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Research Article

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Measurement of Cytoplasmic Free Ca^{2+} Concentration in Rabbit Aorta Using the Photoprotein, Aequorin

Effect of Atrial Natriuretic Peptide on Agonist-induced Ca^{2+} Signal Generation

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Abstract

Addition of norepinephrine, angiotensin II, or histamine leads to a transient rise in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), as measured with aequorin, in rabbit aortic strips. Each induces a $[Ca^{2+}]_i$ transient which peaks in 2 min and then falls either back to baseline (angiotensin II) or to a plateau (norepinephrine and histamine). The $[Ca^{2+}]_i$ transient is due to the mobilization of Ca^{2+} from a caffine-sensitive, intracellular pool. An elevation of $[K^+]$ to 35 mM leads to a monotonic sustained rise in $[Ca^{2+}]_i$ which depends entirely on extracellular Ca^{2+}, but an increase to 100 mM leads to a $[Ca^{2+}]_i$ transient from the mobilization of intracellular Ca^{2+}. Atrial natriuretic peptide does not alter basal $[Ca^{2+}]_i$ nor inhibit the $[Ca^{2+}]_i$ transient induced by either histamine or angiotensin II, but blocks that induced by norepinephrine, and blocks the plateau phase induced by either histamine or norepinephrine. The peptide inhibits the contractile response to all three agonists and to K^+.

Introduction

Previous studies have demonstrated that changes in intracellular Ca^{2+} concentration play a central role in the regulation of smooth muscle contraction (1–5). The rabbit aorta is one of the most well-characterized and commonly used systems for vascular smooth muscle studies. The metabolism of Ca^{2+} during the action of agonists or relaxants on rabbit aorta has been investigated mainly by Ca^{2+}-flux studies (6–9). There has been no report of the direct measurements of changes in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to agonists or relaxants in this tissue. The measurement of $[Ca^{2+}]_i$ would provide indispensable information for further understanding the role of Ca^{2+} in contraction of this type of muscle.

Recently, Morgan and Morgan (10–12) introduced a procedure for loading vascular smooth muscle strips, from ferret portal vein, with the photoprotein, aequorin. They have reported measurements of $[Ca^{2+}]_i$ with a concomitant measurement of the tension generation. Other investigators have reported the measurement of $[Ca^{2+}]_i$ using quin-2 or fura-2 in cultured vascular smooth muscle cells (13–17) or freshly isolated single vascular smooth muscle cells (18), but in these instances no correlation with contraction was made. The method for $[Ca^{2+}]_i$ measurement developed by Morgan and Morgan (10–12) allows one to monitor changes in $[Ca^{2+}]_i$ of intact tissues with normal integrity in response to agonists or relaxants and to correlate changes in $[Ca^{2+}]_i$ with changes in the contractile state.

In the present study we have measured changes in $[Ca^{2+}]_i$ of rabbit aortic strips in response to various agonists or relaxants, employing aequorin as a Ca^{2+} indicator, in order to gain further insight into the messenger function of Ca^{2+} in vascular smooth muscle contraction. It was a matter of particular interest to investigate what effects a recently discovered potent vasorelaxant, atrial natriuretic peptide (ANP)[1] (19–21), have on the Ca^{2+} signal induced by agonists. The results indicate that different agonists induce a rise in $[Ca^{2+}]_i$ in a specific way for each agonist and that ANP affects these agonist-induced Ca^{2+} signals in diverse ways.

Methods

Preparation of rabbit aortic strip and aequorin loading. 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 was removed immediately and placed into an ice-cold modified Krebs-Henseleit buffer (119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11 mM glucose) aerated with 95% O_2/5% CO_2. The aorta was cleaned of adhering loose connective tissues and cut open longitudinally. Endothelial cells were removed by gently rubbing the intimal surface with a wooden stick. The aortic muscle strips were fixed at their ends to a plastic holder, so that they were not free to move during measurement of aequorin signal and developed tension when treated with agonists. The muscle strips were then loaded with aequorin by procedures employing a modification of the method of Morgan and Morgan (10, 11) as developed by W. Apfeldorf and H. Rasmussen (unpublished work). This method reversibly permeabilizes the plasma membrane using EGTA and adenosine 5′-trisphosphate and introduces aequorin into cells. The method allows one to load aequorin into multicellular preparations more efficiently and easily compared to the microinjection method. Briefly, this procedure consists of incubating aortic strips in a series of four solutions at 4°C for 30–120 min. The first step is three 10-min incubations in the following solution: 120 mM K glutamate, 20 mM *N*-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid (TES), 10 mM EGTA, 5 mM adenosine 5′-trisphosphate (NaATP), 2 mM $MgCl_2$, pH 7.1. The second step is an incubation of 1 h in the following solutions: 120 mM K glutamate, 20 mM TES, 0.1 mM EGTA, 5 mM NaATP, 2 mM $MgCl_2$, 100 μg/ml aequorin, pH 7.1. The third step is an incubation of 1 h in the following solution: 120 mM K glutamate, 20 mM TES, 0.1 mM EGTA, 5 mM NaATP, 10 mM $MgCl_2$, pH 7.1. The fourth step is a 2-h incubation at 4°C in the following solution: 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 12.5 mM $MgCl_2$, and 11 mM glucose aerated with 95% O_2/5% CO_2. Readdition of calcium was done by gradually increasing a Ca^{2+} concentration up to 1.5 mM over a 30-min period at room temperature in a modified Krebs-

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1. *Abbreviation used in this paper:* ANP, atrial natriuretic peptide.

Henseleit buffer initially without Ca^{2+} and gassed with 95% O_2/5% CO_2. Overnight recovery was carried out by incubating strips at room temperature in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% calf serum aerated with 95% O_2/5% CO_2.

Measurement of aequorin luminescence. The apparatus for measurement of aequorin luminescence has been described elsewhere by others and us (22, 23). Luminescence was recorded and expressed as nanoamperes (nA) of anode current. In Tables I and II, luminescence values on agonists stimulation were expressed as a ratio of luminescence at each time point to the unstimulated baseline luminescence. The background current was 0.02 nA. The cuvette was continuously perifused at a flow rate of 3.5 ml/min with a modified Krebs-Henseleit buffer containing 1.5 mM Ca^{2+} aerated with 95% O_2/5% CO_2. The buffers containing high K^+ concentrations were prepared by isoosmotic replacement of K^+ with K^+. The test substances were added to the perfusate reservoir. In experiments in which norepinephrine release from nerve endings is likely to occur, an alpha-adrenergic blocker, phentolamine (10^{-5} M), was included in the perfusate to block the action of the neurotransmitter. The temperature of the perfusate was 22°C. The calibration was done using the method described by Allen and Blinks (24) with the assumption that an intracellular Mg^{2+} concentration is 1.0 mM and that $[Ca^{2+}]_i$ is evenly distributed throughout the cytoplasm. Briefly, at the end of the experiments, muscle strips were permeabilized in the perfusion cuvette by perfusing a solution containing 2% Triton X-100, 10 mM $CaCl_2$, 120 mM KCl, and 3 mM Hepes (pH 7.4). This procedure allows all the aequorin present within cells to react with Ca^{2+}. By electrically integrating the resulting flash of aequorin luminescence, the maximal luminescence (L_{max}) was obtained. The aequorin signal (L) at each time point is normalized as fractional luminescence (L/L_{max}). The $[Ca^{2+}]_i$ equivalent to any value of L/L_{max} is calculated by interpolating L/L_{max} values on the calibration curve as developed by Snowdowne and Borle (22).

