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MgADP Promotes a Catch-Like State Developed through Force-Calcium Hysteresis in Tonic Smooth Muscle

Alexander Khromov, Avril V. Somlyo, and Andrew P. Somlyo
Department of Molecular Physiology and Biological Physics, University of Virginia Health Sciences Center, Charlottesville, Virginia 22906-0011 USA

ABSTRACT Tonic rabbit femoral artery and phasic rabbit ileum smooth muscles permeabilized with Triton X-100 were activated either by increasing $[Ca^{2+}]$ from pCa > 8.0 to pCa 6.0 (calcium-ascending protocol) or contracted at pCa 6.0 before lowering $[Ca^{2+}]$ (calcium-descending protocol). The effects of, respectively, high [MgATP]/low [MgADP] [10 mM MgATP + creatine phosphate (CP) + creatine kinase (CK)] or low [MgATP]/[MgADP] (2 mM MgATP, 0 CP, 0 CK) on the "force-[Ca]" relationships were determined. In femoral artery at low, but not at high, [MgATP]/[MgADP] the force and the ratio of stiffness/force at pCa 7.2 were significantly higher under the calcium-descending than calcium-ascending protocols (54% vs. 3% of P_o, the force at pCa 6.0) (force hysteresis); the levels of regulatory myosin light chain (MLC_{20}) phosphorylation (9 ± 2% vs. 10 ± 2%) and the velocities of unloaded shortening V_0 (0.02 ± 0.004 l/s with both protocols) were not significantly different. No significant force hysteresis was detected in rabbit ileum under either of these experimental conditions. [MgADP], measured in extracts of permeabilized femoral artery strips by two methods, was 130–140 μM during maintained force under the calcium-descending protocol. Exogenous CP (10 mM) applied during the descending protocol reduced endogenous [MgADP] to 46 ± 10 μM and abolished force hysteresis: residual force at low $[Ca^{2+}]$ was 17 ± 5% of maximal force. We conclude that the proportion of force-generating nonphosphorylated (AM_{dp}) relative to phosphorylated cross-bridges is higher on the Ca^{2+}-descending than on the Ca^{2+}-ascending force curve in tonic smooth muscle, that this population of positively strained dephosphorylated cross-bridges has a high affinity for MgADP, and that the dephosphorylated AM_{dp} · MgADP state makes a significant contribution to force maintenance at low levels of MLC_{20} phosphorylation.

INTRODUCTION

Contraction and relaxation of smooth muscle, although regulated primarily by calcium-dependent phosphorylation and dephosphorylation of the 20-kDa regulatory myosin light chains (MLC_{20}) can also be modulated by factors that modify, independently of [Ca], the level of MLC_{20} phosphorylation (Somlyo and Somlyo, 1994). In addition, the high affinity of cross-bridges for MgADP (Nishiye et al., 1993; Fuglsang et al., 1993) and the effective competition of low concentrations of MgADP with physiological concentrations of MgATP in slowing relaxation of tonic, although not phasic, smooth muscle (Khromov et al., 1995) suggested that physiological levels (20–100 μM) of MgADP could affect contractile properties and, specifically, contribute to force maintenance at low levels of [Ca] and MLC_{20} phosphorylation. These high force/low phosphorylation states (catch-like state, "latch") occur in smooth muscle following the decline in $[Ca^{2+}]$ that initiates dephosphorylation of MLC_{20} (Dillon et al., 1981; Murphy, 1994). A similar state can be produced in permeabilized smooth muscle by a step-down of free $[Ca^{2+}]$ from higher to lower levels: force maintained at this lower level of [Ca] is significantly higher than that attained by a step-up from calcium-free conditions to an equivalent $[Ca^{2+}]$ (Moreland and Murphy, 1986); this phenomenon is known as "force-hysteresis." The purpose of the present study was to determine whether high-affinity MgADP binding to cross-bridges could account for hysteresis and, by implication, contribute to the catch-like state (latch) in smooth muscle. A preliminary report of some of these findings has been presented to the Biophysical Society (Khromov et al., 1997).

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Address reprint requests to Dr. Andrew P. Somlyo, Department of Molecular Physiology and Biological Physics, University of Virginia Health Sciences Center, 1300 Jefferson Park Ave., Jordan Hall 4-7, Charlottesville, VA 22906-0011. Tel.: 804-924-5926; Fax: 804-982-1616; E-mail: aps2n@aemsun.med.virginia.edu.

This manuscript is dedicated to the memory of the late Dr. Fred S. Fay.

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MATERIALS AND METHODS

All experiments were carried out on 160–200-μm wide, 2.5–3.0-mm long strips of tonic rabbit femoral artery and phasic ileum smooth muscles. The apparatus, composition of the solutions, and method of mounting the preparations were published (Fuglsang et al., 1993; Khromov et al., 1995). Triton X-100 permeabilization was used in the present study to reduce the contribution of ecto-ATPase to ATP hydrolysis (Trinkle-Mulcahy et al., 1994). Since [ADP] and [ATP] cannot be buffered simultaneously inside the muscle strip, the mechanical studies were performed under two extreme conditions of [MgATP]/[MgADP] ratio: 1) *high substrate* containing solution: 10 mM [MgATP], 0.5 mM free Mg^{2+}, exogenous 10 mM creatine phosphate (CP), and 100 IU/ml creatine kinase (CK) where the ratio of MgATP to MgADP was expected to be maximized; or 2) *low substrate* containing solution: 2 mM MgATP, 2 mM free Mg^{2+}, no exogenous CP or CK) where the effects of [MgADP] are expected to be prominent.

Solutions

The composition of solutions used is shown in Table 1.

