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A calcineurin-like phosphatase is required for catch contraction

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The ability of certain molluscan smooth muscles to maintain a prolonged state of contraction, termed 'catch', has been correlated with the activity of a calcineurin-like Ca^{2+}-regulated phosphatase. The release of this phosphatase through extensive treatment of fibers with detergent, as shown by Western blots and a calmodulin-binding overlay assay, results in the loss of catch tension maintenance. This effect is reversed by perfusion of the fiber with brain calcineurin. These findings suggest that the activity of the calcineurin-like phosphatase, switched on during the onset of active contraction, plays a critical role in the maintenance of catch.

Molluscan muscle; Ca^{2+}-regulated phosphatase; Muscle regulation

1. INTRODUCTION

Tonic contraction in molluscan smooth muscles is characterized by a stretch-resistant state — called catch — in which myosin crossbridges attach to actin, but cycle very slowly [1]. In detergent-skinned fiber preparations of the anterior byssus retractor muscle (ABRM) of *Mytilus edulis* — a typical catch muscle — calcium (about 10^{-5} M) induces active contraction, and its removal locks the muscle into catch. Relaxation is achieved by addition of cAMP or by perfusion into the fiber of the catalytic subunit of cAMP-dependent kinase [2–5]. These findings are consistent with the earlier discovery that serotonin, a relaxant of catch contraction in intact fibers, increases intracellular cAMP by activation of adenylate cyclase [6–8]. Taken together, these findings indicate that phosphorylation/dephoshorylation processes play a critical role in the regulation of catch in these muscles.

In contrast to previous studies, which have focused on the role of protein kinases in inducing relaxation, we have attempted to determine the state(s) of the contractile cycle in which phosphatases are involved. In the case of ABRM, these states could be either activation or catch. During activation such a phosphatase could be regulated by the level of Ca^{2+} in the cytoplasm; if, however, the phosphatase activity were switched on during catch, when the Ca^{2+} concentration is close to resting level [9], a second messenger different from Ca^{2+} would be required. Several phosphatases have recently been isolated and characterized; they have been grouped into classes according to their mode of action and regulation (for review see [10]). Ca^{2+}/calmodulin-regulated phosphatases, classified as type 2B (PP2B), have been identified in vertebrates, invertebrates and lower eukoryotes (for review see [11]). Brain calcineurin, the first of this class to be isolated, is made up of two subunits: the catalytic subunit (calcineurin A), responsible also for the binding of calmodulin (CaM) in the presence of micromolar calcium; and the Ca^{2+} binding subunit (calcineurin B) [12].

In this report we show that ABRM fibers display an accelerated relaxation of catch tension with prolonged incubation in detergent-containing solutions. The loss of catch tension maintenance has been correlated with the release from the fiber of a calcineurin-like phosphatase. Addition of calcineurin to the bathing medium restores the ability of the fiber to maintain catch tension, whereas trifluoroperazine (TFP) reversibly accelerates catch tension relaxation. Taken together, these results indicate that the activity of a Ca^{2+}-regulated phosphatase, switched on during the onset of active contraction, plays a critical role in the maintenance of catch.

2. MATERIALS AND METHODS

Calcineurin purified from bovine brain [13], polyclonal antibodies raised in rabbit to brain calcineurin [14] and CaM from bovine testis were a kind gift of Dr. Claude Klee. CaM was also purchased from Calbiochem and calcineurin from Sigma. [^{125}I]CaM was labeled according to the procedure of Klee et al. [13]. Trifluoroperazine was kindly provided by Dr. Stefano Alemá (Institute of Cell Biology, C.N.R., Rome, Italy).

Mussels were purchased at the local fish market and kept alive in aerated artificial sea water at 4°C.