Statistical significance was analyzed by t test.

Tension measurement. After recording aequorin luminescence, each strip was used for a tension measurement. Each strip was cut into 5-mm-wide transverse strips. These were mounted under 2 g of resting tension in 10-ml static incubation muscle chambers. Strips were bathed in a modified Krebs-Henseleit buffer at 37°C and gassed with 95% O_2/5% CO_2. Tissues were allowed to equilibrate for 90 min before experiments were begun. Tension was measured isometrically with Grass FT-03 force-displacement transducers (Grass Instrument Co., Quincy, MA) and was displayed on a Gould 2400 S recorder (Gould Inc., Cleveland, OH) with built-in preamplifiers. In the preliminary experiment in which aequorin-loaded strips were repeatedly contracted with 10^{-6} M norepinephrine every 2 h, the contractile response was found to be maintained throughout these periods (the second and the third contraction were 116 and 117% of the first contraction, respectively [a mean of three determinations]).

Materials. Norepinephrine and salarasin were purchased from Sigma Chemical Co. (St. Louis, MO), angiotension II from Carbiochem-Behring Corp. (San Diego, CA), histamine hydrochloride from Matheson Coleman and Bell (Cincinnati, OH), phentolamine mesylate from Chiba (Summit, NJ). Human (4-28) ANP was a generous gift of Dr. J. Lewicki of California Biotechnology (Mountain View, CA). BAY K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) was a generous gift of Dr. A. Scriabine of Miles Institutes for Preclinical Pharmacology (New Haven, CT). Aequorin was purchased from Dr. Blinks of the Mayo Clinic (Rochester, MN).

Results

Effect of various agonists on $[Ca^{2+}]_i$. The addition of norepinephrine to aequorin-loaded strips induces a prompt and transient increase in luminescence (Fig. 1). The $[Ca^{2+}]_i$ reaches a peak at 1–2 min, then falls to a plateau level by 2–5 min. It remains at this plateau level, slightly but significantly above the unstimulated basal value, for at least 30 min (Table I). The transient nature of the initial rise in $[Ca^{2+}]_i$ is not a consequence of aequorin depletion owing to its consumption, because the readdition of norepinephrine after a washout period generates a similar level of luminescence. The existence of the plateau during norepinephrine stimulation was observed in 12 of 12 experiments using 10^{-6} M norepinephrine. There is no appreciable qualitative difference between the pattern of the norepinephrine-induced changes in $[Ca^{2+}]_i$ among three doses tested (10^{-7}, 10^{-6}, and 10^{-5} M) as shown in Fig. 1. The absolute value of $[Ca^{2+}]_i$ is 200±60 nM (n = 11) for the resting value, 460±70 nM (n = 4) for a peak transient value of stimulation with 10^{-6} M, and 250±40 nM (n = 4) for the plateau phase (at 30 min).

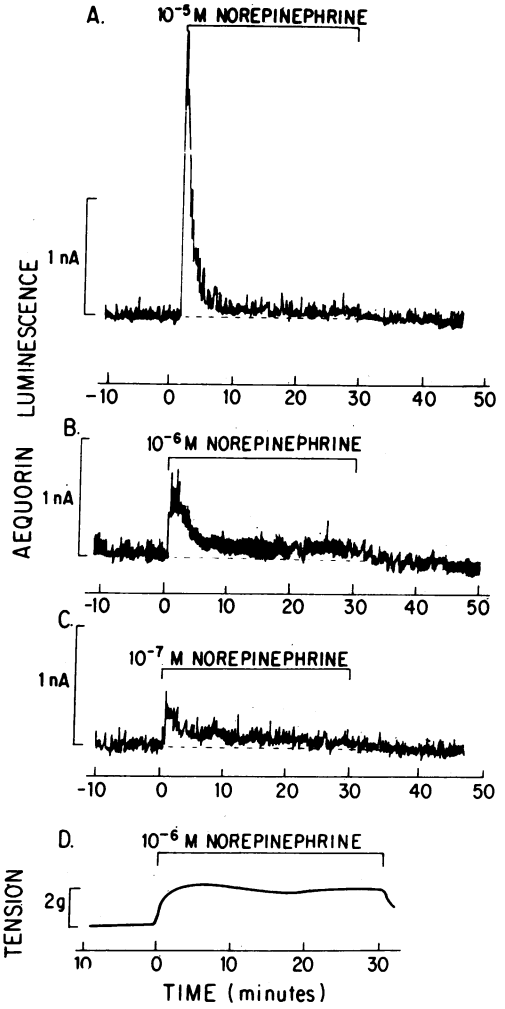

Figure 1. Effect of norepinephrine on cytoplasmic free Ca^{2+} and tension. Aequorin-loaded rabbit aortic strips were stimulated with various concentrations of norepinephrine for 30 min. (*A*) A change in $[Ca^{2+}]_i$ in response to 10^{-5} M norepinephrine. (*B*) A change in $[Ca^{2+}]_i$ in response to 10^{-6} M norepinephrine. (*C*) A change in $[Ca^{2+}]_i$ in response to 10^{-7} M norepinephrine. (*D*) A change in the tension in response to 10^{-6} M norepinephrine of the same strip as used in *B*. The dashed lines show the level of unstimulated baseline luminescence.

The addition of angiotensin II also generates a sharp but transient rise in $[Ca^{2+}]_i$ as shown in Fig. 2. However, unlike the case with norepinephrine stimulation, the $[Ca^{2+}]_i$ returns to a value indistinguishable from the unstimulated baseline value within 15 min of the addition of angiotensin II of three doses tested (5×10^{-9}, 5×10^{-8}, and 5×10^{-7} M). Thus, there is no

Table I. Changes in Cytoplasmic Free Ca^{2+} in Response to Norepinephrine, Angiotensin II, or Histamine

Agonists	n	Peak luminescence of initial Ca^{2+} transients	Luminescence at 30 min after the agonist addition
10^{-6} M norepinephrine	12	2.82±1.03	1.15±0.11*
5×10^{-8} M angiotensin II	12	3.27±1.65	1.01±0.02
10^{-5} M histamine	8	3.91±0.74	1.18±0.10‡
5×10^{-8} M angiotensin II + 2×10^{-8} M BAY K 8644	7	2.74±0.52	1.10±0.06§

Values (means±SD) are expressed as a ratio (L/L_{basal}) of luminescence at each time point to the unstimulated baseline luminescence. The symbols denote statistically significant difference compared with the baseline values (* $P < 0.001$, ‡ $P < 0.002$, § $P < 0.005$).

obvious plateau phase of $[Ca^{2+}]_i$ in response to angiotensin II (Table I). The absolute value of a peak after the addition of 5×10^{-8} M angiotensin II is 450±120 nM ($n = 5$).

