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Some effects of nipradilol, a β-antagonist possessing a nitroxy group, on smooth muscle of the pig coronary artery

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1 The effects of nipradilol, a β-adrenoceptor antagonist which possesses a nitroxy group, on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and on tension development were simultaneously measured by front-surface fluorometry and fura-2-loaded strips in the proximal portion of pig coronary arteries.

2 Nipradilol reduced in a concentration-dependent manner both the $[Ca^{2+}]_i$ and tension, irrespective of whether the strips were unstimulated or exposed to either high K^+ or histamine containing solutions. However, both in the case of contractions induced by high K^+-depolarization and histamine stimulation, for a given $[Ca^{2+}]_i$ elevation the tension which developed in the presence of nipradilol was smaller than that generated in its absence, so that the $[Ca^{2+}]_i$-tension curves during the contraction were shifted to the right.

3 In the absence of extracellular Ca^{2+}, the $[Ca^{2+}]_i$ elevation due to the release of Ca^{2+} from histamine-sensitive store was inhibited by nipradilol. Nipradilol had no effect on the $[Ca^{2+}]_i$ elevation due to the release of Ca^{2+} from caffeine-sensitive stores; however, it did inhibit the caffeine-induced increase in tension. A derivative of nipradilol, which lacked a nitroxy molecule (Nip(-N)), had no effect on the $[Ca^{2+}]_i$ and tension elevated by histamine or caffeine in the absence of extracellular Ca^{2+}.

4 The β-adrenoceptor agonist, isoprenaline, reduced $[Ca^{2+}]_i$ and tension when applied to steady state contractions induced by high K^+, or at the peak level of tension to histamine. The reduction of $[Ca^{2+}]_i$ and tension induced by isoprenaline was inhibited by Nip(-N) in a concentration-dependent manner and nipradilol inhibited the isoprenaline-induced relaxation with bell-shaped concentration-response curves. At lower concentrations, nipradilol acted as a β-blocker, the IC_{50} value being smaller than that of Nip(-N), and at higher concentrations, it acted as a nitrovasodilator.

5 Thus, it is suggested that, at lower concentrations, nipradilol, an antianginal drug, acts as a β-adrenoceptor antagonist. At higher concentrations, it relaxes the proximal portion of the coronary artery by directly reducing $[Ca^{2+}]_i$ and the Ca^{2+}-sensitivity of the myofilaments, apparently due to the presence of the nitroxy molecule.

Keywords: Fura-2; smooth muscle; nipradilol; β-adrenoceptor antagonists; nitrate

Introduction

Nipradilol (3, 4-dihydro-8 (2-hydroxy-3-isopropylamino)-propoxy-3-nitroxy-2*H*-1-benzopyran) is a relatively new agent with an antihypertensive and an antianginal action (Uchida *et al.*, 1983; Sakanashi *et al.*, 1984). It was synthesized with the aim of allowing the compound to possess both the actions of β-adrenoceptor antagonist and a nitrate (Uchida *et al.*, 1983). The direct relaxant action of nipradilol on vascular smooth muscle has been demonstrated in isolated arteries and vein (Uchida 1983; Kou & Suzuki, 1983; Sakanashi *et al.*, 1984). Since a nitroxy group is present within the nipradilol molecule, a portion of its dilator effect may share some characteristics with other nitroxy group-containing vasodilators (Uchida *et al.*, 1983; Kou & Suzuki, 1983; Sakanashi *et al.*, 1984). Nipradilol increases guanosine 3′:5′-cyclic monophosphate (cyclic GMP) production and relaxes smooth muscle (Sugiyama 1988; Imai *et al.*, 1990; Nakazawa *et al.*, 1992). All these effects can be attributed to the nitroxy group of nipradilol (Sugiyama *et al.*, 1988; Imai *et al.*, 1990; Nakazawa *et al.*, 1992).

In the present study, using front-surface fluorometry and fura-2 loaded medial strips of the proximal portion of the pig coronary artery, the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and tension development were monitored simultaneously in an attempt to determine the mechanisms of action of nipradilol. In addition, we also compared the action of nipradilol with that of Nip(-N), a denitrated derivative of nipradilol. Using similar techniques, we have already determined the mechanism of vasorelaxations induced by isoprenaline (Ushio-Fukai *et al.*, 1993) and by nitroglycerin (Abe *et al.*, 1990).

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Methods

Tissue preparation

The left circumflex coronary arteries were dissected from the hearts of pigs at slaughter and segments 2 to 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. The medial preparations thus obtained were cut into approximately 0.5 × 4 mm circular strips 0.1 mm thick.

Fura-2 loading

The strips were loaded with fura-2, in the form of acetoxymethyl ester (fura-2/AM), then were incubated in oxygenated (95% O_2 and 5% CO_2) Dulbecco's modified Eagle's medium containing 25 μM fura-2/AM dissolved in dimethyl sulphoxide and 0.25% foetal bovine serum for 3–4 h at 37°C. After loading with fura-2, the strips were rinsed with normal physiological salt solution (PSS) to remove the dye in the extra-

cellular space and to equilibrate the strips for at least 60 min at 37°C before starting the measurements. Fura-2 loading did not affect the resting tension nor did it produce any change in tension development or the time course. Thus, fura-2 loading produced no change inthe contractility of the vascular strips (Hirano *et al.*, 1990).

Measurement of tension development

The strips were mounted vertically in a quartz organ bath and were connected to a strain gauge (TB-612T, Nihon Koden, Japan). During 60 min fura-2 equilibration period after loading with fura-2, the strips were stimulated with 118 mM K^+-depolarization every 15 min, and the resting tension was adjusted to obtain an optimal length in 118 mM K^+ PSS. The mean ± s.e.mean values of resting tension were 261 ± 2 mg ($n = 69$). The steady state of the response of each strip to 118 mM K^+-depolarization was recorded before starting the experimental protocol. The developed tension was expressed as a percentage, by assigning the values in normal PSS (at rest: 5.9 mM K^+) and at steady state of the response to 118 mM K^+ PSS to be 0% and 100%, respectively.

Front-surface fluorometry

Changes in the fluorescence intensity of the fura-2-Ca^{2+} complex were monitored using a front-surface fluorometer specifically designed by us for fura-2 fluorometry (model CAM-OF-1) with the collaboration of Japan Spectroscopic Co. (Tokyo, Japan). The details of this method of fluorometry have been given elsewhere (Abe *et al.*, 1990; Hirano *et al.*, 1990; Ushio-Fukai *et al.*, 1993).

The ratio of the fluorescence intensities at 340 nm excitation to those at 380 nm excitation was monitored. Before starting each experimental protocol, the responsiveness of each strip to 118 mM K^+-depolarization was recorded, and the values at rest in normal (5.9 mM K^+) and at the steady state of the depolarization with 118 mM K^+ PSS were assigned to be 0% and 100%, respectively. The observations and the statistical analysis of the $[Ca^{2+}]_i$ levels were performed using the fluorescence ratio (%).

To estimate the absolute value of $[Ca^{2+}]_i$ for 0% and 100% of fluorescence ratios, separate measurements were made by the method described by Grynkiewicz *et al.* (1985). The mean values of 8 different measurements of $[Ca^{2+}]_i$ at rest (0%) and 118 mM K^+-depolarization (100%) were 99.2 ± 7.2 nM and 758.0 ± 50.7 nM, respectively. Since the absolute values of $[Ca^{2+}]_i$ were determined in separate measurements, and were not determined in any of experimental protocol, the estimated absolute $[Ca^{2+}]_i$ levels, calculated from the 0% and 100% levels of fluorescence ratios, are shown in the Figures only as the secondary axis associating with the axis of the fluorescence ratio.