Protocol

After permeabilization with 0.5% Triton X-100 at 20°C for 10–15 min at pCa 6.5 in the presence of 1 mM dithiothreitol (DTT), 1 mM NaN_3, the

TABLE 1 Composition of solutions (total mM) at pH 7.1

	Na_2ATP	$MgMS_2$	CP	Ca-EGTA	Pipes	$[Mg^{2+}]_f$	KMS	EGTA
Calcium activating (low substrate)	2.3	4.0	0	1.0	30.0	2.0	118.4	1.0
Calcium activating (high substrate)	15.45	10.7	10.0	1.0	30.0	0.5	43.5	1.0
Relax (G1)	4.6	6.07	0	0	30.0	2.0	106.2	1.0
Rigor	0	2.07	0	0	30.0	2.0	130.7	1.0

The pH of all solutions was 7.1 at 20°C; ionic strength was adjusted to 0.2 M with potassium methanesulfonate (KMS). The calcium-activating solutions (pCa 7.2–6.0) were constructed by mixing the G1 (pCa > 8) and Ca-EGTA (pCa 4.5) solutions in proper proportions, having the same pH and ionic strength. Exogenous calmodulin (4 μM) was added to all calcium-activating solutions. CP, creatine phosphate; $MgMS_2$, magnesium methanesulfonate; PIPES, piperazine-*N*,*N*″-bis(2-ethanesulfonic acid); EGTA, ethyleneglycol-bis(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid).

strips were relaxed for 10–15 min in 4 mM MgATP, pCa > 8 relaxing solution. The dependence of force developed in response to different $[Ca^{2+}]$ was determined by activating the strips either by increasing $[Ca^{2+}]$ from pCa > 8 to a higher $[Ca^{2+}]$ ("calcium-ascending" protocol) or by first contracting them at pCa 6.0 and then reducing $[Ca^{2+}]$ to a lower level (to pCa 7.0; see Fig. 1 *B*). The strips remained in activating solution until force reached a plateau during the ascending protocol and for an equivalent time (10 min) during the descending protocol in order to fix the time during which ATP was hydrolyzed under both conditions. Although force continued to decline slowly with the descending protocol, even after 40 min it reached only 35% of the maximum force at pCa 6.0, compared to 50% at 10 min, and not the low value reached with the ascending protocol. The strips were then transferred to the solution with different $[Ca^{2+}]$. The velocity (V_0) of unloaded shortening at each [Ca] was measured at the plateau of force by the "slack test" (Edman, 1979): three different releases (7–15% of initial length, at a release rate of >200 l/s) were applied with a "servo motor" operating in length control mode (6800, Cambridge Technology, Inc. Watertown, MA) and velocity was estimated as the slope of release length to time to onset of force ("slack length versus time"). The shortening velocity V_0 of smooth muscle is not constant following a length release (Arner and Hellstrand, 1985). Therefore, to standardize conditions, V_0 was measured during the 0.2–1.0 s interval after release and the slack versus time dependence was approximated by linear regression.

Force hysteresis was also measured by rapidly reducing $[Ca^{2+}]$ by photolysis of diazo-2 (Khromov et al., 1995) to avoid changes in nucleotide concentration due to solution exchange. The calcium-descending protocol was performed by activating the femoral artery in the dark with Ca^{2+} buffered to pCa 6.15 with diazo-2 (1 mM diazo-2 + 190 μM calcium); free $[Ca^{2+}]$ was calculated from the known dissociation constants of diazo-2 with Ca^{2+} and Mg^{2+} (Adams et al., 1989). Upon photolysis with a 50-ns pulse of near UV (347 nm) light at the plateau of the pCa 6.15 contraction (~80% of force at pCa 6.0), the Ca^{2+} concentration decreased rapidly to pCa 6.9–7.0 and force fell.

In-phase stiffness was measured simultaneously with isometric force by detecting the force response to sinusoidal length changes (500 Hz, 4 μm) using a piezoelectric element (P2L-060–10, Burley Instruments, Fishers, NY) driven by an amplifier (pz-150M, Burley Instruments). The in-phase component of sinusoidal force changes was demodulated by a lock-in module phase-sensitive detector (Model 4110, Evans Electronics) as determined previously (Somlyo et al., 1988).

[ADP] determination

Two methods, radioisotopic and "subtraction," were used for measuring intracellular [MgADP]. The radioisotopic method was performed according to Trinkle-Mulcahy et al. (1994). Briefly, permeabilized muscle samples (five strips tied together) were activated at appropriate $[Ca^{2+}]$ and, after the time required to reach steady tension with the ascending protocol, incubated for 10 min in the same Ca^{2+}-activating solution containing ^{3}H-ATP (20 μCi/ml) and ^{14}C-sucrose (10 μCi/ml) as a volume marker. After the 10-min incubation, tissue samples were blotted on a damp filter paper and homogenized in 100 volumes of ice-cold 0.4 M perchloric acid containing 1 mM of nonlabeled ATP and ADP. After neutralization with 5 N KOH and precipitation of proteins by perchlorate (2 min × 14,000 rpm), the nucleotides were separated by HPLC on an 8PSAX 10-μ column (Waters, Milford, MA) using a mobile phase of 0.25 M NH_4PO_4 + 13% MEOH pH 5.4 at a flow rate of 3 ml/min (Khromov et al., 1995). Nucleotide-containing and sucrose (which eluted before ADP and ATP) fractions were collected, mixed with scintillant (Ecoscint ICN, Costa Mesa, CA) and counted on LS6000SC (Beckman Instruments, Inc., Fullerton, CA) scintillation counter. To determine the absolute concentration of nucleotides the ratio of disintegrations per minute (dpm) of nucleotides to dpm of sucrose was compared to the ratio of dpm (ATP) to dpm (sucrose) determined by the same protocol performed on the initial incubation solution, which was not exposed to tissue and in which [ATP] was known. (Trinkle-Mulcahy et al., 1994).

