, the first response was slightly reduced but it recovered during subsequent applications. The tension development was not apparently reduced during continuous perfusion with Ca-free solution containing 2 mM EGTA for more than 20 hrs, as shown in Fig. 2, although it slowly decreased when the perfusion was extended to more than 40 hrs, as reported previously (Ashoori & Tomita, 1983).

Recovery of mechanical response following Ca readmission was slow. In the experiment shown in Fig. 3, EGTA was removed 10 min before the addition of Ca. After observing the NA response in the presence of Ca (a) and in Ca-free solution (b, c), NA was applied together with 2.4 mM Ca. This simultaneous application produced some phasic activity which was much

Fig. 2. The NA ($100\ \mu\text{M}$) responses plotted against the time of exposure to Ca-free solution in two different preparations (empty and filled circles) (vas deferens). The preparations were continuously superfused with Ca-free solution containing 2 mM EGTA.

Fig. 3. Recovery of NA response after Ca ($2.4\ \text{mM}$) readmission (vas deferens). a: control response, b and c: NA responses in Ca-free solution at 20 min intervals. d: Ca was applied simultaneously with NA. e-h: Ca application was preceded NA as indicated in each record which was taken at 20 min intervals.

smaller than the control (a). When Ca readmission was preceded, the NA response became progressively larger as the NA application was delayed, but preincubation with Ca of more than 10 min was necessary for complete recovery of the NA response.

The NA response produced in the presence of Ca had two steps in the falling phase when washed out with Ca-free solution (d-h). The second step seems to correspond to the response observed in Ca-free solution (b, c), judging from the time course. This part was also suppressed by Ca readmission was prolonged (e-h).

Fig. 4 shows effects of readmission of Ca in 40 mM K solution. Readmission of 0.1 mM Ca increased the basal tension of the preparation and gradually increased the early phase of the NA response (b, c). In the presence of 40 mM K, the Ca treatment did not significantly affect the following NA response in Ca-free solution (d), although small suppression could be observed in other preparations. When Ca concentration was increased to 0.3 mM, contraction was

Fig. 4. Effects of Ca readmission in 40 mM K solution (vas deferens). NA ($100\ \mu\text{M}$) was applied for 4 min at constant intervals of 20 min. After observing the 4th response to NA in Ca-free solution (a), EGTA was removed and 10 min later 0.1 mM Ca was added. b and c: NA response 10 min and 34 min after Ca readmission, respectively, and d: 10 min after removal of Ca. e-g: similar experiments to a-d, but 0.3 mM Ca was readmitted in another preparation. In (g), Ca was removed and 2 mM EGTA was added 8 min after NA application and 8 min later NA was applied.

quickly developed by Ca itself and NA produced a large transient contraction which increased with the time of exposure to Ca (f, g). There was a slow contraction following wash-out of NA. The NA response observed 8 min after Ca-removal (g) was similar to that produced before the Ca treatment (e).

Some phenothiazine derivatives are known to have an antagonistic action on calmodulin. They strongly suppressed the NA response, but this is likely resulting from a blockade of an alpha-adrenergic receptor (Takayanagi, 1964; Cocks *et al.*, 1981). Another type of calmodulin antagonist, N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide (W-7) suppressed both the NA response in Ca-free solution and the contraction evoked by readmission of Ca (Ashoori & Tomita, 1983). A vasodilative compound, N-(2-guanidinoethyl)-5-isoquinoline sulphonamide (HA-1004) has a similar chemical structure to W-7, but it is known to lack anticalmodulin action (Asano & Hidaka, 1984). HA-1004 markedly reduced the NA response in Ca-free solution, while it had a weak suppressing effect on Ca-induced contractions as shown in Fig. 5. There was a slow recovery in the Ca-induced contraction, but a very poor recovery in the NA response.

Fluoride ion (F^-) is known to produce contraction in the absence of the external Ca in vascular smooth muscle (Fermum *et al.*, 1977; Casteels *et al.*, 1981), vas deferens (Ashoori *et al.*, 1983) and myometrium (Mironneau *et al.*, 1984). F^- slowly increased the basal tension and potentiated the NA response at concentrations between 3 and 10 mM (Fig. 6e-h). W-7 nearly completely abolished the NA response at 0.1 mM without affecting the contracture caused by F^- .

Fig. 5. Effects of HA-1004 on NA response in Ca-free solution and Ca-induced contraction (vas deferens). NA (4 min) and 0.5 mM Ca (6 min) were alternately applied at 20 min intervals. b and c: the 4th response to NA and Ca, respectively. HA compound was applied at 10 μ M, increase to 100 μ M and washed out 10 min before NA application at (d), (f), and (h), respectively. Records were taken in the alphabetical order.

Fig. 6. Effects of fluoride (F) and calmodulin antagonist, W-7 (vas deferens). After observing 3 successive responses to NA at 20 min intervals, 3 mM F was applied and increased to 10 mM before NA application at (e) and (g), respectively. 0.1 mM W-7 was added 10 min before NA at (i).