When the tension is measured after recording aequorin luminescence, these strips show contractile responses qualitatively similar to fresh aortic strips not loaded with aequorin (25). Thus, they show a sustained contraction to norepinephrine (Fig. 1), and a transient contraction to angiotensin II (Fig. 2).

Histamine, which induces a sustained contraction, acts just as does norepinephrine. It induces a Ca^{2+} transient followed by a plateau phase significantly above the unstimulated baseline value (Fig. 3 and Table I).

When the initial rising rate of luminescence of Ca^{2+} transients induced by either 10^{-6} M norepinephrine, 5×10^{-8} M angiotensin II, or 10^{-5} histamine is compared, histamine-induced Ca^{2+} transients show a significantly higher initial rising rate of luminescence than either angiotensin II- or norepinephrine-induced ones (Table II). These results may reflect the difference of diffusion of each agonist into the tissue.

When aortic strips are stimulated with 35 mM K^+, the $[Ca^{2+}]_i$ gradually rises and reaches a plateau within 10 min (Fig. 4 *A*). Then, the $[Ca^{2+}]_i$ stays at that level for at least 30 min. The application of 35 mM K^+ induces a higher plateau level in the $[Ca^{2+}]_i$, but an equal contractile response to those seen after 10^{-6} M norepinephrine or 10^{-5} M histamine (2.6±0.6 g [$n = 5$] for 35 mM K^+, 2.9±1.2 g [$n = 5$] for 10^{-6} M norepinephrine, 3.1±0.8 g [$n = 5$] for 10^{-5} norepinephrine). The absolute value of $[Ca^{2+}]_i$ for the plateau level induced by 35 mM K^+ is 380±100 nM ($n = 5$). The application of 100 mM K^+ induces a different temporal pattern of the $[Ca^{2+}]_i$ response (Fig. 4 *B*). The $[Ca^{2+}]_i$ promptly rises and reaches a peak within 2 min. Then, it gradually decays and reaches a plateau level. The addition of 100 mM K^+ produces a similar pattern of contractile response to that induced by 35 mM K^+ (Fig. 4 *B*). High K^+-induced changes in the $[Ca^{2+}]_i$ are dependent on K^+ concentration and the $[Ca^{2+}]_i$ increases with increasing concentrations of extracellular K^+ (Fig. 4 *C*).

Effect of extracellular Ca^{2+} removal and caffeine treatment on agonist-induced changes in $[Ca^{2+}]_i$. Fig. 5 *A* shows that norepinephrine can cause a transient increase in $[Ca^{2+}]_i$ in muscle strips perfused in a Ca^{2+}-free buffer containing 1 mM EGTA. However, the plateau phase of the norepinephrine-induced rise in $[Ca^{2+}]_i$ is abolished in the absence of extracellular Ca^{2+}. These results suggest that the initial Ca^{2+} transient is mainly due to

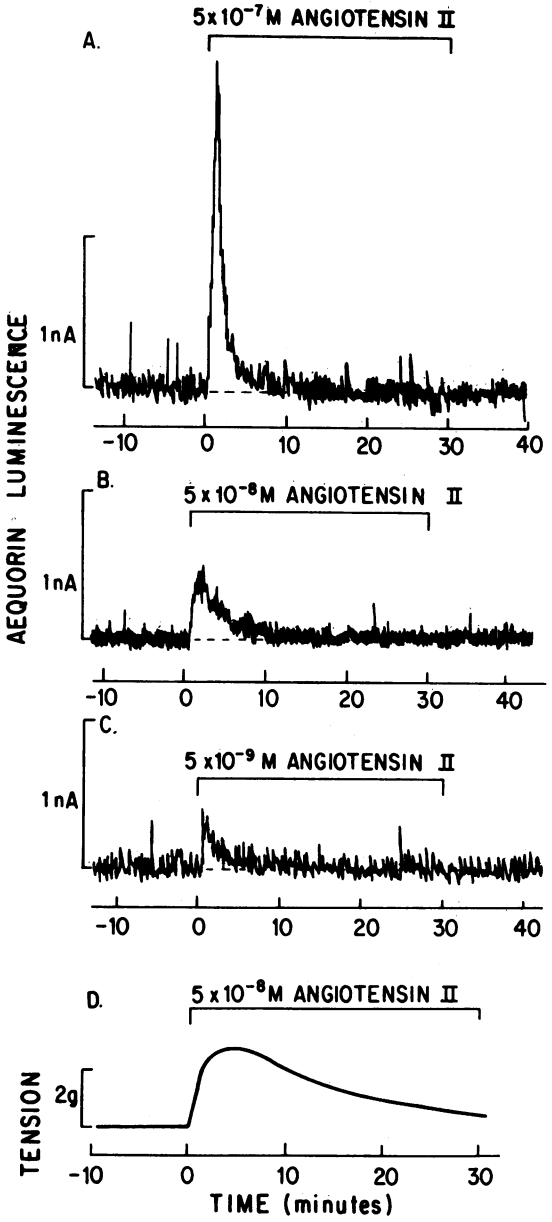

Figure 2. Effect of angiotensin II on cytoplasmic free Ca^{2+} and tension. Aequorin-loaded rabbit aortic strips were stimulated with various concentrations of angiotensin II for 30 min. (*A*) A change in $[Ca^{2+}]_i$ in response to 5×10^{-9} M angiotensin II. (*B*) A change in $[Ca^{2+}]_i$ in response to 5×10^{-8} M angiotensin II. (*C*) A change in $[Ca^{2+}]_i$ in response to 5×10^{-7} M angiotensin II. (*D*) A change in the tension in response to 5×10^{-8} M angiotensin II of the same strip as used in *B*.

mobilization of intracellular free Ca^{2+} and that the following plateau phase is probably due to Ca^{2+} influx across the plasma membrane. When aortic strips are first exposed to 25 mM caffeine in the absence of extracellular Ca^{2+}, a transient rise in $[Ca^{2+}]_i$ is seen (presumably due to the mobilization of Ca^{2+} from sarcoplasmic reticulum) (26, 27). The subsequent addition of norepinephrine causes no Ca^{2+} transient in these caffeine-pretreated muscles (Fig. 5). This disappearance of a norepinephrine-induce rise in $[Ca^{2+}]_i$ is not due to the consumption of aequorin, because the same strip shows a Ca^{2+} transient in response to norepinephrine stimulation after extracellular Ca^{2+} is replenished

