Magnesium Relaxes Arterial Smooth Muscle by Decreasing Intracellular Ca^{2+} Without Changing Intracellular Mg^{2+}

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Abstract

Elevations in extracellular $[Mg^{2+}]$ ($[Mg^{2+}]_o$) relax vascular smooth muscle. We tested the hypothesis that elevated $[Mg^{2+}]_o$ induces relaxation through reductions in myoplasmic $[Ca^{2+}]$ and myosin light chain phosphorylation without changing intracellular $[Mg^{2+}]$ ($[Mg^{2+}]_i$). Histamine stimulation of endothelium-free swine carotid medial tissues was associated with increases in both Fura 2- and aequorin-estimated myoplasmic $[Ca^{2+}]$, myosin phosphorylation, and force. Elevated $[Mg^{2+}]_o$ decreased myoplasmic $[Ca^{2+}]$ and force to near resting values. However, elevated $[Mg^{2+}]_o$ only transiently decreased myosin phosphorylation values: sustained $[Mg^{2+}]_o$-induced decreases in myoplasmic $[Ca^{2+}]$ and force were associated with inappropriately high myosin phosphorylation values. The elevated myosin phosphorylation during $[Mg^{2+}]_o$-induced relaxation was entirely on serine 19, the Ca^{2+}/calmodulin-dependent myosin light chain kinase substrate. Myoplasmic $[Mg^{2+}]$ (estimated with Mag-Fura 2) did not significantly increase with elevated $[Mg^{2+}]_o$. These results are consistent with the hypothesis that increased $[Mg^{2+}]_o$ induces relaxation by decreasing myoplasmic $[Ca^{2+}]$ without changing $[Mg^{2+}]_i$. These data also demonstrate dissociation of myosin phosphorylation from myoplasmic $[Ca^{2+}]$ and force during Mg^{2+}-induced relaxation. This finding suggests the presence of a phosphorylation-independent (yet potentially Ca^{2+}-dependent) mechanism for regulation of force in vascular smooth muscle. (*J. Clin. Invest.* 1992. 89:1988–1994.) Key words: aequorin • Fura 2 • Mag-fura 2 • myosin phosphorylation • smooth muscle

Introduction

Much of the research on magnesium has centered on the clinical effects of altered extracellular $[Mg^{2+}]$ ($[Mg^{2+}]_o$)[1] on vascular, cardiac, and uterine smooth muscle. Mg^{2+} deficiency is associated with a high frequency of cardiac arrhythmias, hypertension, and sudden ischemic myocardial death (1, 2), while Mg^{2+} supplementation decreases arterial blood pressure and reduces the frequency of cardiac arrhythmias after myocardial infarction (1, 3). Magnesium sulfate is widely used in the cessation of premature labor and in the treatment of preeclampsia/eclampsia (4).

In this study, we sought to examine the mechanism responsible for $[Mg^{2+}]_o$-induced smooth muscle relaxation. Myoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$)-dependent activation of myosin light chain kinase and its phosphorylation of myosin's 20-kD regulatory light chain appear to be the primary determinant of the contractile response in smooth muscle (5, 6). In vitro studies have demonstrated relaxation of precontracted vascular smooth muscle in solutions with increased $[Mg^{2+}]_o$ (7–9), and augmentation of agonist-induced contractions in Mg^{2+}-free solutions (7, 8, 10, 11). Several proposed mechanisms for the effects of increased $[Mg^{2+}]_o$ include decreases in ($[Ca^{2+}]_i$) due to alteration of membrane permeability, disruption of agonist-receptor interactions, and/or blockage of Ca^{2+} channels (7). However, increases in intracellular $[Mg^{2+}]$ ($[Mg^{2+}]_i$) are associated with contraction in skinned smooth muscle (12, 13). We tested the hypothesis that elevated $[Mg^{2+}]_o$ produces relaxation of agonist-stimulated vascular smooth muscle by decreasing myoplasmic $[Ca^{2+}]$ (with resultant decreases in myosin light chain phosphorylation and force) without changes in $[Mg^{2+}]_i$.

Our laboratory uses aequorin to estimate myoplasmic $[Ca^{2+}]$ in smooth muscle tissues. Aequorin signals are very sensitive to small changes in $[Mg^{2+}]$ (higher $[Mg^{2+}]$ decreases aequorin light production) (14). If changes in $[Mg^{2+}]_o$ produce changes in $[Mg^{2+}]_i$, then aequorin light signals would not accurately reflect changes in $[Ca^{2+}]_i$. To overcome these problems, we also measured $[Ca^{2+}]_i$ with Fura 2 (which is not very sensitive to changes in $[Mg^{2+}]_i$) and directly measured $[Mg^{2+}]_i$ with Mag-fura 2. Previously, data on the myoplasmic $[Mg^{2+}]$ had been obtained from nuclear magnetic resonance, electron probe analysis, and microelectrodes (15–17).