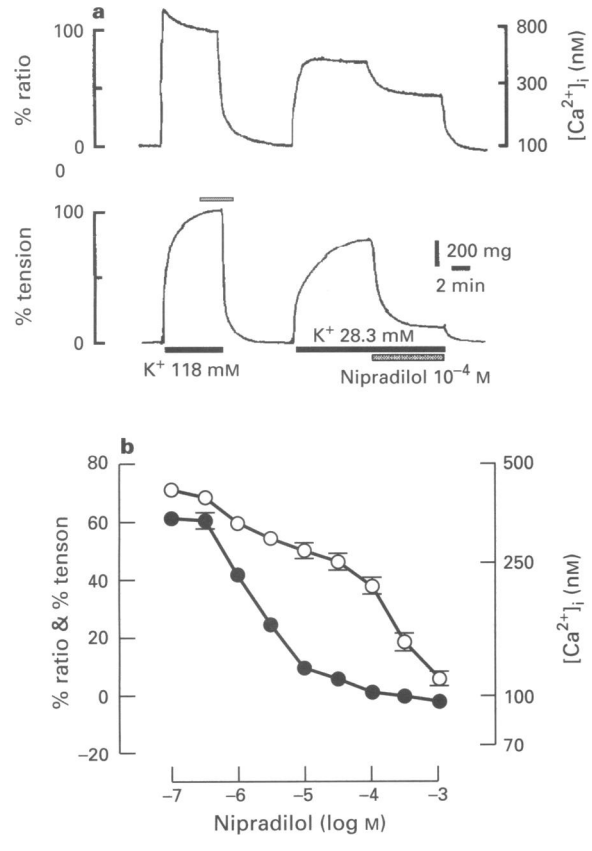

Figure 1 Effects of nipradilol on the increases in $[Ca^{2+}]_i$ and tension induced by depolarization with the high K^+-solution. (a) Representative recordings of the effects of 10^{-4} M nipradilol on the increases in $[Ca^{2+}]_i$ and tension induced by depolarization with the 28.3 mM K^+-solution. Nipradilol was applied when a steady state of tension had developed, induced by 28.3 mM K^+-depolarization. (b) Concentration-dependent effects of nipradilol on the increases in $[Ca^{2+}]_i$ (○) and tension (●) induced by 28.3 mM K^+-depolarization. Data are mean ± s.e.mean ($n = 5$). Nipradilol inhibited the increases in $[Ca^{2+}]_i$ and tension induced by 28.3 mM K^+-depolarization in a concentration-dependent manner.

Solutions and chemicals

The composition of 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; this mixture was bubbled with 95% O_2 and 5% CO_2, with a resulting pH of 7.4 at 37°C. A high external K^+ solution was prepared by replacing NaCl with KCl, isosmotically. The composition of the Ca^{2+}-free solution was the same as that in normal PSS, except that it contained 2 mM EGTA instead of 1.25 mM $CaCl_2$.

Fura-2/AM was purchased from DOJINDO (Kumamoto, Japan), the ionomycin was from Calbiochem (Frankfurt, Germany), histamine hydrochloride was from Wako (Osaka, Japan) and (-)-isoprenaline hydrochloride was from Nikken-Kagaku (Tokyo, Japan). Nipradilol and Nip(-N) were kindly donated by the Kowa Pharmaceutical Co. (Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± s.e.mean of n experiments. The statistical assessment of the data was made by Student's t test, a two-way ANOVA or analysis of covariance (ANOCOVA). P values < 0.05 were considered to be significant. The EC_{50} or IC_{50} values were determined by a four-parameter logistic equation of DeLean *et al.* (1978).

Results

Effects of nipradilol on the increases in $[Ca^{2+}]_i$ and tension induced by high K^+-depolarization

When the vascular strips were exposed to 28.3 mM external K^+ solution, $[Ca^{2+}]_i$ and the tension rapidly increased and reached steady-state levels within 10 min ($[Ca^{2+}]_i = 71.4 \pm 0.5\%$, tension $= 60.3 \pm 2.0\%$, $n = 7$, respectively) (Figure 1a). The application of 10^{-4} M nipradilol at the steady-state caused a rapid reduction of $[Ca^{2+}]_i$ and tension, which reached new steady levels ($[Ca^{2+}]_i = 40.3 \pm 3.0\%$, tension $= 1.5 \pm 0.4\%$, $n = 7$). As shown in Figure 1b, nipradilol induced a concentration-dependent reduction of $[Ca^{2+}]_i$ and tension. The IC_{50} values for $[Ca^{2+}]_i$ and tension were $(1.5 \pm 0.2) \times 10^{-4}$ M and $(1.9 \pm 0.1) \times 10^{-6}$ M, respectively.

Effects of nipradilol on the increases in $[Ca^{2+}]_i$ and tension induced by changes in the external Ca^{2+} during 118 mM K^+-depolarization

As shown in Figure 2a and c, in response to the stepwise increment of external Ca^{2+} (0.05 to 5.0 mM) during 118 mM K^+-depolarization, $[Ca^{2+}]_i$ and tension increased stepwise in a concentration-dependent manner. EC_{50} values of the external Ca^{2+} concentration for the increases in $[Ca^{2+}]_i$ and tension were 0.20 ± 0.03 mM and 0.15 ± 0.01 mM, respectively. As shown in Figure 2b and c, pretreatment with 5×10^{-5} M nipradilol significantly inhibited the increases in $[Ca^{2+}]_i$ and tension ($P < 0.001$ for both by two-way ANOVA, $n = 5$). EC_{50} values of the external Ca^{2+} concentration for the increases in $[Ca^{2+}]_i$ and tension were 0.42 ± 0.05 mM and 1.04 ± 0.15 mM ($n = 5$), respectively.

Effects of nipradilol on the increases in $[Ca^{2+}]_i$ and tension induced by histamine

When 10^{-5} M histamine was applied in normal PSS, $[Ca^{2+}]_i$ and tension rapidly increased (Figure 3a). The levels of $[Ca^{2+}]_i$ and tension at peak were $91.4 \pm 3.9\%$ and $111.3 \pm 4.7\%$ ($n = 5$), respectively. The application of 10^{-5} M nipradilol at the peak of the tension development caused a rapid reduction of $[Ca^{2+}]_i$ and tension, which reached new steady levels of $[Ca^{2+}]_i$ and tension ($[Ca^{2+}]_i = 5.3 \pm 1.6\%$, tension $= 2.0 \pm 0.7\%$, $n = 5$), respectively (Figure 3b). The extent of the reductions by nipradilol of the histamine-induced increases in $[Ca^{2+}]_i$ and tension was concentration-dependent. The IC_{50} values of nipradilol for $[Ca^{2+}]_i$ and tension were $(1.2 \pm 0.6) \times 10^{-7}$ M and $(5.0 \pm 1.6) \times 10^{-8}$ M ($n = 5$), respectively (Figure 3c). Additionally, Nip (-N) ($< 10^{-3}$ M) had no effect on the $[Ca^{2+}]_i$ and tension elevated by high K^+-depolarization or histamine in the presence of extracellular Ca^{2+} (data are not shown).

