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Nitroglycerin inhibits the phosphorylation of intermediate filament proteins rather than myosin light chain on porcine coronary artery sustained contraction

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Abstract. The smooth muscle relaxation induced by nitroglycerin is hypothesized to be mediated by an increase in the cytoplasmic concentration of guanosine 3′,5′-monophosphate (cGMP) and subsequent dephosphorylation of the 20-kilodalton myosin light chain (MLC). We investigated this hypothesis in procine coronary arterial smooth muscle stimulated with histamine (3 μM) or K^+ (30 mM). Stimulation of [^{32}P]Pi-labeled muscle with histamine or K^+ for 2 min resulted in a four- or 6.2-fold increase, respectively, in the incorporation of ^{32}P into MLC. After 48 min of exposure to histamine, MLC phosphorylation decreased to the basal level and the phosphorylation of desmin, synemin, and of three unidentified cytosolic proteins was increased. K^+ stimulation resulted in a sustained increase of MLC phosphorylation but had no effect on the phosphorylation of desmin, synemin, or the three unidentified cytosolic proteins. Application of nitroglycerin (1 μM) 48 min after histamine stimulation inhibited the phosphorylation of desmin, synemin, and the three cytosolic proteins. The sustained phase of histamine-induced contraction was also inhibited to a greater extent then the acute phase of histamine-induced contraction and both the acute and sustained phases of K^+-induced contraction. These results suggest that MLC phosphorylation is required for both phases of K^+-induced contraction, whereas phosphorylation of intermediate filament proteins is required for the sustained phase of histamine-induced contraction. Intermediate filament proteins, rather than MLC, may also be the target for the relaxant action of nitroglycerin during histamine-induced sustained contraction.
Key words. Nitroglycerin; smooth muscle; protein phosphorylation; intermediate filament proteins; myosin light chain; histamine; potassium.

Although nitroglycerin has been used in treating ischemic heart disease for more than a century[1,2], the detailed mechanism of nitroglycerin-induced dilation of vascular smooth muscle is still unknown. Thus, although nitroglycerin has been shown to increase the intracellular concentration of guanosine 3′,5′-monophosphate (cGMP), it is not clear how this increase leads to smooth muscle relaxation, although other effects of nitroglycerin on smooth muscle in addition to an increase of cGMP have been reported[3]. It is also not clear how vascular smooth muscle contraction develops in response to agonists, or how force is maintained at the molecular level. Evidence suggests that phosphorylation of the 20 kDa myosin light chain (MLC) by the enzyme MLC kinase, which is activated by Ca^{2+} and the Ca^{2+}-binding protein calmodulin[4], initiates the contraction of vascular smooth muscle. Several studies have demonstrated a relationship between the stimulus-induced increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), measured with fura-2 or the photoprotein aequorin, and MLC phosphorylation[5–7], or between MLC phosphorylation and the mechanical response[8] during vascular smooth muscle contraction. However, during sustained smooth muscle contraction, tension is maintained while MLC phosphorylation and the $[Ca^{2+}]_i$ decrease to near-basal levels; this mechanical tension maintenance, characterized by slowly cycling cross-bridges, has been called the 'latch state' by Hai and Murphy[9], but the molecular mechanism responsible for this state is unknown. Recently, phosphorylation of desmin, the most abundant protein in vascular smooth muscle, was observed during sustained smooth muscle contraction induced by K^+, histamine, norepinephrine, or phorbol dibutyrate[10]. Two actin-binding proteins, caldesmon and calponin, have also been identified as potential thin filament-based contraction regulatory proteins in smooth muscle[11–14]. These observations suggest that interactions between actin and proteins other than myosin may also modulate contractility, particularly in the sustained phase of smooth muscle contraction.

The effect of nitroglycerin on smooth muscle contraction has been investigated with respect to the myosin-actin interaction[8,15,16]. It has been proposed that nitroglycerin relaxes canine coronary arterial smooth muscle after K^+-induced contraction by reducing MLC phosphorylation without reducing $[Ca^{2+}]_i$[16], or that nitroglycerin relaxes porcine common carotid arteries after histamine-induced contraction by decreasing $[Ca^{2+}]_i$

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and uncoupling stress from MLC phosphorylation[8]. However, the effects of nitroglycerin on other proteins that may regulate smooth muscle contraction have not been evaluated.