Alternatively, tissue nucleotide content was determined by the subtraction method. Briefly, after 10 min incubation, when tension had plateaued in each calcium-activating solution or after 10 min in relaxing solution, duplicate strips (two strips tied together) were frozen in liquid N_2 and thawed while homogenizing for 5 min in 100 volumes of ice-cold 0.4 M perchloric acid (Passonneu et al., 1979). After neutralization and precipitation of proteins, the nucleotides were separated by HPLC as described above. Reextraction of the homogenate by the same protocol yielded <5% of the total extracted metabolites. This method extracts some of the ADP bound to actin (Seraydarian et al., 1962), and so overestimates [ADP]. Therefore, the amount of ADP bound to sites other than myosin was assumed to equal the ADP extracted by the same method from identical-size strips in rigor that were intensively washed (30 min) with nucleotide- and Ca^{2+}-free solution containing 0.1 IU/ml Apyrase (Grade VII, Sigma). The [ADP] remaining bound to myosin in cardiac muscle after such treatment was shown to be very low (<10 μM) (Martin and Barsotti, 1994).

The amount of ADP extracted from rigor preparations was numerically subtracted for the values obtained by the subtraction method presented in Fig. 5 to account for extraction of actin-bound ADP that is not measured with the isotopic method due to the very slow exchange of actin-bound ADP. Inasmuch as MgADP, rather than ADP, is the ligand affecting cross-bridges, its concentration was calculated assuming an association constant of magnesium to ADP as $6.24 \times 10^2\ M^{-1}$.

Strips were frozen in liquid N_2 at the plateau of force at appropriate $[Ca^{2+}]$ and MLC_{20} phosphorylation was measured by 2-D electrophoresis (Kitazawa et al., 1991). All experiments were performed at 20°C.

Chemicals

Creatine phosphate (CP), magnesium methanesulfonate ($MgMS_2$), piperazine-*N,N*″-bis(2-ethanesulfonic acid) (PIPES), and ethyleneglycol-bis(β-aminoethyl)-*N,N,N*′,*N*′-tetraacetic acid) (EGTA) were obtained from Sigma (St. Louis, MO). Triton X-100 was purchased from Boehringer-Mannheim (Germany), diazo-2 from Molecular Probes (Eugene, OR), and radioactive ^{3}H-ATP and ^{14}C-sucrose from DuPont NEN (Boston, MA).

Statistics

The data are presented as means ± SEM and *n* equals the number of observations. Statistical comparison was made by Student's *t*-test and $p < 0.05$ was taken as a significant difference.

RESULTS

Force-[Ca^{2+}] and force-stiffness relationships

Force traces of femoral artery strips obtained with the two different protocols (calcium-ascending and calcium-descending) at low ATP conditions are shown in Fig. 1. The force (relative to force at pCa 6.0) developed at low [Ca^{2+}] (pCa 7.0) under the Ca^{2+}-ascending protocol was significantly less than that developed at the same [Ca^{2+}] under the calcium-descending protocol. Force measurements in the range pCa 7.2–6.0 in femoral artery (Fig. 2 *A*) showed significant "hysteresis" in the force-[Ca] relationships at low [MgATP]: the relative forces developed at pCa 7.2 were 0.03 ± 0.01 and 0.54 ± 0.06 with, respectively, ascending and descending protocols. This difference was highly significant ($p < 0.001$). The averaged force hysteresis did not depend on whether the calcium-ascending or the calcium-descending protocol was used for the first contraction. Force hysteresis was not observed under high ATP conditions, 10 mM MgATP, low free [Mg^{2+}], and exogenous CP and CK (the average force developed at pCa 6.0 was lower by ~10–20% than that at 2 mM MgATP in the absence of exogenous creatine phosphate and creatine phosphokinase): the relative forces at pCa 7.2 under the ascending and descending protocols (0.10 ± 0.06 and 0.2 ± 0.1) were not significantly different, nor did increasing free [Mg^{2+}] to 2 mM cause significant hysteresis under the high [ATP] protocol (data not shown).

Ileum smooth muscle, in contrast to femoral artery, showed no significant hysteresis in the force-[Ca] relationship within the range of pCa 7.0–6.0 under either of the above ATP conditions (Fig. 2 *B*); however, the average force developed at pCa 6.5 at 2 mM [MgATP] was slightly higher than that at 10 mM MgATP. Because of the absence of force hysteresis in ileum, further studies were performed only on femoral artery smooth muscle.

The relationship between force and stiffness in femoral artery strips undergoing the calcium-ascending and the calcium-descending protocols is shown in Fig. 3, where both are normalized to their maximal values reached at the peak of pCa 6.0 activated contraction. While stiffness followed the changes in force during the calcium-ascending and calcium-descending protocols, the stiffness/force ratio attained was higher with the calcium-descending protocol. This difference was abolished in the presence of exogenous creatine phosphate and creatine phosphokinase during the calcium-descending protocol, as shown in Fig. 3, where the data are plotted with the 5% confidence limits.

Velocity-[Ca^{2+}] and phosphorylation-[Ca^{2+}] relationships

Force hysteresis was somewhat greater at pCa 7.2 (Fig. 2 *A*) than at pCa 7.0, but the velocity of shortening at pCa 7.2 was below the lower limit of detection with the slack test. Therefore, we measured V_0 at pCa 7.0. The velocity of unloaded shortening, V_0 in femoral artery under the low-ATP conditions conducive to force hysteresis (low [MgATP]/[MgADP]; Fig. 3) at pCa 7.0 was not significantly different, whether the ascending or the descending protocol was used: $V_0 = 0.02 \pm 0.004$ and 0.02 ± 0.006 lengths/second (l/s) respectively. In order to compare V_0 at similar levels of force reached with the ascending, as at pCa 7.0 with the descending protocol, [Ca^{2+}] was increased