Effects of nipradilol on $[Ca^{2+}]_i$-tension relation

Figure 4a shows $[Ca^{2+}]_i$-tension curves for contractions induced by high K^+-depolarization and the incremental application of external Ca^{2+} during 118 K^+ PSS, in the absence and presence of nipradilol. Nipradilol shifted the $[Ca^{2+}]_i$-tension curves of the steady state of the high K^+-depolarization-induced contraction to the right (Figures 1 and 2; $P < 0.01$ by ANOCOVA). Figure 4b shows the $[Ca^{2+}]_i$-tension curves for histamine-induced contraction in the absence and presence of nipradilol. Nipradilol shifted the $[Ca^{2+}]_i$-tension curve of the peak level of the histamine induced contraction to the right ($P < 0.01$ by ANOCOVA).

Effects of nipradilol on the increases in $[Ca^{2+}]_i$ and tension induced by histamine or by caffeine in Ca^{2+}-free solution

When the vascular strips were exposed to Ca^{2+}-free solution, the level of $[Ca^{2+}]_i$ gradually declined to reach a steady state within 15 min (0% to $-15.1 \pm 1.9\%$). The application of 10^{-5} M histamine in the Ca^{2+}-free solution induced transient

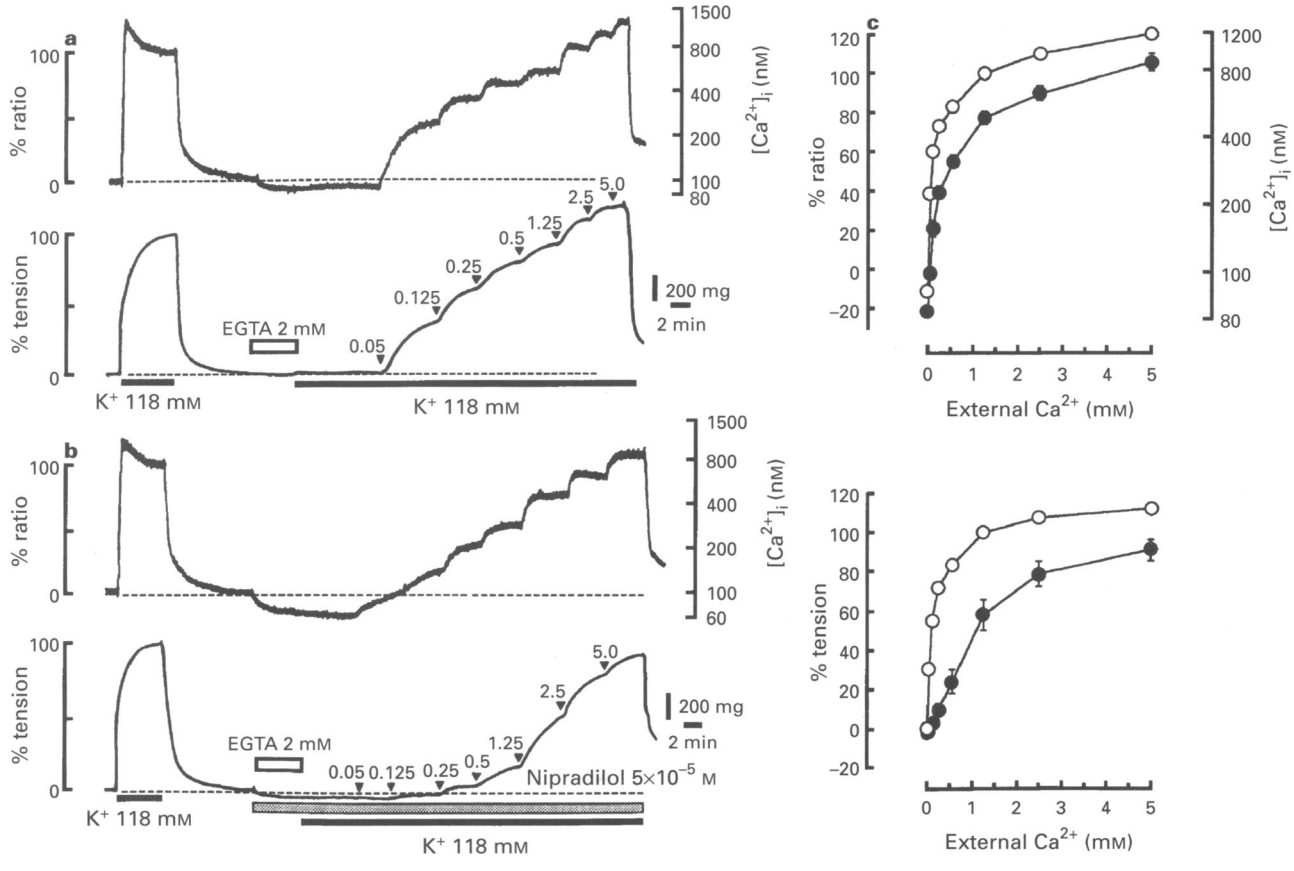

Figure 2 Effects of nipradilol on the increases in $[Ca^{2+}]_i$ and tension induced by the cumulative application of various concentrations of external Ca^{2+} (0.05 to 5 mM) during 118 mM K^+-depolarization. (a,b) Representative recordings of changes in $[Ca^{2+}]_i$ and tension induced by cumulative application of extracellular Ca^{2+} during 118 mM K^+-depolarization in the absence (a) and presence (b) of nipradilol. The numbers at each triangle (▼) indicate the concentration of extracellular Ca^{2+} (in mM). Nipradilol (5×10^{-5} M) was applied for 15 min before and during incremental application of external Ca^{2+}. The broken line indicates the 0% levels of $[Ca^{2+}]_i$ and tension. (c) Summary of the changes in $[Ca^{2+}]_i$ and tension obtained from 4 separate measurements, with (●) or without (○) treatment with 5×10^{-5} M nipradilol. Data are mean ± s.e.mean ($n = 4$). Pretreatment with nipradilol inhibited the increases in $[Ca^{2+}]_i$ and tension induced by the stepwise increment of external Ca^{2+} during 118 mM K^+-depolarization.

elevations of $[Ca^{2+}]_i$ and tension with a peak ($[Ca^{2+}]_i = 14.6 \pm 0.8\%$, tension $= 43.6 \pm 1.7\%$). When 10^{-5} M nipradilol was applied to unstimulated vascular strips in Ca^{2+}-free solution, the $[Ca^{2+}]_i$ and tension significantly decreased ($[Ca^{2+}]_i = -21.0 \pm 0.6\%$ and tension $= -2.6 \pm 0.4\%$; $P < 0.01$ for both by Student's t test) (Figure 5b and d). As shown in Figure 5b and d, pretreatment with 10^{-5} M nipradilol in the Ca^{2+}-free solution strongly inhibited the elevation of $[Ca^{2+}]_i$ ($-10.2 \pm 2.0\%$) and tension ($13.3 \pm 2.4\%$) induced by 10^{-5} M histamine ($P < 0.001$ for both by Student's t test, respectively). Nip(-N) had no effect on the levels of $[Ca^{2+}]_i$ and tension induced by histamine in Ca^{2+}-free solution (Figure 5c and d).