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2.1. *Mechanical measurements*

Freshly dissected ABRM fiber bundles (0.6–0.8 mm in diameter) were kept in seawater for 20–30 min with the ends attached to a perspex support to allow the fibers to relax. The bundle was then attached horizontally to a tension transducer connected with a Gould 2400 recorder, as described by [5]. Prior to treatment with detergent, the bundle was activated with 0.55 μM acetylcholine (Ach) and relaxed with 0.55 μM serotonin (5HT) to verify its contractile behavior. The bundles were skinned in 20 mM EGTA, 8 mM $MgCl_2$, 5 mM MgATP, 50 mM PIPES, pH 6.8 with 0.1% saponin or 0.1–0.2% β-escin. The length of treatment with detergent varied according to the size of the bundle and the source of the animals. Solution for washing the fiber was 0.5 mM EGTA, 3 mM $MgCl_2$, 2 mM MgATP, 50 mM PIPES, pH 6.8. Various free-Ca^{2+} concentrations were obtained by changing the ratio of EGTA to Ca^{2+} as calculated by using a program kindly supplied by Dr. P.D. Chantler (The Medical College of Pennsylvania, Philadelphia, PA) [15]. Typical EGTA/Ca ratios were 0.25 in pre-activating solution (pCa~6.5) and 0.75 in activating solution (pCa~5.5). Catch was induced by removal of Ca^{2+} with 20 mM EGTA, 3 mM $MgCl_2$, 2 mM MgATP, 50 mM PIPES, pH 6.8. It should be noted that 20 mM EGTA is used to insure rapid removal of Ca^{2+} throughout the fiber. Relaxation of catch tension was obtained by adding 5.5 μM cAMP. All solutions contained 0.5 μM leupeptin and 0.1 mM DTT.

2.2. *Protein analysis*

Permeabilized ABRM bundles used for mechanical measurements were homogenized in SDS sample buffer [16] using a glass homogenizer and boiled for 5 min. The solution used for skinning the bundles and the first washing solution were pooled (a total volume of about 1 ml) and concentrated on a centricon 10 microconcentrator (Amicon) to a volume of 50–80 μl. Note that the use of microconcentrators also allowed removal of excess detergent, which otherwise interferes with SDS-PAGE.

Total ABRM extract was obtained by homogenizing the tissue in low salt buffer (40 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 0.5 μM leupeptin, 10 mM NaP, pH 7.0). Myofibrils were obtained by centrifuging the extract in a Beckman microfuge for 15 min. Both supernatant (low salt extract) and pellet (myofibrils) were analysed.

SDS-PAGE was carried out according to Laemmli [16]. Western blots were carried out as described by Towbin et al. [17]. Binding of [^{125}I]CnM was measured using the solid phase assay described by Hubbard and Klee [18].

3. RESULTS

3.1. *Effect of detergent on tension*

ABRM fiber bundles, permeabilized with 0.1% saponin in relaxing solution for 20–30 min at room temperature (20°C), display mechanical properties similar to those of intact fibers (Fig. 1) [5]. Prolonged incubation with saponin for up to 60 min, depending on the fiber's size, does not appear to affect either tension development induced by calcium, or relaxation induced by cAMP, but this treatment accelerates the rate of relaxation of catch tension. In an extensively skinned bundle, about 50% of the catch tension is quickly lost during the first 2–3 min after removal of Ca^{2+}, residual tension decays at a slower rate (Fig. 2). Control fiber bundles skinned for up to 30 min and kept relaxed, or maintained in catch for prolonged periods show only limited acceleration of catch tension decay. Control experiments show that saponin per se has no effect on the contraction cycle.

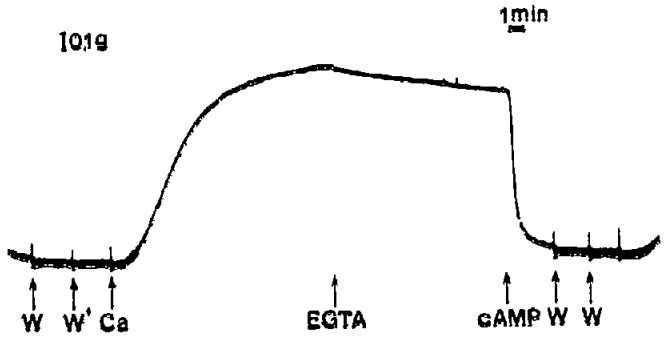

Fig. 1. Typical trace of tension development in an ABRM fiber bundle after chemical skinning with 0.1% saponin for 25 min. Arrows indicate change of solution: wash (W); pre-activating solution, pCa ~6.5 (W'); activating solution, pCa ~5.5 (Ca); catch solution (EGTA); relaxing solution (cAMP).

