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POSSIBLE INVOLVEMENT OF ACTOMYOSIN ADP COMPLEX IN REGULATION OF Ca^{2+} SENSITIVITY IN α-TOXIN PERMEABILIZED SMOOTH MUSCLE

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SUMMARY: The effects of substrate condition and ADPβS on the pCa^{2+}-tension relationships were investigated, using α-toxin permeabilized rabbit mesenteric artery at 37°C. The contraction induced by 10 μM Ca^{2+} solution after permeabilization was as large as that induced by 145 mM K^+ PSS solution containing 10 μM NE in the intact tissue, indicating that the majority of the cells were permeabilized. The Ca^{2+} sensitivity was greatly affected by the substrate condition and increasing the ratio of ATP/CP induced a leftward shift of the pCa^{2+}-tension curve. Addition of 100 μM ADPβS had a similar effect. When the ATP/CP ratio was high, the 0.1 μM Ca^{2+} solution relaxed the tissue precontracted by 10 μM Ca^{2+} solution more slowly showing hysteresis. One mM vanadate, which is reported to relax muscle by forming actomyosin-ADP-Vi (AM-ADP-Vi), completely inhibited both contractions induced by 0.18 μM Ca^{2+} solution containing 2 mM MgADP and 0.3 μM Ca^{2+} solution containing 0.3 μM PDBu. These results indicated that the population of AM-ADP complex in the crossbridge had increased due to the accumulation of ADP inside the tissue or activation of PKC and that the inhibition of ADP release from AM-ADP complex may be playing a key role in increasing Ca^{2+} sensitivity of myofilaments.

Muscle contraction is thought to occur as the result of a cyclic association and dissociation of crossbridges formed between myosin and actin molecules at the expense of hydrolysis of ATP. Some aspects of energy metabolism are similar in smooth and skeletal muscle, but smooth muscle is characterized by its ability to maintain tension at a low cost of ATP hydrolysis (1). It is postulated that not only the number of cycling crossbridges but also their cycling rate can be regulated in smooth muscle (2). However, the mechanisms underlying this high economy and the regulation of crossbridge cycling rate are not well understood. It is well known that smooth muscle contraction is regulated by intracellular [Ca^{2+}] and [Ca^{2+}] dependent myosin light chain (MLC) phosphorylation (3). However, a state in which tension is maintained while shortening velocity, MLC phosphorylation and Ca^{2+} levels are low has been described and termed "latch" state (4-6).

ABBREVIATIONS
ADP; adenosine diphosphate, ADPβS; adenosine-5'-O-(2-thiodiphosphate), AM-ADP; actomyosin-ADP, [Ca^{2+}]; Ca^{2+} concentration, CP; creatine phosphate, CSS; cytoplasmic substitution solution, EGTA; ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid, GTPγS; guanosine 5-(3-O-thio) triphosphate, HMM; heavy meromyosin, MLC; myosin light chain, NE; norepinephrine, PDBu; phorbol 12,13 dibutyrate, Pi; phosphate, PKC; protein kinase C, PSS; phisiological saline solution, Vi; vanadate.

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Recently Hai and Murphy (7, 8) concluded that MLC phosphorylation is both necessary and sufficient for development of the latch state, assuming that latch bridges are formed by dephosphorylation of an attached cross bridge. Accordingly, the function of MLC phosphatase as well as MLC kinase has been promoted as important regulatory mechanisms for smooth muscle contraction (7-10). On the other hand, recent biochemical studies have revealed that the Ca^{2+}-sensitive protein, caldesmon, which inhibits actomyosin ATPase activity at low Ca^{2+} concentration (11), can increase the affinity of the smooth muscle myosin for actin filaments (12). Caldesmon is now being regarded as one of the strongest candidates for a second Ca^{2+} regulatory system which may explain the mechanism of "latch" state (13).