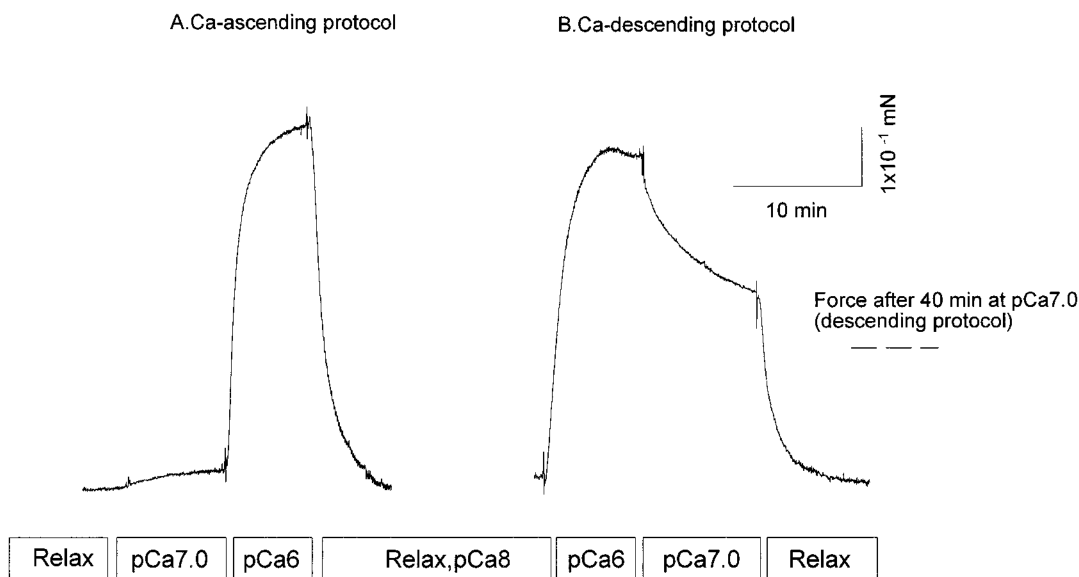

FIGURE 1 Force records showing the experimental protocol. In the first trace the permeabilized femoral artery strip was activated by transfer from relaxing to pCa 7.0 solution containing 2 mM MgATP, 0 CP followed by activation in pCa 6.0 solution (calcium-ascending protocol, *A*). At the plateau of force the strip was relaxed by transfer to pCa 8.0 solution. In the second trace the strip was first activated in pCa 6.0 solution followed by transfer into pCa 7.0 solution (calcium-descending protocol, *B*). Note the different levels of force at pCa 7.0 in the two traces. The interrupted horizontal line in (*B*) shows force level (35%) reached after 40 min in pCa 7.0 solution.

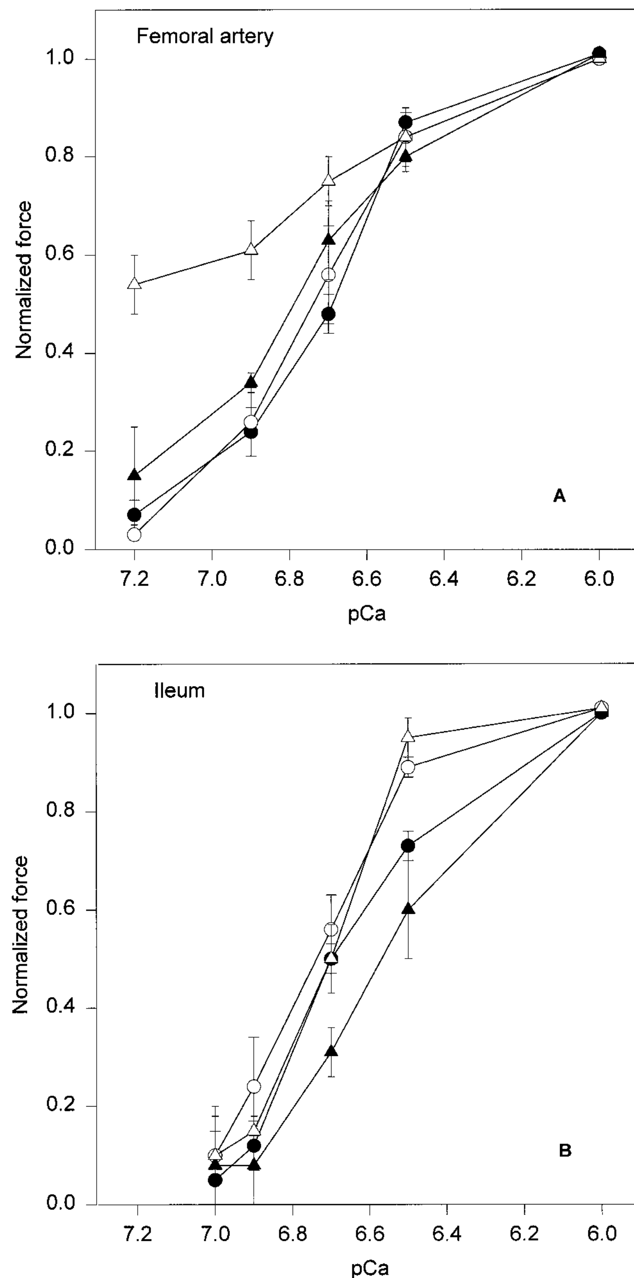

FIGURE 2 Normalized force-[Ca] plots for the permeabilized femoral artery (*A*) and ileum (*B*) strips. The force data (normalized at pCa 6.0) were collected under calcium-ascending (*circles*) and calcium-descending (*triangles*) protocols at two different [MgATP] in the calcium-activating solution low [MgATP] (*open symbols*) and high [MgATP] (*closed symbols*) conditions.

during the calcium-ascending protocol to ~pCa 6.6, to match the force at pCa 7.0 under the calcium-descending protocol. The velocity reached at ~pCa 6.6 with the calcium-ascending protocol was $V_0 = 0.05 \pm 0.005$ l/s ($n = 4$), significantly higher than V_0 at similar amplitude of force (~50% of P_o) developed at pCa 7.0 under the calcium-descending protocol ($V_0 = 0.02 \pm 0.006$, see above). At high [Ca^{2+}] (pCa 6.0), V_0 was 0.07 + 0.01 l/s, significantly ($p < 0.05$) higher than at pCa 7.0.