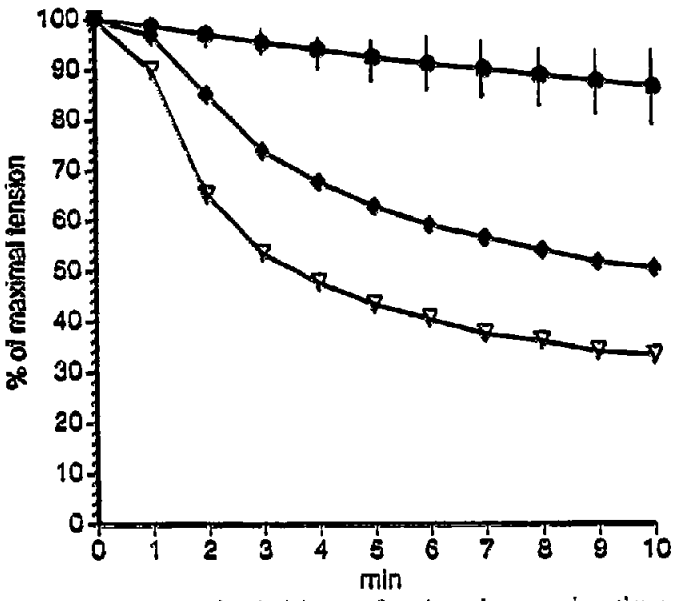

Fig. 2. Time course of typical decays of catch tension at various times of treatment with 0.1% saponin. Tension is expressed as percentage of the maximal tension developed during active contraction; (● – ●) average of four contraction cycles, two after 20 min and two after additional 40 min of treatment with detergent, using the same skinning solution; error bars indicate maximal error. (◆ – ◆) first and (▽ – ▽) second cycle after additional treatment with saponin for 30 min. Note that once acceleration of catch tension decay is observed, subsequent cycles of contraction show increased acceleration.

The protein content of the solution used to permeabilize the fiber was analysed by SDS-PAGE and compared both to skinned and intact ABRM fibers. Weak bands corresponding to myosin, paramyosin, actin, and a protein of about 19 kDa chain weight were observed in the skinning solution (Fig. 3A). Polyclonal antibodies to brain calcineurin were used to assay for the presence of a phosphatase 2B-like protein. Western blots show that anti-calcineurin antibodies cross-react with the 19 kDa band, comigrating with calcineurin B; the decreasing cross-reactivity of the antibody with the skinned ABRM bundles (Fig. 3A, lanes a* and c*) and the increasing cross-reactivity in the corresponding skin-

