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THE INFLUENCE OF Mg^{2+} ON THE PHOSPHORYLATION AND DEPHOSPHORYLATION OF MYOSIN BY AN ACTOMYOSIN PREPARATION FROM VASCULAR SMOOTH MUSCLE [1]

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SUMMARY Previous work has shown that Mg^{2+} levels modulate the net level of myosin light chain phosphorylation in bovine aortic smooth muscle actomyosin preparations. The goal of this study was to determine the precise step, i.e. phosphorylation or dephosphorylation, where Mg^{2+} modulates the net phosphorylation reaction. The technique using [γ^{35}S]ATPγS to monitor the phosphorylating step yielded no effect of either Mg^{2+} or Ca^{2+}. Unfortunately the lack of Ca^{2+}-dependence did not allow conclusions about the influence of Mg^{2+} on myosin light chain kinase activity. The study of the effect of Mg^{2+} on dephosphorylation showed that phosphatase activity in the actomyosin preparation exhibited a Mg^{2+} modulation only when the actomyosin was previously exposed to activating levels ($3x10^{-5}$M) of Ca^{2+}, suggesting the presence of a Ca^{2+} -regulation system for myosin light chain phosphatase.

INTRODUCTION

The actual mechanisms underlying activation and relaxation of the contractile proteins of smooth muscle are still controversial. The most generally accepted mechanism associates a calcium dependent phosphorylation of the 20,000 M_r light chain of myosin with activation and subsequent dephosphorylation with relaxation (1). Other workers have suggested different mechanisms of activation-relaxation involving proteins associated with the thin filament (2,3). The only mutually accepted step is that activation is initiated by a rise in sarcoplasmic calcium levels.

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Certain factors have been shown to influence one of the biochemical equivalents of tension development, calcium stimulated actomyosin ATPase[4] activity. Among these factors are pH (4), cyclic AMP (5), and Mg^{2+} (6). We have recently demonstrated that vascular smooth muscle actomyosin ATPase activity is maximal at free Mg^{2+} levels of approximately 3-5mM with depressions of activity at levels of Mg^{2+} both higher and lower than this range. In addition, we demonstrated that myosin light chain phosphorylation levels exhibited the same Mg^{2+} dependence as the actomyosin ATPase activity, suggesting Mg^{2+} may exert at least part of its influence at the level of the phosphorylation-dephosphorylation sequence.

METHODS

Calcium-sensitive actomyosin was prepared from bovine thoracic aorta by the method of Litten and coworkers (7). To obtain a ^{32}P-labeled myosin, the initial actomyosin was first incubated under conditions compatible with maximum phosphorylation (6), i.e. 5mM Mg^{2+}, 30μM Ca^{2+}, 3mM MgATP, and with [γ-^{32}P]ATP (100 Curies/mmole specific activity in the incubation bath). After five minutes of incubation, the incubation mixture was added to a solution composed of 150mM $MgCl_2$, 7.5mM ATP, 4mM EDTA, 40mM Imidazole (pH 7.0) and 0.5mM dithiothrietol to achieve a protein concentration of 3mg/ml. Myosin was then isolated from this mixture by the method of Persechini and co-workers (8).

Incubation conditions for all experiments were determined by the use of a computer program for the solution of the appropriate conditions as described by Solaro and Shiner (9). For these experiments, the following parameters were held constant: ionic strength = 0.1, pH = 7.0, temperature = 37° C, MgATP = 3mM and actomyosin = 1mg/ml. The various free calcium (Ca^{2+}) and magnesium (Mg^{2+}) levels were achieved primarily by varying the amounts of $CaCl_2$ and $MgCl_2$ added as dictated by the solutions of the appropriate equilibrium equations. All protein concentrations were determined by the method of Lowry, et al. (10) using bovine serum albumin as the standard.

Myosin phosphorylation or dephosphorylation was followed by monitoring the gain or loss of ^{32}P (or ^{35}S) incorporated by a modification of the method of Aksoy, et al. (11). Briefly, a sample of the reaction mixture is taken from the bath and the reaction stopped by rapid addition of the sample to an equal volume of ice-cold 10% trichloroacetic acid containing 2% pyrophosphate. This mixture was then incubated for 20 minutes at 90° C. The precipitate was captured by filtration through Millipore filters which had been pre-washed with 5 volumes of 100mM ATP containing 1mM cysteine. The filters and precipitate were then washed with 5 volumes of 5% trichloroacetic acid containing 1% pyrophosphate and 1mM cysteine, 1 volume of 100mM ATP containing 1mM cysteine, and 5 volumes of deionized water in that order. Filters were then dried for 3 hours at 50° C and added directly

4. Abbreviations used: ATPase, adenosinetriphosphatase (EC 3.6.1.3.); EGTA, ethylene glycol bis(2-aminoethyl)N,N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; ATPγS, adenosine-5'-O-(3-thiotriphosphate).