Methods

Swine carotid arteries were obtained, the adventitia removed, the tissues mounted for isometric stress measurement, and the optimal length for stress development determined (5). Physiologic saline (PSS) contained (mM): NaCl, 140; KCl 5; 3-[*N*-morpholino] propanesulfonic acid (MOPS) 2; $CaCl_2$, 1.6; $MgCl_2$, 1.2; $NaHPO_4$, 1.2; D-glucose, 5.6 (pH 7.4 at 37°C).

Fura 2 estimated myoplasmic $[Ca^{2+}]$. $[Ca^{2+}]_i$ was estimated in a set of tissues with Fura 2 that was loaded intracellularly by incubation of the tissue in the acetomethoxy ester of Fura 2 (Fura 2-AM, Molecular Probes, Inc., Eugene, OR), as previously described (18, 19). Fura 2-AM is not easily solubilized; therefore, a strict protocol was necessary to

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, myoplasmic $[Ca^{2+}]$; L, photon count per second; L_{max}, total aequorin; $[Mg^{2+}]_i$, intracellular $[Mg^{2+}]$; $[Mg^{2+}]_o$, extracellular $[Mg^{2+}]$; MOPS, 3-[*N*-morpholino] propanesulfonic acid; PSS, physiologic saline; R_{max}, the maximal 340/380 ratio observed; R_{min}, the minimal 340/380 ratio observed.

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ensure adequate loading. 0.25 ml of DMSO with 5 mg pluronic F-127 was repeatedly sonicated with a probe sonicator (model H1 at setting 4; Heat Systems-Ultrasonics, Plainview, NY) and cooled in ice water until the pluronic was completely in solution. The DMSO/pluronic solution was added to 50 μg Fura 2-AM and sonicated six times for 1 s with cooling in ice water between sonications. The resulting solution was added to 2.5 ml of PSS that contained neostigmine (to inhibit breakdown of Fura 2-AM by extracellular esterases), and this loading solution was mixed and sonicated. The final loading solution contained 20 μM Fura 2-AM and 250 μM neostigmine. The arterial strips were loaded at 37°C for 3–5 h and washed in PSS for 30 min before each experiment.

The experimental apparatus is very similar to that of Himpens and Somlyo (19), and consists of a Nikon xenon light source, the output of which is directed through a rotating filter wheel that has both 340±5 and 380±5 nm optical filters (Omega Optical, Inc., Brattleboro, VT). The excitation light passes through one arm of a bifurcated light guide, which is placed 0.5–1 mm from the luminal surface of the smooth muscle tissue. The tissue is mounted isometrically to a Harvard Bioscience (South Natick, MA) capacitive force transducer, and bathed in a 3-ml jacketed tissue bath. The second arm of the bifurcated light guide passes emission light through a 525±25-nm filter to an EMI 9828 photomultiplier tube. The output of the photomultiplier tube is electronically demodulated (University of Pennsylvania Biomedical Instrumentation Group) and the 340 and 380 fluorescence and raw force signals are converted to digital signals and stored on a personal computer. Stress was calculated as the total force minus the passive force divided by the cross sectional area. Attempts to calibrate these Fura 2 loaded tissues with ionomycin were unsuccessful (there were very small changes in the 340- and 380-nm signals when $[Ca^{2+}]_o$ was varied between 0 and 500 nM after incubation in 50 μM ionomycin at pH 7.1 or 8.6). After completing experiments, a low $[Ca^{2+}]$ hypotonic solution (EGTA, 2 mM MOPS, pH 7.1 at 37°C) was added to lyse cells, chelate Ca^{2+}, and determine R_{min} (the minimal 340/380 ratio observed). Further incubation in 8 mM EGTA, 10 mM $CaCl_2$, and 2 mM MOPS (pH 7.1 at 37°C) allowed determination of R_{max} (the maximal 340/380 ratio observed). Incubation in 100 mM $MnCl_2$ permitted measurement of background fluorescence (19) that was subtracted from all fluorescence measurements before calculation of the fluorescence ratio. Fura 2 signals were calibrated using the method of Grynliewicz et al. (20).

Aequorin-estimated $[Ca^{2+}]_i$. Myoplasmic $[Ca^{2+}]$ was estimated in a second set of tissues with the photoprotein aequorin that was loaded intracellularly by reversible hyperpermeabilization (5). The aequorin-derived light was collected with a photomultiplier tube, and the photon count per second (L) was divided by an estimate of the total [aequorin] (L_{max}). The logarithm of this ratio ($\log L/L_{max}$) is a function of $[Ca^{2+}]$. Light signals are reported as a change in $\log L/L_{max}$ in which the resting $\log L/L_{max}$ is subtracted from all subsequent $\log L/L_{max}$ values. This normalization markedly decreased interexperimental variability and provided enhanced sensitivity to small changes in $[Ca^{2+}]_i$ (5). Aequorin light signals were calibrated in Ca^{2+}/EGTA buffers at 37°C with $[Mg^{2+}]$ = 0.5 mM.