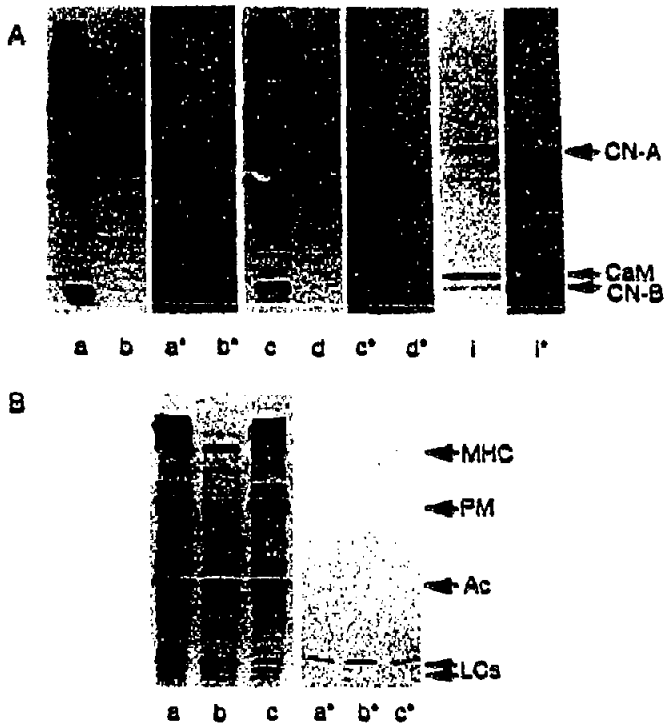

Fig. 3. SDS-PAGE (6–15%) of ABRM and corresponding Western blot with rabbit polyclonal antibody to brain calcineurin. (A) Fiber bundle (a and c) and corresponding skinning solution (b and d) of ABRM treated with 0.1% saponin for 30 min and 2 h respectively. Corresponding Western blots are shown in (a*, c*) and (b*, d*). Mixtures of calcineurin A and B (CN-A and CN-B) and calmodulin (CaM) used to rescue catch tension (i) and corresponding Western blot (i*). Antibodies to brain calcineurin show cross-reactivity with a 19 kDa band in ABRM comigrating with calcineurin B. (B) Total ABRM extract (a), soluble (b) and insoluble fraction (myofibrils) (c) at low ionic strength and corresponding Western blots (a*, b*, c*). Myosin heavy chain (MHC), paramyosin (PM), actin (Ac) and myosin light chains (LCs) are indicated. Poor reaction is observed in myofibrils (c*) in relation to the low ionic strength soluble fraction (b*).

ning solutions (Fig. 3A, lanes b* and d*) agree well with the loss of catch tension maintenance exhibited by these fibers. Since calcineurin antibodies did not cross- react with a band corresponding to calcineurin A (61 kDa), a [^{125}I]CaM binding ovelay assay was used to detect this subunit (Fig. 4): increasing amounts of CaM bound to a ~60 kDa polypeptide were observed in the skinning solutions as the time of detergent treatment increased (Fig. 4, lanes b and d), confirming the Western blot results. The presence of a calcineurin-like phosphatase in ABRM was also verified by comparing total ABRM extracts with washed myofibrils preparations (Figs. 3B and 4). Positive reactions with a 19 kDa (Fig. 3B, lanes a* and b*) and a ~60 kDa polypeptide (Fig. 4, lanes e and f) were observed in the extract and in the fraction soluble at low ionic strength. Weaker reactions were observed in the myofibril action (Fig. 3B, lane c* and Fig. 4, lane g), suggesting that this protein is highly soluble.

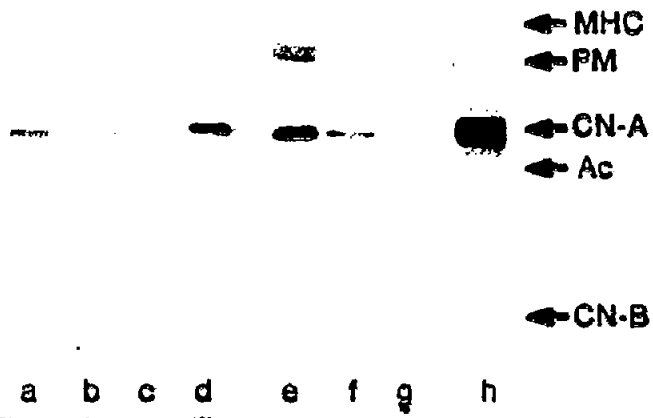

Fig. 4. Binding of [^{125}I]CaM (50 nM) to ABRM samples electrophoresed on SDS-PAGE (6–15%) and electrotransferred to 0.45 μm nitrocellulose. Fiber bundle (a and c) and corresponding skinning solution (b and d) of ABRM treated with 0.1% saponin for 30 min and 2 h, respectively. Total ABRM extract (e), soluble (f) and insoluble fraction (g) at low ionic strength. Calcineurin, purchased from Sigma (h). Binding was detected in the region of the autoradiogram corresponding to calcineurin A (~60 kDa).

