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The mechanism of relaxation induced by atrial natriuretic peptide in the porcine renal artery

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1 The mechanisms underlying the relaxation of the porcine renal artery induced by atrial natriuretic peptide (ANP) were investigated, using front-surface fluorimetry with fura-2 and receptor-coupled permeabilization by α-toxin.

2 ANP decreased the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and tension during the contraction induced by a high external K^+ solution, in a concentration-dependent manner. This ANP-induced decrease in $[Ca^{2+}]_i$ during the contraction induced by high K^+ solution was composed of two phases, an initial rapid phase, followed by a maintenance phase. The initial rapid decrease in $[Ca^{2+}]_i$, but not the maintained decrease in $[Ca^{2+}]_i$, was inhibited when the tissue was treated with thapsigargin, a selective Ca^{2+} pump inhibitor of the sarcoplasmic reticulum. When the tissues were treated with thapsigargin and external Ca^{2+} was replaced by Ba^{2+}, which cannot be transported by the Ca^{2+} pump, ANP did not induce a decrease in $[Ba^{2+}]_i$, even though the elevation of tension induced by Ba^{2+} was strongly inhibited.

3 In the absence of extracellular Ca^{2+}, ANP inhibited the release of Ca^{2+} from the intracellular store induced by noradrenaline (NA).

4 The $[Ca^{2+}]_i$ (abscissa scale)-tension (ordinate scale) relationship observed during the contraction induced by various concentrations of high external K^+ solution was shifted downwards by the addition of 10^{-8} M ANP, indicating that, at any given $[Ca^{2+}]_i$, the tension generated by high K^+ solution was considerably inhibited by the additon of 10^{-8} M ANP. The $[Ca^{2+}]_i$-tension curve of the contraction obtained by the cumulative application of external Ca^{2+} (0-3.75 mM) during depolarization with 118 mM K^+ solution was shifted to the left by 3×10^{-7} M NA. This NA-induced $[Ca^{2+}]_i$-tension relationship was shifted to the right by 10^{-8} M ANP, indicating that the ANP-induced reduction of Ca^{2+}-sensitivity operates during the contraction induced by NA.

5 In α-toxin-permeabilized preparations, ANP induced relaxation of tissues precontracted with a mixture of 3×10^{-7} M Ca^{2+}, 10^{-5} M guanosine 5'-triphosphate (GTP) and 10^{-6} M NA. Thus a component of ANP-induced relaxation took place by way of a reduction in the Ca^{2+} sensitivity of the myofilaments, independent of changes in $[Ca^{2+}]_i$.

6 These results indicate that ANP induces relaxation of the porcine renal artery by: (1) reducing $[Ca^{2+}]_i$ mainly via the activation of the Ca^{2+} pumps located on the sarcoplasmic reticulum and sarcolemma, as well as via inhibition of agoinist-induced release of Ca^{2+} from the intracellular store; and (2) decreasing the Ca^{2+}-sensitivity of the contractile elements.

Keywords: α-Toxin; atrial natriuretic peptide; calcium sensitivity; cyclic GMP; intracellular calcium concentration; renal artery; skinned fibre; vascular smooth muscle

Introduction

Atrial natriuretic peptide (ANP) is a potent relaxant of vascular smooth muscle (Garcia *et al.*, 1985), especially of the renal artery (Currie *et al.*, 1983; Ishihara *et al.*, 1985). The underlying mechanism for this vasorelaxant effect induced by ANP is still not fully understood, although it has been pointed out that ANP-induced vasorelaxation closely resembles that produced by nitro-compounds (Winquist *et al.*, 1984), which increase the intracellular content of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Ohlstein & Berkowitz, 1985). Indeed, it has been reported that ANP relaxes smooth muscle while increasing the intracellular concentration of cyclic GMP in a time- and concentration-dependent manner (Fiscus *et al.*, 1985). Complementary DNA cloning of the ANP receptor revealed that the receptor includes the guanyl cyclase domain (Chinkers *et al.*, 1989). It has thus been suggested that the vasorelaxant effect of ANP is mediated by cyclic GMP as a second messenger.

Cyclic GMP is an important regulator of smooth muscle function. The mechanisms underlying cyclic GMP-mediated vasorelaxation have been mainly attributed to effects on Ca^{2+} mobilization, and thus, a decrease in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) (Kamm & Stull, 1989; Lincoln, 1989; Marin & Sanchez-Ferrer, 1990; Rand, 1992). These inlcude: (1) inhibition of generation of inositol trisphosphate (IP_3); (2) increased sequestration of cytosolic Ca^{2+} into stores; (3) inhibition of Ca^{2+} influx; (4) stimulation of membrane Ca^{2+}-adenosine trisphosphatase (ATPase); (5) opening of the K^+ channels; and (6) inhibition of adenosine 3':5'-cyclic monophosphate (cyclic AMP) phosphodiesterase. In addition to a decrease in $[Ca^{2+}]_i$, simultaneous measurements of $[Ca^{2+}]_i$ and tension (Morgan & Morgan, 1984; Karaki *et al.*, 1988; Yanagisawa *et al.*, 1989; Abe *et al.*, 1990) along with receptor-coupled permeabilization of smooth muscle (Nishimura & van Breemen, 1989), have indicated that cyclic GMP and/or nitro-compounds induce a decrease in the Ca^{2+} sensitivity of the contractile elements.

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It is thus likely that ANP may decrease the Ca^{2+} sensitivity of the contractile elements. However, there has been no report, to our knowledge, which describes an ANP-induced decrease in Ca^{2+} sensitivity of the contractile elements in the smooth muscle. In ferret aortic smooth muscle contracted by a high external K^+ solution, it was reported that the relaxant effects of ANP were mainly due to a decrease in $[Ca^{2+}]_i$, while, in addition, the Ca^{2+}-force curve was not changed by ANP (Suematsu *et al.*, 1991). In the present study, we investigated the effects of ANP on Ca^{2+} mobilization and on the Ca^{2+} sensitivity of the contractile elements of porcine renal artery, using front-surface fluorimetry (Hirano *et al.*, 1989) and receptor-coupled permeabilization by α-toxin (Nishimura *et al.*, 1988), respectively.

Methods

Tissue preparation

The first branches of the renal artery were dissected from pig kidney obtained from a slaughter-house and segments 2–3 cm from the origin were excised and cut longitudinally. The endothelium was removed by rubbing the inner surface with a cotton swab and then the adventitia was trimmed away under a binocular microscope. The medial preparations thus obtained were cut into approximately 1 × 5 mm strips, 0.2 mm thick. The strips were loaded with fura-2 by incubation in oxygenated (95% O_2 : 5% CO_2) Dulbecco's modified Eagle's medium containing 2.5×10^{-5} M fura-2/AM (an acetoxymethyl ester form) dissolved in dimethyl sulphoxide (final concentration: 5%) and 5% foetal bovine serum for 3–4 h at 37°C (Hirano *et al.*, 1989). After loading with fura-2, the strips were rinsed with normal physiological salt solution (PSS) for at least 60 min at 37°C to remove the dye in the extracellular space and to equilibrate the strips before starting the measurements.