Mag-Fura 2–estimated myoplasmic $[Mg^{2+}]$. $[Mg^{2+}]_i$ was estimated for a third set of tissues incubated in the acetomethoxy ester of Mag-fura 2 (Mag-fura 2 AM; Molecular Probes, Inc.). The loading solution was prepared as in the Fura 2 methods, except the final [Mag-fura 2 AM] was 15 μM, and the final [DMSO] was 50 μl/ml. Experiments were performed using the experimental apparatus, filters, and tissue bath described in the Fura 2 methods above. Attempts to calibrate the Mag-fura 2–loaded tissues with calcium ionophores were unsuccessful. After completing experiments, a zero $[Mg^{2+}]$ hypotonic solution (8 mM EGTA, 2 mM MOPS, pH 7.1 at 37°C) was added to lyse cells, chelate Mg^{2+}, and determine R_{min}. Next, the tissue was incubated in a high Mg^{2+} solution (8 mM EGTA, 2 mM MOPS, 20 mM Mg^{2+}, pH 7.1 at 37°C) for determination of R_{max}. Background fluorescence was measured during incubation with 100 mM $MnCl_2$ for subtraction from all measurements before calculation of the fluorescence ratio.

Myosin phosphorylation. Myosin light chain phosphorylation was measured in a fourth set of tissues that underwent identical protocols, except that the tissues were not loaded with Fura 2 AM or aequorin. Tissues were frozen by immersion in an acetone/dry ice slurry at −78°C, and the percent total myosin phosphorylation determined (21, 22) using two-dimensional separation of the light chains by isoelectric focusing and SDS-polyacrylamide gel electrophoresis. Fura 2 or aequorin loading has been shown not to alter phosphorylation determination (5, 18).

Tryptic ^{32}P-phosphopeptide mapping. A fifth set of tissues was incubated in oxygenated PSS containing $^{32}PO_4$ (0.4 mCi/ml, 2 h) for subsequent tryptic digestion and peptide mapping of myosin light chain variants (23). The strips were rinsed in PSS, equilibrated, and subjected to the above protocol for myosin light chain phosphorylation. After two-dimensional separation of the myosin light chain isoforms and coomassie blue staining, proteins were transferred electrophoretically to nitrocellulose membranes which were then subjected to autoradiography. The area containing the smooth muscle-specific monophosphorylated myosin light chain isoforms was excised, subjected to tryptic digestion, and the tryptic fragments separated by thin layer electrophoresis and chromatography, as described previously (24). The tryptic fragments were also subjected to acid hydrolysis, and the phosphopeptides separated by thin layer electrophoresis (24).

Determination of cAMP and cGMP. A sixth set of tissues were stimulated, frozen by immersion in an acetone/dry ice slurry, and homogenized in 0.1 N HCl for cAMP and cGMP immunoassay by the Diabetes Core Lab, University of Virginia Health Sciences Center (25).

Results

In this study, we found that the estimated resting $[Ca^{2+}]_i$ was greater in the Fura 2-loaded preparations. If we assume the K_d of Fura 2 in the cytoplasm of the swine carotid was 224 nM, the resting $[Ca^{2+}]_i$ in the four preparations studied with Fura 2 was 188±28 nM. This value was significantly greater than the resting $[Ca^{2+}]_i$ estimate obtained in the four aequorin-loaded preparations (69±13 nM). In a prior study, we found that Fura 2 estimates of resting $[Ca^{2+}]_i$ were slightly, but not significantly, greater than aequorin $[Ca^{2+}]_i$ estimates (106±15 vs. 73±9 nM, n = 18 [18]). The discrepancy in resting $[Ca^{2+}]_i$ estimates in the current study is likely caused by multiple factors (reviewed in references 26–28): (*a*) Calibration of Fura 2 is inaccurate if Fura 2 is loaded into intracellular organelles (which would significantly increase basal Fura 2 $[Ca^{2+}]_i$ estimates), or if the cells contain partially deesterified Fura 2 AM (which is fluorescent but not $[Ca^{2+}]$ sensitive). (*b*) The excitation and emission characteristics, and the K_d of Fura 2 observed in solution are different than these values observed in intact cells (secondary to changes in ionic composition, viscosity, and binding to proteins or other cellular constituents). If the K_d of Fura 2 in the cytoplasm of swine carotid were 82 nM, the two basal $[Ca^{2+}]_i$ estimates would be identical. (*c*) Aequorin light signal calibrations are very sensitive to changes in temperature and $[Mg^{2+}]_i$. If resting $[Mg^{2+}]_i$ were less than 0.5 mM, then the resting aequorin $[Ca^{2+}]_i$ estimate would be larger. Since there are so many uncertainties in calibration, we have chosen to present Fura 2 and aequorin data as background subtracted 340/380 ratio and $\log L/L_{max}$ change. For comparison, the estimated $[Ca^{2+}]_i$ and the percent change in estimated $[Ca^{2+}]_i$ above basal levels are also shown in Table I.