3.2. *Rescue of catch tension*

ABRM fiber bundles, skinned for up to 30 min with saponin, maintain 85–90% of the maximal tension developed during active contraction after 10 min of catch. Fibers exposed to skinning solution for an additional 60–90 min, however, show activation by Ca^{2+} comparable to fibers treated for 30 min, but display poor catch tension, which is reduced to 30–50% of the maximum. In order to verify whether the loss of the calcineurin-like protein was responsible for the accelerated decay of tension, fibers which display poor catch tension were incubated with a mixture of calcineurin and CaM. (The incubation was carried out for 20 min prior to activation with Ca^{2+} to allow diffusion into the fiber). In this case, a full recovery of catch tension was obtained when the fibers still retained about 50% of the catch tension prior to incubation with calcineurin (Fig. 5A). Moreover, a recovery of catch tension to about 70% of the maximum (as compared to 85–90% of control) was obtained when calcineurin was added to fibers which display only about 30% of catch tension (Fig. 5B). Repeated incubations with calcineurin and CaM prior to each cycle of contraction maintain catch tension. Omission of the calcineurin-CaM mixture results in poor catch tension, suggesting that this phosphatase is easily released from the fiber (Fig. 5). Incubation with up to 10 μM CaM without calcineurin does not result in recovery of catch tension, suggesting that the phosphatase is required to lock the muscle into catch.

The involvement of this or a related phosphatase was further investigated using the phenothiazine tranquilizer trifluoroperazine (TFP), known to bind CaM and to inhibit its activity ([19], for review see [20]). When permeabilized ABRM bundles were incubated with increasing concentrations of TFP (0.1–0.5 mM) prior to