The application of 20 mM caffeine after 15 min incubation in the Ca^{2+}-free solution induced transient elevations of $[Ca^{2+}]_i$ and tension with a peak ($[Ca^{2+}]_i = 3.3 \pm 0.2\%$, tension $= 3.0 \pm 0.3\%$, respectively) (Figure 6a). As shown in Figure 6b and d, pretreatment with nipradilol (10^{-5} M) had no effect on the elevation of $[Ca^{2+}]_i$ ($3.4 \pm 0.4\%$) induced by caffeine. However, the tension development ($-2.7 \pm 0.8\%$) induced by caffeine was significantly inhibited by nipradilol ($P < 0.01$ by Student's t test).

Effects of nipradilol on $[Ca^{2+}]_i$ and tension in the presence of isoprenaline

When 2×10^{-7} M isoprenaline was applied during 28.3 mM K^+-depolarization, $[Ca^{2+}]_i$ and tension decreased rapidly from the plateau level ($[Ca^{2+}]_i = 71.4 \pm 0.9\%$, tension $= 60.0 \pm 1.5\%$, $n = 56$) to reach a new steady-state level ($[Ca^{2+}]_i = 58.4 \pm 1.1\%$, tension $= 14.3 \pm 0.9\%$, $n = 56$). As shown in Figure 7a, the subsequent application of 10^{-5} M Nip(-N) almost completely abolished the inhibiting effect of isoprenaline on $[Ca^{2+}]_i$ and tension ($[Ca^{2+}]_i = 64.3 \pm 2.5\%$, tension $= 56.2 \pm 5.2\%$). As shown in Figure 8a, the reversal effect of Nip(-N) on tension was concentration-dependent (10^{-8} M $<$ Nip(-N) $< 10^{-4}$ M, $IC_{50} = (7.6 \pm 1.5) \times 10^{-7}$ M), and the maximum reversal of the isoprenaline effect on tension was observed at 10^{-5} M Nip(-N). On the other hand, concentration-response curves of nipradilol on the inhibitory effect of isoprenaline were bell-shaped (Figure 8a). Nipradilol interfered with the isoprenaline-induced reduction of tension at lower concentrations ($\leqslant 3 \times 10^{-7}$ M, $IC_{50} = (8.1 \pm 6.8) \times 10^{-8}$ M), and the maximum reversal effect

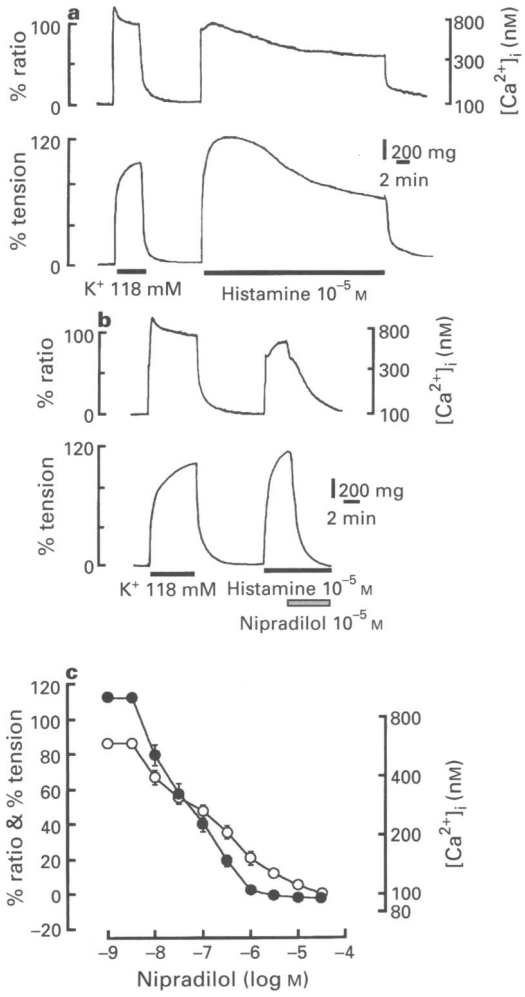

Figure 3 Effects of nipradilol on the increases in $[Ca^{2+}]_i$ and tension induced by histamine in the presence of extracellular Ca^{2+}. (a, b) Representative recordings of the changes in $[Ca^{2+}]_i$ and tension induced by 10^{-5}M histamine (a). Nipradilol (10^{-5}M) was applied when the histamine-induced tension reached peak levels (b). (c) Concentration-dependent effects of nipradilol on the increases in $[Ca^{2+}]_i$ (○) and tension (●) induced by 10^{-5}M histamine. Data are mean ± s.e.mean ($n = 5$). Nipradilol inhibited the increases in $[Ca^{2+}]_i$ and tension induced by 10^{-5} M histamine in a concentration-dependent manner.

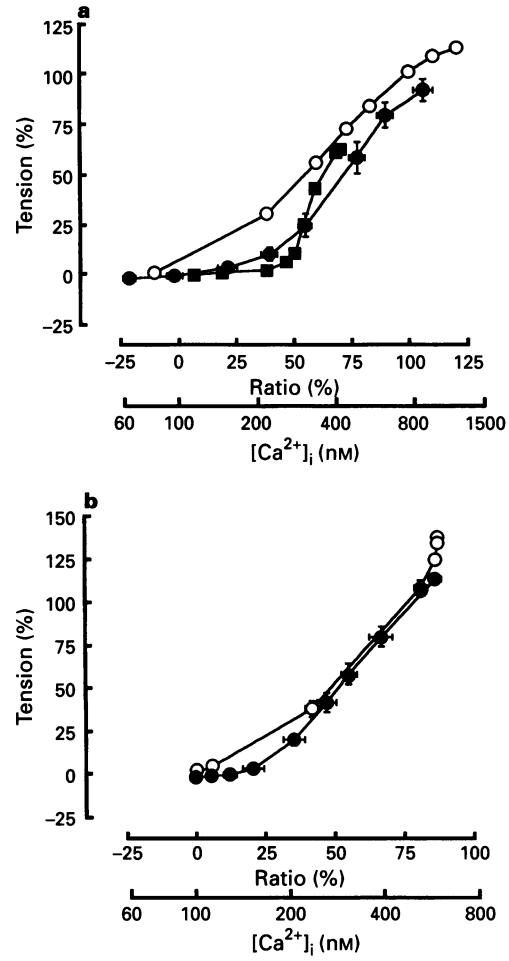

Figure 4 Effects of nipradilol on the $[Ca^{2+}]_i$-tension relation. (a) The $[Ca^{2+}]_i$-tension relation curve of high K^+-depolarization-induced contraction in the presence of nipradilol obtained from the data in Figures 1b (■) and 3c (●). The control (○) indicates the values obtained with contraction induced by the cumulative application of external Ca^{2+} during 118 mM K^+-depolarization in the absence of nipradilol (Figure 2c). Data are mean ± s.e.mean ($n = 4 \sim 7$). (b) The $[Ca^{2+}]_i$-tension relationship of histamine-induced contraction in the presence of nipradilol obtained from the data in Figure 3c (●). The control (○) $[Ca^{2+}]_i$-tension relationship was obtained by a stepwise application of histamine (10^{-7} to 10^{-4}M) without nipradilol. Data are mean ± s.e.mean ($n = 5$). Nipradilol shifted the $[Ca^{2+}]_i$-tension curves to the right in both high K^+-depolarization and histamine stimulation.