The approach of permeabilizing smooth muscle tissues by staphylococcal α-toxin has provided further evidence that receptor stimulation, GTPγS, and phorbol esters do increase the Ca^{2+} sensitivity of contractile elements (14-16). In α-toxin permeabilized tissues, the intracellular regulatory proteins are retained and experiments can be performed at 37°C (16), which is more physiological and was not possible in detergent skinned smooth muscle fibers. This α-toxin treated muscle model should be helpful in exploring the mechanisms underlying the regulation of Ca^{2+} sensitivity and perhaps the regulation of crossbridge cycling rate. We report herein that merely changing the substrate condition, namely changing the concentration of ATP and/or CP, can cause a dramatic change of the Ca^{2+} sensitivity of the α-toxin permeabilized rabbit mesenteric artery. The data showed that increasing the population of AM-ADP complex by the accumulation of ADP inside the fiber (or in the crossbridge) may be the cause of this Ca^{2+} sensitivity change. The possible role of caldesmon inhibition of ADP release from the AM-ADP complex in the regulation of Ca^{2+} sensitivity and crossbridge cycling rate has been discussed.

MATERIALS AND METHODS

Small rings (about 250 μm diameter and about 300 μm long) from the third or forth degree branch of the superior mesenteric artery from New Zealand white rabbit were prepared in physiological saline solution (PSS which contained in mM : NaCl 140, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1, glucose 10, HEPES 5, pH= 7.2) under binocular microscope, and two tungsten wires (40 μm diameter, California Fine Wire Company) were passed through the lumen. One wire was fixed to the chamber and the other was attached to a force transducer(U gage, Sinko Co. Ltd, Tokyo, Japan). After recording the maximal intact contraction by 145 mM K^+ PSS (NaCl was substituted for KCl in PSS) with 10 μM NE, Staphylococcal α-toxin (30 μg protein/ml; α-toxin was a generous gift from Dr. R.J. Hohman, NIH) was applied for 10 minutes in a 2 mM EGTA, 0 M Ca^{2+}-containing cytoplasmic substitution solution (CSS). This experimental CSS solution contained 2 mM EGTA, 130 mM K propionate, $MgCl_2$ 3.1 mM, 2 mM Na_2ATP, 20 mM creatine phosphate, 20 mM Tris maleate (pH = 6.8), and indicated concentrations of free Ca^{2+}. The apparent binding constant used for the Ca^{2+}-EGTA complex was 10^6/M. The concentrations of ATP and CP in the CSS solution were changed as indicated in the Figures or Figure legends. In case of changing the ATP concentration, the $MgCl_2$ concentration was also changed as to keep the Mg^{2+} concentration 1.5 mM, using the binding constant 4 x 10^3 for MgATP complex. All tension experiments were carried out at 37°C.

RESULTS

Fig. 1A shows the contraction induced by 145 mM K^+ PSS solution plus 10 μM NE in the intact tissue. The amplitude of this contraction was nearly the same as that of the 10 μM Ca^{2+}-

