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G protein-mediated inhibition of myosin light-chain phosphatase in vascular smooth muscle

(calcium sensitization/protein phosphatase/α-adrenergic/guanosine 5′-[γ-thio]triphosphate/*Staphylococcus aureus* α toxin)

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ABSTRACT **The mechanism of G protein-mediated sensitization of the contractile apparatus of smooth muscle to Ca^{2+} was studied in receptor-coupled α-toxin-permeabilized rabbit portal vein smooth muscle. To test the hypothesis that Ca^{2+} sensitization is due to inhibition of myosin light-chain (MLC) phosphatase activity, we measured the effect of guanosine 5′-[γ-thio]triphosphate and phenylephrine on the rate of MLC dephosphorylation in muscles preactivated with Ca^{2+} and incubated in Ca^{2+}- and ATP-free solution containing 1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine (ML-9) to block MLC kinase activity. Guanosine 5′-[γ-thio]triphosphate alone (300 μM) or in combination (3 μM) with phenylephrine decreased the rates of relaxation and dephosphorylation of MLC to about half of control values; this inhibition is sufficient to account for maximal G protein-mediated Ca^{2+} sensitization of MLC phosphorylation. The rate of thiophosphorylation of MLC with adenosine 5′-[γ-thio]-triphosphate was not affected by guanosine 5′-[γ-thio]triphosphate. We suggest that inhibition of protein phosphatase(s) by G protein(s) may have important regulatory functions.**

Phosphorylation of myosin light chain (MLC) of smooth muscle by Ca^{2+}/calmodulin-regulated MLC kinase activates actomyosin ATPase and is the major regulatory mechanism of smooth muscle contraction (1). However, although the rise in cytoplasmic Ca^{2+} and consequent activation of MLC kinase is the primary mechanism triggering MLC phosphorylation and contraction, the relationship between cytoplasmic Ca^{2+} and phosphorylation and force is not constant (2–14). Thus, a variety of agonists can sensitize the contractile regulatory apparatus to Ca^{2+} to increase the force/Ca^{2+} ratio (9, 10), whereas desensitization to Ca^{2+} causes a decline in force, without, necessarily, any decrease in $[Ca^{2+}]$ (11, 12). The sensitizing effect of agonists is mediated by a G protein, as shown by the ability of guanosine 5′-[γ-thio]triphosphate (GTP[γS]) to induce and guanosine 5′-[β-thio]diphosphate to inhibit Ca^{2+} sensitization (6–8, 10, 13).

We had proposed that Ca^{2+} sensitization by agonists is mediated by the inhibition of MLC phosphatase (14) and have supported this hypothesis by demonstrating that Ca^{2+} sensitization was accompanied by, and presumably the result of, increased MLC phosphorylation (8, 12). The major purpose of the present study was to determine whether the sensitization-induced increase in MLC phosphorylation is from inhibition of MLC phosphatase or from the alternate possibility, stimulation of MLC kinase. We used smooth muscle permeabilized with α-toxin because this preparation retains both coupled receptors and soluble intrinsic proteins (6, 8, 12) and a MLC kinase inhibitor, 1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine (ML-9) (15), under ATP- and Ca^{2+}-free conditions, to determine rates of MLC dephosphorylation uninfluenced by phosphorylation. Similarly, we measured the rate of (thio)phosphorylation with adenosine 5′-[γ-thio]triphosphate (ATP[γS]) because that rate is not expected to be affected by phosphatases (16).

EXPERIMENTAL PROCEDURES

Small strips (50–70 μm thick, 200–350 μm wide, and 2–3 mm long) of longitudinal muscle of rabbit portal vein were dissected and freed from connective tissue. Single (for only force measurements) or duplicate (for measurements of both force and phosphorylation) strips were tied with monofilament silk to the fine tips of two tungsten needles, one of which was connected to a force transducer and mounted in a well on a bubble plate, as described (7).

