The effect of atrial natriuretic peptide (ANP) on angiotensin II- and histamine-induced contraction and muscle light chain phosphorylation was examined in strips of rabbit aorta smooth muscle. Preincubation of strips with $10^{-5}$ M ANP prior to addition of either agonist inhibits both the increase in extent of myosin light chain phosphorylation and the contractile response to either $5 \times 10^{-6}$ M angiotensin II or $10^{-5}$ M histamine without inhibiting the agonist-induced increase in the intracellular free Ca$^{2+}$ concentration. Furthermore, in muscle strips precontracted with either angiotensin II or histamine, addition of ANP leads to a prompt relaxation and a prompt decrease in the extent of myosin light chain phosphorylation. These data argue that ANP uncouples the initial agonist-induced Ca$^{2+}$ transient from the increase in extent of myosin light chain phosphorylation either by inhibiting the Ca$^{2+}$-dependent activation of myosin light chain kinase or stimulating the activity of a phosphoprotein phosphatase capable of bringing about the rapid dephosphorylation of phosphorylated myosin light chains.

The peptide hormone ANP is known to inhibit the contractile response of vascular smooth muscle induced by angiotensin II, histamine, norepinephrine, or an elevation of extracellular K$^+$ concentration (1, 2). One of the ways by which ANP is thought to exert its effects is by activating a receptor-linked guanylate cyclase in the plasma membrane (3). The resulting rise in cGMP concentration is thought, in turn, to activate a cGMP-dependent protein kinase which by catalyzing the phosphorylation of specific cellular proteins brings about a relaxation of the contracted muscle (4, 5). However, the specific phosphoproteins responsible for this relaxation are not known, nor is it clear at which point ANP acts in the sequence of events, which begin with the interaction of angiotensin II or histamine with their receptors and ends in the contractile response. At present this sequence is thought to be as follows: hormone-receptor interaction, activation of a specific phospholipase C, hydrolysis of phosphatidylinositol 4,5-bisphosphate with the generation of diacylglycerol and inositol 1,4,5-trisphosphate (6, 7), a transient rise in intracellular Ca$^{2+}$ concentration due to inositol 1,4,5-trisphosphate-induced release of Ca$^{2+}$ from the sarcoplasmic reticulum (8-10), an activation of the Ca$^{2+}$-calmodulin-dependent myosin light chain kinase, and the phosphorylation of the 20-kDa myosin light chain (11, 12) which brings about the interaction of myosin with actin and thus contraction.

In a previous study (13), we found that even though ANP inhibits angiotensin II- and histamine-induced contraction in rabbit aorta, it does not inhibit the transient rise in intracellular [Ca$^{2+}$] induced by these hormones, as measured with the photoprotein aequorin. A similar finding has been reported by Hassid (14) measuring intracellular Ca$^{2+}$ concentration with Fura 2. The present experiments were undertaken to analyze the effect of ANP on a biochemical event which is associated with elevated levels of intracellular Ca$^{2+}$ concentration, e.g. the increase in the extent of myosin light chain phosphorylation.

**EXPERIMENTAL PROCEDURES**

Preparation of Rabbit Aortic Strips—New Zealand White rabbits weighing 2.0-2.5 kg were killed by an intravenous injection of sodium pentobarbital. The descending portion of thoracic aorta smooth muscle was removed immediately and placed into a modified Krebs-Henseleit buffer containing 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.5 mM MgSO$_4$, 1.5 mM CaCl$_2$, 25 mM NaHC$_3$, 11 mM glucose and aerated with 95% O$_2$, 5% CO$_2$ or into a Krebs-Ringer Heps buffer containing 139 mM NaCl, 4.6 mM KCl, 12 mM MgCl$_2$, 1.5 mM CaCl$_2$, 5.5 mM glucose, 10 mM Heps, 0.3 mM NaH$_2$PO$_4$, pH 7.4, oxygenated with 100% O$_2$.