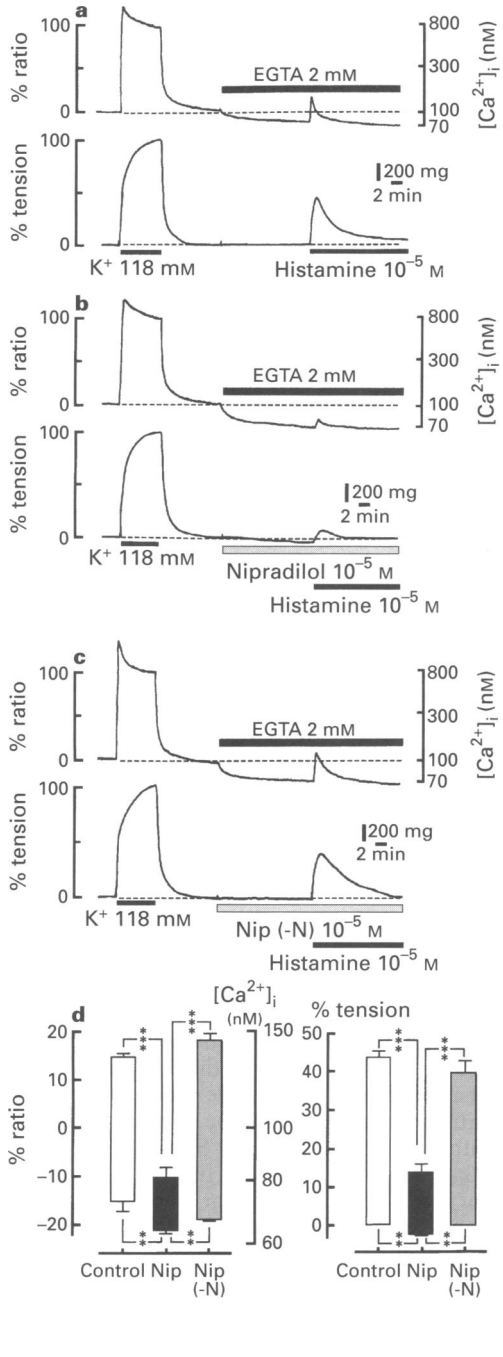

Figure 5 Effects of nipradilol and Nip(-N) on the increases in $[Ca^{2+}]_i$ and tension induced by histamine in a Ca^{2+}-free solution containing 2 mM EGTA. (a) Representative recordings of 10^{-5} M histamine-induced $[Ca^{2+}]_i$ and tension in Ca^{2+}-free solution (control). The broken lines indicate the 0% levels of $[Ca^{2+}]_i$ and tension. (b, c) Representative recordings of the effect of 10^{-5} M nipradilol (b) and 10^{-5} M Nip(-N) on the increases in $[Ca^{2+}]_i$ and tension induced by 10^{-5} M histamine in the absence of extracellular Ca^{2+}. Nipradilol or Nip(-N) was applied 15 min before as well as during the application of histamine. (d) Summary of the changes in $[Ca^{2+}]_i$ and tension obtained from 5 separate measurements. Control: in the absence of nipradilol or Nip(-N); Nip: 10^{-5} M nipradilol; Nip(-N): 10^{-5} M Nip(-N). The bottom and top of each column indicate the levels of $[Ca^{2+}]_i$ and tension just before and at the peak levels obtained by the application of histamine, respectively. Data are mean ± s.e.mean ($n=5$). $^{**}P<0.01$, $^{***}P<0.001$. In the absence of extracellular Ca^{2+}, pretreatment with nipradilol inhibited the elevation of $[Ca^{2+}]_i$ and tension induced by histamine, while Nip(-N) had no effect on the levels of $[Ca^{2+}]_i$ and tension induced by histamine.

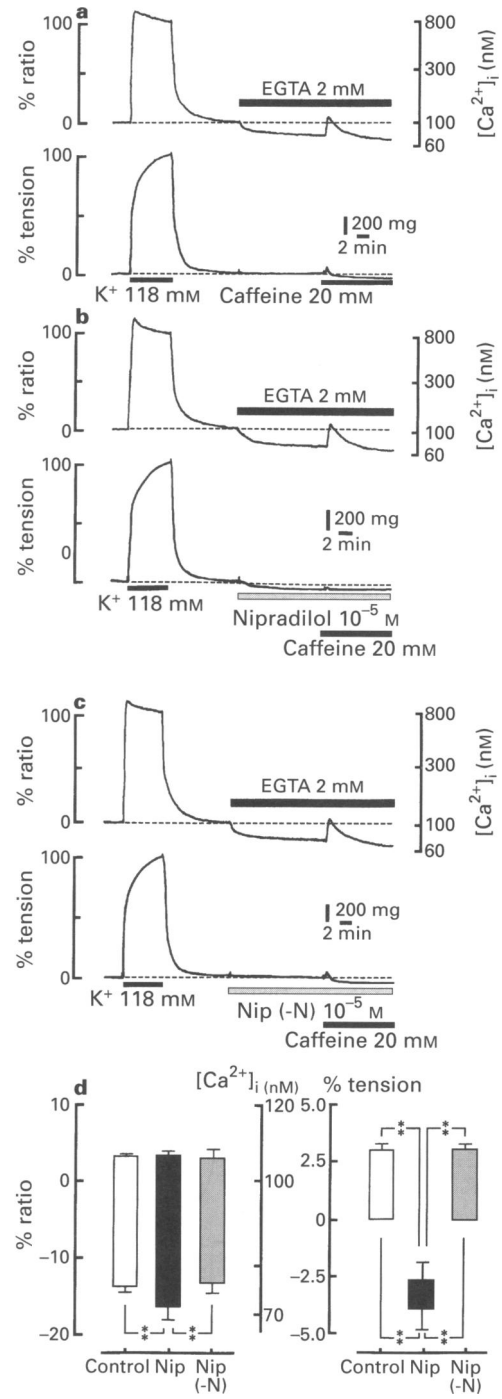

Figure 6 Effects of nipradilol and Nip(-N) on the increases in $[Ca^{2+}]_i$ and tension induced by caffeine in a Ca^{2+}-free solution containing 2 mM EGTA. (a) Representative recordings of the changes in $[Ca^{2+}]_i$ and tension induced by 20 mM caffeine in Ca^{2+}-free solution (control). The broken lines indicate the 0% levels of $[Ca^{2+}]_i$ and tension. (b, c) Representative recordings of the effect of 10^{-5} M nipradilol (b) and 10^{-5} M Nip(-N) (c) on the changes in $[Ca^{2+}]_i$ and tension induced by 20 mM caffeine in the absence of extracellular Ca^{2+}. Nipradilol or Nip(-N) was applied 15 min before as well as during the application of 20 mM caffeine. (d) A summary of the changes in $[Ca^{2+}]_i$ and tension obtained from 5 separate measurements. Control: in the absence of nipradilol or Nip(-N); Nip: 10^{-5} M nipradilol; Nip(-N): 10^{-5} M Nip(-N). The bottom and top of each column indicate the levels of $[Ca^{2+}]_i$ and tension just before and at the peak levels obtained by the application of 20 mM caffeine, respectively. Data are mean ± s.e.mean ($n=5$). $^{**}P<0.01$. In the absence of extracellular Ca^{2+}, nipradilol had no effect on the $[Ca^{2+}]_i$ elevation induced by caffeine, while it did inhibit the caffeine-induced increase in tension.