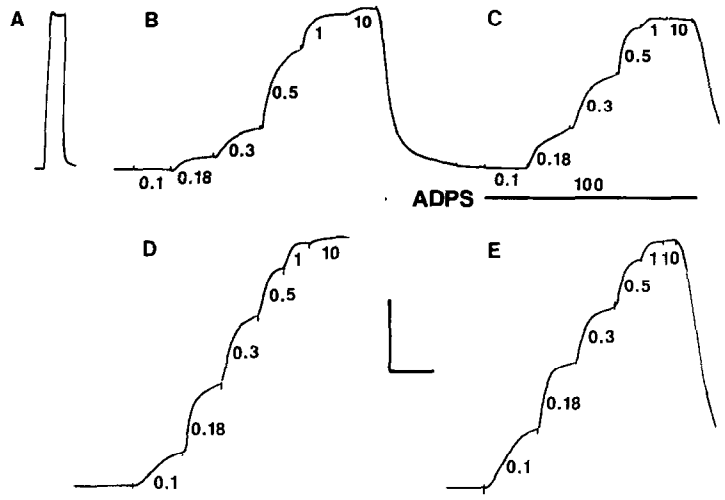


Figure 1.
Effect of substrate condition (B, D, E) and ADPβS (C) on the pCa^{2+}-tension relationship.
A. Contraction of the intact tissue induced by 145 mM K^+ PSS solution containing 10 μM NE.
B. pCa^{2+}-tension relationship in the control condition (2 mM ATP, 20 mM CP, and 1.5 mM Mg^{2+}).
C. Effect of 100 μM ADPβS (ADPS) on pCa^{2+}-tension relationship in the control condition.
D. pCa^{2+}-tension relationship under the condition of 8 mM ATP, 20 mM CP, and 1.5 mM Mg^{2+}.
E. pCa^{2+}-tension relationship under the condition of 8 mM ATP, 10 mM CP, and 1.5 mM Mg^{2+}.
The numbers illustrated under the traces are the concentration of Ca^{2+} and ATPβS in μM. Vertical and horizontal calibrations represent 50 mg and 5 min, respectively. Experiments were done at 37°C. A, B and C were done in the same tissue. D and E were done in tissues different from each other and also different from A, B, and C. Traces shown are representative of 3 experiments.

induced contraction after α-toxin permeabilization (Fig. 1B), indicating that the majority of cells are permeabilized by α-toxin. Fig. 1 B-E shows the change of Ca^{2+} sensitivity due to changing substrate conditions. Fig. 1B shows the pCa^{2+}-tension relationship in the control condition (2 mM ATP, 20 mM CP, and 1.5 mM Mg^{2+}). If the ATP concentration was raised to 8 mM, while CP and Mg^{2+}concentrations were unchanged (20 mM and 1.5 mM, respectively), the pCa^{2+}-tension relationship was shifted to the left (Fig. 1D). If the increase of Ca^{2+} sensitivity observed in Fig. 1D is due to the rise in ATP concentration, one would expect that the Ca^{2+} sensitivity should be decreased by lowering the CP concentration, since less ATP will be regenerated from ADP and CP catalyzed by endogenous creatine phosphokinase. However, as shown in Fig. 1E, reduction of the CP concentration from the condition described in Fig. 1D (20 mM) to 10 mM, while other components were kept the same, caused a further leftward shift of the pCa^{2+}-tension curve. From these results, we considered that the leftward shift of the pCa^{2+}-tension curve might be due to the accumulation of ADP. In other words, CP functions not only to regenerate ATP but also to reduce the ADP concentration. This hypothesis was confirmed in Fig. 1C, where 100 μM ADPβS, a nonhydrolyzable ADP analog, caused a similar leftward shift of the pCa^{2+}-tension curve, while the concentrations of ATP and CP were unchanged (2 mM and 20 mM, respectively).

The protocols illustrated in Fig. 2 were designed to further show the effect of substrate condition and ADPβS on the contraction induced by a fixed Ca^{2+} concentration. In Fig. 2A, the contraction induced by 0.18 μM Ca^{2+} was recorded under various substrate conditions, while Mg^{2+}

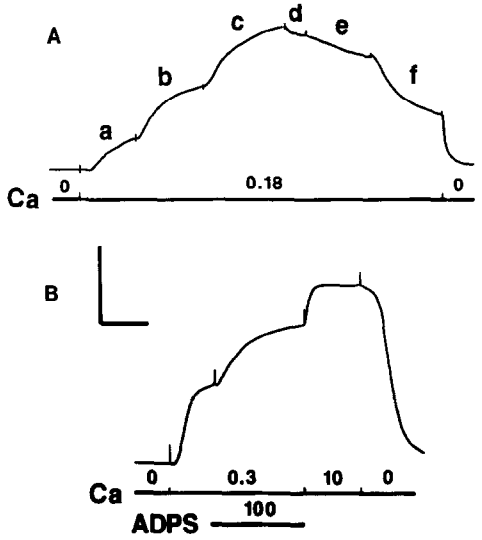


Figure 2.
Effect of substrate condition and ADPβS on the contraction induced by a fixed Ca^{2+} concentration.
A. Contraction induced by 0.18 μM Ca^{2+} solution under various substrate conditions, while keeping the Mg^{2+} concentration at 1.5 mM. a: 2 mM ATP, 10 mM CP. b: 4 mM ATP, 10 mM CP. c: 8 mM ATP, 10 mM CP. d: 8 mM ATP, 20 mM CP. e: 4 mM ATP, 20 mM CP. f: 2 mM ATP, 20 mM CP.
B. Effect of 100 μM ADPβS (ADPS) on the contraction induced by 0.3 μM Ca^{2+} solution.
The numbers illustrated under the traces are the concentration of Ca^{2+} and ADPβS in μM. Vertical and horizontal calibrations represent 50 mg and 5 min, respectively. Experiments were done at 37°C. Trace shown is representative of 3 experiments.