The present study attempted to clarify the roles of various proteins in the acute and sustained phases of smooth muscle contraction induced by histamine or K^+. We also investigated whether nitroglycerin-induced smooth muscle relaxation was mediated by the dephosphorylation of MLC or other muscle proteins.

Materials and methods

Preparation of porcine coronary artery strips. Fresh porcine hearts were obtained from a local slaughterhouse and transported at 4 °C in a physiological salt solution containing 140 mM NaCl, 4.7 mM KCl, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 30 μM EDTA, 0.5 μM ascorbic acid, 10 mM glucose, and 10 mM Hepes (pH adjusted to 7.4 at 37 °C). Physiological salt solution containing 30 mM K^+ was prepared by reducing the NaCl concentration in an equimolar manner. The buffer was gassed with 100% oxygen. Te arterial segments were collected from proximal portion of the right coronary arteries and were cleaned of most adherent tissues. The intimal surface was rubbed with a cotton swab to remove the endothelium, and the arterial segments were opened longitudinally and cut into transverse strips (2.5 mm wide, 7 mm long, and 0.7 mm thick). These strips were then mounted in 10-ml water-jacketed muscle chamber with sewing cotton thread. Changes in isometric tension were measured with an isometric strain gauge transducer (TB611T, Nihon Kohden Kogyo, Tokyo, Japan) and displayed on a recorder with a built-in preamplifier (AP600G; Nihon Kohden Kogyo).

Phosphorylation studies. Phosphorylation experiments were performed as described previously for carotid arterial smooth muscle[17,18]. Carrier-free [^{32}P]Pi (5.55 MBq/ml) was added to each flask and incubated with the tissue for 4 h at 37 °C. Samples were treated with histamine (3 μM), or physiological salt solution containing 30 mM K^+ for 2 or 48 min before application of nitroglycerin. Incubations were terminated 3 min after addition of nitroglycerin by aspirating the buffer and immersing the muscle strips in an acetone-dry ice slurry containing 10% (w/v) trichloroacetic acid and 20 mM dithiothreitol. Strips were thawed in 200 ml of ice-cold stop buffer and then homogenized in a motor-driven conical glass-glass homogenizer. The homogenates were centrifuged and the supernatants collected. The protein content of each supernatant was determined by the method of Lowry et al.[19] with bovine serum albumin as standard. The supernatants were mixed with 0.5 volume of isoelectric focusing sample buffer. Appropriate dilutions were made to equalize the protein concentrations of the samples so that 50 μg of protein were loaded onto each gel.

Two-dimensional gel electrophoresis. Muscle supernatant proteins were resolved by two-dimensional gel electrophoresis as described by O' Farrell[20] with minor modifications. Isoelectric focusing was performed for 9000 volt hours with LKB ampholines (2%, v/v); one part pH 3 to 10 and four parts pH 5 to 7. The second dimension of electrophoresis was performed on a 12.5% polyacrylamide gel. Gels were stained with Coomassie blue, on filter paper, and subjected to autoradiography for seven or 24 h with Kodak X-Omat-AR film. ^{32}P-labeled proteins were quantitated with an AMBIS Beta Scanning System (Automated Microbiology Systems, San Diego, CA, USA). Twelve tissue incubations (four controls and eight experimental) were performed in each experiment.

The isoelectric focusing pH gradient was determined by equilibration 0.5 cm pieces of the tube gels in 2 ml of degassed and deionized water for one hour[20]. The apparent isoelectric point of each phosphoprotein resolved in the two-dimensional gels was determined from the pH gradient curve by comparison of the distance of the protein spot from the alkaline end of the gel. Apparent molecular mass was determined by comparison with standard proteins.