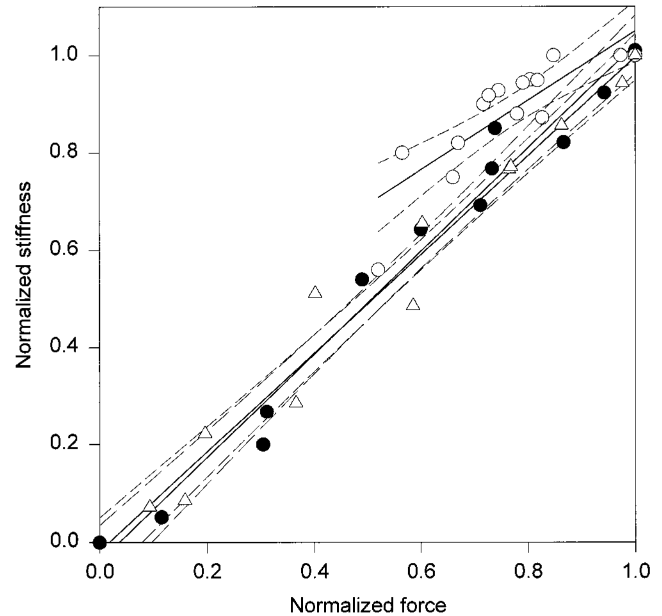

FIGURE 3 The relationship between stiffness and force throughout the calcium-ascending (●) and calcium-descending (○) protocols in permeabilized femoral artery strips in the absence and presence (△) of an ATP regenerating system (10 mM CP and 100 IU/ml CK) during calcium-descending protocol. The stiffness and force data were normalized to maximal values achieved at pCa 6.0. The stiffness-force dependencies were extrapolated as linear and are shown together with confidence limits ($p < 0.05$).

The level of MLC_{20} phosphorylation was determined in femoral artery (Fig. 4) during maximal force hysteresis (in the presence of low [MgATP]/[MgADP], pCa 7.2). At pCa 7.2 MLC_{20} phosphorylation was 10 ± 2% and reached 70 ± 15% when [Ca^{2+}] was increased to pCa 6.0, but returned to its low level (9 ± 2%) when [Ca^{2+}] was decreased ($n = 4$).

ADP content

Extraction of ADP from muscles in rigor that were extensively washed to remove ADP, other than bound to actin, yielded 320 ± 20 μM ADP ($n = 16$), most of it presumably representing ADP bound to actin. This amount was subtracted from the [ADP] determined at different [Ca^{2+}]. Measured by the radioisotopic method, [ADP] during rigor was ~20 μM after apyrase treatment and ~50 μM without apyrase treatment. The concentration of ADP and ATP in the tissue extracts was measured after 10 min, by which time tension reached steady levels at pCa 7.0 and pCa 6.0 (see Methods).

The calculated total [MgADP] (myosin-bound and free, but excluding actin-bound) estimated by the two methods appeared to be very similar in all studied conditions and is presented in Fig. 5. The [MgADP] determined by the subtraction and radioisotopic methods, respectively, in the permeabilized femoral artery was 140 ± 20 μM and 180 ± 40 μM in the absence of calcium; following the rise in [Ca^{2+}] to pCa 7.0 it was 165 ± 20 μM and 180 ± 50 μM. At high

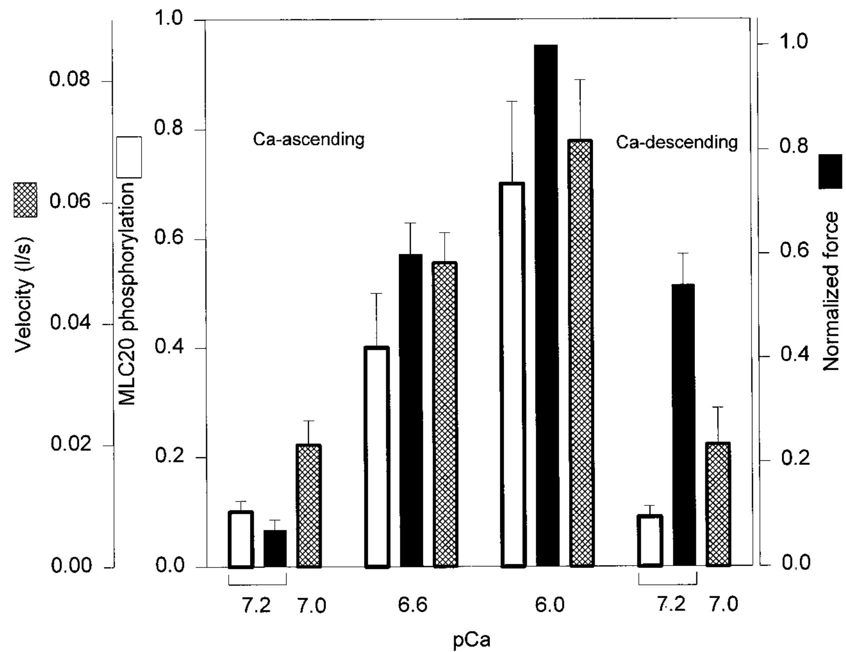

FIGURE 4 Summary of force, unloaded velocity of shortening, and MLC_{20} phosphorylation. Measurements were made on permeabilized femoral artery strips at low (pCa 7.2 for force and phosphorylation, pCa 7.0 for velocity measurements) and high (pCa 6.0) [Ca] under calcium-ascending and calcium-descending protocols, as well as at pCa 6.6, where the calcium-activated force is equal to the force at pCa 7.0 during the descending protocol. The force values under either protocol were normalized to maximal force developed at pCa 6.0 under the same protocol. MLC_{20} and velocity measurements made at pCa 6.0 are the reference values obtained by transferring the strips directly from relaxing to calcium-activating solutions.