Measurement of tension development

The fura-2 loaded strips were mounted vertically to arrange the smooth muscle cells longitudinally in a quartz organ bath as the luminal side of the strip faced the front of the bath and was connected to a strain gauge (TB-612-T, Nihon Koden, Japan). During a 60 min fura-2 equilibration period, the strips were stimulated with 118 mM K^+ solution every 15 min and then the resting tension was increased in a stepwise manner to obtain a maximum contraction. The resultant resting tension levels were about 300 mg (=2.97 mN). The responsiveness of each strip to 118 mM K^+ was recorded before starting the experimental protocol. The developed tension was expressed as a percentage, assuming the values in normal PSS (at rest: 5.9 mM K^+) and 118 mM K^+ PSS (at the steady state of contraction) to be 0% and 100%, respectively. The simultaneous measurements of $[Ca^{2+}]_i$ and tension experiments were carried out at 37°C.

Front-surface fluorometry

Changes in the fluorescence intensity of the fura-2-Ca^{2+} complex were monitored with a front-surface fluorometer specifically designed for fura-2 fluorometry (CAM-OF-2), with the collaboration of the Japan Spectroscopic Co., Tokyo, Japan. Briefly, strips were illuminated by guiding the alternating (400 Hz) 340 and 380 nm excitation light from a xenon light source through quartz optic fibres arranged in a concentric inner circle (diameter, 3 mm). Surface fluorescence of the strip was collected by glass optic fibres arranged in an outer circle (diameter, 7 mm) and introduced through a 500 nm bandpass filter (bandwidth, 10 nm) into a photomultiplier.

The ratio (R) of the fluorescence intensities at 340 nm excitation to those at 380 nm excitation was monitored with a sampling rate of 400 Hz and expressed as a percentage, assuming the steady-state values in normal (5.9 mM K^+) and 118 mM K^+ PSS to be 0 and 100%, respectively. The absolute values of $[Ca^{2+}]_i$ for 0% and 100% levels of R were determined in separate measurements, using the equation derived by Grynkiewicz *et al.* (1985) and the dissociation constant of 224 nM for Ca^{2+}-fura-2 complex, as follows: R_{max} was determined by the addition of 2.5×10^{-5} M ionomycin to normal PSS (containing 1.25 mM Ca^{2+}) and R_{min} was determined in Ca^{2+}-free (2 mM EGTA) PSS. The mean values of $[Ca^{2+}]_i$ at rest (0%) and at the steady state of 118 mM K^+ depolarization (100%) were 112±3.3 and 679±21 nM ($n=7$), respectively. Since $[Ca^{2+}]_i$ was calculated on the assumption that the dissociation constant of fura-2 for Ca^{2+} in the cytosol of vascular smooth muscle cells was 224 nM, which was obtained in buffer that mimicked the internal ionic composition at pH 7.05 and 37°C, the absolute $[Ca^{2+}]_i$ values obtained are simply an approximation; therefore, the R values were used in the statistical analysis of $[Ca^{2+}]_i$ levels.

Permeabilization by α-toxin

Permeabilization of the porcine renal artery was performed as previously described (Nishimura *et al.*, 1988). Briefly, small rings from third branches of the porcine renal arteries were dissected and trimmed. Two tungsten wires were passed through the lumen. One wire was fixed to the chamber while the other was attached to a force transducer (U gage, Minebea Co Ltd., Japan). Permeabilization was accomplished by incubating the arterial segments with Staphylococcal α-toxin (5000 unit ml^{-1}, CALBIOCHEM, La Jolla, CA, U.S.A.), for 40 min in a Ca^{2+}-free cytoplasmic substitution solution (CSS) containing 2 mM EGTA. The apparent binding constant used for the Ca^{2+}-EGTA was 10^6/M (Saida & Nonomura, 1978). After permeabilization, the tissues were stretched by a manipulator connected to the force transducer, to the appropriate resting tension which gave the maximum contraction by 10^{-5} M Ca^{2+} containing CSS solution. Tension development measurements in α-toxin permeabilized tissues were carried out at room temperature.

Drugs and solutions

The composition of the normal physiological salt solution (normal PSS) was (mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25 and D-glucose 11.5. High-K^+ PSS was made by an equimolar substitution of KCl for NaCl; 118 mM K^+ PSS was made by an equimolar substitution of KCl for NaCl and $BaCl_2$ for $CaCl_2$. The Ca^{2+}-free solution (Ca^{2+}-free PSS) contained 2 mM EGTA instead of 1.25 mM $CaCl_2$ and was produced by an exclusion of $CaCl_2$ from the normal PSS. All solutions were gassed with a mixture of 5% CO_2 : 95% O_2 (pH 7.4 at 37°C). The composition of the cytoplasmic substitution solution (CSS) was (mM): EGTA 10, K-methanesulphonate 100, $MgCl_2$ 3.38, Na_2ATP 2.2, creatine phosphate 10, Tris maleate (pH=6.8) 20 and the concentration of free Ca^{2+} indicated in the results. (−)-Noradrenaline hydrochloride and thapsigargin were purchased from SIGMA (St. Louis, MO, U.S.A.). Atrial natriuretic peptide (Human, 1-28) was from Peptide Institute Co. Ltd. (Osaka, Japan). Fura-2/AM (an acetoxymethyl ester form) and EGTA were from DOJINDO (Kumamoto, Japan). Fura-2/AM was dissolved in dimethylsulphoxide (DMSO) as a stock solution and diluted in the medium immediately before loading the dye. The final concentration of DMSO was 5%.

Statistical analysis

The measured values are expressed as the means±s.e. mean based on the observations of different tissue specimens ($=n$) taken from at least 4 different animals. Student's t test was used to determine the statistical significance. Analysis of co variance was used to determine the non-overlapping (or shift) of the $[Ca^{2+}]_i$-force relationship. P values <0.05 were considered to be significant.

Results

The effects of ANP on the increase in $[Ca^{2+}]_i$ and tension development induced by high external K^+ solutions

Figure 1a and b shows representative time courses of the effect of 10^{-8} M ANP on the increase in $[Ca^{2+}]_i$ and tension during the contractions induced by 118 mM and 40 mM K^+ solutions, respectively. When strips were depolarized with high external K^+ solutions, $[Ca^{2+}]_i$ and tension rapidly increased and reached steady-state levels in 10 and 20 min, respectively. The steady-state levels were dependent on the concentration of the external K^+ solution, and were maintained for at least 30 min during the observations. When the external bathing solution was changed from a high K^+ solution to a normal PSS, both the $[Ca^{2+}]_i$ and tension rapidly returned to the resting levels. The application of 10^{-8} M ANP during the steady-state contraction induced by 118 mM (Figure 1a) or 40 mM (Figure 1b) K^+ solutions caused significant, though small, reductions of $[Ca^{2+}]_i$ levels, composed of two phases, consisting of an initial rapid phase followed by a maintenance phase.