was kept constant at 1.5 mM. Although the Ca^{2+} concentration was fixed, the contractile state was significantly different under each condition. A higher contractile force could be obtained by raising the ATP concentration (Fig. 2 a, b, c) and relaxation could be induced by raising the CP concentration (d) and decreasing the ATP concentration (d, e, f). Fig. 2B shows that 100 μM ADPβS increased the contractile state induced by a 0.3 μM Ca^{2+} CSS solution containing 2 mM ATP and 20 mM CP.

We also noticed that relaxation of the fiber becomes much slower or even incomplete at higher ATP and/or lower CP concentrations, conditions under which ADP may more easily accumulate. As shown in Fig. 3, 0.1 μM Ca^{2+} CSS solution which contained 2 mM ATP and 20 mM CP did not cause contraction and rapidly relaxed the fiber to the basal level, even if it was preincubated by 10 μM Ca^{2+} CSS solution (first contraction). However, under the conditions of 4 mM ATP and 10 mM CP (second contraction in Fig. 3) or 8 mM ATP and 10 mM CP (third contraction), 0.1 μM Ca^{2+} solution only slowly relaxed the fiber precontracted by 10 μM Ca^{2+} solution. The steady state force induced by 0.1 μM Ca^{2+} solution after the 10 μM Ca^{2+} induced contraction appeared to be higher than that before the 10 μM Ca^{2+}-induced contraction. This slow relaxation and hysteresis in the latter condition may be also due to the accumulation of ADP.

Fig. 4A shows that a large contraction can be induced by a 0.18 μM Ca^{2+} solution containing 8 mM ATP and 2 mM MgADP and that this contraction was almost completely inhibited by vanadate, which is known to inhibit muscle contraction by binding to the actomyosin ADP complex (17). As we reported recently (16), PDBu induces a marked leftward shift of pCa^{2+}-tension curve,

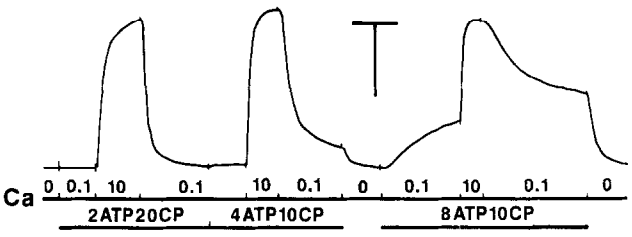


Figure 3.
Effect of substrate condition on the relaxation rate and hysteresis of the permeabilized muscle at 37°C. First, 0.1 μM Ca^{2+} solution was applied and then 10 μM Ca^{2+} solution was applied. After this contraction reached steady state, the tissue was then relaxed by re-adding 0.1 μM Ca^{2+} solution. This protocol was performed in the 2 mM ATP, 20 mM CP condition (first contraction, illustrated "2ATP20CP"), the 4 mM ATP, 10 mM CP condition (second contraction, "4ATP10CP") and in 8 mM ATP, 10 mM CP condition (third contraction, "8ATP10CP"). Ca^{2+} concentrations are given under the trace in μM. Vertical and horizontal calibrations represent 50 mg and 5 min, respectively. Traces shown are representative of 3 experiments.

in rat mesenteric artery. This effect was also observed in rabbit mesenteric artery. Fig. 4B shows a contraction induced by 0.3 μM Ca^{2+} solution containing 0.3 μM PDBu. The addition of 1 mM vanadate also completely inhibited this contraction, suggesting that the PDBu-induced increase of Ca^{2+} sensitivity might also be related to the increased population of actomyosin ADP complex within the crossbridges.