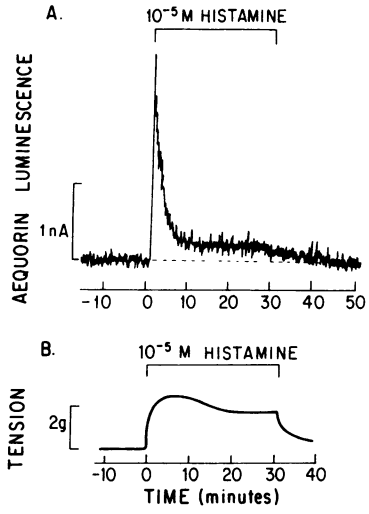

Figure 3. Effect of histamine on cytoplasmic free Ca^{2+} and tension. Aequorin-loaded rabbit aortic strip was stimulated with 10^{-5} M of histamine for 30 min. (*A*) A change in $[Ca^{2+}]_i$. (*B*) A change in the tension of the same strip.

(data not shown). These results are reproduced in five of five experiments.

Angiotensin II and histamine also elicit a Ca^{2+} transient in the absence of extracellular Ca^{2+}. Likewise, caffeine pretreatment abolishes the angiotensin II- or histamine-induced $[Ca^{2+}]_i$ transient in the absence of extracellular Ca^{2+} (data not shown). These data suggest that norepinephrine, angiotensin II, and histamine act on the caffeine-sensitive intracellular Ca^{2+} pool to cause the release of Ca^{2+}.

The high K^+-induced rise in $[Ca^{2+}]_i$ is differently affected by removal of extracellular Ca^{2+} depending on the K^+ concentrations employed (Fig. 6). The 35 mM K^+-induced rise in $[Ca^{2+}]_i$ is totally abolished when extracellular Ca^{2+} is removed. However, a stimulation with 100 mM K^+ still elicits a rise in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} although the amplitude of luminescence is markedly diminished compared to that in the presence of extracellular Ca^{2+} (Fig. 4 *B*). These results were reproduced in four of four experiments.

Effect of BAY K 8644, a Ca^{2+}-channel agonist, on angiotensin II-induced $[Ca^{2+}]$ response and contractile response. Unlike norepinephrine or histamine, angiotensin II does not induce an obvious plateau phase in the $[Ca^{2+}]_i$ response (Figs. 1–3). Because the plateau phase in the $[Ca^{2+}]_i$ response to norepinephrine or to histamine appears to be dependent on extracellular Ca^{2+} (Fig. 5), the effect of BAY K 8644, an activator of voltage-dependent Ca^{2+} channels (28), on the $[Ca^{2+}]_i$ response and the contractile response to angiotensin II was studied. As shown in Fig. 7, the

Table II. Comparison of Initial Rising Rates of Ca^{2+} Transients Induced by Norepinephrine, Angiotensin II, or Histamine

Agonists	*n*	Initial rising rate of luminescence
10^{-6} M norepinephrine	12	2.75±2.13*
5×10^{-8} M angiotensin II	12	2.50±1.74‡
10^{-5} M histamine	8	5.28±1.07

Values (means±SD) are measures of the slope of an initial rise of luminescence and are expressed as a change in L/L_{basal} per minute. L/L_{basal} was calculated as explained in Table I. The symbols denote statistically significant difference compared with the value with histamine stimulation (* $P < 0.001$; ‡ $P < 0.002$).

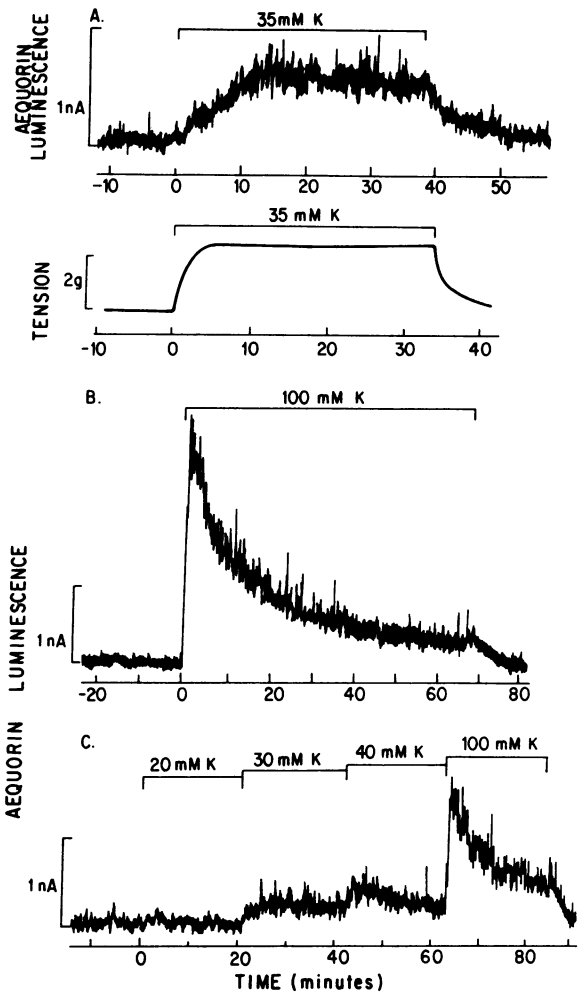

Figure 4. Effect of extracellular high K^+ on cytoplasmic free Ca^{2+} and tension. (*A*) A change in cytoplasmic free Ca^{2+} and tension in response to 35 mM K^+. (*B*) A change in cytoplasmic free Ca^{2+} in response to 100 mM K^+. (*C*) Changes in cytoplasmic free Ca^{2+} in response to various concentrations of K^+. In these experiments the perfusate contained 10^{-5} M phentolamine. In *A*, aequorin luminescence shows a more gradual increase than tension. This dissociation may reflect the difference between the perfusion system for measurement of aequorin luminescence and the static incubation system for tension measurement (see Methods).

combined addition of 5×10^{-8} M angiotensin II and 2×10^{-8} M BAY K 8644, which by itself does not cause any contraction, induces a sustained contraction which is totally blocked by salarasin, an antagonist of angiotensin II. The addition of both drugs also induces a Ca^{2+} transient which is followed by a plateau phase significantly higher than that seen in the unstimulated tissue (Table I). Thus, the stimulation of Ca^{2+} influx by BAY K 8644 during angiotensin II action changes the responses induced by angiotensin II to become similar to those induced by norepinephrine or histamine, suggesting an important role of the plateau phase of the $[Ca^{2+}]_i$ in sustaining the contractile response in the case of both norepinephrine and histamine.