Identification of cytoskeletal proteins. The major cytoskeletal proteins (MLC, actin, desmin, synemin, and vimentin) were identified in the two-dimensional gel system by their comigration with the appropriate standard proteins (provided by Dr. Howard Rasmussen, Yale University) pruified from bovine tracheal smooth muscle.

Chemicals. Nitroglycerin was obtained from Nihon Kayaku (Tokyo, Japan); [^{32}P]Pi was obtained from New England Nuclear (Boston, MA, USA); and electrophoresis supplies were from Bio-Rad (Rockville Center, NY, USA), with the exception of ampholytes,

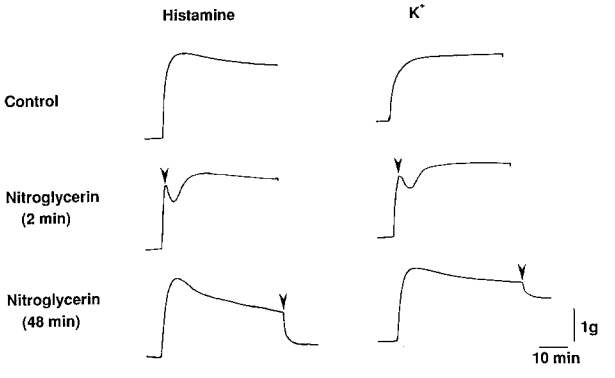

Figure 1. Representative contraction curves for porcine coronary arterial smooth muscle generated by 3 μM histamine (left traces) or 30 mM K^+ (right traces) under control conditions (upper traces), and the effects of nitroglycerin (1 μM) (arrow heads) added 2 min (middle traces) or 48 min (lower traces) after stimulation.

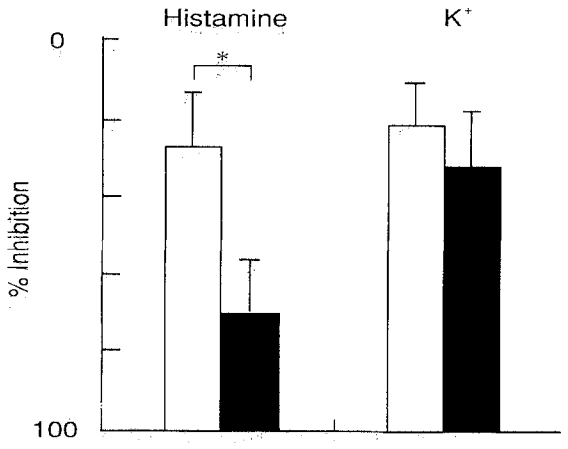

Figure 2. Effect of nitroglycerin (1 μM) on 3 μM histamine- or 30 mM K^+-induced contraction. Nitroglycerin was applied 2 min (open bars) or 48 min (solid bars) after stimulation. *$p < 0.01$ vs. control, Student's t-test.

which were from Pharmacia (Piscataway, NJ, USA). All other chemicals, including histamine, were from Sigma (St. Louis, MO, USA).

Statistical analysis. Results are expressed as means ±SD and were analyzed with Student's t-test for paired or unpaired observations, as appropriate. A p value <0.05 was considered statistically significant.

Results

Effects of nitroglycerin on histamine- and K^+-induced contraction. Porcine coronary arterial smooth muscle strips responded to 3 μM histamine or 30 mM K^+ with an initial rapid contraction (acute phase) followed by a gradually decreasing tonic contraction (sustained phase) (fig. 1, upper trace). Treatment with nitroglycerin (1 μM) inhibited contraction by histamine or K^+ by 26 ± 11% or 19 ± 10% (n = 8) at 2 min and by 69 ± 11% or 30 ± 12% (n = 8) at 48 min, respectively (fig. 1, middle and lower traces). The percent inhibition by nitroglycerin was significantly greater ($p < 0.01$) at 48 min than at 2 min for histamine-induced contraction, but did not differ significantly between 2 and 48 min for K^+-induced contraction (fig. 2).