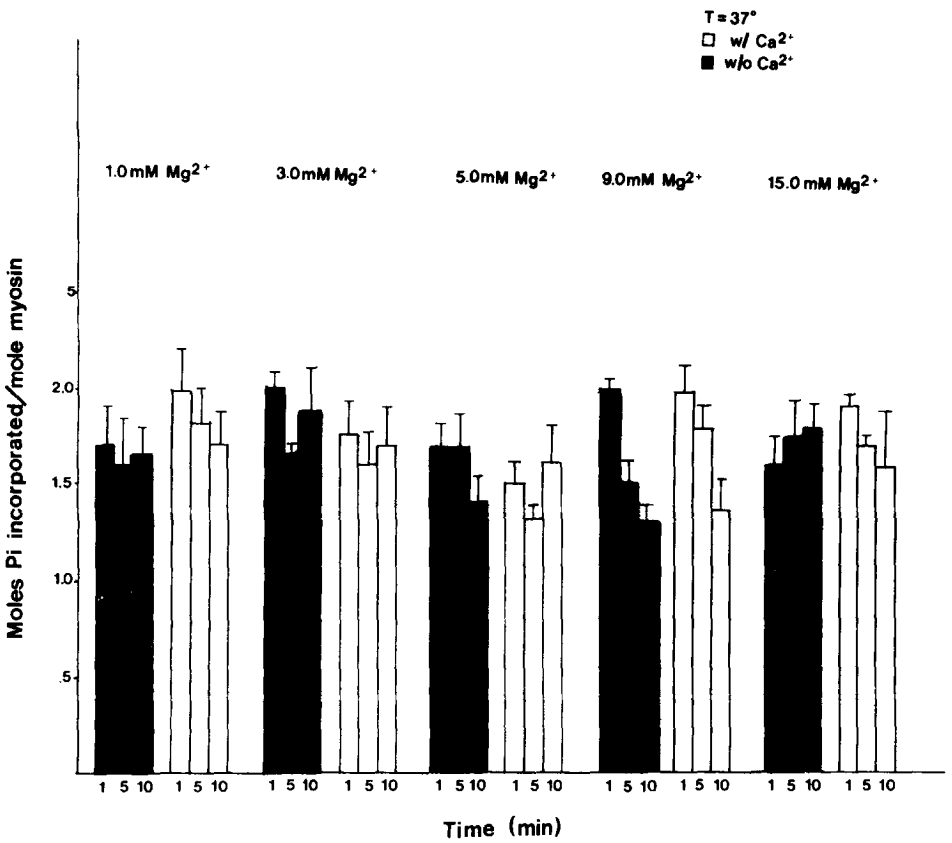

Figure 1.
Thiophosphorylation of vascular smooth muscle actomyosin at various Mg^{2+} levels at high ($3x10^{-5}$, open bars) and low ($1x10^{-7}$), closed bars) Ca^{2+}. Assay conditions were T = 37° C, 1mg/ml actomyosin, 20mM Imidazole (pH 7.0), 3mM EGTA, KCl as necessary to achieve u = 0.1, 2mM MgATPγS, $MgCl_2$ and $CaCl_2$ as necessary to achieve the indicated Ca^{2+} and Mg^{2+} levels, and [γ-^{35}S]ATPγS to achieve a specific activity of 2 Curie/mmole. The affinity constants for metal ions for ATPγS were assumed to be the same as for ATP. Activity is expressed as mole thiophosphate incorporated/mole myosin determined from the ^{35}S bound. Error bars represent ± SEM of at least 4 experimental determinations using at least two separate actomyosin preparations.

to liquid scintillation medium for subsequent counting. Pre-washing of the filters with cold ATP was found to be necessary to prevent any labeled ATP from binding to the filters.

[γ-^{32}P]ATP was obtained from ICN and [γ-^{35}S]ATPγS was obtained from New England Nuclear. Unlabeled ATPγS was obtained from Boehringer-Mannheim.