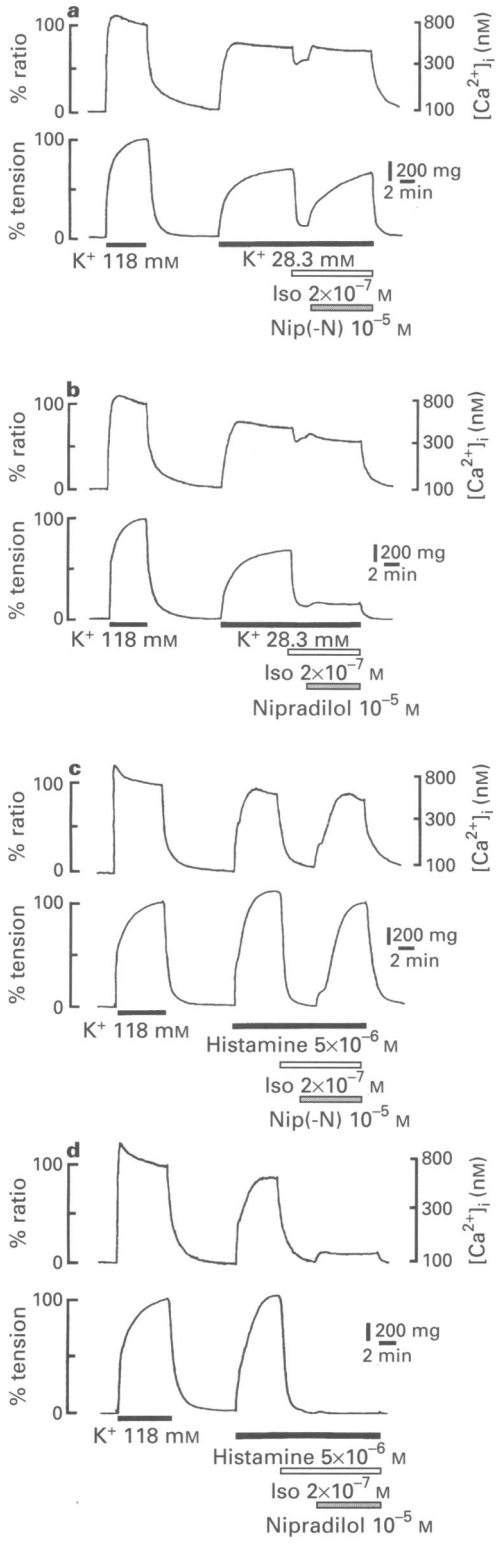

Figure 7 Effects of Nip(-N) and nipradilol on the reduction of $[Ca^{2+}]_i$ and tension development induced by isoprenaline during the contraction induced by high K^+-depolarization and histamine stimulation. (a, b) Representative recordings of effect of 10^{-5} M Nip(-N) (a) and 10^{-5} M nipradilol (b) on the reduction of $[Ca^{2+}]_i$ and tension induced by 2×10^{-7} M isoprenaline during the contractions induced by 28.3 mM K^+-depolarization. Isoprenaline (Iso) was applied at a steady state of contraction induced by K^+-depolarization. Subsequently, nipradilol or Nip(-N) was applied when $[Ca^{2+}]_i$ and tension were reduced to reach a steady state by isoprenaline. (c, d) Representative recordings of the effect of 10^{-5} M Nip(-N) (c) and 10^{-5} M nipradilol (d) on the reduction of $[Ca^{2+}]_i$ and tension induced by 2×10^{-7} M isoprenaline during contraction induced by 5×10^{-6} M histamine. Isoprenaline was applied at the peak level of tension induced by histamine. Subsequently, nipradilol or Nip(-N) was applied when $[Ca^{2+}]_i$ and tension were reduced to reach a steady state by isoprenaline. The concentration of isoprenaline applied (2×10^{-7} M) was one which induced a submaximal inhibitory effect on the increases in $[Ca^{2+}]_i$ and tension induced by 28.3 nM K^+-depolarization or 5×10^{-6} M histamine stimulation. At a high concentration (10^{-5} M), nipradilol had almost no inhibitory effect on the isoprenaline-induced relaxation during 28.3 mM K^+-depolarization or 5×10^{-6} M histamine stimulation.

of nipradilol on tension against isoprenaline was observed at 3×10^{-7} M nipradilol ($44.5 \pm 5.3\%$, $n = 4$). However, at higher concentrations ($> 10^{-6}$ M), the inhibitory effects on the reduction of tension are decreased in a concentration-dependent manner. As shown in Figures 7b and 8a, 10^{-5} M nipradilol only transiently and slightly, if at all, inhibited the reduction of $[Ca^{2+}]_i$ and tension induced by isoprenaline during 28.3 mM K^+-depolarization.

When isoprenaline was applied during 5×10^{-6} M histamine stimulation, both $[Ca^{2+}]_i$ and tension rapidly decreased from peak levels ($[Ca^{2+}]_i = 89.5 \pm 1.0\%$, tension $= 108.0 \pm 0.09\%$, $n = 45$) to reach a new steady-state level ($[Ca^{2+}]_i = 4.0 \pm 0.8\%$, tension $= -0.6 \pm 0.2\%$, $n = 45$). As shown in Figure 7c, the subsequent application of 10^{-5} M Nip(-N) almost completely abolished the inhibiting effect of isoprenline on $[Ca^{2+}]_i$ and tension ($[Ca^{2+}]_i = 67.0 \pm 4.0\%$, tension $= 65.8 \pm 8.6\%$, $n = 5$). As shown in Figure 8b, the reversal effect of Nip(-N) on $[Ca^{2+}]_i$ and tension against isoprenaline was concentration-dependent (10^{-8} M < Nip(-N) < 10^{-4} M, IC_{50} for $[Ca^{2+}]_i = (7.6 \pm 6.3) \times 10^{-7}$ M and tension $= (9.0 \pm 1.0) \times 10^{-7}$ M), and the maximum reversion of isoprenaline effect was observed at 3×10^{-6} M Nip(-N) ($[Ca^{2+}]_i = 75.4 \pm 3.1\%$, tension $= 74.9 \pm 2.8\%$, $n = 5$) (Figure 8b). On the other hand, nipradilol showed bell shaped-concentration-effect on the isoprenaline-induced reduction of $[Ca^{2+}]_i$ and tension. At lower concentrations ($\leq 3 \times 10^{-7}$ M), nipradilol inhibited the reduction of $[Ca^{2+}]_i$ and tension in a concentration-dependent manner (IC_{50} for $[Ca^{2+}]_i = (6.2 \pm 5.8) \times 10^{-8}$ M and tension $= (2.5 \pm 1.3) \times 10^{-8}$ M, Figure 8b), and the maximum reversal of the isoprenaline effect was observed at 3×10^{-7} M nipradilol ($[Ca^{2+}]_i = 69.1 \pm 4.1\%$, tension $= 58.1 \pm 6.0\%$, $n = 4$). However, at higher concentrations ($> 3 \times 10^{-7}$ M), the inhibitory effect on the isoprenaline-induced reduction of $[Ca^{2+}]_i$ and tension is decreased in a concentration-dependent manner. As shown in Figures 7d and 8b, 10^{-5} M nipradilol only transiently and slightly, inhibited the reduction of $[Ca^{2+}]_i$ and tension induced by isoprenaline during 5×10^{-6} M histamine stimulation.