[Ca^{2+}] (pCa 6.0) the [MgADP] was significantly higher (220 ± 20 μM and 250 ± 50 μM, respectively).

There was no significant difference in the MgADP content of strips at pCa 7.0 undergoing the calcium-ascending or calcium-descending protocols: 165 ± 20 μM/180 ± 50 μM, $n = 5$ and 140 ± 20 μM/130 ± 50 μM, $n = 4$ for subtraction and radioisotopic methods, respectively. Exogenous CP (10 mM) and CK (100 IU/ml) in the low Ca^{2+}-activating solution (pCa 7.0) during the descending protocol reduced [MgADP] significantly, to 46 ± 10 μM ($n = 7$); this was accompanied by a significant decrease in the amplitude of maintained force (17 ± 5% of force at pCa 6.0).

Because an exogenous ATP regenerating system was not included in the femoral artery strips throughout the experimental protocol, a gradient of [MgATP] between the intracellular space and surrounding solution can be expected to

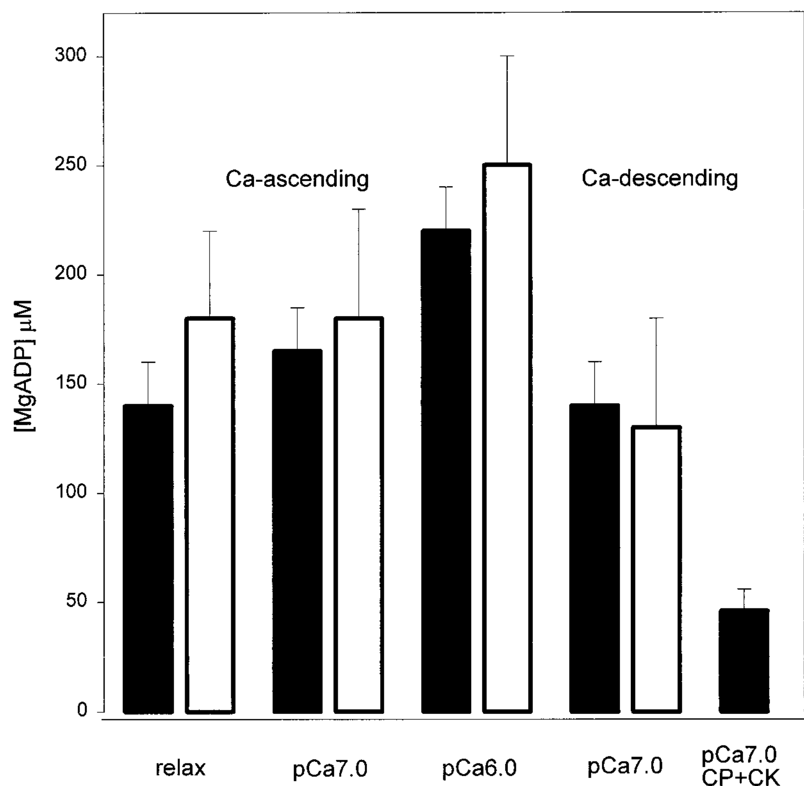

FIGURE 5 The concentration of MgADP in permeabilized femoral artery strips in the presence of 2 mM MgATP at various [Ca^{2+}] under the calcium-ascending and calcium-descending protocols. The data shown represent the results obtained by the two methods: radioisotopic (*open bars*) and "subtraction" (*closed bars*) for permeabilized muscles.

develop at high [Ca^{2+}] activating conditions. As determined by the radioisotopic method at pCa 6.0, the [MgATP] was 1.5 ± 0.2 mM and 1.8 ± 0.3 mM, in the cellular space and surrounding media, respectively. At low [Ca^{2+}] there was no significant gradient of [MgATP]: the intracellular [MgATP] was 1.8 ± 0.2 mM and 2.0 ± 0.3 in the surrounding media ($n = 3$, radioisotopic method).

To compare our results with the previously published values of [ADP] in smooth muscle, we also determined the nucleotide content of relaxed and K^+-stimulated (140 mM for 30 min at 20°C) intact femoral artery strips by the subtraction method. The concentration of ATP was 1.0 ± 0.1 mM ($n = 3$) or ~2 mM, when corrected for extracellular space, somewhat higher than [ATP] determined in intact porcine carotid artery and portal vein (Krisanda and Paul, 1983; Hellstrand and Paul, 1983), but within the range reported for other rabbit smooth muscles (Butler and Davies, 1980). The total concentration of ADP in intact femoral artery at 20°C was 160 ± 20 μM ([MgADP] ~35–60 μM, the lower and upper limits calculated by assuming 0.5 or 1.0 mM free magnesium; Tashiro and Konishi, 1997) and 40% or 60% extracellular space. After correction for extracellular space [ranging from 40% wet wt in Cohen and Murphy (1978) to 60% in Gong et al. (1992)], the total intracellular [ADP] was estimated within the range of 270 ± 30 μM to 400 ± 50 μM, respectively. However, we found only a trend, but no statistically significant increase in [ADP] during prolonged K^+ contraction in this very small number of experiments: 160 ± 20 μM in relaxed femoral artery and 200 ± 20 μM ($n = 3$) after 30 min of contraction, respectively. [ADP] was reported previously as 220 μM, rising to 270 μM after prolonged contraction in intact porcine carotid artery at 20°C (Krisanda and Paul, 1983).

The MgADP values determined by us include both free myosin and bound nucleotide in solution, but exclude slowly exchanging (actin-bound) MgADP. It has been suggested, based on the similar concentrations of myosin heads and MgADP, that myosin contains bound MgADP in smooth muscle (Vyas et al., 1992). However, the precision of currently available methods is insufficient for directly measuring changes in the partitioning between free and myosin-bound MgADP under different experimental conditions. Implied in the hypothesis presented by us and based on the effects of MgATP/MgADP ratio on hysteresis, is that the proportion of MgADP-bound cross-bridges is increased during hysteresis. Furthermore, in comparing the MgADP concentrations measured by us (Fig. 5) with published in vivo values, note should be taken of the large contribution to ADP by the ecto-ATPase activity in permeabilized smooth muscle, as indicated by the "relaxed" value (Fig. 5) obtained under Ca^{2+} free conditions.