RESULTS

The ATP analog, ATPγS, has been demonstrated to be a suitable substrate for the myosin light chain kinase (MLCK) catalyzed phosphorylation of the 20,000 M_r light chain of myosin (LC_{20}) with the subsequent thiophosphorylated LC_{20} being phosphatase resistant (12,13). It would appear, therefore, that the specific influence of Mg^{2+} on the MLCK-catalyzed

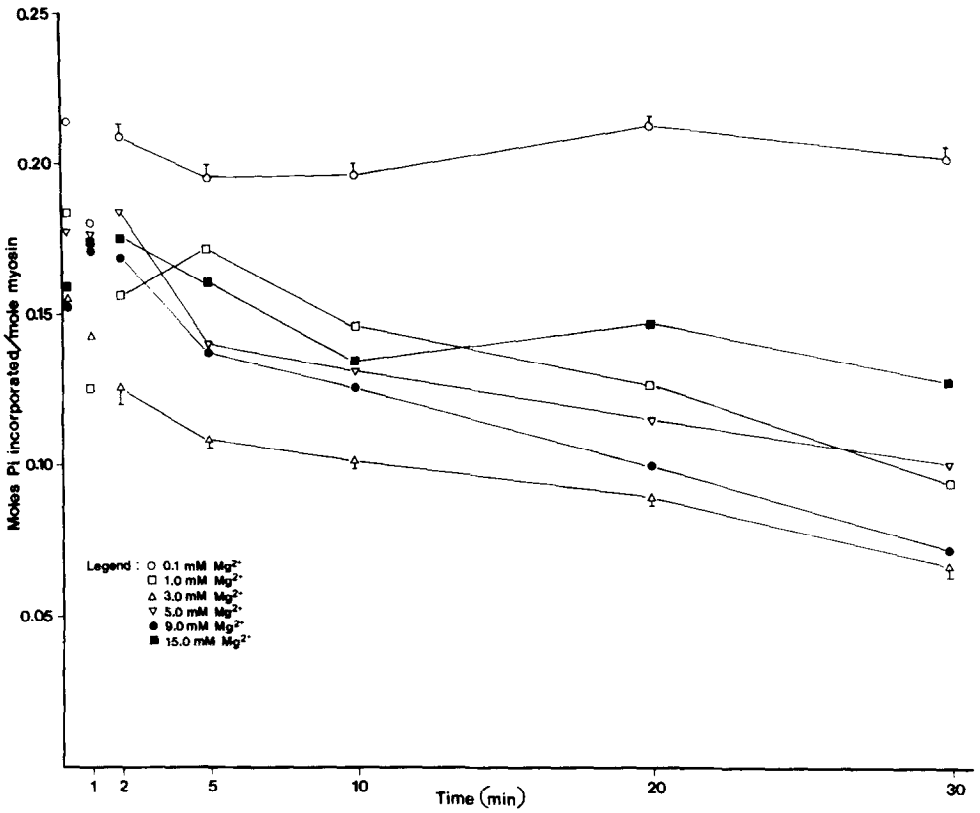

Figure 2.
Myosin light chain phosphatase activity as a function of time at varying Mg^{2+} levels using added exogenous myosin as substrate. Conditions were the same as in Figure 1 except that the Ca^{2+} level was always $1x10^{-7}$ and no added ATP. The reaction was started by the addition of 0.7mg/ml of exogenous myosin previously labeled with ^{32}P. The ordinate is the level of ^{32}P bound expressed as mole Pi/mole exogenous myosin. Error bars represent ± SEM of at least 4 different preparations.

reaction could be determined by following the incorporation of ^{35}S from [γ^{35}S]ATPγS at varying Mg^{2+} levels in an actomyosin preparation. The results, shown in Figure 1, were quite disappointing in this regard. Over the range of 0.1 to 15mM Mg^{2+}, we could observe no difference in the thiophosphorylation in either the presence or absence of Ca^{2+} at the various Mg^{2+} levels.