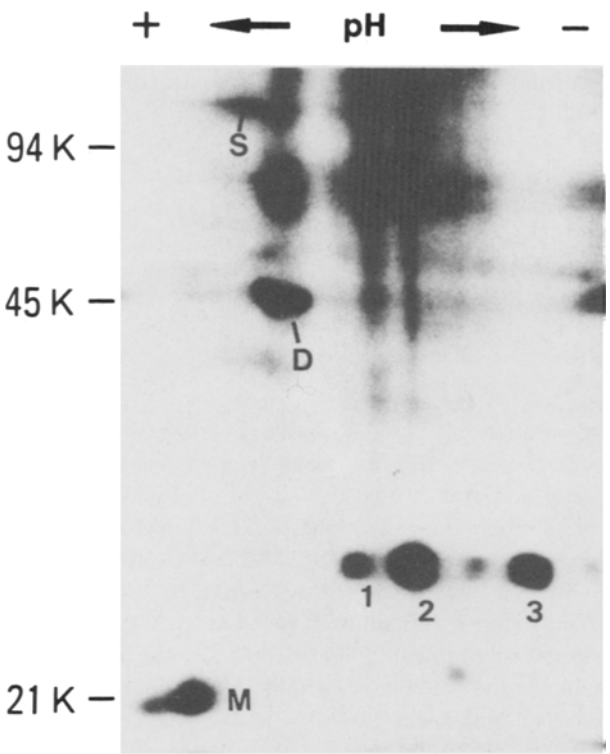

Figure 3. Representative electrophoretic pattern obtained from the total homogenate of porcine coronary arterial strips 48 min after stimulation with 3 μM histamine. ^{32}P-labelled proteins were separated by two-dimensional gel electrophoresis and detected by autoradiography (exposure for 24 h). S, synemin; D, desmin; M, MLC (myosin light chain); 1, 2, and 3, unidentified cytosolic proteins. Molecular size calibration is shown in kilodaltons (K).

Effect of histamine and K^+ on MLC phosphorylation. Figure 3 shows a respresentative electrophoretic pattern obtained with the total homogenate of porcine coronary arterial strips 48 min after stimulation with 3 μM histamine. The major proteins detected by autoradiography were identified as contractile and cytoskeletal proteins based on their comigration with purified protein standards. These proteins included 20 kDa

Table 1. Time-dependent changes induced by 3 μM histamine in the phosphorylation of MLC and intermediate filament proteins in porcine coronary artery smooth muscle.

Identified proteins	Mr/pI	Relative change (-fold) vs. control			
		2 min (n = 8)	8 min (n = 7)	16 min (n = 7)	48 min (n = 8)
20 kDa MLC	20 kDa/5.2	4.09 ± 0.36**	2.05 ± 0.36 **	1.81 ± 0.27**	1.25 ± 0.25
Desmin	57 kDa/5.4	1.42 ± 0.14	1.54 ± 0.68	2.09 ± 0.51	1.90 ± 0.35**
Synemin	230 kDa/5.3	1.15 ± 0.17	0.98 ± 0.17	1.09 ± 0.25	1.50 ± 0.14*
Unidentified cytosolic proteins					
# 1	27 kDa/5.7	1.14 ± 0.07	1.02 ± 0.21	1.10 ± 0.12	1.30 ± 0.13*
# 2	27 kDa/5.8	1.31 ± 0.10	1.40 ± 0.18	1.61 ± 0.30	1.80 ± 0.18*
# 3	27 kDa/6.1	1.23 ± 0.15	1.25 ± 0.10	1.25 ± 0.25	1.45 ± 0.14*

Data are means ±SD for the indicated number (n) of experiments. *$p < 0.05$; **$p < 0.01$ vs. a ratio of 1.0 [cpm (histamine)/cpm (control)], Student's t-test.

Table 2. Time-dependent changes induced by 30 mM K^+ in the phosphorylation of MLC and intermediate filament proteins in porcine coronary artery smooth muscle.