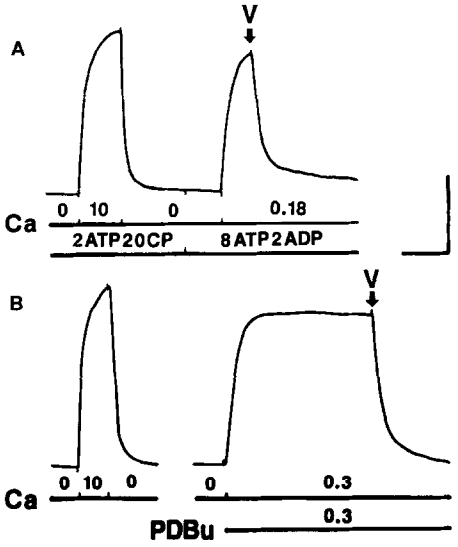


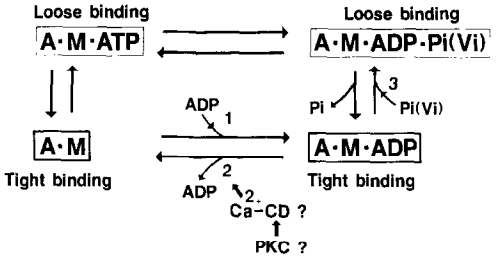
Figure 4.
Effect of 1 mM vanadate (V) on a 2 mM Mg ADP (A) and a 0.3 μM PDBu induced contraction.
A. First, a control 10 μM Ca^{2+}-induced contraction was recorded in 2 mM ATP, 20 mM CP condition. Then, 0.18 μM Ca^{2+} solution containing 8 mM ATP and 2 mM MgADP was added. After this contraction reached steady state, 1 mM vanadate (V) was applied.
B. First, a control 10 μM Ca^{2+}-induced contraction was recorded. Then, a 0.3 μM Ca^{2+} solution containing 0.3 μM PDBu was added. After this contraction reached steady state, 1 mM vanadate (V) was applied. The substrate condition was the same as control (2 ATP, 20 CP) throughout the record.
The numbers illustrated under the traces are the concentration of Ca^{2+} and PDBu in μM. Vertical and horizontal calibrations represent 50 mg and 5 min, respectively. Experiments were done at 37°C. Traces shown are representative of 3 experiments.

DISCUSSION

Studies using isolated contractile proteins have shown that actomyosin hydrolyzes ATP via a series of reactions (18-20), a simplified version of which is illustrated in scheme 1. Since AM-ADP is the force generating intermediate in the normal crossbridge cycle, this scheme predicts that increasing the concentration of ADP should increase the population of the AM-ADP complex and the tension generated at a fixed intermediate Ca^{2+} concentration.

This prediction has been verified by Ventura-Clapier et al. in the heart (21), Hoar et al. in skeletal muscle (22) and Kerrick and Hoar in smooth muscle (23). The latter authors pointed out that the ADP effect in smooth muscle would be independent of MLC phosphorylation and would slow ATP consumption and shortening velocity, properties characteristic of the "latch" state. However, millimolar concentrations of ADP were required to generate force in chicken gizzard smooth muscle permeabilized by Triton X. Thus, the reservations in explaining smooth muscle "latch" contracture on the basis of ADP accumulation were that unphysiologically high ADP concentrations were required and that this effect was not specific for smooth muscle.