Discussion

In the present study, we investigated the cellular mechanisms by which nipradilol induces vasorelaxation in the proximal portion of the coronary artery of the pig. Previous studies have shown that nipradilol is a unique β-adrenoceptor antagonist with a nitroglycerin-like action because it possesses a nitroxy group within its molecule (Koh & Suzuki, 1983; Uchida *et al.*, 1983; Sakanashi *et al.*, 1984). It has been demonstrated that nipradilol directly relaxes vascular smooth muscle in isolated arteries and veins (Koh & Suzuki, 1983; Uchida *et al.*, 1983; Sakanashi *et al.*, 1984). Therefore, in the present study, we have determined how nipradilol modifies the elevation of $[Ca^{2+}]_i$ induced by K^+-depolarization and histamine stimulation and Ca^{2+} sensitivity of the contractile element, and what will happen when nipradilol is applied during relaxation induced by a β-agonist, i.e. isoprenaline, in the proximal portion of the coronary artery of the pig.

Nip(-N), which lacks a nitroxy molecule, had no effect on the increases in $[Ca^{2+}]_i$ and tension induced by high K^+-depolarization or histamine stimulation, in the presence (data are not shown) or absence of extracellular Ca^{2+}. Therefore, it is apparent that the nitroxy molecule of nipradilol plays an important role in the relaxation of the vascular smooth muscle

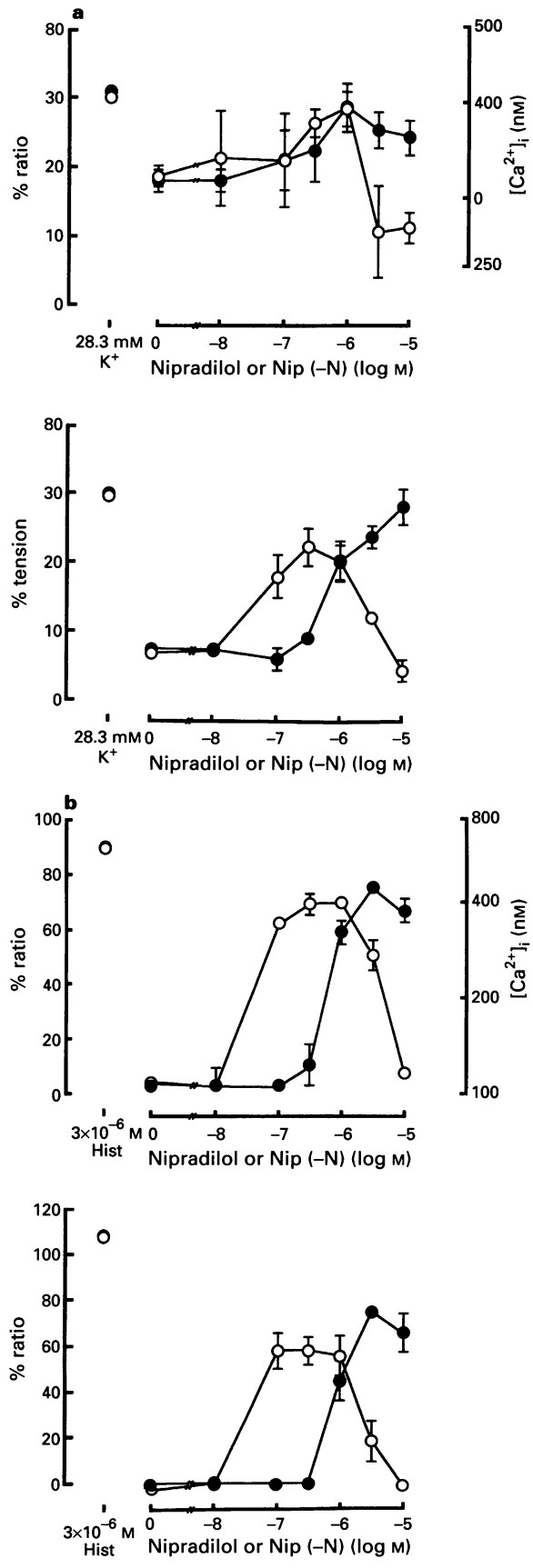

Figure 8 Concentration-dependent effect of nipradilol or Nip(-N) on the decreases of $[Ca^{2+}]_i$ and tension induced by 2×10^{-7} M isoprenaline during the contraction with 28.3 mM K^+-depolarization (a) and 5×10^{-6} M histamine (b). (a, b) (○) Nipradilol, (●) Nip(-N); data are mean ± s.e. ($n = 4-6$). Data were obtained with an experimental protocol similar to Figure 7. Labels on the abscissa scale: 28.3 mM K^+: steady state of the contraction induced by 28.3 mM K^+-depolarization; 5×10^{-6} M Hist: a peak of the tension induced by 5×10^{-6} M histamine; 0: steady state of the relaxation induced by 2×10^{-7} M isoprenaline. Nipradilol inhibited isoprenaline-induced relaxation during high K^+-depolarization or histamine stimulation with a bell-shaped concentration-response curve.

cells. We have reported that nitroglycerin inhibits the extracellular Ca^{2+}-dependent elevation of $[Ca^{2+}]_i$, Ca^{2+} influx, under stimulation with either high K^+-depolarization or histamine. In addition, nitroglycerin induced much greater reduction of tension than that expected from the reduction of $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$-tension relation curve shifted to the right in the pig isolated coronary artery (Abe *et al.*, 1990). Yanagisawa *et al.* (1989) reported that nitroglycerin relaxed canine coronary arterial smooth muscles without reducing $[Ca^{2+}]_i$ in contractions induced by very high external K^+-depolarization. Sodium nitroprusside, another nitro-vasodilator, was found to be more effective in relaxing vascular smooth muscle than in decreasing Ca^{2+} (Morgan & Morgan, 1984; Karaki *et al.*, 1988). The results of the present study in which nipradilol shifted the $[Ca^{2+}]_i$-tension relation curve to the right is compatible with previous studies on nitro vasodilators. Thus, it is likely that nipradilol has a nitroglycerin-like action on the Ca^{2+}-sensitivity of the contractile apparatus in vascular smooth muscle. It has been reported that nipradilol increased intracellular cyclic GMP levels during relaxation (Sugiyama *et al.*, 1988; Imai *et al.*, 1990; Nakazawa *et al.*, 1992), and this relaxing effect was attenuated by methylene blue (Imai *et al.*, 1990; Nakazawa *et al.*, 1992), an inhibitor of soluble guanylate cyclase (Gruetter *et al.*, 1981). It has been reported that the activation of cyclic GMP-dependent protein kinase may be responsible for the vasodilator effect of nitrate (Waldman & Murad, 1987), and evidence has been presented that cyclic GMP-dependent protein kinase phosphorylates myosin light chain kinase (Rapoport *et al.*, 1982; 1983; Pfitzer *et al.*, 1983). Myosin light chain kinase phosphorylates myosin and leads to smooth muscle contraction. However, the phosphorylation of the myosin light chain kinase by cyclic GMP or cyclic GMP-dependent protein kinase diminishes the phosphorylation of myosin light chain, which inhibits contraction (Rapoport *et al.*, 1982; 1983; Pfitzer *et al.*, 1983). Consistent with this idea, Nishimura & van Breemen (1989) reported that cyclic GMP and sodium nitroprusside induced a rightward shift in the pCa-tension curve in an α-toxin permeabilized smooth muscle preparation. Thus, evidence is accumulating that nitro vasodilators do decrease the Ca^{2+} sensitivity of the contractile apparatus, mediated through a second messenger, which could possibly be cyclic GMP and cyclic GMP-dependent protein kinase activation.