Force hysteresis induced by flash photolysis of diazo-2

In the standard hysteresis protocol used in the above experiments, the relatively high [MgADP] reached in the myofilament lattice and surrounding solution during the plateau of the pCa 6.0-activated contraction would not be maintained following transfer of the strip into the low [Ca], nominally ADP-free (~20 μM, probably due to ATP contamination) solution. Therefore, we could not exclude the possibility that this initial difference in [MgADP] affected the force reached at low [Ca] with the descending protocol. To avoid the simultaneous change in both parameters, [Ca^{2+}] and [MgADP], we used flash photolysis of diazo-2 to reduce [Ca^{2+}] in the descending protocol from pCa 6.15 to pCa 6.9–7.0 (Khromov et al., 1995). Force fell to a level 50 ± 5% ($n = 4$) of the force at pCa 6.15; this value was not significantly different from that (55–60% of maximal force at pCa 6.0) reached by the standard protocol utilizing solution exchange (Fig. 2 *A*). For the calcium-ascending protocol the strips were activated in the same Ca^{2+}-diazo-2 buffer (pCa 6.15) prephotolyzed with the same light energy as during the calcium-descending protocol described above. The force developed under this condition (pCa 6.9–7.0) was 20 ± 5% of P_o at pCa 6.0, in agreement with the results shown in Fig. 2 *A*. Therefore, the results of these control experiments show that the initial difference in [MgADP] had no significant effect on the residual tension after reducing [Ca^{2+}], presumably because the system was saturated regardless of how [Ca^{2+}] was reduced.

DISCUSSION

Our major finding is that the development of a high-force, low-MLC_{20} phosphorylation state, generated in permeabilized tonic smooth muscle during cross-bridge cycling at relatively low substrate low ([MgATP]/[MgADP] ratio), can be prevented by high ATP conditions (relative to MgADP). We show (Fig. 1) that force generated by a small increment in [Ca^{2+}]$_i$ from a nominally Ca^{2+}-free condition is significantly lower than the force reached by descending from a relatively high [Ca^{2+}]$_i$ to the identical, low level [Ca^{2+}]$_i$, as reported previously in permeabilized carotid artery by Moreland and Murphy (1986, but cf. Chatterjee et al., 1987). The high-force, low-velocity and relatively low MLC_{20} phosphorylation state generated by the descending protocol of changing [Ca^{2+}]$_i$ is phenomenologically indistinguishable from the catch-like state (Somlyo and Somlyo, 1967) or latch (Dillon et al., 1981) observed in nonpermeabilized smooth muscle and develops at [MgATP]/[MgADP] ratios expected to be present in vivo: ~2 mM [MgATP] (present study; 1 mM total ATP measured and corrected for 40–60% extracellular space) and 58–150 μM [MgADP] (are, respectively, the lower and upper limits based on 35 μM MgADP and 0.5 M free Mg^{2+} and 60% extracellular space compared with 60 μM MgADP and 1 mM free Mg^{2+} corrected for 40% extracellular space).

The presence of MgADP-dependent force hysteresis in tonic, but not in phasic, smooth muscle is consistent with the myosin isoform-specific (Somlyo, 1993) higher affinity of cross-bridges for MgADP in tonic smooth muscle. In

theory, two mechanisms could account for MgADP-dependent development of a high-force, relatively low-phosphorylation and low-shortening-velocity state: a build-up of [MgADP] and/or an increase in a cross-bridge population having high affinity for [MgADP].

The first of the above mechanisms is unlikely to be dominant, because hysteretic force was maintained regardless of whether [MgADP] was reduced during solution exchange of [Ca^{2+}] (from ~230 to 140 μM after 10 min and, presumably, to <140 μM immediately following solution exchange containing only 20 μM contaminant ADP) or maintained at constant, elevated levels (when [Ca^{2+}] was reduced during flash photolysis). Therefore, although there may be a modest increase in [MgADP] during contraction, we favor the explanation that the fall in [Ca^{2+}]$_i$ generates, in tonic smooth muscles, a population of dephosphorylated cross-bridges having very high affinities for physiological [MgADP], and MgADP inhibits escape from this (AM_{dp}ADP) state. Considering simple competitive inhibition between the two nucleotides and [MgATP] = 2.0 mM (K_m ~100 μM; Iino, 1981; Arner and Hellstrand, 1985), the presence of 100 μM [MgADP] (K_i = 1 μM) will significantly inhibit (Khromov et al., 1995) the rate of the ATP-mediated reaction (? slow component of relaxation) by ~10-fold. This interpretation is supported by the faster rate of MLC_{20} dephosphorylation than relaxation in femoral artery smooth, and the slowing of the slow, dephosphorylation independent phase of relaxation by MgADP (Khromov et al., 1995). The ability of high (10 mM) [MgATP] to abolish force hysteresis (present study) probably explains the frequent absence of latch (Chatterjee et al., 1987; Haeberle, 1987) in permeabilized smooth muscles examined in the presence of high [MgATP]. In phasic smooth muscles little or no force hysteresis, resulting in catch-like state or latch, would be expected (present study) and, indeed, it is found to be minimal (Himpens et al., 1988). This interpretation accounts for the previously unexplained absence of force hysteresis in taenia coli (Paul et al., 1987).