Effect of ANP on agonist-induced changes in $[Ca^{2+}]_i$. The effects of ANP on the $[Ca^{2+}]_i$ responses of aortic strips to the various agonists was examined. In these experiments, to facilitate comparison, paired-aequorin loaded aortic strips were prepared

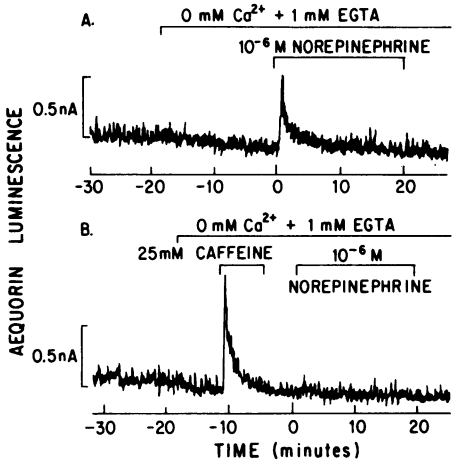

Figure 5. Effect of extracellular Ca^{2+} removal and caffeine pretreatment on agonist-induced changes in cytoplasmic free Ca^{2+}. (*A*) A change in $[Ca^{2+}]_i$ in response to 10^{-6} M norepinephrine in the Ca^{2+}-free buffer containing 1 mM EGTA. (*B*) Effect of pretreatment with 25 mM caffeine on 10^{-6} M norepinephrine-induced change in $[Ca^{2+}]_i$ in the Ca^{2+}-free buffer containing 1 mM EGTA.

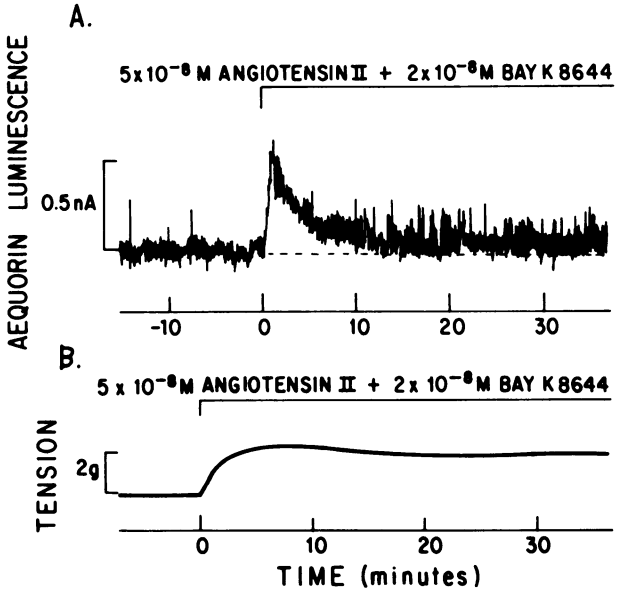

Figure 7. Effect of the combined addition of angiotensin II and BAY K 8644 on cytoplasmic free Ca^{2+} and tension. Aequorin-loaded rabbit aortic strip was stimulated with both 5×10^{-8} M angiotensin II and 2×10^{-8} M BAY K 8644 for 30 min. (*A*) A change in $[Ca^{2+}]_i$. (*B*) A change in the tension of the same strip. In this experiment the perfusate contained 10^{-5} M phentolamine.

from the same aorta. One strip was pretreated with ANP for 10 min and the other strip was not, before each strip was stimulated with a particular agonist. The doses of agonists used in these experiments were chosen on the basis of preliminary studies indicating that ANP at 10^{-7} M caused an 80% or greater inhibition of the early phase of the contractions of rabbit aortae induced by standard doses of the particular agonists.

As shown in Fig. 8, pretreatment with 10^{-7} M of ANP does not change the baseline value of $[Ca^{2+}]_i$, but nearly completely inhibits both the $[Ca^{2+}]_i$ signal and the contraction induced by 10^{-6} M norepinephrine. When the same strip is again challenged with 10^{-6} M norepinephrine after a 4-h washout period, the strip responds with both a Ca^{2+} transient and a contractile response. The inhibition of both a Ca^{2+} transient and a contractile response by ANP was overcome by the addition of a higher dose of norepinephrine (data not shown). If 10^{-7} M ANP is added to a strip prestimulated with 10^{-6} M norepinephrine, the plateau phase of the $[Ca^{2+}]_i$ response is probably immediately abolished with a concomitant inhibition of the contractile response as shown in Fig. 9.

In contrast to its effect in norepinephrine-treated strips, 10^{-7} M ANP does not inhibit the Ca^{2+} transient induced by 5×10^{-8} M angiotensin II as shown in Fig. 10 in spite of the fact that this dose of ANP completely blocks the contractile response to this dose of angiotensin II. The different effects of ANP on norepinephrine- or angiotensin II-induced Ca^{2+} transients are typically shown in Fig. 11, which demonstrate that when a strip pretreated with ANP is sequentially stimulated by norepinephrine and then angiotensin II, stimulation with only angiotensin II induces a Ca^{2+} transient. Likewise, 10^{-7} M ANP does not block the Ca^{2+} transient induced by 10^{-5} M histamine despite having a profound inhibitory effect on the contractile response to this agent (Fig. 12). However, 10^{-7} M ANP appears to abolish the plateau phase of the $[Ca^{2+}]_i$ response to histamine just as it does in the case of norepinephrine (Fig. 12).

As reported previously by other investigators (19, 21), ANP is less effective in inhibiting high K-induced contractions than in inhibiting agonist-induced contractions. It is of interest to examine if this smaller effect of ANP on high K-induced contraction is based on a smaller effect of ANP on a high K-induced rise in the $[Ca^{2+}]_i$. As shown in Fig. 13, ANP induces a transient and partial decrease in luminescence (36±7% [n = 4]) when ANP is added to strips prestimulated with 35 mM K. This dose of ANP induces a sustained but partial inhibition (26±7% [n = 4]) of contractile response induced by 35 mM K. Thus, ANP appears to have a smaller inhibitory effect on both the increase in $[Ca^{2+}]_i$ and the contractile response induced by high K in contrast to its effect on norepinephrine- or histamine-induced responses.

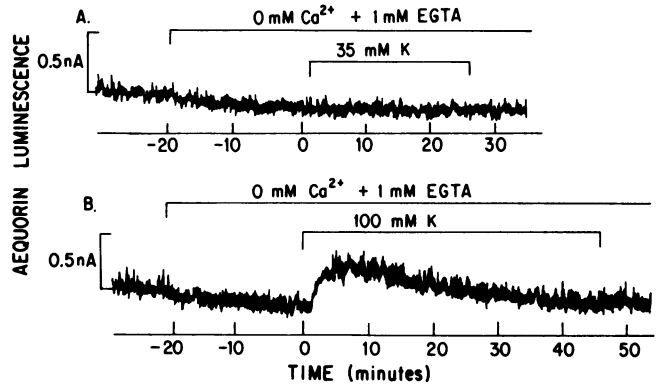

Figure 6. Effect of extracellular Ca^{2+} removal on high K^+-induced changes in cytoplasmic free Ca^{2+}. (*A*) A change in $[Ca^{2+}]_i$ in response to 35 mM K^+ in the Ca^{2+}-free buffer containing 1 mM EGTA. (*B*) A change in $[Ca^{2+}]_i$ in response to 100 mM K^+ in the Ca^{2+}-free buffer containing 1 mM EGTA. In these experiments the perfusate contained 10^{-5} M phentolamine.