The first buffer was used in the experiment depicted in Fig. 4 where the level of phosphorylated MLC was measured during isometric contraction. The second buffer was used in experiments where the protocol called for long incubation time, in order to prevent the need for continuous gassing with CO$_2$, e.g. in the experiments depicted in Fig. 2 where the tissue was labeled for 3 h in [$^{35}$P]PO$_4$ and in the matching contraction experiments shown in Fig. 1. Both the measurements of isometric tension and the examination of the effects of ANP on MLC phosphorylation were carried out the same in Krebs-Henselit buffer or in Krebs-Ringer Heps. The aorta was cleaned of adhering loose connective tissues and cut open longitudinally. The endothelium was removed by gentle rubbing with a wooden applicator and each aorta was cut into transverse strips.

The Tension Measurement—The strips were mounted under resting tension in 10-mL muscle chambers and were allowed to equilibrate for 90 min in the buffer at 37°C before experiments were begun. Tension was measured isometrically with Grass FT-03 force displace-
The aortic strips were fixed on a plastic support so that they were not free to move and isometric tension developed. Loading of the strips with aequorin was carried out by a minor modification of the method of Morgan and Morgan (8). The apparatus for measurement of aequorin luminescence has been described elsewhere (13). The aequorin-loaded strips were perfused with the modified Krebs-Henseleit buffer aerated with 95% O₂, 5% CO₂ at 37 °C. Luminescence was recorded as nanocuries of anode current and expressed as a ratio to baseline current.

**Measurement of Myosin Light Chain Phosphorylation with a [³²P]PO₄ Labeling Method**—The dissected aorta was cut transversely into 3-mm wide strips. Strips were incubated for 3 h at 37 °C in 1 ml of Krebs-Ringer Heps containing 200 µCi carrier-free [³²P]PO₄ and were then oxygenated every 30 min. The hormones were added at the final concentration and for the period specified for the legend to Fig. 2. The reaction was terminated by replacing the incubation buffer with dry ice-cold acetone as described by Driska et al. (15). The tissue was then homogenized in ice-cold stop buffer (20 mM sodium phosphate, 10 mM NaF, 15 mM EDTA, 2 mM EGTA, pH 7.4) with a motor-driven conical glass-glass homogenizer. Sodium dodecyl sulfate was added to a final concentration of 1.3%, and further homogenization was carried out using a Teflon glass homogenizer. The homogenate was centrifuged for 2 min at 16,000 g. Equal amounts of the supernatant proteins (60 µg) were resolved by two-dimensional gel electrophoresis according to published procedures (16). The gels were silver stained and dried onto filter paper. Autoradiography was done (16). The MLC was identified on the basis of its comigration with purified MLC from rabbit aorta (17). For quantitation of [³²P]PO₄ incorporation into MLC, the spot corresponding to phosphorylated MLC was excised from the gels and the radioactivity measured with a liquid scintillation spectrometer.

**Measurement of the Absolute Amount of Phosphorylated Myosin Light Chain**—Transverse rabbit aortic strips (1-mm wide) were allowed to equilibrate in the modified Krebs-Henseleit buffer aerated with 5% O₂, 95% CO₂ at 37 °C for 90 min under approximately 1 g of basal tension. Strips were treated with ANP and/or ANP for the indicated time periods and quick-frozen by using metal clamps cooled in liquid nitrogen. The extent of MLC phosphorylation was measured by an immunoblotting technique (18). Briefly, frozen tissues were homogenized in pyrophosphate buffer. Proteins in the 7000 X g supernatant were precipitated with trichloroacetic acid, washed with diethyl ether, and resuspended in urea buffer. The phosphorylated and nonphosphorylated forms of MLC were separated by electrophoresis in polyacrylamide gels containing 40% glycerol. Light chains were visualized on nitrocellular blots of glyceraldehydes after incubation with serum raised against purified bovine tracheal smooth muscle light chain and reaction with peroxidase-linked second step antibody. Relative areas of the bands were quantitated with a scanning laser densitometer.