Identified proteins	Mr/pI	Relative change (-fold) vs. control			
		2 min (n = 7)	8 min (n = 6)	16 min (n = 6)	48 min (n = 7)
20 kDa MLC	20 kDa/5.2	6.22 ± 1.73**	3.28 ± 0.80**	2.30 ± 0.40**	2.23 ± 0.44**
Desmin	57 kDa/5.4	1.14 ± 0.15	1.40 ± 0.16	1.28 ± 0.06	1.41 ± 0.09
Synemin	230 kDa/5.3	0.99 ± 0.21	1.04 ± 0.36	1.00 ± 0.23	0.98 ± 0.24
Unidentified cytosolic proteins					
# 1	27 kDa/5.7	1.19 ± 0.19	0.99 ± 0.13	0.98 ± 0.11	1.11 ± 0.18
# 2	27 kDa/5.8	1.36 ± 0.26	1.45 ± 0.28	1.12 ± 0.20	1.02 ± 0.14
# 3	27 kDa/6.1	1.26 ± 0.11	1.33 ± 0.18	1.19 ± 0.09	1.12 ± 0.08

Data are means ± SD for the indicated number (n) of experiments. **$p < 0.01$ vs. a ratio of 1.0 [cpm (K^+)/cpm (control)], Student's *t*-test.

MLC, topomyosin, desmin (α and β), and synemin. Three unidentified cytosolic proteins which also showed changes in the extent of phosphorylation in response to various stimuli are numbered 1 to 3. The molecular weight of all three proteins was 27,000 and the pI values were 5.7, 5.9, and 6.1, respectively. Stimulation of [^{32}P]Pi-labeled porcine cornary artery smooth muscle with histamine or K^+ for 2 min resulted in a four- or 6.2-fold increase, respectively, in the incorporation of ^{32} into MLC (tables 1 and 2). After 8 min, however, the increase in ^{32}P incorporation into MLC was 2.1- or 3.3-fold, respectively, relative to control. Forty-eight min after stimulation with histamine, the increase in MLC phosphorylation was only 1.3-fold. In contrast, a 2.2-fold increase in MLC phosphorylation was still apparent 48 min after stimulation with K^+.

Since all the phosphorylation changes noted in this study are based on a change in radioactivity, it is possible that they reflect a change in the rate of dephosphorylation or a change in the radiolabeled ATP pool induced by metabolic changes in the cell. These possibilities are difficult to disprove, but they are unlikely explanations for the observed changes. If tissue is 'pure labeled' with [^{32}P]PO_4^{3-} during the first hour of incubation, the [^{32}P]ATP pool subsequently gradually decreases as shown by the decrease in radioactivity of phosphorylation in control samples. Under these condition, histamine treatment results in phosphorylation changes that are attenuated but otherwise identical to those observed in the presence of extracellular [^{32}P]PO_4^{3-}.

Effect of histamine and K^+ on the phosphorylation of intermediate filament proteins. With histamine stimulation, the phosphorylation of desmin and of cytosolic proteins 2 and 3 was increased 1.4-, 1.3-, and 1.2-fold respectively after 2 min, following which there was a gradual 1.9-, 1.8-, and 1.5-fold increase after 48 min. The phosphorylation of synemin and cytosolic protein 1 was slightly less extensive, showing an increase of 1.5- and 1.3-fold after 48 min, respectively (table 1). However, all of the intermediate filament proteins were significantly phosphorylated at 48 min. In contrast, K^+ stimulation produced similar phosphorylation of the intermediate filament proteins up to 8 min, it did not produce a significant increase of phosphorylation at 48 min (table 2).