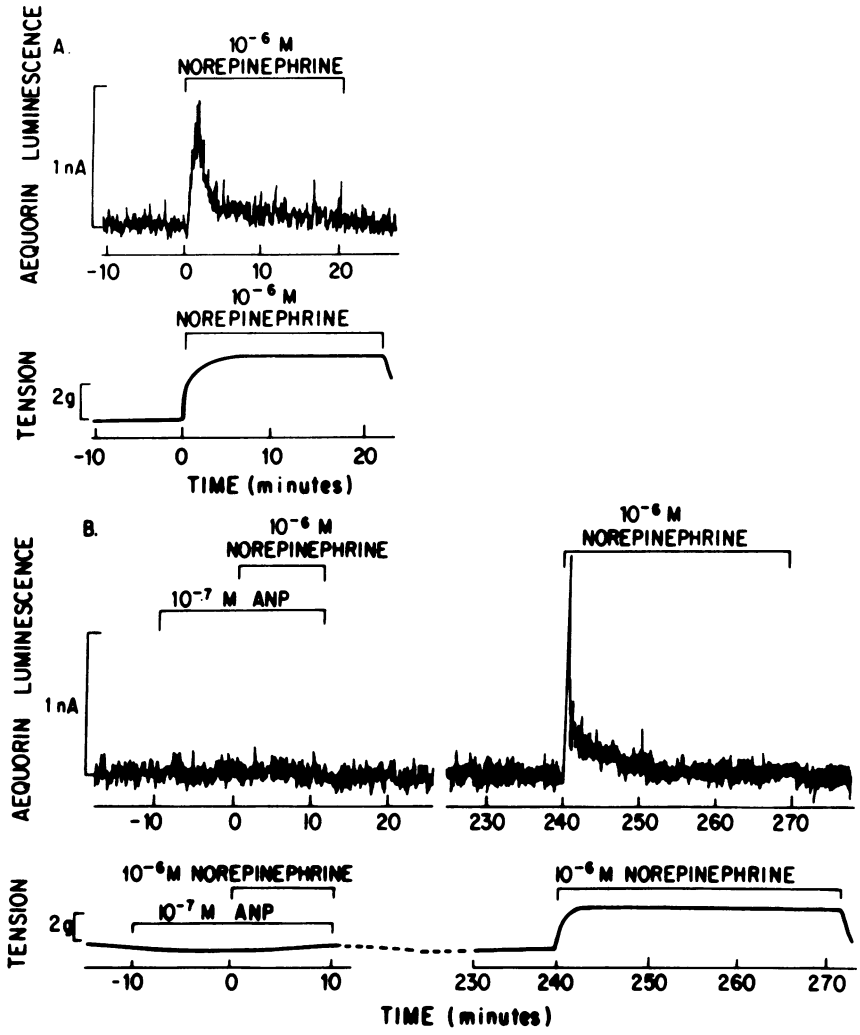

Figure 8. Effect of ANP on norepinephrine-induced changes in cytoplasmic free Ca^{2+} and tension. (*A*) 10^{-6} M norepinephrine-induced $[Ca^{2+}]_i$ response and contraction in a control strip. (*B*) 10^{-6} norepinephrine-induced $[Ca^{2+}]_i$ response and contraction in a strip pretreated with 10^{-7} ANP. The same strip was challenged again with norepinephrine after a washout period.

Discussion

The results of the present study demonstrate by direct measurements of $[Ca^{2+}]_i$ that the actions of three different agonists, norepinephrine, angiotensin II, and histamine differ in important ways. The addition of norepinephrine to aequorin-loaded aortic strips generates a rapid rise in $[Ca^{2+}]_i$ followed by a plateau phase significantly higher than the unstimulated baseline value (Fig. 1), and these changes in $[Ca^{2+}]_i$ are correlated with a prompt and sustained contractile response. The experiments in which

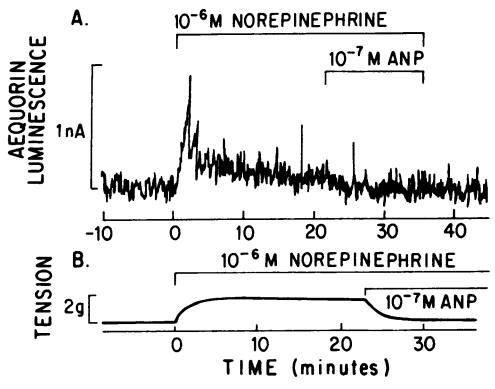

Figure 9. Reversal of norepinephrine-induced responses by ANP. Aequorin-loaded rabbit aortic strip was stimulated with 10^{-6} M norepinephrine, then treated with 10^{-7} M ANP. (*A*) A change in $[Ca^{2+}]_i$. (*B*) A change in the tension of the same strip.

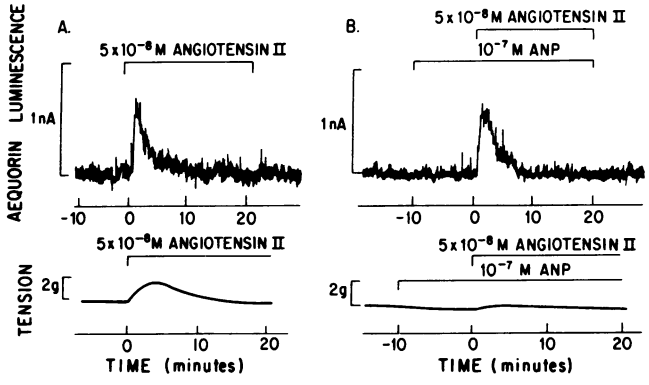

Figure 10. Effect of ANP on angiotension II-induced changes in cytoplasmic free Ca^{2+} and tension. (*A*) 5×10^{-8} M-induced $[Ca^{2+}]_i$ response and contraction in a control strip. (*B*) 5×10^{-8} M angiotensin II-induced $[Ca^{2+}]_i$ response and contraction in a strip pretreated with 10^{-7} M ANP.