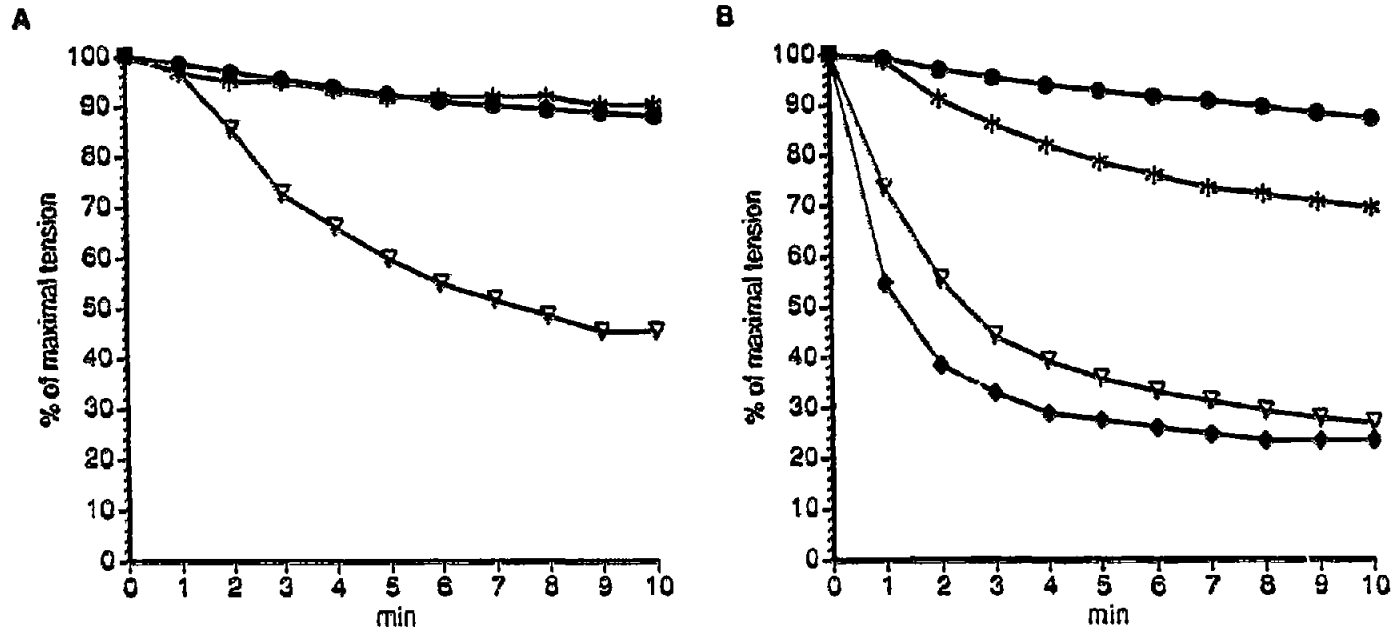

Fig. 5. Time course of catch tension in ABRM bundles incubated with 0.62 μM calcineurin and 5.5 μM CaM prior to activation by Ca^{2+}. (A) (●–●) catch tension decay after treatment with saponin for 25 min shown as control and (◆–◆) after additional 45 min of skinning; catch tension is completely regained (*–*) after incubation with calcineurin. (B) (●–●) control (average from three contraction cycles, two after 20 min and one after additional 40 min treatment with saponin); catch tension after treatment with saponin for 120 min, (◆–◆) before and (*–*) after incubation with calcineurin (average of three cycles with calcineurin; catch tension (▽–▽) in a subsequent cycle, without incubation with calcineurin.

activation (to allow the drug to diffuse into the fiber), as well as during activation by Ca^{2+}, the decay of catch tension was accelerated and only about 50% of the maximal tension was retained at high TFP concentrations (measured after 10 min of catch) (Fig. 6). Extensive washing rescued catch tension in fibers exposed to up to 0.125 mM TFP (Fig. 6A), but was ineffective in fibers incubated with higher concentrations of TFP (Fig. 6B).