Histamine stimulation of swine carotid tissues increased both Fura 2 fluorescence (340/380 ratio) and aequorin light

Table I. Calibration of Aequorin and Fura 2-estimated $[Ca^{2+}]_i$

Stimulus	$[Mg^{2+}]_o$	Aequorin-estimated $[Ca^{2+}]_i$	Aequorin-estimated % Change above basal	Fura 2-estimated $[Ca^{2+}]_i$	Fura 2-estimated % Change above basal
	mM	*nM*		*nM*	
Control	1.2	69±13	—	188±28	—
Histamine	1.2	157±11	149±29	539±143	175±44
Histamine	2	131±6	110±27	483±129	149±43
Histamine	5	108±13	66±15	384±90	102±31
Histamine	10	68±10	6±17	247±24	37±14
Histamine	0	132±7	111±28	649±137	242±43*
Control	1.2	75±13	14±16	228±14	28±12

Log L/L_{max} change (aequorin) and background corrected 340/380 ratios (Fura 2) were averaged over the 1 min interval that occurred just before each solution change in Fig. 1 (i.e., each measurement was collected between 9 to 10 min after the preceding solution change). $[Ca^{2+}]_i$ was calculated as described in the methods. Percent change above basal $[Ca^{2+}]_i$ was calculated individually for each tissue and then averaged. Data is presented as mean±1 SEM with $n = 4$. * Significantly different vs. aequorin-estimated % change (unpaired t test with $P < 0.05$).

production (log L/L_{max} change; Fig. 1). After 10 min of histamine stimulation, estimated $[Ca^{2+}]_i$ was 175±44% (Fura 2) and 149±29% (aequorin) greater than resting $[Ca^{2+}]_i$ estimates (the difference between the change in Fura 2 and aequorin $[Ca^{2+}]_i$ estimates was not statistically significant). The increased myoplasmic $[Ca^{2+}]$ was associated with significant increases in myosin light chain phosphorylation and stress (force normalized to cross-sectional area).

Sequential increases in $[Mg^{2+}]_o$ from 1.2 to 2, 5, and 10 mM incrementally decreased both Fura 2 fluorescence and aequorin light production, and the tissues proportionally relaxed (Fig. 1). After 10 min of incubation in 3 μM histamine and 10 mM $[Mg^{2+}]_o$ (50 min in Fig. 1), stress decreased by 65±10% and estimated $[Ca^{2+}]_i$ decreased by 79±8% (Fura 2) and 96±11% (aequorin) when compared with the values measured after the first 10 min of histamine stimulation (20 min in Fig. 1). The Fura 2 and aequorin estimated change in $[Ca^{2+}]_i$ did not significantly differ during high $[Mg^{2+}]_o$-induced relaxation (Table I). Myosin phosphorylation values transiently decreased 2 min after addition of 10 mM $[Mg^{2+}]_o$. However, despite sustained decreases in $[Ca^{2+}]_i$ and stress, myosin light chain phosphorylation values (measured 10 min after addition of 10 mM $[Mg^{2+}]_o$) only decreased by 38±15%.

Removal of $[Mg^{2+}]_o$ increased $[Ca^{2+}]_i$ and stress associated with sustained high myosin phosphorylation values. The change in Fura 2-estimated $[Ca^{2+}]$ in 0 mM $[Mg^{2+}]_o$ was significantly greater than the change in aequorin-estimated $[Ca^{2+}]_i$, potentially because the Fura 2 signal gradually was drifting upward. Washout of histamine with normal PSS ($[Mg^{2+}]$ 1.2 mM) returned $[Ca^{2+}]_i$, myosin phosphorylation, and stress to near resting values. These results suggest that elevated $[Mg^{2+}]_o$ relaxes vascular smooth muscle by decreasing $[Ca^{2+}]_i$, since both aequorin and Fura 2 showed significant and parallel decreases in $[Ca^{2+}]_i$ associated with the relaxation.