After measuring contractions induced by high (154 mM) K^+ and by agonists, the strips were incubated at room temperature (21–22°C) in relaxing solution containing 4.5 mM MgATP and 1 mM EGTA for several minutes and treated for 30–60 min with 5,000–10,000 units of *Staphylococcus aureus* α-toxin (GIBCO/BRL) per ml at pCa 6.3 buffered with 10 mM EGTA. To deplete the sarcoplasmic reticulum of calcium and maintain cytoplasmic Ca^{2+} constant, every strip was also treated with 10 μM A23187 (Calbiochem) for 30–45 min in the relaxing solution. The composition of solutions was essentially the same as described (7), except that the relaxing and activating solutions used for permeabilized strips contained 2.1 mM free Mg^{2+} and 4.4 mM MgATP at pH 7.1 and 15°C. All activating solutions contained 10 mM EGTA. To eliminate MLC kinase activity during measurements of the rates of dephosphorylation, ML-9 (Calbiochem), an inhibitor of MLC kinase and other kinases (15) was added to the solutions that contained neither ATP nor Ca^{2+} (10 mM EGTA added).

Tissue preparations frozen for measurements of MLC phosphorylation have been described in detail (8). MLC was separated by two-dimensional isoelectric focusing/SDS gel electrophoresis, stained with colloidal gold, and analyzed as described (8).

RESULTS

MLC Phosphorylation and Force at Steady State and the Effect of GTP[γS]. To measure the rate of MLC phosphorylation/dephosphorylation reactions accurately, these experiments were done at 15°C, at which temperature the time courses of contraction and relaxation, induced by $[Ca^{2+}]$ changes, were slower and less phasic (12). For example, the $t_{1/2}$ of relaxation of maximum force, after Ca^{2+} removal, was 2.8 ± 0.3 min ± SE (n = 4) at 15°C, four times slower than

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Abbreviations: MLC, 20-kDa myosin light chain; GTP[γS], guanosine 5′-[γ-thio]triphosphate; ML-9, 1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine; ATP[γS], adenosine 5′-[γ-thio]triphosphate; *M* and *PM*, concentrations of unphosphorylated and phosphorylated MLC, respectively.

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that at 25°C (0.7 ± 0.1 min, n = 4). The high temperature dependence of the relaxation rate seems to correlate with the high Q_{10} (≥4; M. Ikebe, personal communication) of MLC phosphatase, indicating that relaxation at 15°C is not limited by the rate of EGTA diffusion and Ca^{2+} binding. Ca^{2+}-activated maximum force was increased at lower temperature: force induced by pCa 5 at 15°C was 120 ± 7% of the force at 25°C ± SE (n = 10).

The dose dependence of the Ca^{2+}-sensitizing effect of GTP[γS] on contraction in Fig. 1 (also see Fig. 3*A*) shows that 300 μM GTP[γS] causes near-maximal sensitization. Therefore, we used this concentration to determine GTP[γS] effects on MLC phosphorylation and dephosphorylation. Phenylephrine (100 μM) shifted the dose–response curve by ≈15-fold, presumably by accelerating the exchange of GTP[γS] for GDP on the G protein (17). With the agonist, 3 μM GTP[γS] sensitized the contractile response to Ca^{2+} by approximately the same extent as did 300 μM GTP[γS] without any agonist. Therefore, we also used this combination (phenylephrine plus 3 μM GTP[γS]) to measure an effect on MLC dephosphorylation.

To compare the calculated steady-state kinetic constants (see below) with measured ones, we determined the maximum effect of GTP[γS] on steady-state MLC phosphorylation at various [Ca^{2+}] values. Table 1 shows that 300 μM GTP[γS] significantly increased phosphorylation to 19% at pCa >8 and to 41% at pCa 6.5 but caused no significant increase at pCa 5. A small increase (2% of maximum force) in force by GTP[γS] was detectable at pCa >8, whereas force was markedly increased to 76% at pCa 6.5 and moderately to 112% at pCa 5. Control responses to Ca^{2+} increases alone were comparable to results reported (8) with guinea pig portal vein.