Phosphatases from smooth muscle are quite labile and their isolation in native form has proven quite difficult (14). To circumvent this problem, we attempted to assay the phosphatase endogenous to the actomyosin preparation and see if we could discern an effect of Mg^{2+} on this portion of the phosphorylation-dephosphorylation sequence. One such approach is to add a

labeled, phosphorylated myosin to the actomyosin preparation and follow the decrease in myosin phosphorylation. The results of this approach are shown in Figure 2. Note that at 0.1mM Mg^{2+} there is no discernible loss of myosin phosphorylation, but at levels of at least 1mM Mg^{2+} there is a steady loss of myosin phosphorylation, a result consistent with the presence of a Mg^{2+}-requiring phosphatase in the actomyosin preparation. Another approach to this same question is to allow the actomyosin preparation to phosphorylate its own myosin, stop the reaction by adding excess EGTA to inhibit the Ca^{2+}-dependent MLCK (15,16) and follow the subsequent loss of myosin phosphorylation. The results, shown in Figure 3, again demonstrate that no significant dephosphorylation occurs at 0.1mM Mg^{2+}. At levels of 1mM Mg^{2+} or higher, there is an initial rapid loss of myosin phosphorylation followed by a slow, but steady loss. This initial phase was not observed when purified myosin was added as substrate to the actomyosin preparation. In addition this rapid initial phase exhibited a dependence on the Mg^{2+} level present in the bath. This dependence is shown by comparing the rates of dephosphorylation under the varying conditions as in Figure 4.

DISCUSSION

The studies examining the phosphorylation of myosin light chain using ATPγS were surprising but disappointing from the standpoint of the purpose of this report. The apparent lack of either Ca^{2+} or Mg^{2+} dependence of thiophosphorylation is consistent with the observation by Peterson (17) that ATPγS produced a Ca^{2+}-insensitive, irreversible contracture of "skinned" vascular smooth muscle. These results are not consistent with the results of similar studies using visceral smooth muscle (18), suggesting that there may be fundamental differences in the myosin light chain kinase or the biochemical mechanisms producing myosin light chain phosphorylation in these two different types of smooth muscle.

The studies examining the dephosphorylation of myosin light chain were also surprising to some extent but much more consistent with the overall purpose of this report. Mg^{2+} levels exhibited one or two effects

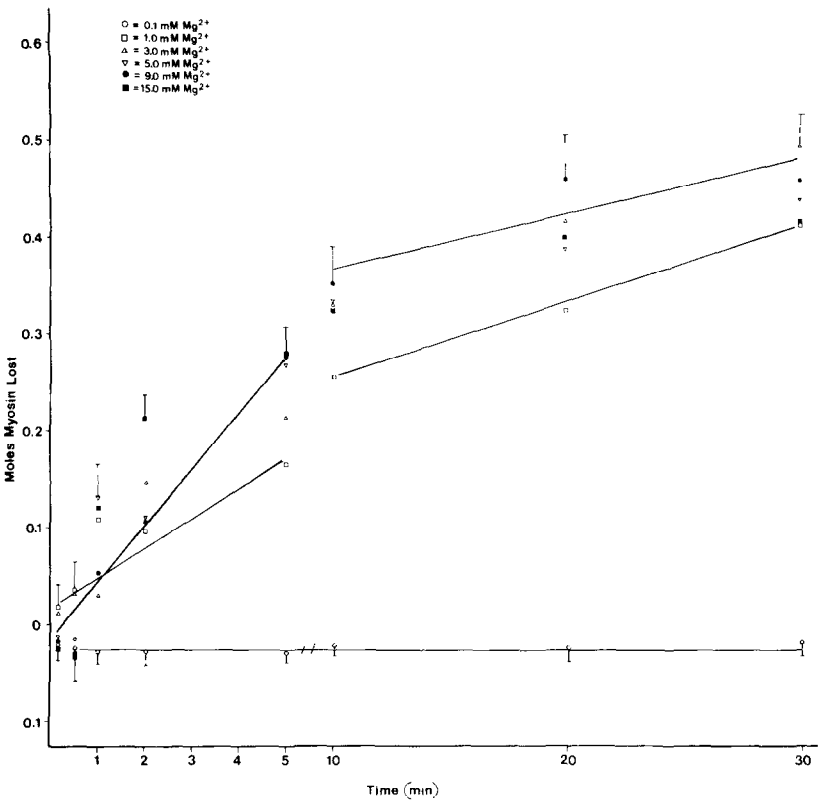

Figure 3.