Figure 2 summarizes the levels of $[Ca^{2+}]_i$ (Figure 2a) and tension (Figure 2b) observed in the maintenance phase induced by high (118 mM or 40 mM) K^+ solutions, following decreases of $[Ca^{2+}]_i$ (a) and tension (b) induced by various concentrations of ANP. The application of ANP ($3 \times 10^{-10} - 10^{-7}$ M) reduced $[Ca^{2+}]_i$ and tension in a concentration-dependent manner, and the maximal reductions of $[Ca^{2+}]_i$ and tension were observed at a concentration greater than 10^{-8} M. During this relaxation, the levels of tension were much smaller than expected from the levels of $[Ca^{2+}]_i$, suggesting a decrease in Ca^{2+} sensitivity of the contractile elements.

Figure 3a and b shows the effects of 10^{-8} M ANP on $[Ca^{2+}]_i$ and tension at the steady-state of the contractions induced by various concentrations of external K^+ solutions (5.9, 20, 30, 40, 60 and 118 mM). Since Figure 2 indicated that the ANP-induced decreases in tension are much greater than those expected from the ANP-induced decrease in $[Ca^{2+}]_i$, the $[Ca^{2+}]_i$ (abscissa scale)-tension (ordinate scale) relationships (Figure 3c) were determined by plotting the data shown in Figure 3a and b. As shown in Figure 3c, the $[Ca^{2+}]_i$-tension relationship of the contraction obtained by depolarization with high external K^+ solutions was shifted downwards by ANP ($P<0.01$ by analysis of covariance, $n=6$). This indicated that ANP relaxed the porcine renal artery not only by reducing $[Ca^{2+}]_i$, but also by markedly reducing in the contractile response, at any given elevation of $[Ca^{2+}]_i$.

The effects of ANP on $[Ca^{2+}]_i$ and tension development induced by NA in normal PSS

Figure 4a and b shows the effects of 10^{-8} M ANP on the time course of changes in $[Ca^{2+}]_i$ and tension induced by NA (10^{-7} M) in normal PSS. The application of NA (10^{-7} M) induced rapid increases in $[Ca^{2+}]_i$ and tension, which then reached peak levels at about 5 min, and thereafter, slightly decreased to reach a steady state at about 15 min. At steady-state, the tension levels (120%) were greater than those expected from the $[Ca^{2+}]_i$ levels (60%), which indicated that, at a given level of $[Ca^{2+}]_i$, NA induces a greater degree of tension than depolarization with K^+-solution, and thereby, increases the Ca^{2+} sensitivity of the contractile elements. These elevations of $[Ca^{2+}]_i$ and tension induced by NA were markedly inhibited by incubation of the strips with 10^{-8} M ANP, 10 min before and during the application of 10^{-7} M NA. It was apparent that the extent of the reduction of tension (Figure 4b) was much greater than that of $[Ca^{2+}]_i$ (Figure 4a), which indicated that ANP also decreases the Ca^{2+} sensitivity of the contractile element in the presence of NA. It should be noted that the $[Ca^{2+}]_i$ level just before the application of NA (0 time point) was significantly lower with ANP present, than in normal PSS although the tension remained unchanged, which indicated that ANP decreased the $[Ca^{2+}]_i$ levels, but not the tension, when the tissue specimens were under resting conditions.

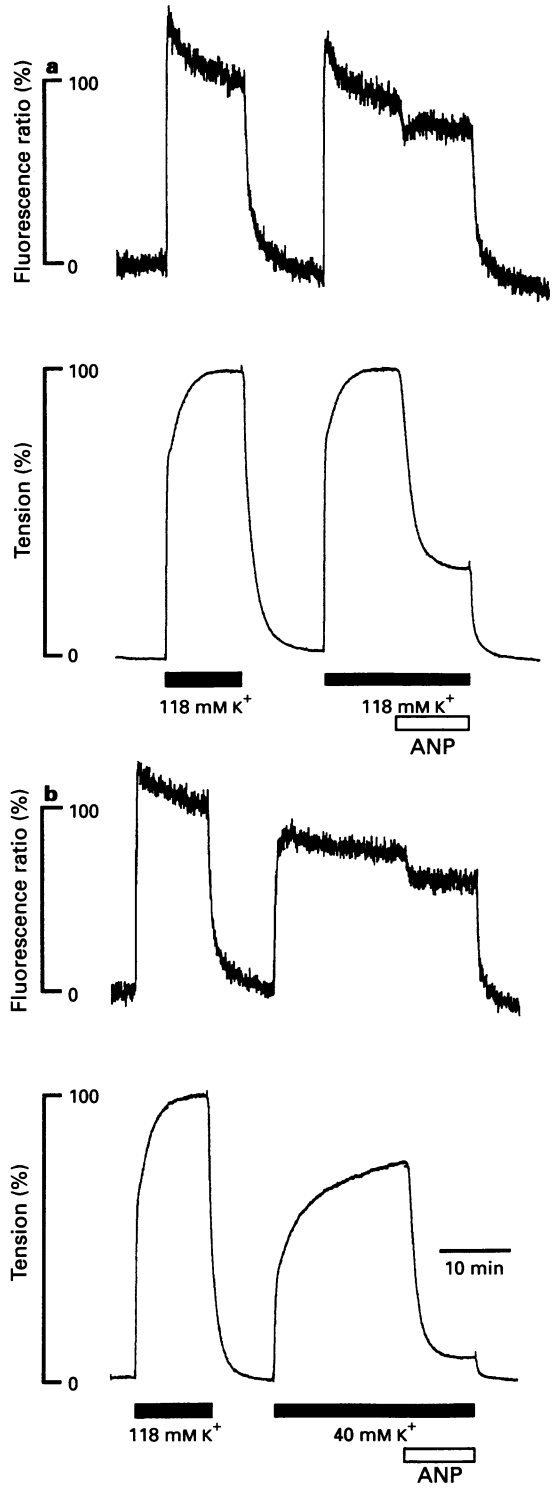

Figure 1 Representative recordings of the effect of 10^{-8} M ANP on the elevations of $[Ca^{2+}]_i$ and tension during contraction induced by 118 mM K^+ solution (a) and 40 mM K^+ solution (b). ANP was applied during the steady-state of the contraction. The responses of the fluorescence ration and the tension to 118 mM K^+ were recorded before each experiment to determine the 100% levels. The traces shown are representative of 6 similar independent experiments.