Myosin light chain phosphorylation only transiently de-

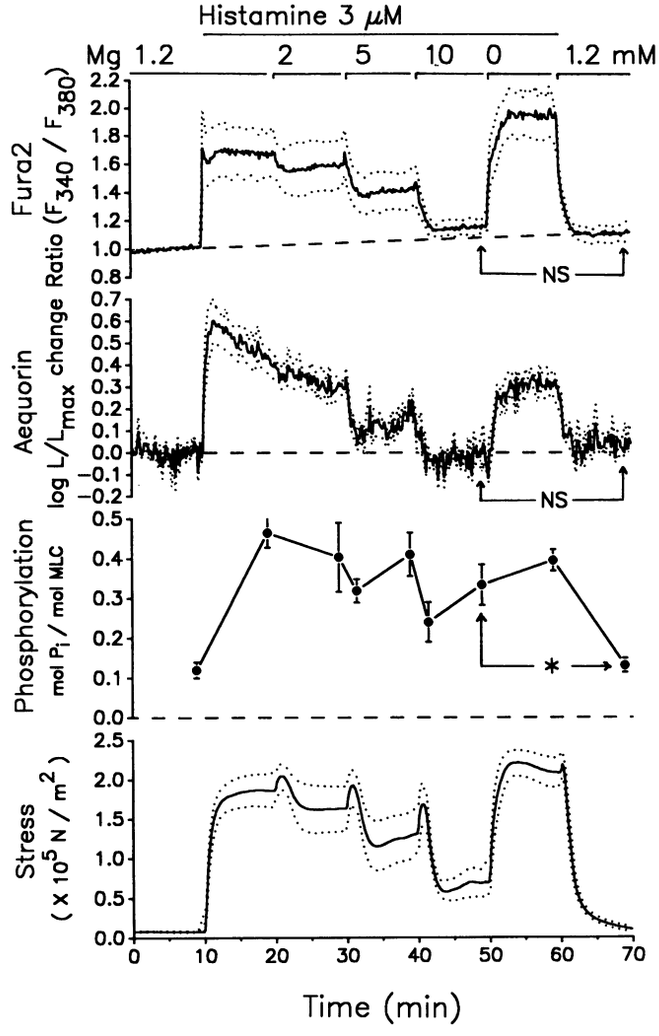

Figure 1. Mean data during $[Mg^{2+}]_o$-induced relaxation of submaximally stimulated tissues. The time courses of Fura 2-estimated myoplasmic $[Ca^{2+}]$ (background corrected 340/380 fluorescence ratio, $n = 6$), aequorin-estimated myoplasmic $[Ca^{2+}]$ (log L/L_{max} change units, $n = 4$), myosin light chain phosphorylation ($n = 4$–5), and active stress were measured in swine carotid medial tissues. Tissues in PSS ($[Mg^{2+}] = 1.2$ mM) were submaximally stimulated at 10 min with 3 μM histamine, and solutions with $[Mg^{2+}]_o$ of 2, 5, 10, and 0 mM were added at 10-min intervals. Histamine was washed out at 60 min to show the change in baseline observed with Fura 2 (aequorin signals are stable over longer periods). Data were collected at 1-s intervals and averaged over 10 s for plotting. Data are shown as mean (*solid line*)±1 SEM (*dotted line*) with the exception that phosphorylation is shown as circles with error bars (myosin phosphorylation was measured 10 min after each solution change and also 2 min after 5 and 10 mM Mg^{2+}). Symbols without error bars represent SEM smaller than the symbol size. Stress is shown from the Fura 2 experiments (*solid lines*) at 10-s intervals. Stress from aequorin and phosphorylation experiments was similar to that observed with Fura 2-loaded tissues, and was omitted for clarity. Fura 2 calibration had a mean R_{min} of 0.51, and a mean R_{max} of 13.1. The intracellular [Fura 2] was 5.2±0.8 μM. Estimated resting $[Ca^{2+}]$ was 181±30 nM in the Fura 2-loaded and 69±14 nM in the aequorin-loaded preparations. NS, not significant. *Significantly different at the $P < 0.05$ level. The rank order correlation for $[Mg^{2+}]_o$ vs. Fura 2 ratio when stimulated with histamine was 1.0 ($P < 0.01$), indicating that the change in Fura 2 fluorescence ratio was highly dependent on changes in $[Mg^{2+}]_o$.

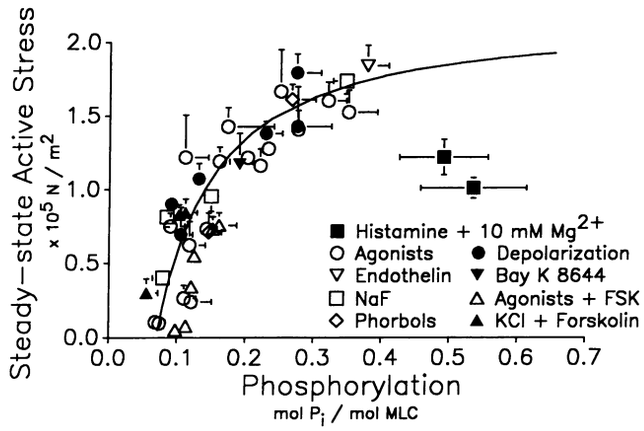

Figure 2. The relationship between steady-state active stress and percent myosin light chain phosphorylation. Vascular smooth muscle contraction by agonists (*open circles*), depolarization (*filled circles*), and various activators of contraction demonstrate a hyperbolic relationship between percent phosphorylation and steady-state stress (data replotted from reference 31). Relaxation of 3 or 10 μM histamine-induced contractions with 10 mM $[Mg^{2+}]_o$ for 2 h (*filled squares*) was associated with elevated phosphorylation ($\sim$ 50%) at relatively low stresses ($\sim$ 60% of maximal stress).

creased during high $[Mg^{2+}]_o$-induced relaxation. We evaluated whether the relatively low stress with high phosphorylation observed 10 min after application of 10 mM $[Mg^{2+}]_o$ was time dependent. We previously showed that various stimuli and stimuli plus forskolin induced a unique relationship between steady-state phosphorylation and stress in the swine carotid (Fig. 2). Tissues were stimulated with 3 or 10 μM histamine and relaxed with 10 mM $[Mg^{2+}]_o$ for 30 min. Stress was constant for the last 15 min of this protocol, indicating a steady-state relationship had been achieved. In the presence of 10 mM $[Mg^{2+}]_o$, significantly less steady-state stress was observed than would be expected for the high levels of myosin phosphorylation (Fig. 2). This result suggests that this finding is not time dependent.