Fig. 7. Effects of verapamil on contractions produced by oxytocin and 40 mM K in the rat myometrium. Oxytocin (20 nM) (a) and 40 mM K (b) were alternately applied in the presence of Ca (normal solution) at 20 min intervals in the same preparation. Verapamil was cumulatively applied from 0.1 to 10 μ M 10 min before oxytocin. Records (c) were obtained from another preparation, showing verapamil effects on the oxytocin response in Ca-free solution produced at 20 min intervals. Records of the response in Ca-free solution are shown from the 4th application of oxytocin.

2. The response of non-pregnant rat myometrium to oxytocin in Ca-free solution

The responses of myometrium to oxytocin in the presence and absence of Ca are considered to be fundamentally similar to those of the vas deferens to NA (Ashoori *et al.*, 1985; Sakai *et al.*, 1980, 1981, 1982). The oxytocin response consisted of phasic and tonic components in the presence of Ca, but only the tonic component remained in Ca-free solution in a reduced size. The response in Ca-free solution increased gradually for several hrs and reached a steady state when oxytocin application was repeated. No clear decrease in the response was observed even after more than 40 hrs perfusion with Ca-free solution containing 2 mM EGTA. However, it was suppressed by a transient readmission of Ca, as observed for the vas deferens.

A Ca channel blocker, verapamil (0.1–10 μ M), significantly suppressed the contractions produced by oxytocin (20 nM) and 40 mM K in the presence of Ca (Fig. 7). On the other hand, the oxytocin response in Ca-free solution was not affected by verapamil at 1 μ M and only slightly reduced at 10 μ M, in accord with the results on the vas deferens (Ashoori & Tomita, 1983).

Fig. 8. The rate of loss of tissue Ca content in Ca-free 40 mM K solution (myometrium). Filled circle: measured with atomic absorption spectrophotometry, and empty circle: measured with ^{45}Ca -efflux,

Fig. 9. Effects of pH on the oxytocin response (myometrium). The pH of the external solution was changed from 8 to 6 with a buffer containing 2 mM each of Tris, maleic acid and acetyl glycine titrated with NaOH. The preparation was equilibrated for 10 min at each pH before oxytocin application given at 20 min intervals. Two different preparations were used in the presence and absence of HCO_3^- separately.

The rate of loss of Ca contained in the tissue exposed to Ca-free solution containing 40 mM K and 2 mM EGTA was measured with atomic absorption spectrophotometry and ^{45}Ca -efflux. The results are shown in Fig. 8. Both methods gave much the same results; the tissue Ca decreased exponentially between 2 and 6 hrs and their half time was 130–180 min ($n=6$). These values were similar to those obtained in the vas deferens. If the rate of decrease is assumed to be constant after 6 hrs, the tissue Ca content after 24 hrs exposure to Ca-free medium is estimated to be approximately $0.3 \mu\text{moles/kg}$ wet weight, which is critical for contraction.

The plasma membrane of rat myometrium and rabbit aortic muscle has a high-affinity Ca-binding protein. This binding is pH-dependent, being reduced at low pH (Grover *et al.*, 1983a, b). At pH 7.3, the rabbit aorta is contracted by NA in Ca-free solution, but pretreatment at pH 5.1 reduces the contractions, probably due to depletion of Ca at the binding sites (Grover *et al.*, 1983a). As shown in Fig. 9, the oxytocin response decreased as the pH of the external solution was reduced. This tendency was much more stronger when HCO_3^- was omitted from the solution (g–j). However, the recovery from acidic treatment was nearly perfect and some

Fig. 10. Effects of HA-1004 on the myometrium. a and b: contractions produced alternately by oxytocin and 40 mM K, respectively, in the presence of 2.4 mM Ca (normal solution). c: oxytocin responses in another preparation in normal and Ca-free solution. The responses in Ca-free solution are shown from the 4th oxytocin application. HA compound was cumulatively applied from 1 to 10 μ M 10 min before oxytocin.

potentiation was transiently observed when pH was raised again from 6 to 8 (e, k).

The oxytocin response of myometrium was resistant to calmodulin antagonists, such as chlorpromazine, trifluoperazine and fluphenazine which are derivatives of phenothiazine, while the Ca-induced contraction was markedly reduced by these compounds (Ashoori *et al.*, 1985). However, W-7 suppressed both the oxytocin response in Ca-free solution and Ca-induced contraction, although the recovery was better for the Ca-induced contraction. Fig. 10 shows effects of HA-1004 on the contractions produced by oxytocin (a), 40 mM K (b) in the presence of 2.4 mM Ca and the oxytocin response in Ca-free solution (c). The HA compound had very weak effects on the phasic contractions but reduced the tonic component slightly in the solution containing Ca. On the other hand, the oxytocin response in Ca-free solution was very strongly suppressed (c).