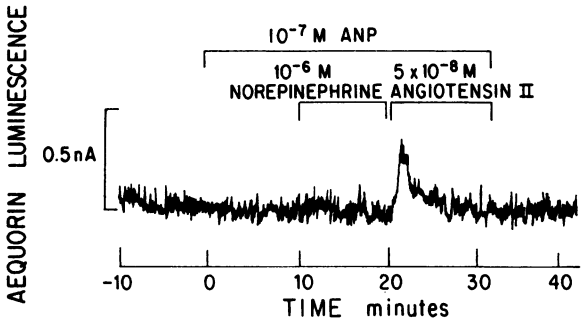

Figure 11. Effect of sequential addition of norepinephrine and angiotensin II on cytoplasmic free Ca^{2+} in the presence of ANP. Aequorin-loaded rabbit aortic strip was pretreated with 10^{-7} M ANP for 10 min, then sequentially stimulated with 10^{-6} M norepinephrine and 5 $\times$ 10^{-8} M angiotensin II.

extracellular Ca^{2+} was removed or caffeine pretreatment was done suggest that the source of mobilized Ca^{2+} for initial Ca^{2+} transients is mainly from an intracellular pool (presumably the sarcoplasmic reticulum) and that the source of mobilized Ca^{2+} for the plateau phase of $[Ca^{2+}]_i$ response probably is an extracellular pool (Fig. 4). These results not only have confirmed previous observations (6–9) based upon $^{45}Ca^{2+}$ flux studies showing that norepinephrine mobilizes Ca^{2+} from both intra- and extracellular pools in this tissue, but also have shown that the agonist-induced mobilization of Ca^{2+} from both pools results in a temporal change in $[Ca^{2+}]_i$ that consists of initial Ca^{2+} transients and a following plateau phase. Because aequorin gives unequally large weight to regions of cells where a high Ca^{2+} concentration exists (29, 30), these data do not necessarily mean that during the plateau phase of $[Ca^{2+}]_i$, there is a uniform increase in $[Ca^{2+}]_i$ within the cytoplasm. The plateau phase may reflect an increase in $[Ca^{2+}]_i$ in localized regions within the cytoplasm.

The addition of either angiotensin II or histamine also generates Ca^{2+} transients (Figs. 2 and 3). However, it should be noted that stimulation with angiotensin II produces no obvious sustained plateau phase of $[Ca^{2+}]_i$ response unlike stimulation with norepinephrine or histamine. Both norepinephrine and histamine induce a sustained contraction of rabbit aortic strips (Figs. 1 and 3). On the other hand, angiotensin II causes a transient contraction as shown in Fig. 2. The plateau phase of $[Ca^{2+}]_i$ in response to norepinephrine appears to reflect an increase in Ca^{2+} influx across the plasma membranes as evidenced by the results shown in Fig. 5 and the sustained phase of norepinephrine-induced contraction is dependent on the presence of extracellular Ca^{2+} (6, 8). Further, stimulation of Ca^{2+} influx with a low dose of BAY K 8644 (28) during angiotensin II action converts the angiotensin II-induced contractile response to a sustained one which is associated with a plateau phase of the $[Ca^{2+}]_i$ response (Fig. 7). These data suggest that an increase in Ca^{2+} influx plays an important role in sustaining the contractile response to norepinephrine and histamine and that the inability of angiotensin II to produce a sustained contraction is due to a smaller effect of this agonist on Ca^{2+} influx rate in rabbit aortic smooth muscle.

Recent studies in many types of cells have revealed that the biochemical basis of the Ca^{2+} mobilization from the intracellular storage site by Ca^{2+}-mobilizing hormones is the generation of inositol 1,4,5-trisphosphate and its actions on the intracellular storage site (presumably endoplasmic reticulum) (31). Several investigators have shown that inositol 1,4,5-trisphosphate indeed causes a release of Ca^{2+} from the intracellular storage site in permeabilized vascular smooth muscle cells (32–35). Because norepinephrine, angiotensin II, and histamine are known to cause polyphosphoinositide hydrolysis and the generation of inositol 1,4,5-trisphosphate in vascular smooth muscle (36–39), Ca^{2+} transients elicited by stimulation with these agonists are most likely caused by Ca^{2+} release from the intracellular storage site on which inositol 1,4,5-trisphosphate acts. In fact, the data

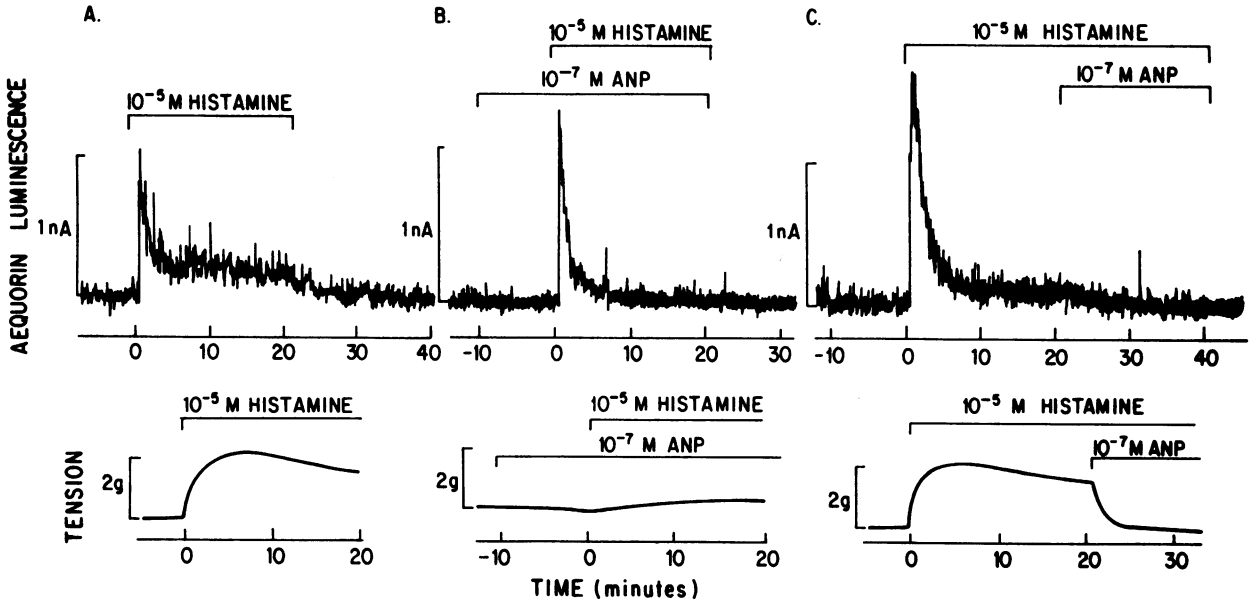

Figure 12. Effect of ANP on histamine-induced changes in cytoplasmic free Ca^{2+} and tension. (*A*) 10^{-5} M histamine-induced $[Ca^{2+}]_i$ response and contraction in a control strip. (*B*) 10^{-5} M histamine-induced $[Ca^{2+}]_i$ response and contraction in a strip pretreated with 10^{-7} M ANP. (*C*) Inhibition of 10^{-5} M histamine-induced $[Ca^{2+}]_i$ response of the plateau phase and the tonic phase of contraction by 10^{-7} M ANP.