Histamine induces elevation of $[Ca^{2+}]_i$ due to both Ca^{2+} influx from extracellular space and Ca^{2+} release from intracellular store site; but K^+-depolarization induces elevation due to Ca^{2+} influx from the extracellular space (Abe *et al.*, 1990; Hirano *et al.*, 1991). In the present study, nipradilol inhibited extracellular Ca^{2+}-dependent elevation of $[Ca^{2+}]_i$ caused by both K^+-depolarization and histamine. In addition to the inhibition of Ca^{2+} influx, nipradilol inhibited the transient elevation of $[Ca^{2+}]_i$ and tension induced by histamine in the absence of extracellular Ca^{2+}. As we have already reported, nitroglycerin depleted Ca^{2+} from the histamine-sensitive store (Abe *et al.*, 1990) and noradrenaline-sensitive store (Kai *et al.*, 1990; Abe *et al.*, 1994) and inhibited the release of Ca^{2+} from these stores in the absence of extracellular Ca^{2+}. Nipradilol appeared to deplete the stored Ca^{2+} and also to inhibit Ca^{2+} release from the histamine-sensitive store. In smooth muscle, receptor-mediated Ca^{2+} release from the store can play a major role in the excitation-contraction coupling (Somlyo, 1985). It is mediated by activation of the phospholipase C-inositol trisphosphate (IP_3) cascade, coupled to receptors by G-proteins. Inhibitors of phospholipase C (neomycin) and IP_3 (heparin), block the release of intracellular Ca^{2+} induced by agonist, but not by caffeine (Kobayashi *et al.*,

1988; 1989; Yamamoto *et al.*, 1990). In the present study, nipradilol had no effect on the release of Ca^{2+} from the caffeine-sensitive Ca^{2+} stores. However, nipradilol inhibited the tension development induced by caffeine. This resulted in a shift of the $[Ca^{2+}]_i$-tension relation curves to the right, which may be due to a nipradilol-induced reduction of $[Ca^{2+}]_i$ sensitivity of the contractile apparatus in vascular smooth muscle. Thus, the observation that nipradilol inhibits the histamine-induced but not the caffeine-induced release of intracellular Ca^{2+} store site suggests that nipradilol also inhibits intracellular Ca^{2+} release through the inhibition of the receptor-coupled signal transduction pathway. It was reported that nitroglycerin enhanced the Ca^{2+} uptake into and/or the accumulation of Ca^{2+} in the store. However, it is unlikely that nipradilol enhances the uptake of Ca^{2+} in the store, because nipradilol apparently decreased Ca^{2+} in the histamine-sensitive store.

In the present study, we have found that nipradilol inhibits the extracellular Ca^{2+}-dependent elevation of $[Ca^{2+}]_i$. Kou & Suzuki (1983) showed that both nipradilol and nitroglycerin induced vasorelaxation without any electrophysiological changes of the sarcolemmal membrane in canine coronary artery. It is reported that nitroglycerin-induced relaxation is to some extent related to an inhibition of Ca^{2+} influx, but is mainly due to increased extrusion of Ca^{2+} (Ito & Kuriyama, 1983). It has also been reported that nitroglycerin and/or cyclic GMP-mediated systems stimulate sarcolemmal Ca^{2+} ATPase resulting in pump activation and an enhanced Ca^{2+} extrusion (Vrolix *et al.*, 1988; Popescu *et al.*, 1985a,b). Thus, it remains to be elucidated how nipradilol- and/or the nitroglycerin-cyclic GMP-mediated mechanism controls the influx and the efflux of Ca^{2+} through the sarcolemmal membrane in relaxation of the vascular smooth muscle.

We have previously reported that the proximal portion of the porcine coronary artery is β-adrenoceptor dominant (Nishimura *et al.*, 1987), and activation of these receptors by isoprenaline results in a reduction of cytosolic calcium concentrations (Shogakiuchi *et al.*, 1991; Ushio-Fukai *et al.*, 1993). Isoprenaline relaxes almost all varieties of smooth muscle by increases in the intracellular cyclic AMP levels due to the activation of adenylate cyclase (Bülbring & Tomita, 1987). Isoprenaline relaxes pig coronary arteries precontracted by high K^+-depolarization and histamine by reducing $[Ca^{2+}]_i$ and also by directly controlling Ca^{2+}-sensitivity of the contractile elements through a second messenger, possibly cyclic AMP (Ushio-Fukai *et al.*, 1993). In the present study, isoprenaline decreased the elevation of $[Ca^{2+}]_i$ and tension induced by K^+-depolarization and histamine stimulation. Nipradilol inhibited isoprenaline-induced relaxation during high K^+-depolarization or histamine stimulation with a bell-shaped concentration-response curve. IC_{50} value as a β-blocker for nipradilol (around 10^{-7} M) was much lower than that of Nip(-N) (around 10^{-6} M). At a higher concentration ($\geqslant 10^{-6}$ M), nipradilol, but not Nip(-N), induced relaxation in a concentration-dependent manner. The present study indicates that the effects of Nip(-N) on vascular smooth muscle cells is only as a β-adrenoceptor antagonist, which is consistent with the report by Kou & Suzuki (1983). Thus, it was revealed that nipradilol had a β-blocking activity with an IC_{50} value smaller than that of Nip(-N) at lower concentrations and a nitrovasdilator-related activity at higher concentrations. However, it has been reported that the beneficial effect of nipradilol is mainly attributable to its β-antagonist action on the canine myocardium because of the similarity in the action of Nip(-N) and propranolol (Noguchi & Sakanashi, 1987; Hiro *et al.*, 1989). In the heart, stimulation of β-adrenoceptors leads to positive inotropic and chronotropic response, while it leads to relaxation in vascular smooth muscle. These findings may, in part, contribute to the differences between the effects of nipradilol on the myocardium and vascular smooth muscle. As has been suggested by Borg *et al.* (1991), the prevalence of one of the two mechanisms of action or the coexistence of both may depend on which vascular bed is investigated.

In conclusion, nipradilol, a β-blocker possessing a nitroxy group, relaxes the proximal portion of the pig coronary artery both by directly reducing $[Ca^{2+}]_i$ and by reducing the Ca^{2+} sensitivity of the contractile apparatus in the vascular smooth muscle through a second messenger. The mechanism of reduction of $[Ca^{2+}]_i$ involves the inhibition of the receptor-mediated release of stored Ca^{2+} and Ca^{2+} influx. Furthermore, it was revealed that, at lower concentrations, nipradilol has a β-blocking activity, its IC_{50} value against isoprenaline being smaller than that of Nip(-N), and a nitrovasodilator-related activity at higher concentrations. The reason why the IC_{50} value of nipradilol as a β-blocker is smaller than that of Nip(-N), a derivative of nipradilol lacking nitroxy molecule, has not been elucidated in the present study.

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