To examine the effect of ANP on the Ca^{2+}-tension relation in the presence of NA quantitatively, changes in $[Ca^{2+}]_i$ and tension were monitored with a stepwise increment of extracellular $Ca^{2+}]_o$), during the depolarization with 118 mM K^+

solution containing NA. Figure 5 shows the representative time courses of the changes in $[Ca^{2+}]_i$ and tension induced by the cumulative application of $CaCl_2$ during depolarization with 118 mM K^+ solution in the absence (a) and presence of 3×10^{-7} M NA (b), and in the presence of 3×10^{-7} M NA and 10^{-8} M ANP (c). When the bathing solution was changed from normal PSS to Ca^{2+}-free PSS containing 2 mM EGTA, $[Ca^{2+}]_i$ decreased to reach new steady levels although the tension remained at the resting levels. The application of NA (3×10^{-7} M) induced only transient elevations of $[Ca^{2+}]_i$ and tension from these levels; the detailed analysis of this phenomenon is shown in Figure 6. In all three cases (Figure 5a, b, c), the stepwise increase in $[Ca^{2+}]_o$ (0.01–3.75 mM) induced a stepwise increase in $[Ca^{2+}]_i$ and tension in a concentration-dependent manner. Without NA and ANP treatment (Figure 5a, Figure 6), $[Ca^{2+}]_i$ increased from $-51.6 \pm 4.1\%$ (at $[Ca^{2+}]_o = 0$) to $112.5 \pm 4.0\%$ (at 3.75 mM $= [Ca^{2+}]_o$), while the tension increased to $120.0 \pm 4.0\%$. In the presence of NA, but not ANP, $[Ca^{2+}]_i$ increased from $-54.4 \pm 1.5\%$ at 0 mM $[Ca^{2+}]_o$ to $114.9 \pm 3.6\%$ at 3.75 mM $[Ca^{2+}]_o$, and tension increased to $160.7 \pm 9.6\%$ (Figure 5b; Figure 6, $n = 9$). In the presence of both NA and ANP, $[Ca^{2+}]_i$ increased from $-59.7 \pm 3.3\%$ at 0 mM $[Ca^{2+}]_o$ to $117.0 \pm 7.4\%$ at 3.75 mM $[Ca^{2+}]_o$, while the tension increased to $147.4 \pm 8.3\%$ (Figure 5c; Figure 6, $n = 9$). Treatment with 10^{-8} M ANP inhibited increases in both $[Ca^{2+}]_i$ ($P < 0.05$ by Student's t test at 0.03 and 0.1 mM $[Ca^{2+}]_o$), and tension ($P < 0.05$ by Student's t test at all $[Ca^{2+}]_o$ range except 3.75 mM) (Figure 6a, b). Figure 6c shows the $[Ca^{2+}]_i$-tension relationships plotted using the data points shown in Figure 6a and b. The $[Ca^{2+}]_i$-tension curve in the presence of NA ($n = 9$) was shifted to the right by the addition of ANP ($n = 9$) ($P < 0.01$ by analysis of covariance), but was still located to the left of that observed in the absence of both NA and ANP ($n = 6$) ($P < 0.01$ by analysis of covariance).

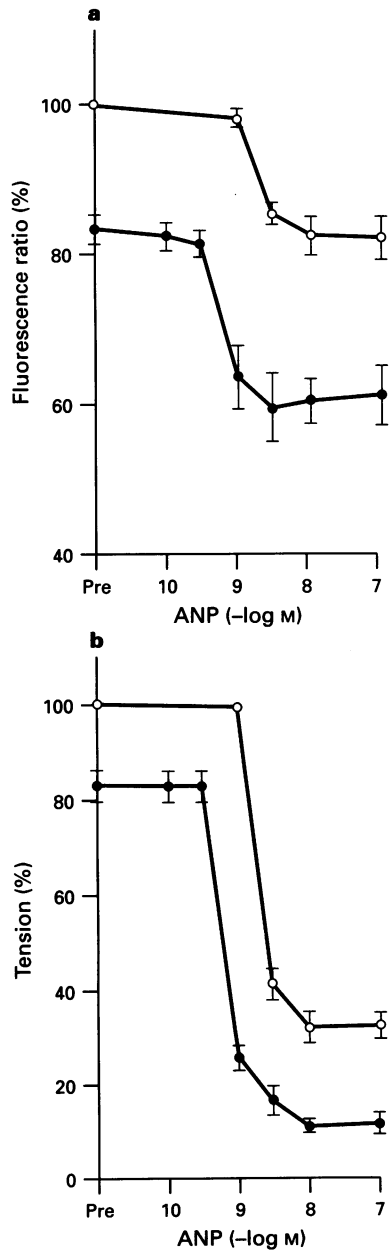

Figure 2 The effects of various concentrations of ANP on the increases in $[Ca^{2+}]_i$ (a) and tension (b) during contraction induced by 118 mM (○) and 40 mM (●) K^+ solutions. All measurements were performed as noted in Figure 1, while $[Ca^{2+}]_i$ and tension were determined at the steady-state levels after ANP application ($n = 5$). Pre: values just before the application of ANP.

The effects of ANP on the tension development in α-toxin-permeabilized preparations

To confirm further that ANP decreases the Ca^{2+} sensitivity of the contractile elements, we permeabilized the smooth muscle with α-toxin (Nishimura *et al.*, 1988). Figure 7 shows representative recordings of the effect of 10^{-7} M ANP on tension

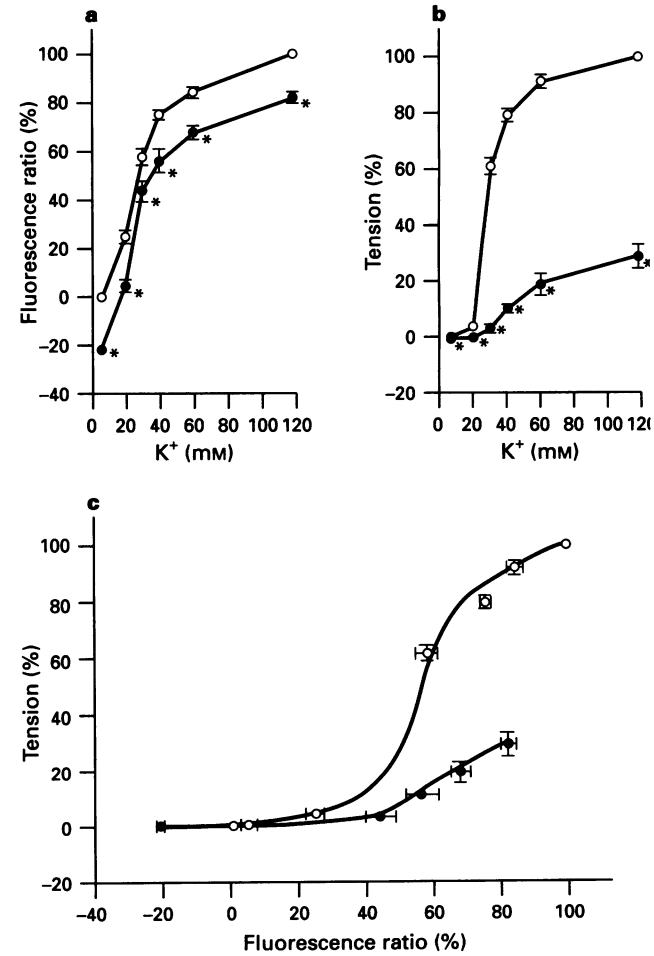

Figure 3 The effects of ANP on $[Ca^{2+}]_i$ (a) and tension (b) during contraction induced by various concentrations of high K^+ solution (5.9, 20, 30, 40, 60, 118 mM). At the steady state of contractions (○, $n = 8$), 10^{-8} M ANP was applied and measurements were performed at 10 min after the application of ANP (●, $n = 6$). In (c) the $[Ca^{2+}]_i$-tension relationships were plotted, using the data points as shown in (a) and (b). *Significantly different from control ($P < 0.05$).

developed by 3×10^{-7} M Ca^{2+}, 10^{-5} M GTP and 10^{-5} M NA in α-toxin-permeabilized porcine renal artery. The application of ANP during the steady-state contraction by the mixture of 3×10^{-7} M Ca^{2+}, 10^{-5} M GTP and 10^{-5} M NA induced relaxation although $[Ca^{2+}]_i$ was kept constant.