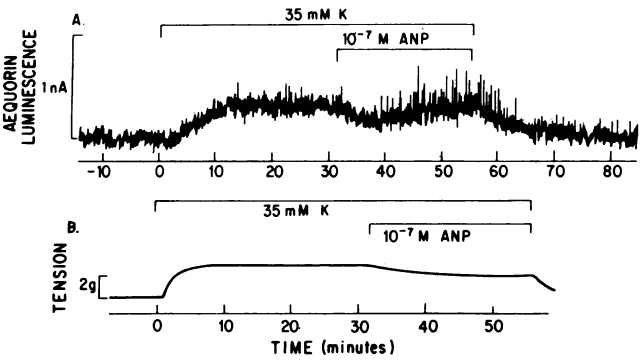

Figure 13. Effect of ANP on high K^+-induced changes in cytoplasmic free Ca^{2+} and tension. Aequorin-loaded rabbit aortic strip was stimulated with 35 mM K^+, then treated with 10^{-7} M AMP. (*A*) A change in $[Ca^{2+}]_i$. (*B*) A change in the tension. In this experiment the perfusate contained 10^{-5} M phentolamine.

presented in Fig. 5 indicate that these three agonists all act on the caffeine-sensitive Ca^{2+} pool suggesting that the mobilized Ca^{2+} pool is present in the sarcoplasmic reticulum.

The present studies have shown that the high K^+ stimulation generates two different patterns of Ca^{2+} signal, depending upon the concentration of K^+ used. As depicted in Fig. 4, a lower concentration of K^+ (35 mM K^+) produces a monophasic rise in $[Ca^{2+}]_i$. In contrast, a higher concentration of K^+ (100 mM K^+) induces a biphasic response. The results shown in Fig. 6 indicate that the mechanism of an increase in $[Ca^{2+}]_i$ differs between stimulation with 35 mM K^+ and 100 mM K^+. The rise in $[Ca^{2+}]_i$ induced by 35 mM K^+ is totally due to Ca^{2+} influx across the plasma membrane and the rise in $[Ca^{2+}]_i$ by 100 mM K^+ is due to both Ca^{2+} influx and a release of Ca^{2+} from the intracellular storage sites. Because stimulation with high K^+ by itself does not cause polyphosphoinositide hydrolysis (38–40), Ca^{2+} release from the intracellular storage site during stimulation with high K^+ must occur through a different mechanism than the formation of inositol 1,4,5-trisphosphate. Several investigators have suggested that the mechanism of Ca^{2+}-induced Ca^{2+} release or depolarization-induced Ca^{2+} release from the intracellular storage site may operate in smooth muscle just as it does in skeletal and cardiac muscle (15, 26, 27). However, a Ca^{2+}-induced Ca^{2+} release cannot account for the effect of 100 mM K^+ which induces a rise in $[Ca^{2+}]_i$ even in muscle strips incubated in the absence of extracellular Ca^{2+}. Rather, some other mechanism must operate to link the K^+-evoked depolarization of the plasma membrane to the release of Ca^{2+} from the sarcoplasmic reticulum.

ANP is a potent vasorelaxant and has a greater effect on aorta and its main branches than on peripheral resistance vessels (41). In rabbit aorta, ANP inhibits contraction induced by agonists or high K^+ to various degrees (19). There is now considerable evidence that the vasorelaxant effect of ANP may be mediated by the activation of a particulate guanylate cyclase (20, 42). However, its mechanism of action is not yet fully understood. In the present study, we undertook to determine whether ANP acts at a site of Ca^{2+}-signal generation in response to agonists or at a site beyond Ca^{2+}-signal generation.

ANP at 10^{-7} M causes a 80% or greater inhibition of the contraction induced by either norepinephrine, angiotensin II, or histamine at the doses used in the present experiments (Figs. 8–12). At this concentration, ANP inhibits the Ca^{2+} transient induced by norepinephrine, but not the transients induced by either angiotensin II or histamine (Figs. 8–12). In that ANP has been reported to block norepinephrine-induced production of inositol monophosphate in rat aorta (43), the inhibitory effect of ANP on Ca^{2+} transients induced by norepinephrine is most likely mediated by the inhibition of receptor-mediated phospholipase C stimulation and, thereby, inhibition of inositol 1,4,5-trisphosphate production. This result is consistent with recent observations by Meisheri et al. (44) and Chiu et al. (45) that ANP blocks $^{45}Ca^{2+}$ efflux induced by norepinephrine in rabbit aorta. However, a paradox exists. On the one hand, it appears that all three agonists act via the phosphoinositide system, and mobilize the same pool of Ca^{2+}, but on the other hand, ANP inhibits selectively the effect of norepinephrine on phospholinositide hydrolysis. These results suggest that ANP acts to inhibit the coupling of the norepinephrine receptor to phospholipase C, but not the coupling of either the histamine or angiotensin II receptor.

We have found that ANP does not inhibit angiotensin II-induced Ca^{2+} transients in adrenal glomerulosa strips (Apfeldorf, W., and P. Barrett, unpublished work). Kurtz et al. (46) have recently reported that ANP does not inhibit angiotensin II-induced Ca^{2+}-transients in juxtaglomerular cells. Likewise, in the present study it was found that ANP does not inhibit the angiotensin II-induced Ca^{2+} transient in vascular smooth muscle (Fig. 10). Thus, the site of inhibitory actions of ANP on angiotensin II action is apparently beyond Ca^{2+}-signal generation in various tissues.

Another effect of ANP on Ca^{2+}-signal generation by these agonists is an inhibition of the plateau phase in the $[Ca^{2+}]_i$ response to norepinephrine or histamine with concomitant inhibition of the contractile response as shown in Figs. 9 and 12. In contrast, the 35 mM K^+-induced increase in $[Ca^{2+}]_i$ is only weakly and transiently inhibited by ANP (Fig. 13). Because the plateau phase of the $[Ca^{2+}]_i$ response to norepinephrine or histamine appears to be related to Ca^{2+} influx across the plasma membrane (Fig. 4), these data suggest that this inhibitory effect of ANP results from antagonizing the agonist-induced increase in Ca^{2+} influx and/or stimulating Ca^{2+} efflux. Clearly, ANP has another site of action beyond Ca^{2+}-signal generation because ANP inhibits contraction induced by angiotensin II, but not the Ca^{2+} transients induced by angiotensin II. In addition, ANP exerts a sustained inhibition of contraction induced by high K^+, but a transient inhibition of $[Ca^{2+}]_i$ response. The third site of ANP action appears to play a major role in exerting its vasorelaxant effect on at least the early phase of angiotensin II- or histamine-induced contraction. Its molecular mechanism is still unknown although some studies have suggested that a cyclic GMP-dependent activation of myosin light chain phosphatase may be involved (47, 48).

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