A potential explanation for high myosin light-chain phosphorylation values at low $[Ca^{2+}]_i$ and force is that the myosin light chains may be phosphorylated on an inactive residue during Mg^{2+}-induced relaxation (i.e., phosphorylation may not be on serine 19, the residue phosphorylated by myosin light chain kinase and associated with increases in myosin ATPase activity). Tryptic ^{32}P-phosphopeptide mapping of monophosphorylated myosin light chains was performed on tissues stimulated with histamine alone (Fig. 3 *A*) or stimulated with histamine and then relaxed with high $[Mg^{2+}]_o$ (Fig. 3 *B*). In both cases, the tryptic peptides entirely comigrated with peptides derived from myosin light chains phosphorylated by purified myosin light chain kinase. Amino acid analysis revealed that all the phosphorylation was on serine residues. These data suggest that the myosin phosphorylation was entirely at serine 19 during either histamine stimulation or high $[Mg^{2+}]_o$-induced relaxation.

Changes in $[Mg^{2+}]_o$ could potentially increase [cAMP] or [cGMP], both known relaxants of vascular smooth muscle. We found that 10 mM Mg^{2+} (in a protocol identical to Fig. 1) did not change these levels significantly above resting controls ([cAMP] was 0.204±0.005 pg/mg wet wt vs. the control value of 0.227±0.010 pg/mg wet wt, [cGMP] was 0.070±0.003 pg/mg wet wt vs. the control value of 0.078±0.013 pg/mg wet wt, both not significantly different, n = 4).

We evaluated whether Mg^{2+}-induced relaxation was associated with a change in $[Mg^{2+}]_i$. Four tissues were loaded with Mag-Fura 2, and the ratio of 340 (magnesium-bound) over 380 (magnesium-free) fluorescence and stress were recorded (Fig. 4). Stimulation with 3 μM histamine in normal $[Mg^{2+}]_o$ (1.2 mM) contracted, and addition of 10 mM $[Mg^{2+}]_o$ relaxed the tissues. Subsequent removal of $[Mg^{2+}]_o$ in the presence of 3 μM histamine and depolarization with 109 mM KCl in $[Mg^{2+}]_o$ = 1.2 mM induced near maximal stress. None of these manipulations significantly changed the Mag-Fura 2 ratio. Calibration of the Mag-Fura 2 loaded tissues was performed by lysis of the cells in hypotonic solutions that were Mg^{2+}-free or had high $[Mg^{2+}]_o$ (20 mM). Note the relatively small, nonsignificant changes in Mag-Fura 2 signals during the pharmacologic manipulations, as compared with the large changes in signal during the calibration.

Discussion

In histamine-precontracted swine carotid arterial tissues, both Fura 2 and aequorin-estimated myoplasmic $[Ca^{2+}]$ decreased when extracellular $[Mg^{2+}]$ was increased (Fig. 1). These data are consistent with the hypothesis that increased $[Mg^{2+}]_o$ relaxes arterial smooth muscle primarily by decreasing $[Ca^{2+}]_i$. Potentially, elevated $[Mg^{2+}]_o$ may decrease $[Ca^{2+}]$ influx through Ca^{2+} channels, stabilize the plasma membrane, or inhibit agonist-receptor interactions. In rat and rabbit vascular smooth muscle, sustained contractions from high K^+ and norepinephrine are inhibited by either removal of extracellular Ca^{2+} or addition of extracellular Mg^{2+}, while the addition of verapamil more selectively inhibits K^+ contractions (7). Thus, it appears that increased $[Mg^{2+}]_o$ is a nonselective and competitive inhibitor of both receptor-mediated Ca^{2+} entry and voltage-dependent Ca^{2+} channels.