**Materials**—Human atrial natriuretic peptide (4-28) was a generous gift of Dr. J. Lewicki of California Biotechnology (Mountain View, CA). Angiotensin II was purchased from Behring Diagnostics.

**RESULTS**

**Aequorin Luminescence**—Shown in Fig. 1 are experiments similar to those reported previously (12) in which the effects of ANP on the Ca²⁺ transient induced by angiotensin II was examined. In aequorin-loaded muscle strips, addition of 5 × 10⁻⁸ M angiotensin II (AII) led to a similar Ca²⁺ transient in both control (Fig. 1A) and ANP-pretreated (Fig. 1B) muscle strips. As shown by the measurement of tension in the lower traces, this concentration (10⁻⁸ M) of ANP, which had no effect on the angiotensin II-induced transient, completely inhibited the angiotensin II-induced contraction. A similar result was observed when histamine rather than angiotensin II was the agonist (13). In both cases ANP inhibits the agonist-induced contraction without altering appreciably the initial agonist-induced Ca²⁺ transient (13).

**ANP and Agonist-induced Contraction**—As shown in the upper panels of Fig. 2, either 10⁻⁷ M histamine or 5 × 10⁻⁸ M angiotensin II lead to a rapid and sustained contraction of aortic muscle strips. Pretreatment of muscle strips with 10⁻⁷ M ANP blocks the contractile response to either 10⁻⁴ M histamine (Fig. 2A, center panel) or to 5 × 10⁻⁸ M angiotensin II (Fig. 2B, center panel). In addition, as shown in the lower panel of Fig. 2, the effect of 5 × 10⁻⁸ M angiotensin II on the time course of the contractile response induced by 5 × 10⁻⁴ M atrial natriuretic peptide (ANF) beginning 5 min prior to the addition of angiotensin II.
frames of Fig. 2, addition of $10^{-7}$ M ANP 1.5–2 min after the addition of the agonist leads to a rapid relaxation of muscle strips contracted as a result of exposure to either $10^{-6}$ M histamine (Fig. 2A, bottom panel) or $5 \times 10^{-8}$ M angiotensin II (Fig. 2B, bottom panel).

**ANP and Extent of Myosin Light Chain Phosphorylation—** Studies of the change in extent of myosin light chain phosphorylation were carried out using protocols similar to those employed in the contractile studies (Fig. 2), i.e. studies of the effect of ANP on the extent of MLC phosphorylation was examined in two ways: (a) the effect of prior addition of ANP on the ability of subsequently added agonist to increase the extent of MLC phosphorylation, and (b) the effect of ANP on MLC phosphorylation in muscle strips previously exposed to agonist. As shown in Fig. 3A, 2 min after the addition of angiotensin II, there was a nearly 5-fold increase in the extent of phosphorylation which was nearly totally prevented by the prior addition of $10^{-7}$ M ANP 2.5 min before angiotensin II (Fig. 3A). Similarly, 1.5 min after histamine addition there was a 3-fold increase in extent of MLC phosphorylation which again was completely prevented if the muscle strip had been preincubated with $10^{-7}$ M ANP for 2.5 min (Fig. 3A). In contrast, pretreatment of muscle strips with ANP, caused only a partial inhibition of the increase in extent of MLC phosphorylation seen 4 min after the subsequent histamine addition (Fig. 3A).

When muscle strips were first induced to contract by addition of angiotensin and $10^{-7}$ M ANP then added 2 min later, a prompt relaxation ensued (Fig. 2B, bottom panels), and a complete reversal of myosin light chain phosphorylation was found when measured 3 min later (i.e. after 6 min exposure to angiotensin II) (Fig. 3B). Even though $10^{-7}$ M ANP also caused a complete relaxation of the contraction induced by histamine (Fig. 2A, bottom panel), it caused only a partial reversal of the histamine-induced phosphorylation of MLC (Fig. 3B).