Effects of nitroglycerin on histamine- and K^+-induced protein phosphorylation. Nitroglycerin (1 μM) reduced the extent of MLC phosphorylation by 19 and 23% when applied to coronary artery strips 2 and 48 min, respectively, after stimulation with histamine (fig. 4). The phosphorylation of desmin, synemin, and cytosolic proteins 1 to 3 in the presence of histamine was not inhibited significantly by nitroglycerin after 2 min, but was inhibited by 45%, 67%, and 32% (average of proteins 1 to 3), respectively, after 48 min (fig. 4). In contrast, nitroglycerin had no significant effect on MLC or intermediate filament protein phosphorylation when

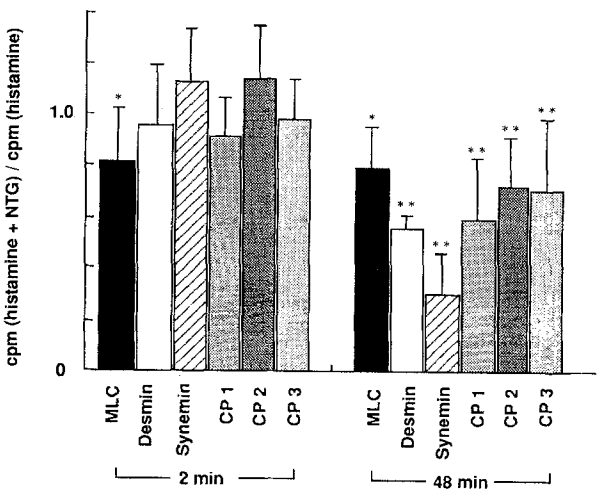

Figure 4. Quantification of the effects of nitroglycerin on protein phosphorylation induced by 3 μM histamine. Nitroglycerin (NTG) (1 μM) was applied 2 or 48 min after stimulation. Data are means ± SD of eight different experiments. *$p < 0.05$, **$p < 0.01$ vs. a ratio of 1.0 [cpm (histamine)/cpm (control)], Student's *t*-test.

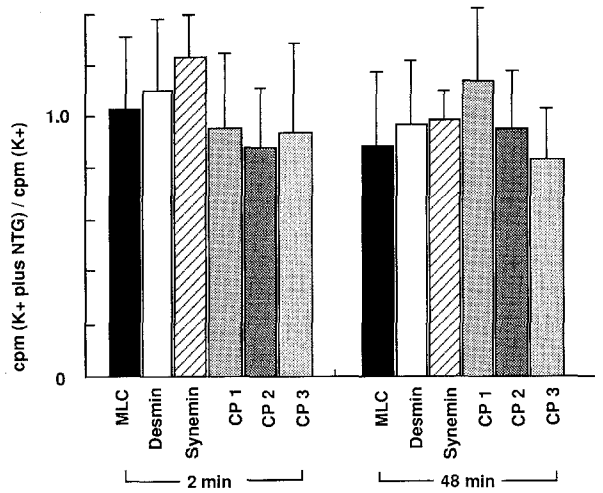

Figure 5. Quantification of the effects of nitroglycerin on protein phosphorylation induced by 30 mM K^+. Nitroglycerin (NTG) (1 μM) was applied 2 or 48 min after stimulation. Data are means ±SD of eight different experiments.

added 2 or 48 min after exposure of muscle to 30 mM K^+ (fig. 5).

Discussion

The major phosphoproteins of vascular smooth muscle included MLC, synemin, and two variants of desmin (α and β). Several additional lower molecular mass (<40 kDa) phosphoproteins have also been described[21]. The inhibitory effect of nitroglycerin on smooth muscle contraction has only been evaluated previously with regard to myosin-actin interaction during the initial rapid phase of contraction[16,22].

In the present study, we investigated the effects of nitroglycerin on contraction and protein phosphorylation induced by 3 μM hsitamine and 30 mM K^+ during both the acute and sustained phases of contraction.