Discussion

Both the response to Na in the vas deferens and the response to oxytocin in the myometrium can be produced repeatedly in Ca-free solution for more than 40 hrs. Measurements of the Ca content of the tissue indicate that Ca is being reduced exponentially in Ca-free solution, and the estimated Ca content from the time course of Ca loss becomes too low for contraction after exposure to Ca-free solution for more than 24 hrs. The response observed in Ca-free solution is not increased, but actually reduced, by short readmission of Ca. These results are difficult to explain that the receptor-mediated contraction is due to release of intracellular Ca.

In vascular and some gastrointestinal smooth muscles, a transient contraction can be evoked by their appropriate agonists after removal of the external Ca. Since these contractions decrease with the time of exposure to Ca-free solution, Ca released from a limited intracellular source is believed to be responsible for the response (Brading & Sneddon, 1980; Casteels & Droogmans, 1981; Deth & Van Breemen, 1977; Droogmans *et al.*, 1977). However, the present experiments on the vas deferens and myometrium of the rat revealed no such transient response suggestive of the intracellular Ca release. It may be argued that filling of intracellular Ca may be very slow, because Ca-dependent contractions recovers very gradually (Fig. 3). However, the contraction which is independent of the external Ca is readily suppressed by a short Ca-readmission. Furthermore, even in excess K solution, a phasic contraction cannot be produced in the subsequent response to NA 8 min after Ca removal, even after producing a large transient contraction by NA in the presence of Ca (Fig. 4).

In the rabbit aortic smooth muscle, NA-sensitive intracellular Ca pool is depleted when the preparation is pretreated by Ca-free solution at low pH (5.1) (Grover *et al.*, 1983a). This is interpreted that the plasma membrane binds a large amount of Ca with high affinity at pH 7.3 but Ca-binding protein releases Ca at low pH. However, in the present experiments, no evidence for depletion of intracellular Ca was found. Although the response to oxytocin was strongly reduced by acidification, a good recovery was observed by raising pH again in the absence of Ca (Fig. 9). In some smooth muscles, caffeine is known to release Ca from the same intracellular store as for receptor-mediated contractions (Deth & Casteels, 1977; Deth & Lynch, 1981; Itoh *et al.*, 1981, 1982, 1983). Caffeine markedly reduces both the NA response in the vas deferens and the oxytocin response in the myometrium in Ca-free solution but the recovery is perfect in the absence of Ca (Ashoori & Tomita, 1983; Ashoori *et al.*, 1985). The suppression of caffeine has been explained by an increase in cyclic AMP resulting from phosphodiesterase inhibition. Thus, these results also throw some doubt about the contribution of intracellular Ca release.

Since the solubility of CaF_2 is low, the contraction induced by F^- is considered to be due to a Ca-independent process (Casteels *et al.*, 1981). F^- may activate the same process as for the receptor-mediated contraction in Ca-free solution, because it potentiates the NA response in the vascular muscle (Casteels *et al.*, 1981) and vas deferens (Ashoori & Tomita, 1983), and the oxytocin response in the myometrium (the present experiment). However, W-7 blocks only the oxytocin response without affecting F^- -induced contraction. Furthermore, in the rat

myometrium, isoprenaline fails to reduce the contraction produced by F^- while it completely relaxes the preparation contracted by acetylcholine or prostaglandin E_2 in Ca-free solution (Mironneau *et al.*, 1984). Thus, it seems that the underlying mechanism of the F^- -induced contraction differs from that of the receptor-mediated contractions which are resistant to Ca-removal.

Contraction of the smooth muscle is thought to be mediated by phosphorylation of myosin light chains and this phosphorylation occurs when light chain kinase is activated by Ca binding to calmodulin which is a subunit of the kinase (Hartshorne & Siemankowski, 1981). Therefore, the effect of calmodulin antagonists is crucial in clarifying the contribution of Ca to the contraction which is very resistant to Ca-removal. However, calmodulin antagonists probably have many side effects. Strong suppressing effects of phenothiazine derivatives on the NA response in the vas deferens is probably due to an α -receptor blocking action (Takayanagi, 1964; Cocks *et al.*, 1981). In the myometrium, these drugs selectively reduce the Ca-induced contraction. It may be argued that the drugs are blocking the Ca channel at the plasma membrane (Karaki *et al.*, 1982). However, the fact that the drugs have very weak effect on the contraction in Ca-free solution may still suggest that this contraction is independent of the Ca-calmodulin system, provided that intracellular drug concentrations are reasonably increased.

W-7 is reported to be a highly selective antagonist of calmodulin, although it acts also on several protein kinase (Asano *et al.*, 1982; Hidaka *et al.*, 1981). This compound suppresses both the Ca-induced contraction and the receptor-mediated contraction in Ca-free solution. A similar compound, HA-1004 which lacks calmodulin antagonistic effect (Asano & Hidaka, 1984) reduces the receptor-mediated contraction in Ca-free solution much strongly than the Ca-induced contraction. The site of action of HA-1004 has to be clarified, but it is possible that W-7 may exert the suppressing effect on the process in common with HA-1004, in addition to its calmodulin antagonistic action. Then, there is no contradiction to the idea that the receptor-mediated contraction observed in the present experiments is mediated by some process independent of the Ca-calmodulin system.

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