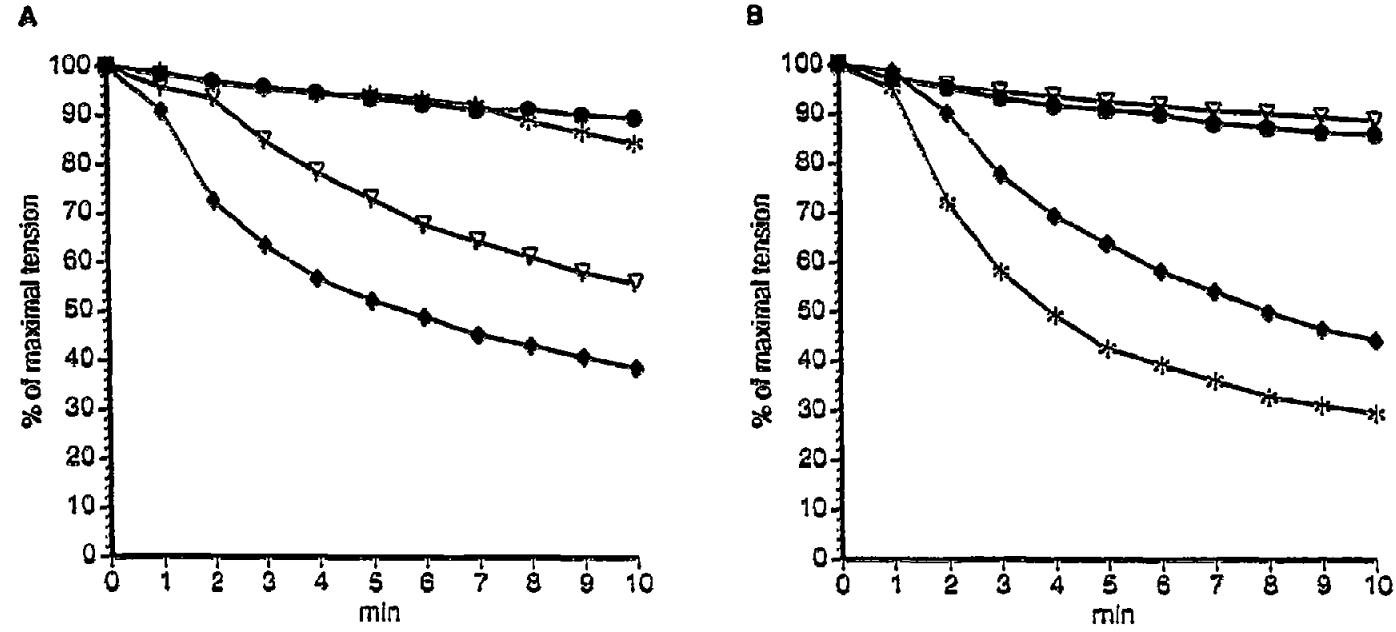

Fig. 6. Time course of catch tension in ABRM bundles incubated with increasing concentrations of TFP and 10 μM CaM. (A) (●–●) catch tension decay shown as control; (◆–◆) after incubation of the fiber with 0.125 mM TFP during active contraction and (*–*) following washing out of the drug; (▽–▽) catch tension in fiber incubated with 0.25 mM TFP. (B) catch tension (●–●) in control cycle and (▽–▽) after incubation with 0.5 mM TFP. (◆–◆) washing out TFP at this concentration is ineffective in recovering catch. Catch tension (*–*) after incubation with 10 μM CaM.

In these fibers, however, catch tension was recovered by adding CaM (10 μM), suggesting a possible involvement of this or related to Ca^{2+}-binding protein with catch maintenance.

4. DISCUSSION

The role of serotonin in relaxing catch contraction in molluscan smooth muscle has long been recognized [1,21]. The finding that the action of this neurotransmitter is mediated by the activation of adenylate cyclase, resulting in an increase of intracellular cAMP, suggested a possible role for phosphorylation in the relaxation of catch [6–8]. This hypothesis has received support from more recent studies on detergent-skinned fibers of the ABRM. In these preparations catch contraction can be relaxed by direct application of cAMP or by perfusion of the fiber with the catalytic subunit of cAMP-dependent protein kinase [2–5]. Further support for the phosphorylation hypothesis has come from in vitro studies showing that several proteins of molluscan smooth muscles can be phosphorylated, including myosin heavy chain, paramyosin and myosin light chains [5,22–27]. In addition to these thick filaments proteins, the recent identification of caldesmon in ABRM [28] has also called attention to thin filament-associated proteins as possible regulators of catch contraction. The precise target(s) of phosphorylation have not yet, however, been identified.

In this report we have approached the question of catch regulation by correlating the mechanical behavior of these muscles with the action of protein phosphatases. We show that prolonged treatment of ABRM fiber bundles with detergent induces the loss of a calcineurin-like phosphatase, as shown by a CaM binding overlay assay and Western blots. Type 2B phosphatases have been found throughout the animal kingdom and, although the A subunit has been shown to be tissue and species specific, the B subunit appears to be highly conserved [11]. ABRM fiber bundles that display poor catch tension maintenance because of extensive skinning (30–50% of the maximal tension developed during active contraction) regain the ability to maintain catch when perfused with brain calcineurin just prior to activation by Ca^{2+}. Since calcineurin is a Ca^{2+}/CaM-regulated phosphatase, these findings suggest that dephosphorylation takes place during the onset of active contraction and that this process is mediated by the increased Ca^{2+} concentration. Whatever the target(s) of phosphorylation in vivo, these results imply that they would be dephosphorylated during active contraction by the action of a Ca^{2+}-dependent phosphatase; and that they would, therefore, remain dephosphorylated during catch. Relaxation would then be brought about by the action of a cAMP-dependent kinase. A more complicated scheme involving a cascade of kinases/phosphatases, is, of course, also possible.

Additional support for the involvement of a Ca^{2+}-regulated phosphatase in the maintenance of catch tension comes from the behavior of ABRM bundles treated with the phenothiazine tranquilizer TFP. We find that this drug induces an accelerated catch tension decay and that this effect can be reversed by washing out the drug, when it is used at moderate concentrations (up to 0.125 mM), or by adding CaM to the bathing medium of the fiber, when TFP is used at higher concentrations (up to 0.5 mM). Phenothiazines, as well as other related drugs, are known to bind to a subclass of Ca^{2+}-binding proteins in the presence of Ca^{2+} and to affect their biological activity [19,20,29]. The number of TFP-binding sites on CaM and their dependence on Ca^{2+} seems to vary depending on the ionic strength used [19,30]. Under the experimental conditions of our study, it is likely that the number of TFP sites is quite high, given the ionic strength of the buffer and the need for exogenous CaM to relieve the effect. It cannot be excluded, however, that the observed effect of TFP on ABRM may be due to pathways different from the one involving CaM or a related Ca^{2+}-binding protein.