Effect of GTP[γS] on Rate of MLC Dephosphorylation and Relaxation. To determine the GTP[γS] effect on MLC phosphatase activity, the effect of guanine nucleotide on the rate of dephosphorylation of MLC was measured in strips preactivated by maximum Ca^{2+} (pCa 5) and incubated in Ca^{2+}- and ATP-free solution containing 100 μM ML-9, a protein kinase inhibitor with strong activity toward MLC kinase (15). This ML-9 concentration reversibly decreased the contraction activated by submaximal Ca^{2+} (pCa 6)—from 55% of maximum force at pCa 5 to 12%. The early phase (up to 50%) of relaxation of muscles in ATP-containing, Ca^{2+}-free (10 mM EGTA) solution was unaffected by ML-9, although this phase was inhibited by GTP[γS] (see below). The later phase of relaxation was slightly accelerated: the time required for 80% relaxation was slightly decreased from 4.8 ± 0.6 (n = 4) to 3.7 ± 0.4 min ($P < 0.05$ by paired t test, but not significant by unpaired t test) (n = 6).

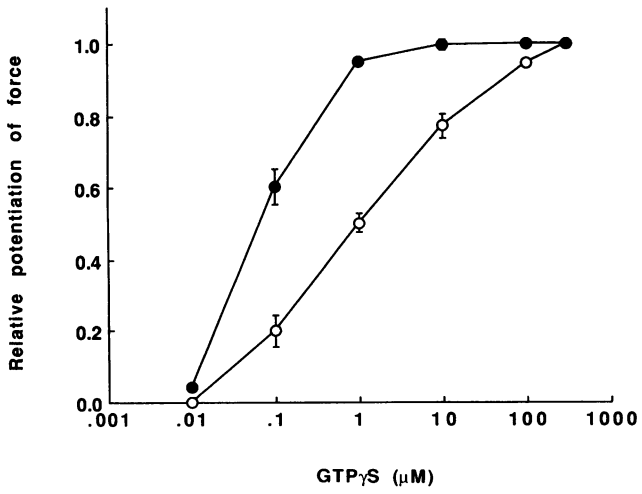

FIG. 1. Dose–response relationship of GTP[γS]-induced potentiation of contraction at pCa 6.5 with (●) and without (○) 100 μM phenylephrine. GTP[γS] was cumulatively added when contraction induced by pCa 6.5 reached a steady-state plateau with or without phenylephrine. Force unit is the difference between forces at pCa 6.5 without and with 300 μM GTP[γS] (see also Table 1). Maximum level of potentiation by GTP[γS] alone differed insignificantly from that with phenylephrine (n = 3–6).

Table 1. Relationship between MLC phosphorylation and force and the effect of 300 μM GTP[γS] on rabbit portal vein at 15°C

pCa condition	Phosphorylation, % of total MLC ± SE (n)	Force, % of force at pCa 5 ± SE (n)	k_1,* min^{-1} ± SE
>8	9 ± 3.1 (3)	0	0.11 ± 0.04
>8 + GTP[γS]	19 ± 2.4 (3)	2 ± 0.2 (3)	
6.5	21 ± 1.3 (4)	13 ± 3.3 (8)	0.27 ± 0.02
6.5 + GTP[γS]	41 ± 2.1 (3)	76 ± 6.0 (4)	
5	77 ± 4.7 (9)	100	3.42 ± 0.94
5 + GTP[γS]	81 ± 5.5 (5)	112 ± 2.7 (8)	

*Data were calculated from Eq. 1; t in the equation is time in min. The average force without GTP[γS] at pCa 5 was 2.0 ± 0.17 × 10^5 N/m^2 strip cross section (n = 7). See text for further detail.