Our hypothesis also stated that the $[Mg^{2+}]_o$-induced relaxation was independent of changes in $[Mg^{2+}]_i$. We suspected $[Mg^{2+}]_i$ was not substantially changing based on the comparison of aequorin and Fura 2 results. Fura 2 is not very sensitive to changes in $[Mg^{2+}]$. In contrast, aequorin is exquisitely sensitive to changes in $[Mg^{2+}]$ (elevated $[Mg^{2+}]$ decreases aequorin light and artifactually lower $[Ca^{2+}]$ estimates) (14). If elevated $[Mg^{2+}]_o$ were to increase $[Mg^{2+}]_i$ from a resting value of 0.5 to 1.25 mM, the aequorin signal would be expected to decrease by 0.3 log L/L_{max} change units (this calculation was based on in vitro calibration curves [29]). However, we found that both the aequorin and Fura 2 $[Ca^{2+}]_i$ signals observed 10 min after addition of 10 mM $[Mg^{2+}]_o$ in the presence of histamine were not significantly different than the signals observed 10 min after washout of histamine in 1.2 mM $[Mg^{2+}]_o$ (50 vs. 70 min in Fig. 1). We would have been able to statistically detect a decrease in aequorin light of 0.18 log L/L_{max} units (based on the standard error of the measurements). These calculations suggest that $[Mg^{2+}]_o$-induced changes in $[Mg^{2+}]_i$ are at best small. Very small changes in Mag-Fura 2 signals during changes in $[Mg^{2+}]_o$ (Fig. 4) confirmed our suspicions that myoplasmic $[Mg^{2+}]$ was not significantly changing during changes in $[Mg^{2+}]_o$. $[Mg^{2+}]_i$ also did not substantially change during agonist- or depolarization-induced contractions, confirming earlier reports that $[Mg^{2+}]_i$ is tightly regulated in smooth muscle (15, 16, 30).

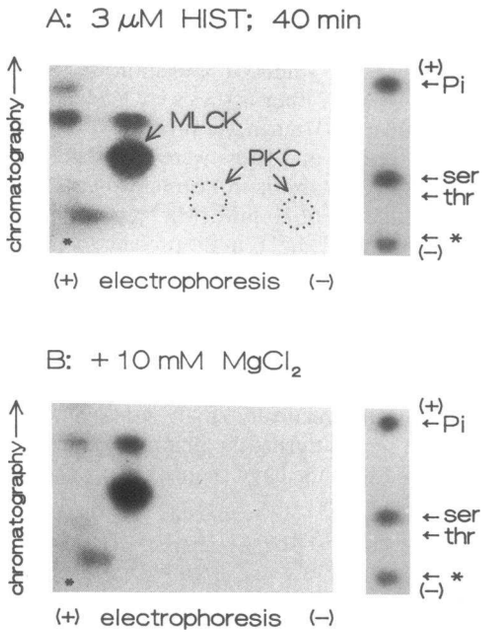

Figure 3. Two-dimensional phosphopeptide mapping of myosin light chains in ^{32}P-labeled strips of swine carotid artery. Autoradiograms of tryptic ^{32}P-phosphopeptide maps of monophosphorylated myosin light chains after 40 min of 3 μM histamine stimulation (*A*, phosphorylation = 0.74), and after 40 min of 3 μM histamine stimulation with sequential increases in $[Mg^{2+}]_o$ to 10 mM $MgCl_2$ (a protocol identical to Fig. 1 *B*, phosphorylation = 0.69). The maps are representative of two separate experiments. *The point of sample application. *MLCK*, the position of the principle phosphopeptide resulting from the phosphorylation of purified 20-kD myosin light chains by myosin light chain kinase (23). *PKC*, the positions of phosphopeptides resulting from the phosphorylation of myosin light chains by purified protein kinase C (23) (which phosphorylates at serine 1, 2, and threonine 9 [46]). The phosphoamino acid analyses at the right of each map confirm that the ^{32}P-labeled residues are phosphoserine (ser), as opposed to phosphothreonine (thr), thus identifying the site of phosphorylation entirely at serine 19.

Two significant differences between the two $[Ca^{2+}]_i$ indicators were observed in this study. During the initial response to histamine stimulation, the initial aequorin-estimated $[Ca^{2+}]_i$ transent was larger than was observed with Fura 2 (Fig. 1). This may represent transient spatial $[Ca^{2+}]_i$ inhomogeneities (see reference 18 for discussion). Additionally, resting $[Ca^{2+}]_i$ estimates were higher with Fura 2.

High extracellular $[Mg^{2+}]$ induced a relaxation with low estimated myoplasmic $[Ca^{2+}]$ and unexpectedly high myosin phosphorylation values (i.e., high extracellular $[Mg^{2+}]$ induced a high $[Ca^{2+}]_i$ sensitivity). Changes in $[Ca^{2+}]_i$ sensitivity have been observed with other stimuli in smooth muscle; e.g., KCl-depolarized swine carotid arterial tissues have a lower $[Ca^{2+}]_i$ sensitivity than histamine-stimulated tissues (i.e., KCl depolarization induced higher $[Ca^{2+}]_i$, yet lower myosin phosphorylation values than were observed with histamine stimulation) (5, 31). Changes in $[Ca^{2+}]_i$ sensitivity could originate from regulation of either myosin light chain kinase or myosin phosphatase (18, 32–35). Myosin light chain kinase is a Ca^{2+}/calmodulin-dependent enzyme that can be phosphorylated, and phosphorylated myosin light chain kinase has a lower $[Ca^{2+}]$ sensitivity (i.e., it is desensitized) (32, 36). Myosin light chain kinase extracted from KCl-depolarized swine carotid artery has a lower $[Ca^{2+}]$ sensitivity than myosin light chain kinase extracted from histamine-stimulated tissues (18), suggesting that myosin light chain kinase may be more highly phosphorylated with KCl depolarization than with histamine stimulation. Because the high values of myosin light chain phosphorylation during $[Mg^{2+}]_o$-induced relaxation occur entirely at the myosin light chain kinase substrate (23, 37), and because $[Ca^{2+}]$ estimates are low, it is possible that elevated $[Mg^{2+}]_o$ may further increase the $[Ca^{2+}]$ sensitivity of myosin light chain kinase, potentially by further decreasing myosin light chain kinase phosphorylation. Alternatively, myosin phosphatase could be regulated (38). Contractile agonists, GTP analogues, and AlF_4^- have been shown to increase $[Ca^{2+}]_i$ sensitivity in smooth muscle (31, 33, 35, 39). Potentially, myosin phosphatase may be inhibited by increases in $[Mg^{2+}]_o$.