The effect of ANP on the intracellular Ca^{2+} release induced by NA in Ca^{2+}-free solution

Figure 8a and b show the effect of 10^{-8} M ANP on $[Ca^{2+}]_i$ and tension induced by 3×10^{-7} M NA in Ca^{2+}-free PSS containing 2 mM EGTA. When the vascular strips were exposed to Ca^{2+}-free PSS, $[Ca^{2+}]_i$ fell to reach a steady state ($-39.8 \pm 3.6\%$, $n=6$), while the tension remained almost unchanged. After a 15 min incubation in Ca^{2+}-free PSS, NA

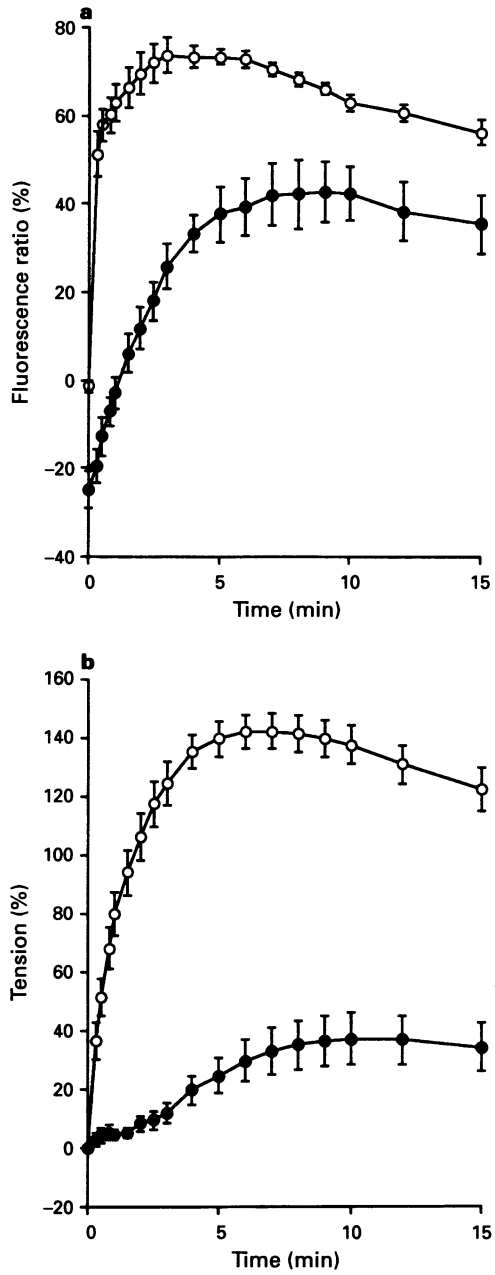

Figure 4 The effects of ANP on the time courses of changes in $[Ca^{2+}]_i$ (a) and tension (b) induced by NA. The strips were incubated either with (●) or without (○; control) 10^{-8} M ANP, 10 min before and during the application of 10^{-7} M NA. The data are expressed as the means ± s.e. mean ($n=6$). Abscissa scale: time after the application of NA.

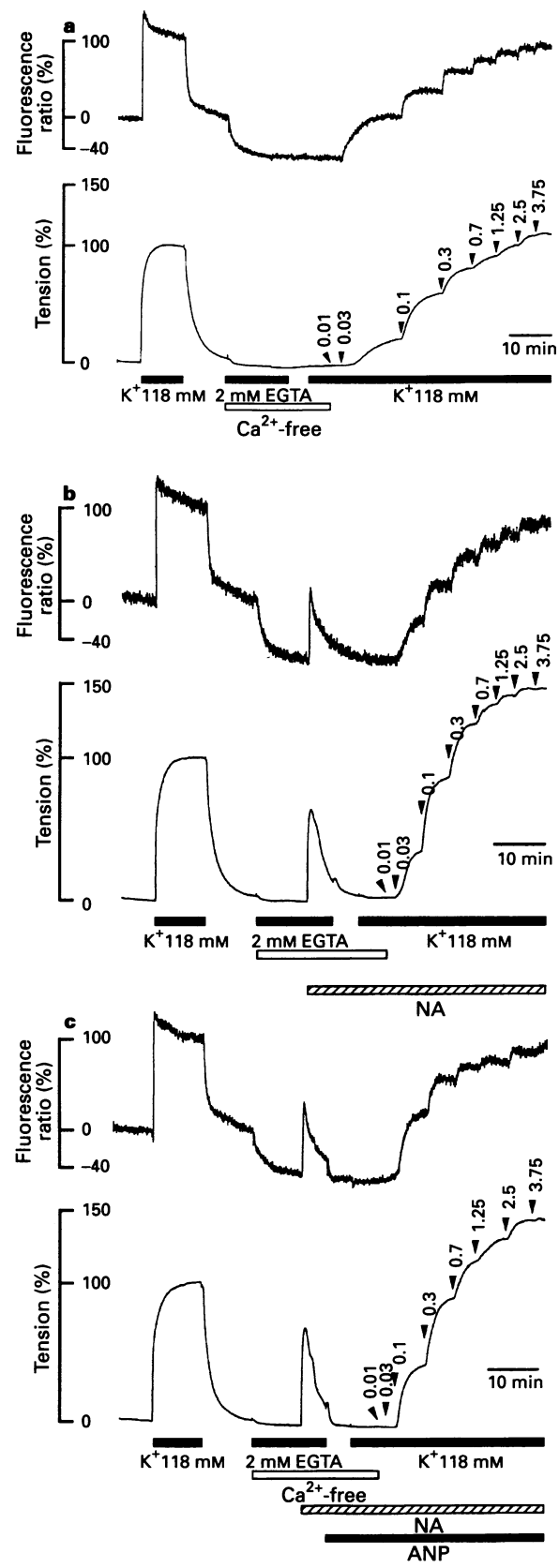

Figure 5 Representative recordings of the changes in $[Ca^{2+}]_i$ and tension induced by the cumulative application of external Ca^{2+} (0.01–3.75 mM) in Ca^{2+}-free, 118 mM K^+ solution (a). Similar recordings in Ca^{2+}-free, 118 mM K^+ solution containing 3×10^{-7} M noradrenaline in the absence (b) and presence (c) of 10^{-8} M ANP. In (c), ANP was applied 10 min before and during the cumulative application of extracellular Ca^{2+}. The numbers with arrow heads on the traces represent the final extracellular Ca^{2+} concentrations expressed in mM.

caused transient elevations of $[Ca^{2+}]_i$ and tension ($[Ca^{2+}]_i$, 30.0±3.5%; tension 70.2±7.0%, $n=6$). As shown in Figure 8, the application of 10^{-8} M ANP in Ca^{2+}-free PSS induced a further decrease in $[Ca^{2+}]_i$ (−50.7±2.9%, $n=6$, $P<0.05$ by Student's t test), but not in tension. Pretreatment with 10^{-8} M ANP in Ca^{2+}-free PSS strongly inhibited the elevations of $[Ca^{2+}]_i$ and contraction induced by NA ($[Ca^{2+}]_i$, −18.0±4.0%; tension 18.9±3.8%, $n=6$, $P<0.001$ by Student's t test).