GTP[γS] (300 μM), present in both activating and relaxing solutions, markedly prolonged the relaxation rate that followed Ca^{2+} removal and ML-9 addition: $t_{1/2}$ increased significantly ($P < 0.01$) from 2.3 ± 0.3 (n = 6) to 4.3 ± 0.3 min (n = 4). Addition of GTP[γS] 10 s after removal of Ca^{2+} and ML-9 application also prolonged relaxation: $t_{1/2}$ was increased to 3.0 ± 0.3 min (n = 4; $P < 0.05$ by paired t test).

Fig. 2 shows the effect of 300 μM GTP[γS] (added 5 min before activation by pCa 5) on the time course of dephosphorylation of MLC. GTP[γS] decreased the rate of MLC dephosphorylation to 47% of control value. The time course of dephosphorylation of phosphorylated MLC (*PM*) between 0 and 2 min was fitted with a single exponential equation: $PM = 101.9 \times \exp(-1.044 \times t)$ for control and $PM = 101.4 \times \exp(-0.494 \times t)$ for 300 μM GTP[γS]. Each regression coefficient was 0.99. Much lower concentrations (3 μM) of GTP[γS] were sufficient to similarly inhibit MLC dephosphorylation with an agonist, phenylephrine (Fig. 2): $PM = 100.9 \times \exp(-0.554 \times t)$. This result is consistent with the effect of phenylephrine on GTP[γS]-induced Ca^{2+} sensitization of contraction (Fig. 1) and confirms that inhibition of MLC phosphatase by GTP[γS] alone at higher concentration is not due to a nonspecific, direct (i.e., not G protein-mediated) effect of GTP[γS] on protein phosphatase. The control rate constant (1.044 min^{-1}) of MLC dephosphorylation, assuming a Q_{10} of 3–4, agreed with reported values (18, 19). The simultaneously measured relaxation was much slower with than without GTP[γS], and the residual (rigor) level of force 10 min after removal of ATP and Ca^{2+} and addition of ML-9 was also higher with than without the guanine nucleotide analogue.

When 300 μM GTP[γS] was applied 10 s after withdrawal of ATP and Ca^{2+} and addition of ML-9 to inhibit MLC kinase activity, levels of phosphorylation (23 ± 4.8% of total MLC at 2 min and 11 ± 2.2% at 4 min) were significantly higher than control values, indicating that GTP[γS] inhibited dephosphorylation, even without Ca^{2+}. There was no significant inhibition at 1 min, probably due to the slow onset of the GTP[γS] effect, even when liberated by photolysis of the caged nucleotide analogue (20).

Extensive permeabilization of muscles with saponin (0.2 mg/ml for 15 min), in contrast to permeabilization with α-toxin (Fig. 3*A*), abolished the Ca^{2+}-sensitizing effect of GTP[γS] on contraction, whereas the "Ca^{2+}-sensitizing" action of microcystin-LR, a direct protein phosphatase in-