Although the stress curves for contraction induced by histamine and K^+ were similar, the patterns of protein phosphorylation induced by these two stimulants differed in the sustained phase of contraction. Thus, the increase in MLC phosphorylation induced by histamine was four-fold after 2 min of stimulation, but only two-fold after 8 min and 1.3-fold after 48 min. The histamine-induced increase in the phosphorylation of desmin, synemin, and three unidentified cytosolic proteins was approximately 1.6-fold after 48 min. In contrast, the sustained phase of K^+-induced contraction was only associated with a sustained increase in MLC phosphorylation. The relative time-dependent changes in MLC phosphorylation were similar after histamine and K^+ stimulation, but MLC phosphorylation was still elevated significantly at 48 min after K^+ stimulation, whereas the increase produced by histamine was not significant at this time. On the other hand, histamine caused a successive development of intermediate filament phosphorylation. These results suggest that the actin-myosin system, which is activated by MLC phosphorylation, may be responsible for the initial rapid phase of contraction, whereas the actin-intermediate filament system, which is specific to smooth muscle and may be activated by phosphorylation of its component proteins, may mediate the tonic phase of histamine-induced contraction. These conclusions are consistent with previous observations indicating that regulation by MLC phosphorylation could not explain all aspects of smooth muscle contractile function, particularly tonic contractile responses[9,23]. Rasmussen et al.[23] have suggested that protein kinase C promotes intermediate filament protein phosphorylation which may be responsible for sustained contraction. A possible reason for the differences in the onset of phosphorylation of the intermediate filament proteins is that they have different sensitivities to protein kinases, such as protein kinase C. An important finding of the present study was that the phosphorylation of various intermediate filament proteins increased gradually along with the decrease in MLC phosphorylation in the sustained phase of histamine-induced contraction, with their level of phosphorylation being significantly increased at 48 min. We considered the time points of 2 min and 48 min to be most representative of the acute and sustained phases of contraction, respectively, and tried to identify the major protein phosphorylation targets involved in contraction as well as the different effects of nitroglycerin at these two times.

In the tension studies, tension redevelopment was noted from 3–4 min after addition of nitroglycerin in the acute phase of both types of stimulation (fig. 1, middle trace). This may have occurred because consumption of nitroglycerin meant that it could no longer overcome the force developed by the high MLC phosphorylation at that time. The histamine-induced sustained contraction was inhibited by nitroglycerin to a greater extent than the histamine-induced initial contraction and the forces associated with both phases of K^+-induced contraction. In addition, intermediate filament protein phosphorylation associated with histamine-induced sustained contraction was inhibited by nitroglycerin to a greater extent than was MLC phosphorylation. These results suggest that intermediate filament proteins, rather than MLC, are the targets for the vasorelaxant effect of nitroglycerin during histamine-induced sustained smooth muscle contraction.

The nitroglycerin-induced relaxation of K^+-stimulated smooth muscle was not accompanied by a change in MLC phosphorylation level either in the acute or the sustained phase of contraction. Yanagisawa et al.[16] showed that nitroglycerin relaxes K^+-stimulated canine coronary arterial smooth muscle without reducing $[Ca^{2+}]_i$, and these researchers suggested that relaxation

is achieved by a decrease in the extent of MLC phosphorylation even in the presence of a high $[Ca^{2+}]_i$ that is maintained by depolarization-induced Ca^{2+} influx. McDaniel et al.[8] showed that nitrovasodilators relax histamine-stimulated porcine arterial smooth muscle by reducing $[Ca^{2+}]_i$ and the uncoupling of stress from MLC phosphorylation. These observations suggests that nitrovasodilators, acting via cGMP or some other mediator, may regulate stress by at least two mechanisms that uncouple 1) $[Ca^{2+}]_i$ from MLC phosphorylation or 2) MLC phosphorylation from the mechanical response.

The latter action may account for our observation that nitroglycerin-induced relaxation of K^+-stimulated smooth muscle was not accompanied by MLC dephosphorylation. Similarly, nitroglycerin may affect the relation between $[Ca^{2+}]_i$ and intermediate filament protein phosphorylation, or the relation between intermediate filament protein phosphorylation and the mechanical response for histamine-stimulated contraction.

In conclusion, MLC phosphorylation may be required for development of the initial rapid phase of histamine-induced contraction and for both the acute and sustained phases of K^+-induced contraction. Intermediate filament proteins such as desmin, synemin, and possibly cytosolic proteins 1, 2, and 3 play an important role in the sustained phase of histamine-induced contraction. Nitroglycerin, acting via cGMP or some other mediator, inhibits the phosphorylation of intermediate filament proteins to a greater extent than that of MLC during the sustained phase of histamine-induced coronary artery contraction.

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