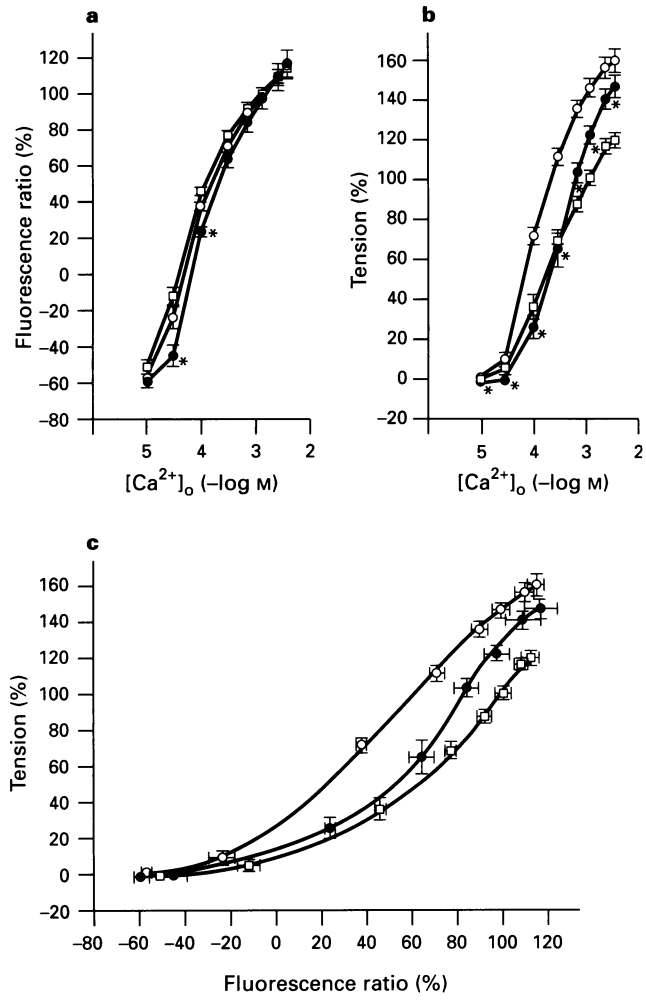

Figure 6 The effects of ANP on the elevations of $[Ca^{2+}]_i$ (a) and tension (b) induced by the cumulative application of external Ca^{2+} during high K^+ solution in the presence of 3×10^{-7} M NA. The data were obtained from the measurements using a protocol as shown in Figure 5 (□; in the absence of NA and ANP, ○; in the absence of ANP, ●; in the absence of ANP). In (c) the $[Ca^{2+}]_i$-tension curves were plotted using the data points shown in (a) and (b). The data are expressed as the means±s.e. mean ($n=9$).

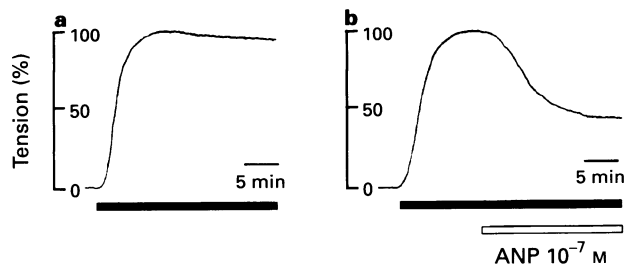

Figure 7 Representative recordings of the changes in tension induced by ANP of α-toxin-permeabilized porcine renal artery. The renal arterial strip was contracted (solid horizontal bars) with 3×10^{-7}M Ca^{2+}, 10^{-5}M GTP and 10^{-5}M NA (a, control; b, 10^{-7}M ANP was applied during the steady state of contraction).

The effect of ANP on the Ca^{2+} pumps on sarcoplasmic reticulum (SR) and sarcolemma

We next explored the mechanism for the ANP-induced reduction of $[Ca^{2+}]_i$. For this purpose, we used thapsigargin (TG), an inhibitor of the sarcoplasmic reticulum (SR) or endoplasmic reticulum (ER) Ca^{2+} ATPase (Thastrup *et al.*, 1989; 1990), to determine the relative contribution of SR to ANP-induced reduction of $[Ca^{2+}]_i$. As shown in Figure 9a, treatment of the strips with 10^{-5} M TG induced a slight sustained increase in $[Ca^{2+}]_i$ (20.2±2.6%; $n=8$) without any significant change in tension (0.95±0.35%, $n=8$). The application of 10^{-8} M ANP during the steady-state contraction induced by 118 mM K^+ solution, after treatment with TG, induced a monophasic reduction of $[Ca^{2+}]_i$ (Figure 9a, b).

We further investigated the mechanism by which ANP reduces $[Ca^{2+}]_i$, using Ba^{2+} which permeates the Ca^{2+} channels, and is a poor activator of the Ca^{2+} pumps of the sarcolemma (Yamaguchi *et al.*, 1989) and the SR (Schilling *et al.*, 1989). As shown in Figure 9c, the strip was first treated by TG and exposed to the 2 mM EGTA containing Ca^{2+}-free solution, followed by the 118 mM K^+ solution containing 1.25 mM Ba^{2+} instead of 1.25 mM Ca^{2+} (118 mM K^+, Ba^{2+} solution). The application of 118 mM K^+, Ba^{2+} solution increased $[Ba^{2+}]_i$ and tension ($[Ba^{2+}]_i$; 102.8±9.0%, $n=6$: tension 44.3±5.5%, $n=6$). The addition of 10^{-8} M ANP during the sustained contraction induced by the 118 mM K^+, Ba^{2+} solution produced relaxation but did not decrease $[Ba^{2+}]_i$ ($[Ba^{2+}]_i$; 101.3±8.5%, $n=6$: tension 4.9±3.6%, $n=6$).

Discussion

In the present study, we investigated the mechanism underlying the ANP-induced relaxation of the porcine renal artery. The major finding is that ANP induces a decrease in Ca^{2+} sensitivity of the contractile elements in vascular smooth muscle. In addition, we also demonstrated that ANP decreases

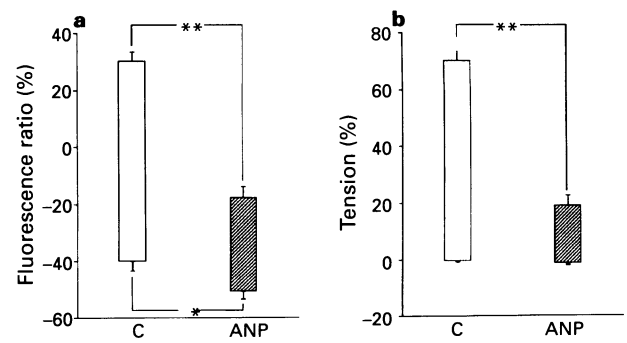

Figure 8 The effects of ANP on the elevations of $[Ca^{2+}]_i$ (a) and (b) induced by NA in Ca^{2+}-free PSS (2 mM EGTA). $[Ca^{2+}]_i$ and tension development induced by the application of 3×10^{-7}M NA in Ca^{2+}-free PSS, in the absence (open columns) and presence (hatched columns) of 10^{-8}M ANP; 3×10^{-7}M NA added 15 min after the application of Ca^{2+}-free PSS and ANP was applied for 10 min before and during the application of NA. The data were obtained from 6 independent experiments carried out with the same protocols. The bottom and top of each column indicate the levels of $[Ca^{2+}]_i$ and tension just before and at the peak obtained by the application of NA, respectively. Data are expressed as the means±s.e. mean ($n=6$). *$P<0.05$, **$P<0.001$.