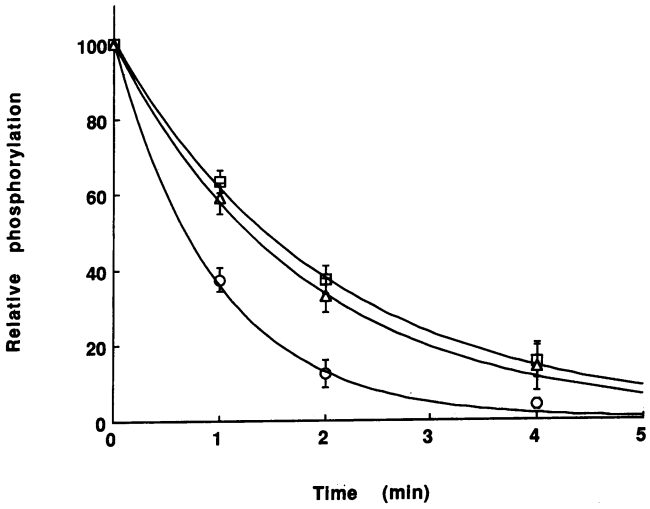

FIG. 2. Time course of MLC dephosphorylation and the effects of 300 μM GTP[γS] alone and in combination (3 μM) with 100 μM phenylephrine at 15°C. α-Toxin-permeabilized rabbit portal vein smooth muscle activated by pCa 5 for 10 min was incubated in Ca^{2+}- and ATP-free solution containing 100 μM ML-9 to block MLC kinase activity. GTP[γS] (300 μM) (□) and 3 μM plus 100 μM phenylephrine (△) were present in both activating and Ca^{2+}-free, rigor solutions. Phosphorylation is expressed as percentage MLC phosphorylation at pCa 5 and 0 min under each condition. One hundred percent of relative phosphorylation corresponds to 77 ± 4.7% of total MLC (mean ± SEM, n = 9) at pCa 5 alone, 81 ± 5.5% (n = 5) with 300 μM GTP[γS], and 74 ± 3.9% (n = 8) with 3 μM GTP[γS] and phenylephrine. The time course was fitted with $PM = 101.9 \times \exp(-1.044 \times t)$ for control (○), $PM = 101.4 \times \exp(-0.495 \times t)$ for GTP[γS] alone (300 μM), and $PM = 100.9 \times \exp(-0.554 \times t)$ for GTP[γS] (3 μM) plus agonist, where PM represents the fraction of phosphorylated MLC, and t is time in min. Values are expressed as mean ± SEM (n = 5–9).

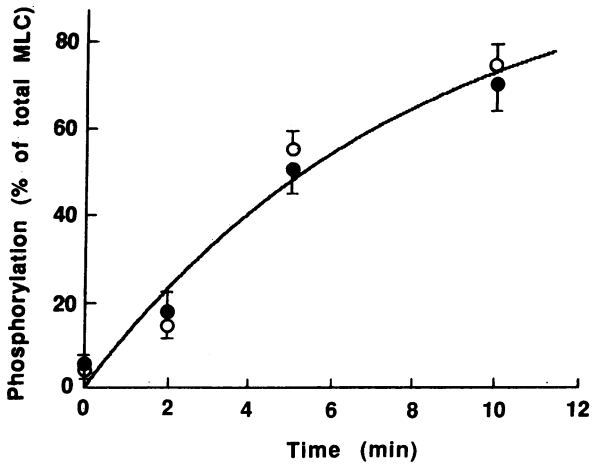

FIG. 4. Time course of ATP[γS]-induced MLC thiophosphorylation at pCa 6.5 and 15°C. ATP[γS] (2 mM) was added to start thiophosphorylation after a 25-min incubation in adenine nucleotide-free solution. Ca^{2+} (pCa 6.5) with (●) and without (○) 300 μM GTP[γS] was applied 5 min before ATP[γS] addition. Ca^{2+} level was clamped with 10 mM EGTA. Solid line represents $PM = 1 - \exp(-0.128 \times t)$ (n = 4–5).

hibitor (21), completely remained (Fig. 3*B*). This result indicates that the GTP[γS] effect depends on G proteins associated with membranes or otherwise removed or inactivated by vigorous saponin permeabilization.

No Effect of GTP[γS] on Rate of Thiophosphorylation. The GTP[γS]-induced increase in MLC phosphorylation and force could also be from stimulation of MLC kinase activity. To determine whether GTP[γS] affects this enzyme, we used ATP[γS] as substrate because MLC thiophosphorylation resists dephosphorylation (16). After incubation in ATP-free solution for 25 min (Ca^{2+}-free condition for the first 20 min and pCa 6.5 with or without GTP[γS] for the last 5 min), 2 mM ATP[γS] was added to initiate thiophosphorylation. Fig. 4 shows that 300 μM GTP[γS] had no effect on the time course of MLC thiophosphorylation, indicating that GTP[γS] had no effect on MLC kinase activity.