In order to demonstrate that the observed inhibition of $[^{32}P]P\text{IPO}_{4}$ incorporation into MLC by ANP truly reflects inhibition of the increased level of phosphorylated MLC and to ensure that these results were not affected by the experimental conditions in which the aortic strips are “free floating” rather than under isometric tension, the effect of ANP on angiotensin II-induced phosphorylation of MLC was studied during an isometric contraction. Isometrically mounted control or ANP-pretreated strips were freeze-clamped 2 min after addition of ANP. The amount of phosphorylated MLC was measured by an immunoblotting procedure (18). As shown in Fig. 4, there was only a very small amount of phosphorylated MLC in the control tissue, and ANP had no effect on this value. Addition of angiotensin II led to a significant increase in isometric tension and in the extent of MLC phosphorylation (approximately 10-fold increase). Pretreatment with ANP led to an inhibition of the angiotensin II-induced increases of in both isometric force and in MLC phosphorylation (>90%).

The results from the two methods agree very closely. They both indicate that ANP causes over a 90% decrease in the extent of MLC phosphorylation seen 2 min after ANP addition.

**DISCUSSION**

In this report we have shown that ANP inhibits or reverses angiotensin II- and histamine-induced contraction and myosin light chain phosphorylation. Previous studies (13) with agonin-loaded rabbit aortic strips have shown that addition of angiotensin II generates a sharp but transient rise in luminescence that reaches a peak at ~2 min and falls to plateau level which is slightly elevated above base-line value by 10 min. A similar result is shown in Fig. 1. Also in previous studies (13), it was shown that addition of histamine induces a transient rise in luminescence that peaks ~1.5 min and then falls to plateau level which is slightly higher than the unstimulated base line. Preincubation of strips with $10^{-7}$ M ANP, prior to addition of $5 \times 10^{-8}$ M angiotensin II or $10^{-5}$ M ANP inhibited or reversed the extent of MLC phosphorylation.
histamine does not affect the transient rise in aequorin luminescence (13, Fig. 1), induced by either agonist, but abolishes the plateau phase seen after histamine addition (13). Since ANP by itself does not cause any change in luminescence and does not affect the transient increase in luminescence caused by either angiotensin II or histamine, one can conclude that ANP does not affect the transient elevation in Ca2+ induced by these agonists. Nonetheless, ANP does inhibit the angiotensin II- or histamine-induced stimulation of MLC phosphorylation (Figs. 3 and 4) measured at a time 2 and 1.5 min, respectively, after their respective additions when the [Ca2+]i transient reaches its respective peak. Furthermore, in already contracted muscle strips in which the extent of myosin light chain phosphorylation is increased, addition of ANP leads to a prompt relaxation (Fig. 2) and a complete or partial reversal of the extent of MLC phosphorylation (Fig. 3) induced by these two agonists.

It thus seems that ANP inhibits the increase in the level of phosphorylated MLC induced by agonist either by the activation of phosphoprotein phosphatase and/or by inhibition of the MLC kinase. The latter could be achieved by altering the distribution of Ca2+ within the cells or by some manner independent of Ca2+.

As mentioned above, ANP elevates cGMP levels within the cells (3). The latter is known to relax smooth muscle (19) and to inhibit [32P]PO4 incorporation into MLC (19). However, in vitro studies have shown that phosphorylation of MLC kinase by cGMP-dependent protein kinase, does not inhibit its activity (20, 21). Therefore, direct phosphorylation of the MLC kinase is less likely to be the mechanism by which ANP exerts its relaxing effect.

As mentioned above, 40% of the MLC phosphorylation observed 4 min after histamine stimulation is resistant to inhibition by ANP. It is thus possible that this fraction represents MLC which is phosphorylated by a kinase other than the Ca2+ calmodulin-dependent myosin light chain kinase.

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