Taken together, these results indicate that the ability of molluscan muscles to maintain catch tension at low Ca^{2+} concentrations is linked to the action of a Ca^{2+}/CaM regulated phosphatase which becomes active during the onset of contraction.

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REFERENCES

[1] Twarog, B.M. in: Motility in Cell Function, F.A. Pepe, J.W. Sanger and V.T. Nachmias, Eds.), Academic Press, New York, 1979, pp. 231–241.
[2] Cornelius, F. (1980) J. Gen. Physiol. 75, 709–725.
[3] Cornelius, F. (1982) J. Gen. Physiol. 79, 821–834.
[4] Pfitzer, G. and Rüegg, J.C. (1982) J. Comp. Physiol. 147, 137.
[5] Castellani, L. and Cohen, C. (1987) Science 235, 334–337.
[6] Cole, R.A. and Twarog, B.M. (1972) Comp. Biochem. Physiol. 43A, 321–330.
[7] Achazi, R.K., Dölling, B. and Hankshorst, R. (1974) Pflügers Arch. 349, 19–27.
[8] Köhler, G. and Lindl, T. (1980) Pflügers Arch. 383, 257–262.
[9] Ishii, N., Simpson, A.W.M. and Ashley, C.C. (1989) Science 243, 1367–1368.
[10] Cohen, P. and Cohen, P.T.W. (1989) J. Biol. Chem. 264, 21435–21438.

[11] Klee, C.B. and Cohen, P. (1988) in: Molecular Aspects of Cellular Regulation (C.B. Klee and P. Cohen, Eds.), Elsevier, Amsterdam, 1988, pp. 225–245.
[12] Klee, C.B., Draetta, G.F. and Hubbard, M.J. (1988) Adv. Enzymol. Relat. Areas Mol. Biol. 61, 149–200.
[13] Klee, C.B., Krinks, M.H., Manalan, A.S. and Cohen, P. and Stewart, A.A. (1983) Methods Enzymol. 102, 227–244.
[14] Manalan, A.S. and Klee, C.B. (1983) Proc. Natl. Acad. Sci. USA 80, 4291–4295.
[15] Chantler, D.P. and Szent-Györgyi, A.G. (1980) J. Mol. Biol. 138, 473–492.
[16] Laemmli, U.K. (1970) Nature, 227, 680–685.
[17] Towbin, H., Stuehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[18] Hubbard, M.J. and Klee, C.B. (1987) J. Biol. Chem. 262, 15062–15070.
[19] Massom, L., Lee, H. and Jarrett, H.W. (1990) Biochemistry 29, 671–681.
[20] Klee, C.B. and T.C. Vanaman (1982) Adv. Prot. Chem. 35, 213.
[21] Twarog, B.M., Muneoka, Y. and Ledgere, M. (1977) J. Pharmac. Exp. Ther. 201, 350–356.
[22] Castellani, L, Elliott Jr., B.W. and Cohen, C. (1988) J. Musc. Res. Cell Motil. 9, 533–540.
[23] Achazi, R.K. (1979) Pflügers Arch. 379, 197–201.
[24] Cooley, L.B., Johnson, W.H. and Krause, S. (1979) J. Biol. Chem. 254, 2195–2198.
[25] Watabe, S. and Hartshorne, D.J. (1990) Comp. Biochem. Physiol. 96B, 639–646.
[26] Sohma, H. and Morita, F. (1986) J. Biochem. 100, 1155–1163.
[27] Sohma, H., Inoue, K. and Morita, F. (1988) J. Biochem. 103, 431–435.
[28] Bennett, P.M. and Marston, S.B. (1990) J. Muscle Res. Cell Motil. 11, 302–312.
[29] Marshak, D.R., Lukas, T.J. and Watterson, D.M. (1985) Biochemistry 24, 144–150.
[30] Levin, R.M. and Weiss, B. (1978) Biochim. Biophys. Acta 540, 192–204.