Model Calculation. We examined whether the measured inhibition of MLC dephosphorylation by GTP[γS] could yield the steady-state increase in MLC phosphorylation during Ca^{2+} sensitization. We assume, from biochemical results, the following simple two-state model for the reaction of MLC phosphorylation: $M \underset{k_2}{\overset{k_1}{\rightleftharpoons}} PM$, where M and PM represent the concentrations of unphosphorylated and phosphorylated MLC, respectively; and k_1 and k_2 are the on and off rate constants, respectively, of phosphorylation. If $k_1 = 0$ (MLC kinase is inactive) and $PM = A$ at time $t = 0$, the amount of

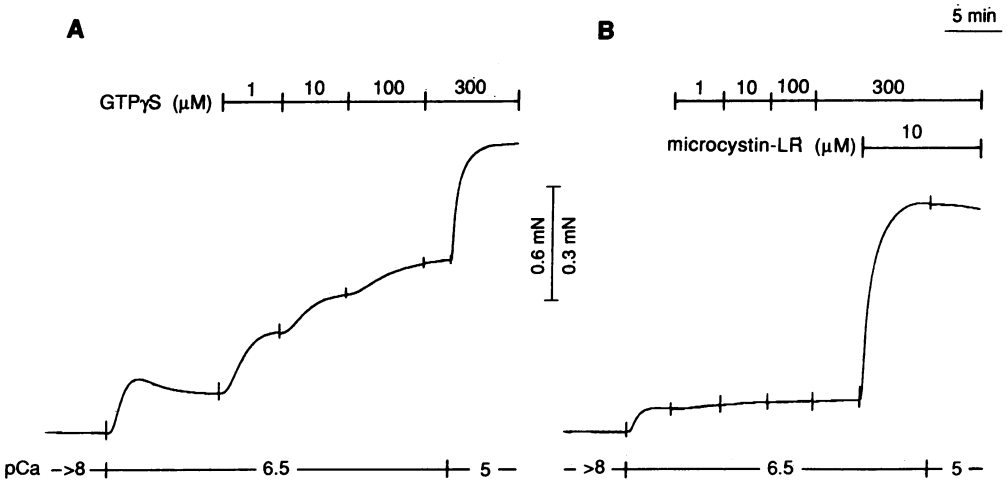

FIG. 3. Abolition of GTP[γS]-induced but not of microcystin-LR-induced potentiation of contraction in portal vein smooth muscle permeabilized with saponin. (*A*) Muscles were permeabilized with α-toxin at 5000 units/ml for 45 min at pCa 6.3 and treated with 10 μM A23187 for 30 min. (*B*) Muscles were permeabilized with saponin at 0.2 mg/ml for 15 min in relaxing (0.2 mM EGTA) solution containing 10 μM A23187. In experiments with saponin, all solutions contained 2.5 μM calmodulin, 100 μM leupeptin, and 1 mM dithiothreitol. Note that the GTP[γS]-induced potentiation of contraction seen in α-toxin-permeabilized muscles (*A*) was abolished in vigorously saponin-permeabilized ones (*B*), and yet a direct inhibitor of protein phosphatase, 10 μM microcystin-LR, markedly potentiated the contraction.

phosphorylated MLC represents $PM = A \times \exp(-k_2 \times t)$. The curves in Fig. 2 represent this case. The measured k_2 with and without GTP[γS] was, respectively, 0.494 and 1.044 min^{-1} at 15°C. When $k_2 = 0$ (when phosphatase activity is inhibited), $PM = 1 - \exp(-k_1 \times t)$. The k_1 of the thiophosphorylation was 0.128 min^{-1} at pCa 6.5 and 15°C, as shown in Fig. 3. In the case of normal phosphorylation,