Myosin light chain phosphatase activity as a function of time at varying Mg^{2+} levels using endogenous myosin as substrate. Conditions were the same as in Figure 2 except initially there was 0.1mM EGTA and $CaCl_2$ to achieve $3x10^{-5}$M Ca^{2+} and 3mM MgATP with 100 Curie/mmole [γ-^{32}P]ATP. At the time of maximal phosphorylation (6), EGTA was added to 5.1mM EGTA final concentration ($1x10^{-7}$M Ca^{2+}) to stop the phosphorylation reaction. The dephosphorylation was then followed as described. Error bars represent + SEM of at least three experimental determinations using at least two different actomyosin preparations.

depending on the assay condition. Using exogenous myosin as substrate, a requirement for at least 1mM Mg^{2+} to produce myosin light chain phosphatase activity was demonstrated but no further effect of Mg^{2+} was observed. Using endogenous myosin as substrate, both a requirement for 1mM Mg^{2+} plus a Mg^{2+} modulation of myosin light chain phosphatase activity over the range of 1 to 9mM Mg^{2+} were demonstrated. The requirement for minimal levels of Mg^{2+} for myosin light chain phosphatase activity is consistent with recent reports on isolated phosphatases (14). The difference in modulatory effect depending on the assay condition could have two explanations, a difference in substrate or a regulatory mechanism

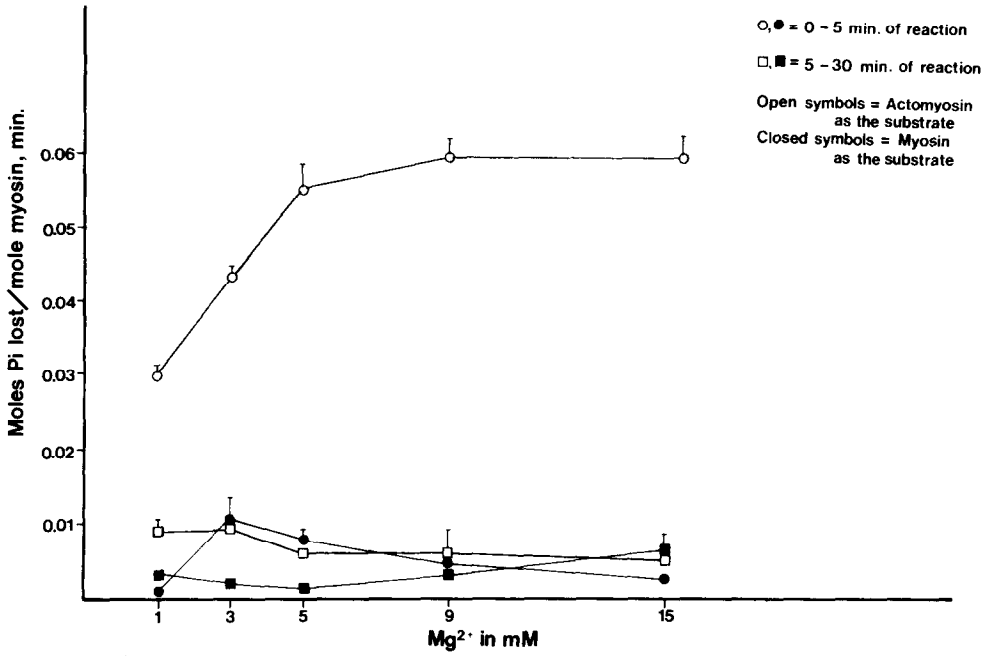

Figure 4.
Myosin light chain phosphatase activity as a function of Mg^{2+} concentration. The data is the same as presented in Figures 2 and 3 except apparent rate constants were determined by fitting the appropiate data points by linear regression analysis. Circles represent slopes from points taken in the first five minutes and squares represent slopes from points taken 5 to 30 minutes after initiation of the reaction.

operating on the myosin light chain phosphatase requiring Ca^{2+} for activation. Difference in substrate could either be due to a non-myosin phosphorylated protein being present in the actomyosin preparation or a difference in the phosphorylated myosin itself, depending on the degree of phosphorylation. The former possibility can largely be ruled since we have previously shown nearly all the incorporated ^{32}P under our assay conditions is associated with the LC_{20} of myosin (6). The latter possibility cannot, however, be ruled out particularly in view of the recent demonstration of cooperativity in the phosphorylation of myosin (19), but it will be difficult to substantiate. Our preferred explanation is that there is a Ca^{2+}-activated regulatory system for myosin light chain phosphatase which is Mg^{2+} modulated in vascular smooth muscle. This would explain why our assay using exogenous myosin in which the actomyosin was never exposed to high Ca^{2+} levels exhibited only a basal phosphatase activity while our assay using endogenous myosin which required exposing the actomyosin to

high Ca^{2+} to induce phosphorylation exhibited a more active, Mg^{2+}-modulated phosphatase activity. It would be both ironic and logical if Ca^{2+} induced a phosphorylation of myosin to produce contraction and a subsequent phosphorylation of myosin light chain phosphatase to induce relaxation.

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