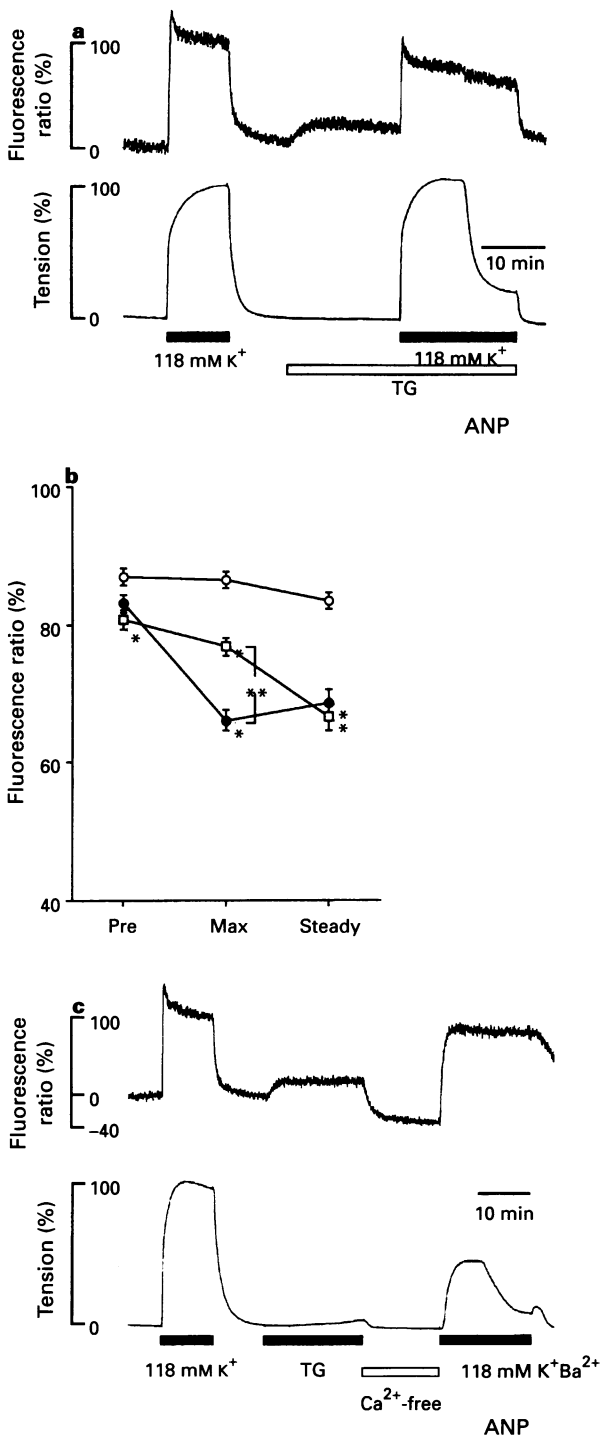

Figure 9 The effect of thapsigargin (TG) and Ba^{2+} on the 10^{-8} M ANP-induced decrease in $[Ca^{2+}]_i$ and tension during the contraction induced by 118 mM K^+ solution. ANP (10^{-8} M) was applied during the steady state of the contraction induced by the depolarization with 118 mM K^+ solution. (a) Representative recordings showing the effect of thapsigargin (TG) on the ANP-induced decreases in $[Ca^{2+}]_i$ and tension. ANP (10^{-8} M) was applied during the steady state of the contraction induced by the depolarization with 118 mM K^+ solution. The initial rapid decrease, but not the maintained decrease in $[Ca^{2+}]_i$, disappeared after treatment with TG. (b) A summary of the data obtained from the measurements as shown in (a). The ANP-induced changes in the fluorescence ratio in the time-matched controls (without TG and ANP treatment; ○), TG-non-treated (●) and TG-treated (□) tissues prior to the application of ANP were plotted at three different time points (Pre, just before ANP application; Max, the time point at which ANP induced maximum decrease in $[Ca^{2+}]_i$; Steady, steady state). The rapid, Max reduction of $[Ca^{2+}]_i$ was inhibited by TG treatment. $^*P<0.05$ vs control, $^{**}P<0.001$. (c) Representative recordings showing the effect of replacement of Ca^{2+} by Ba^{2+} on the ANP-induced decrease in $[Ca^{2+}]_i$. The strips were first treated with TG, followed by the exposure to the 2 mM EGTA containing Ca^{2+}-free solution. Then, 118 mM K^+ solution containing 1.25 mM Ba^{2+} (K^+, Ba^{2+}) was applied, which induced a sustained contraction. The addition of 10^{-8} M ANP during this sustained contraction did not decrease $[Ba^{2+}]_i$, but did decrease tension.

$[Ca^{2+}]_i$ during contraction induced by high external K^+ solution and NA, and that ANP inhibits NA-induced Ca^{2+} release from the intracellular store. These latter two mechanisms have already been described as major mechanisms for ANP-induced vasorelaxation (Takuwa & Rasmussen, 1987; Cornwell & Lincoln, 1988; Suematsu *et al.*, 1991). We thus confirmed that ANP promotes changes in Ca^{2+} mobilization from the intracellular store to decrease $[Ca^{2+}]_i$. However, this mechanism can only partially explain the ANP-induced decrease in $[Ca^{2+}]_i$. Therefore, we tried to explain further the mechanisms involved in the ANP-induced reduction of $[Ca^{2+}]_i$, using thapsigargin, an inhibitor for Ca^{2+} ATPase of the SR, and Ba^{2+}, which permeates the Ca^{2+} channels, but is a poor activator of the Ca^{2+} pump of the sarcolemma. These experiments indicated that the Ca^{2+} pumps located on the SR and sarcolemma were mainly responsible for the ANP-induced reduction of $[Ca^{2+}]_i$.

ANP-induced decrease in Ca^{2+} sensitivity of the contractile elements

The observations in the present study indicated that ANP relaxes the porcine renal artery, not only by reducing $[Ca^{2+}]_i$, but also by decreasing the Ca^{2+}-sensitivity of the contractile elements. As shown in Figures 1, 2 and 3, the extent of the reduction in tension induced by ANP was much greater than that expected from the decrease in $[Ca^{2+}]_i$ during contraction induced by the high external K^+ solution. Additional evidence for the ANP-induced decrease in Ca^{2+} sensitivity could also be seen in the experiment illustrated in Figure 9c, in which ANP markedly reduced tension without any change in $[Ba^{2+}]_i$.