In considering the very high affinity (K_d = 1–5 μM) first detected in smooth muscle in rigor (Fuglsang et al., 1993; Nishiye et al., 1993; see below), and comparing it with the relatively high [MgADP] in intact and permeabilized smooth muscles, it is occasionally overlooked (Clark et al., 1995; Clark and Dillon, 1995) that the low K_d values represent MgADP binding to (largely positively) strained cross-bridges in the absence of ATP (in rigor). Clearly, if MgATP and MgADP compete for the same nucleotide binding site in smooth (Khromov et al., 1996), as in striated muscles (Cooke and Pate, 1985), the [MgADP] required to affect cross-bridges will be higher in the presence of physiological [MgATP] than in its absence. In the presence of 1 mM MgATP the apparent K_d of MgADP will be 11–55 μM for dephosphorylated, and at least 4- to 5-fold higher (Nishiye et al., 1993) for phosphorylated cross-bridges. The validity of the K_d and kinetic constants derived from flash photolysis studies of skinned smooth muscle (Fuglsang et al., 1993; Nishiye et al., 1993; Khromov et al., 1996) is reinforced by solution studies (Cremo and Geeves, 1998; Gollub et al., 1996) that show comparably high affinity (K_d = 5 μM) for MgADP of the dephosphorylated complex of (chicken gizzard) myosin subfragment 1 with actin (acto S_1), as well as an association constant of ATP for actomyosin that is somewhat lower than the association constant for MgADP and very comparable to the values obtained in skinned fibers (Nishiye et al., 1993; Khromov et al., 1996).

Unloaded shortening velocity (V_0) was the same at low force generated by the ascending as at the high force levels generated by the descending protocol (0.02 l/s) for the same [Ca^{2+}] (Fig. 4). Although consistent with the comparable levels of MLC_{20} phosphorylation (~10%), this observation was somewhat surprising under the assumption that cycling rates decreased during the descending protocol.

We can offer two not mutually exclusive mechanisms that would account for this observation. The first explanation depends on the assumption that, at least within the lower ranges of V_0, the latter is a function of not only the rate, but also the number of cross-bridges cycling, presumably reflecting internal load. If so, then a small number of relatively rapidly cycling (phosphorylated) cross-bridges working against little or no internal load could result in the same V_0 as a larger number of more slowly cycling bridges (during hysteresis) working against a larger internal load of attached, nonphosphorylated cross-bridges. This explanation is supported by a higher stiffness/force ratio during hysteresis than during the ascending phase of force development. The second explanation is based on a structural redistribution of the cross-bridges from force generators in series to a larger proportion distributed in parallel (Rüegg, 1971). Although such distribution, perhaps based on the entrance of cooperatively cycling nonphosphorylated bridges into activity, is conceivable, it unfortunately lacks a ready experimental basis for proving or disproving it.

The slow V_0 during force hysteresis could be accounted for by two populations of cross-bridges: one rapidly and the other slowly (or non-) cycling (Murphy, 1994), or by a single population of slowly cycling cross-bridges (Butler et al., 1987). If we consider the slowly cycling cross-bridges to be unphosphorylated, as commonly and reasonably assumed (Murphy, 1994; Somlyo, 1993), then we have to consider the operation of another mechanism, such as cooperative reattachment (Somlyo et al., 1988; Vyas et al., 1994) to account for cycling of this population.

A major technical problem associated with this, as with similar studies of both muscle and nonmuscle cells (e.g., Krisanda and Paul, 1983), is the measurement of [MgADP] that is not actin-bound. The ADP chemically extracted from smooth muscles in rigor is greatly in excess of what could be bound (~50 μM) to myosin and must originate from another source, most likely actin (~1 mM in smooth muscle). We therefore subtracted the average amount of ADP extracted from muscle in rigor from the values obtained under the different experimental conditions, and also measured ADP by a radioisotopic method (Trinkle-Mulcahy et al., 1994). The good agreement between the results obtained

with the two types of measurement inspires us with some confidence about the values obtained.

Evaluation of the sources of [MgADP] in permeabilized smooth muscle is further complicated by the presence of a highly active ecto-ATPase that is thought to account for the relatively high [MgADP] measured in relaxed permeabilized smooth muscle (pCa > 8; Fig. 5). Although the ecto-ATPase activity can be reduced 5- to 10-fold by extensive permeabilization with Triton X-100 (Trinkle-Mulcahy et al., 1994), the protocol employed in the present study achieved a reduction by only 30% of the activity in α-toxin-permeabilized preparations (Khromov et al., 1995). More extensive permeabilization, in our experience, results in significant impairment of the mechanical properties of smooth muscle: even with the present protocol the relative contribution of the nonlinear (presumably non-cross-bridge) compliance to the total compliance of smooth muscle increased significantly (Khromov, Somlyo and Somlyo, submitted). The rate of ADP generation by ecto-ATPase in Triton-treated muscles and measured by the radioisotopic method was 0.72 mM/min (based on the total radiolabeled ADP accumulated over 10 min in the tissue and in the surrounding solution). Calculations based on the diffusion equation and similar to those described earlier (Khromov et al., 1995), assuming a nucleotide diffusion coefficient $D = 10^6$ cm^2/s (Chase and Kushmerick, 1995) show that for radius $r = 100\ \mu m$, $\sim$180 μM MgADP will be accumulated inside the strip as a result of ecto-ATPase activity under these conditions. This value is within the errors of estimate obtained in femoral artery measured with the two methods (Fig. 5).

In conclusion, we find that the force hysteresis observed in smooth muscle, when $[Ca^{2+}]_i$ is reduced from high to intermediate activating levels, occurs at the relatively low, but physiological, [MgATP]/MgADP] ratios. We further consider that, rather than being caused by a large build-up of total [MgADP], this is primarily the result of the development of a population of strained, dephosphorylated cross-bridges having the high affinity for MgADP previously detected in rigor (Fuglsang et al., 1993; Nishiye et al., 1993). This latter cross-bridge population together with a small population of cross-bridges remaining phosphorylated is thought to contribute to the ability of smooth muscle to maintain force at low levels of myosin light chain phosphorylation.

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