$$PM = k_1 \times [1 - \exp\{-(k_1 + k_2) \times t\}]/(k_1 + k_2). \quad [1]$$

Because Ca^{2+}-dependent regulation of the smooth muscle phosphatase has not been demonstrated, we assume k_2 to be independent of $[Ca^{2+}]$. The steady-state values of PM at pCa 6.5 were estimated, using the above measured k_1 and k_2, to be 11% of total MLC in the absence and 21% in the presence of GTP[γS]: both values are about half of the measured values (Table 1). This difference might be caused by k_1 being smaller, rather than k_2 being larger, than estimated from the steady-state values, because ATP[γS] only thiophosphorylates Ser-19, not Thr-18 of MLC (22). Also ATP[γS] is probably not as good a substrate for MLC kinase as is ATP. Alternatively, k_1 can be derived through Eq. 1 from the steady-state phosphorylation values (Table 1). Using the latter values of k_1, we calculate that GTP[γS] can increase the steady-state value to, respectively, 17 ± 5.0, 36 ± 1.8, and 87 ± 2.9%, in good agreement with the measured (Table 1) GTP[γS]-induced increases in phosphorylation (respectively, 19 ± 2.4, 41 ± 2.1, and 80 ± 5.5% of total MLC). Thus, the measured reduction in the off rate constant of phosphorylation by the guanine nucleotide analogue (Fig. 2) appears sufficient to explain the GTP[γS]-induced increase in steady-state phosphorylation (Table 1). It follows that GTP[γS] would have had to cause a 2-fold increase in MLC kinase activity to obtain the measured sensitization; such an easily detectable increase in thiophosphorylation was clearly not seen.

DISCUSSION

The primary finding of this study is the clear-cut inhibition of dephosphorylation of smooth muscle MLC by an α_1-adrenergic agonist and GTP[γS]. These agents, as well as several other agonists, can also increase MLC phosphorylation and force by means of a mechanism that is inhibited by guanosine 5′-[β-thio]diphosphate (8, 10, 12). Taken together, these results indicate that excitatory agonists can increase MLC phosphorylation through inhibition of MLC phosphatase(s). The protein phosphatase or phosphatases modulated by G protein(s) remain to be identified. The ≈50% inhibition of the rate of MLC dephosphorylation (Fig. 2) could quantitatively account for the Ca^{2+}-sensitizing effects of GTP[γS]—the increase in MLC phosphorylation and force at constant free Ca^{2+}. At the same time, we found no evidence of GTP[γS] having any effect on MLC kinase when we used ATP[γS] as the phosphate donor to eliminate the effects of phosphatases. These results are consistent with our hypothesis (14) that the Ca^{2+}-sensitizing action of agonists is mediated through G protein-coupled inhibition of MLC phosphatase. We suggest that similar modulation of protein phosphatases by G proteins may have general functions in other cell systems.

The maximum rate constant of phosphorylation at 15°C is calculated as 3.4 ± 0.9 min^{-1} (Table 1), based on 77 ± 4.7% of total MLC phosphorylated at pCa 5. This rate at steady state is, assuming a Q_{10} of 2 (23), rather lower than the value (1.1 s^{-1}) measured during the initial phase of contraction at 37°C (24), possibly due to desensitization of MLC kinase activity to Ca^{2+} at steady state (11, 12, 25). The rate constant under Ca^{2+}-free (pCa > 8) conditions is similarly calculated to be 0.11 ± 0.04 min^{-1} or ≈3% of the maximum: this rate is sufficient to account for the rate ($t_{1/2}$ of <10 min) of force development of permeabilized smooth muscle by GTP[γS] in Ca^{2+}-free solution. This level (3%) of kinase activity without Ca^{2+} appears much higher than that without Ca^{2+} (0.05% of the maximum activity) of isolated MLC kinase in the presence of 2 μM calmodulin (M. Ikebe, personal communication), suggesting that another kinase, perhaps the autophosphorylated form of Ca^{2+}/calmodulin kinase 2 (26), or a Ca^{2+}-independent, proteolytic fragment (1) of MLC kinase, is responsible for MLC phosphorylation in the absence of Ca^{2+}.

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