It is well known that NA increases Ca^{2+} sensitivity of the contractile apparatus in smooth muscle (Nishimura *et al.*, 1988). Since ANP could relax the peremeabilized preparation during contraction induced by a fixed $[Ca^{2+}]_i$ in the presence of NA and GTP as shown in Figure 7b, and since the rightwards shift of the $[Ca^{2+}]_i$-tension curve was observed in the measurement described in Figure 6c, it is apparent that ANP has the potential to decrease the Ca^{2+} sensitivity of the contractile element even during activation by NA. In contrast to the present study, Suematsu *et al.* (1991) found that the Ca^{2+}-force curve of the contraction induced by high external K^+ solution was unaffected by ANP. This discrepancy could thus be explained by the differences in the efficacy of ANP to relax different types of arteries (the renal artery in the present study vs. the aorta in Suematsu's study). Indeed, there have been several reports which show that the efficacy of ANP in relaxing different types of arteries varies widely, and the renal artery is one of the most sensitive to ANP (Currie *et al.*, 1983; Garcia *et al.*, 1985; Ishihara *et al.*, 1985).

Although the mechanism for the ANP-induced decrease in Ca^{2+} sensitivity of the contractile elements is currently unknown, it is conceivable that it occurs through the action of cyclic GMP as a second messenger. Previous studies in vascular smooth muscle have shown elevations of cyclic GMP concentrations in response to ANP (Fiscus *et al.*, 1985: Rapoport *et al.*, 1985; Fujii *et al.*, 1986; Leitman *et al.*, 1988), and the reduction of Ca^{2+} sensitivity has thus been considered to be one of the characteristic modes of action of cyclic GMP and/or nitro-vasodilators (Morgan & Morgan, 1984; Karaki *et al.*, 1988; Yanagisawa *et al.*, 1989; Nishimura & van Breemen, 1989; Abe *et al.*, 1990).

ANP-induced changes in Ca^{2+} release from the intracellular store

We observed that ANP decreased $[Ca^{2+}]_i$, regardless of whether the strips were at rest (Figure 4) or activated by high external K^+ solution (Figures 1, 2 and 3) or NA (Figure 4). Thus, it is obvious that the reduction of $[Ca^{2+}]_i$ is one of the major mechanisms for the ANP-induced vasorelaxation. However, the mechanism underlying the ANP-induced decrease in $[Ca^{2+}]_i$ is still not fully understood. One possible mechanism might be inhibition of the pharmacomechanical coupling induced by agonists through inhibition of the hydrolysis of phosphatidyl inositol 4,5 bisphosphate (Fujii *et al.*, 1986; Rapoport, 1986; Meyer-Lehnert *et al.*, 1988; Winquist & Hintze, 1990). As shown in Figure 8, ANP inhibited the NA-induced transient increase in $[Ca^{2+}]_i$ and contraction in the absence of extracellular Ca^{2+}, indicating that ANP inhibits the Ca^{2+} release from the intracellular Ca^{2+} stores. Tamm *et al.* (1990) reported that ANP inhibited the increase in protein kinase C activity induced by angiotensin II. These observations are considered to be compatible with the idea that ANP inhibits the hydrolysis of phosphatidyl inositol 4,5 bisphosphate. This mechanism may thus also play a role in the ANP-induced relaxation of the contraction induced by NA. However, the ANP-induced reduction of $[Ca^{2+}]_i$ observed either during resting conditions or during high K^+ contractions cannot be explained by this mechanism, since the hydrolysis of phosphatidyl inositol 4,5 bisphosphate will not be involved under these conditions.

ANP-induced changes in Ca^{2+} pumps

Another possible mechanism for the ANP-induced reduction of $[Ca^{2+}]_i$ observed during resting conditions or during the high K^+ contractions might be the activation of the Ca^{2+} pumps, that is, the acceleration of Ca^{2+} uptake into SR and/or Ca^{2+} extrusion through sarcolemma, and/or the inhibition of Ca^{2+} influx through sarcolemma. In this study, we first examined whether ANP accelerates Ca^{2+} uptake into SR. As shown in Figure 9a, treating the tissue with thapsigargin, an inhibitor of the SR Ca^{2+} ATPase, inhibited the ANP-induced initial rapid decrease in $[Ca^{2+}]_i$. Thus, it appeared that the ANP-induced reduction of $[Ca^{2+}]_i$ involves the acceleration of Ca^{2+} uptake by SR Ca^{2+} pump. However, it is obvious that this mechanism alone will not explain the entire mechanism for the ANP-induced reduction of $[Ca^{2+}]_i$, since the ANP-induced sustained reduction of $[Ca^{2+}]_i$ was not affected by the treatment with TG.

The extrusion of Ca^{2+} from smooth muscle cells most likely involves both the ATP-dependent Ca^{2+}-pump of the sarcolemma and Na^+/Ca^{2+} exchanger (Somlyo, 1985). In order to test whether ANP accelerates the ATP-dependent Ca^{2+}-pump of the sarcolemma, we used thapsigargin together with Ba^{2+} which permeates Ca^{2+} channels, but is a poor activator of the Ca^{2+} pump of the sarcolemma (Yamaguchi *et al.*, 1989) and the SR (Schilling *et al.*, 1989). Since the ANP-induced reduction of $[Ba^{2+}]_i$ was completely abolished by treatment with TG and Ba^{2+} (Figure 9c), it was clear that ANP decreased $[Ca^{2+}]_i$ (or $[Ba^{2+}]_i$) via acceleration of the ATP-dependent Ca^{2+}-pump of the sarcolemma as well as of the SR. Consistent with these findings, Furukawa *et al.* (1988) reported that ANP stimulated sarcolemmal Ca^{2+}-pump activity via cyclic GMP generation. They (Furukawa *et al.*, 1991) also reported that ANP activated Na^+-dependent Ca^{2+} efflux. However, this mechanism may not exist in the porcine renal artery, since it has been reported that Ba^{2+} can be extruded by Na^+-Ca^{2+} exchange (Wagner-Mann *et al.*, 1992). If ANP accelerated Ca^{2+} extrusion via the activation of the Na^+-Ca^{2+} exchange mechanism, $[Ba^{2+}]_i$ would have been decreased by ANP in the protocol described in Figure 9c.

If ANP inhibited the voltage-dependent Ca^{2+} influx mechanism, $[Ba^{2+}]_i$ should have been decreased by ANP in the protocol described in Figure 9c, since Ba^{2+} can permeate voltage-dependent Ca^{2+} channels and can be extruded by Na^+-Ca^{2+} exchange. However, as shown in Figure 9c, no ANP-induced decrease in $[Ba^{2+}]_i$ was observed.

ANP therefore relaxes the porcine renal artery by: (1) reducing $[Ca^{2+}]_i$ via activation of Ca^{2+} pumps located on the SR and sarcolemma, and inhibiting the agonist-induced release of Ca^{2+} from the intracellular store; and (2) decreasing the Ca^{2+} sensitivity of the contractile elements in smooth muscle cells.

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