The high level of myosin light chain phosphorylation observed with elevated $[Mg^{2+}]_o$ was also dissociated from the development of force. This relationship is not irreversibly changed, since complete removal of $[Mg^{2+}]_o$ from the extracellular solution results in the regeneration of force with continued high myosin light chain phosphorylation (Fig. 1). In skinned tissues the opposite result is observed. High $[Mg^{2+}]_i$ induces contraction with little change in phosphorylation (12, 13). These findings suggest that Mg^{2+}-induced contractions in skinned tissues may result from Mg^{2+} interactions with either the actomyosin ATPase, conformational changes of the myo-

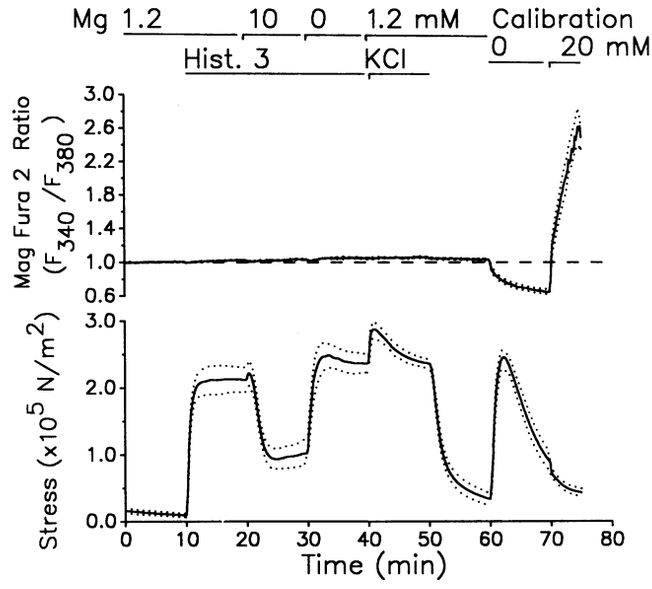

Figure 4. Mean $[Mg^{2+}]_i$ data during Mg^{2+}-induced relaxation of submaximally stimulated tissues. The time course of Mag-Fura 2–estimated myoplasmic $[Mg^{2+}]$ (background corrected 340/380 fluorescence ratio, $n = 4$), and active stress measured in swine carotid medial tissues. Tissues in PSS ($[Mg^{2+}]$ 1.2 mM) were submaximally stimulated with 3 μM histamine, 10 mM $[Mg^{2+}]_o$ was added at 20 min, and zero mM $[Mg^{2+}]_o$ was added at 30 min. Histamine was removed and the tissues depolarized with 109 mM KCl in PSS at 40 min ($[Mg^{2+}]$ 1.2 mM), and then relaxed in normal PSS at 50 min with PSS. Calibration was performed with hypotonic Mg^{2+}-free solution, 20 mM Mg^{2+} solution, and 100 mM $MnCl_2$ (not shown). Mean R_{min} was 0.64 and mean R_{max} was 2.62 with an intracellular [Mag-Fura 2] of 51±17 μM. Data were collected at 1-s intervals and averaged over 10 s for plotting, as in Fig. 1.

sin head, or a Ca^{2+}–Mg^{2+} binding site on myosin itself (12, 40, 41). We have shown that the $[Mg^{2+}]_o$-induced relaxations were not associated with significant increases in $[Mg^{2+}]_i$, [cAMP], or [cGMP], and that the myosin light chains are still phosphorylated at serine 19. These data suggest that there may be a phosphorylation-independent, yet potentially Ca^{2+}-dependent, system for regulating force in vascular smooth muscle. Potential candidates for such a regulatory system include myosin heavy chain phosphorylation (42), the thin filament proteins calponin (43), or caldesmon (44, 45).

In conclusion, elevated $[Mg^{2+}]_o$ relaxes swine carotid by decreasing $[Ca^{2+}]_i$ without significant changes in $[Mg^{2+}]_i$. These relaxations were not associated with a decrease in phosphorylation, suggesting a phosphorylation-independent mechanism for contractile force regulation in arterial smooth muscle.

Note added in proof. We have found a similar dissociation of force from myosin light-chain phosphorylation in histamine-stimulated swine carotid artery relaxed with nitrovasodilators (34).

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