Our present data show that addition of 100 μM ADPβS or increasing the ADP concentration by merely increasing the ATP/CP ratio dramatically increases the Ca^{2+} sensitivity of α-toxin permeabilized smooth muscle. It can thus be concluded that this preparation is very sensitive to ADP bringing the effective concentration (100 μM) within the range of ADP concentrations encountered under physiological conditions. Krisanda and Paul (24) reported a value of 0.27 μmol ADP/g wet weight or approximately 400 μmol/liter cytoplasm (assuming 32% extracellular space, 25) in high K^+ contracted smooth muscle, which was significantly higher than in resting muscle. Kushmerick et al. (26) chemically measured an ADP concentration of 340 μM in perfused rabbit bladder, while NMR analysis of smooth muscle [ADP] yielded a value of 0.16 μmol/g wet weight or 235 μM (27). Thus, it seems plausible to explain the hysteresis observed during K^+ stimulation and subsequent relaxation of the intact ferret aorta by DeFeo and Morgan (28) by an accumulation of ADP in the intact working muscle. However, ADP accumulation probably does not account for the difference in $[Ca^{2+}]_i$ sensitivity measured in the ferret portal vein between phenylephrine stimulation and high K^+ depolarization (28). Neither could it account for the PDBu initiated increase in Ca^{2+} sensitivity of the α-toxin permeabilized rabbit mesenteric artery under conditions which prevent ADP accumulation in the present study (Fig. 4B).



Scheme 1.
Simplified scheme of actomyosin ATPase (ref. 18-20). Abbreviations used are as follows: A: actin, M: myosin, Pi: phosphate, Vi: vanadate, CD: caldesmon, PKC: protein kinase C.

If a latch-like state can be induced by reaction "1" in Scheme 1, one would also predict that the inhibition of ADP release from the AM-ADP complex (inhibition of reaction "2" in the Scheme 1) would increase the population of AM-ADP, slow down the crossbridge cycling rate, and induce a latch-like state, regardless of the energy state of the smooth muscle. This idea has been proposed previously by Lash et al. (12) in a study of the function of caldesmon in a cell free in-vitro system. Recent biochemical studies have shown that, under low Ca^{2+} condition, caldesmon inhibits actomyosin ATPase activity (11), that this inhibition can be reversed by the addition of Ca^{2+} and calmodulin, and that the ATPase inhibition is associated with an increase in the affinity of smooth muscle heavy meromyosin (HMM) for actin filament (12, 29, 30).

Two different hypotheses have been put forward to explain how caldesmon inhibits actomyosin ATPase and causes tight binding between actin and myosin. The first holds that caldesmon inhibits release of ADP from the acto-HMM-ADP complex causing accumulation of tightly bound crossbridges and also inhibiting ATPase activity since ADP release is the rate limiting step (12). In support of this hypothesis, Marston (31) reported that caldesmon inhibits actomyosin ATPase by inhibiting the product release from the AM-ADP-Pi complex. The second hypothesis states that caldesmon cross-links actin and myosin filaments and by so doing inhibits crossbridge cycling (29,30).

Although it is clear that the exact role played by caldesmon in latch formation remains unresolved (13), the data gathered on the α-toxin permeabilized smooth muscle favor a pivotal role for the accumulation of AM-ADP. This preparation has a very high affinity for ADP and the accumulation of AM-ADP by increments of ADP increases force as predicted by *in vitro* studies (12). We have previously reported that activation of α-adrenergic receptors, G proteins or PKC increases force at constant low $[Ca^{2+}]_i$ in the α-toxin permeabilized artery (14, 16). We now report that vanadate has the same relaxing effect on permeabilized smooth muscle contracted by phorbol ester as those stimulated to contract by ADP. Vanadate is an analog of phosphate and is reported to cause relaxation by complexing with AM-ADP (reaction "3" in Scheme 1; 17). Thus, it appears that the PDBu induced contraction is also characterized by accumulation of AM-ADP. Since the effect of PDBu was observed with 20 mM creatine phosphate and 2 mM ATP, it is more likely to be due to an inhibition of ADP release than to a mass action effect of accumulated ADP.

Our data could be explained according to the first hypothesis above (see also ref. 12), if PKC mediated phosphorylation of caldesmon (32,33) decreased its sensitivity to Ca^{2+}, allowing inhibition of ADP release from the AM-ADP complex. This reaction could also be the basis for "latch" type or slowly cycling actomyosin crossbridges.

We conclude that ADP may play a key role in the observed increase in $[Ca^{2+}]_i$ sensitivity of smooth muscle and that caldesmon is a strong candidate for controlling ADP release from AM-ADP.

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