Characterization in rat aorta of the binding sites responsible for blockade of noradrenaline-evoked calcium entry by nisoldipine

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- 1 The effectiveness of the calcium antagonist, 1,4-dihydropyridine nisoldipine, as an inhibitor of contraction and ⁴⁵Ca entry evoked by noradrenaline in rat aorta has been investigated and correlated with binding characteristics in intact artery.
- 2 Contractions evoked by noradrenaline were concentration-dependently depressed by nisoldipine (0.3–300 nM). About 60% of the response was resistant to inhibition, while KCl-induced contractions could be completely blocked. Noradrenaline-induced contractions were also less sensitive to nisoldipine inhibition than were KCl-induced contractions.
- 3 Preincubation of the aorta with nisoldipine in high KCl depolarizing solution increased the inhibition of the contraction evoked by a short application of noradrenaline or KCl to a similar extent.
- 4 The inhibition by nisoldipine of ⁴⁵Ca influx evoked either by KCl depolarizing solution or by noradrenaline correlated well with the inhibition of the contractile responses. However, while KCl-stimulated ⁴⁵Ca influx was totally abolished by nisoldipine (300 nM), 38% of the noradrenaline-stimulated ⁴⁵Ca influx was resistant to inhibition by nisoldipine (300 nM).
- 5 The study of [³H]-(+)-PN 200-100 ([³H]-(+)-isradipine) binding in intact aorta showed the presence of a homogeneous population of specific binding sites. *K_D* values were dependent on the KCl concentration in the bath while *B_{max}* was unaffected. Binding of [³H]-(+)-isradipine was also increased in tissue exposed to noradrenaline; in the presence of 10⁻⁵ M noradrenaline, binding parameters of [³H]-(+)-isradipine were close to the values obtained in aorta bathed in 20 mM KCl solution.
- 6 Displacement of [³H]-(+)-isradipine specific binding by nisoldipine was determined in segments of mesenteric artery and of aorta. The potency of nisoldipine was dependent on the incubation conditions applied to the vessel, as follows: KCl (100 mM) depolarizing solution > noradrenaline (10⁻⁵ M) = KCl (25 mM) solution > physiological solution. The *K_i* value measured in aorta exposed to noradrenaline (10⁻⁵ M) was close to the *IC₅₀* value of nisoldipine on the noradrenaline-evoked contraction.
- 7 The membrane potential value of rat aorta was estimated by the distribution of [³H]-tetraphenylphosphonium bromide ([³H]-TPP⁺). [³H]-TPP⁺ uptake concentration-dependently decreased when the KCl concentration in the bath was increased from 5.9 to 130 mM. Noradrenaline also concentration-dependently decreased [³H]-TPP⁺ uptake; the maximum effect (1–10 μM noradrenaline) was comparable in amplitude to the effect of 25 mM KCl solution.
- 8 It is concluded that in rat aorta, noradrenaline activates voltage-operated calcium channels that contain the specific, voltage-sensitive binding sites for calcium antagonistic dihydropyridines. The existence of a fraction of noradrenaline-stimulated ⁴⁵Ca entry that is resistant to nisoldipine blockade suggests that another Ca²⁺ entry pathway is also opened by the agonist.

Introduction

In rat aorta, stimulation of α₁-adrenoceptors by noradrenaline increases cytosolic free Ca²⁺ and produces contraction (Godfraind, 1976; Cauvin & Malik, 1984; Minneman, 1988). It has been shown that the early fast component of the contraction evoked by a high concentration of noradrenaline persisted in the absence of extracellular calcium, implying that noradrenaline releases Ca²⁺ from intracellular stores. It is now well established that activation of α₁-adrenoceptors causes the hydrolysis of inositol phospholipids, through the coupling of the adrenoceptor with phospholipase C. Inositol 1,4,5-trisphosphate released into the cytosol then induces the release of Ca²⁺ from internal stores, which is responsible for the initial phase of the contraction (Minneman, 1988). The other part of the contraction, which constitutes the tonic, sustained component of the contraction, is dependent on the presence of Ca²⁺ in the bathing solution (Godfraind & Kaba, 1969). Noradrenaline has indeed been reported to enhance ⁴⁵Ca²⁺ influx (Godfraind, 1976). This observation indicates that the stimulation of α₁-receptors opens channels in the membrane, allowing Ca²⁺ to enter the cell. Dihydropyridine calcium antagonists are known to prevent the increased Ca²⁺

entry produced by stimulation in vascular smooth muscle, without affecting resting Ca²⁺ fluxes or intracellular Ca²⁺ release (Godfraind *et al.*, 1986). However, in rat aorta as in most vascular smooth muscles, there are important differences in the potency of calcium antagonists against Ca²⁺ entry stimulated by receptors or by high K⁺ depolarization, implying that separate Ca²⁺ entry pathways are called into play by the two types of stimulation (for review see Spedding & Cavero, 1984; Cauvin & van Breemen, 1985). This observation led to the hypothesis of the existence of Ca²⁺ channels gated by receptor activation (ROCs) and of Ca²⁺ channels gated by potential (POCs) (Bolton, 1979). If the existence of POCs is well documented, there are few reports demonstrating the existence of ROCs (see Rink, 1988), and the events coupling α₁-adrenoceptor stimulation and Ca²⁺ entry have not been elucidated. There is some evidence that, at least in some tissues, noradrenaline evokes a depolarization, although results are conflicting as far as the degree of depolarization and the relation between depolarization and tension generation are concerned (see Bolton & Large, 1986; Haeusler & De Peyer, 1989). On the other hand, Nelson *et al.* (1988) recently reported that at a concentration that did not depolarize, noradrenaline shifted the relationship between *V_m* and contraction to more negative potentials, so that noradrenaline contracts arteries by interacting with POCs, rather than by

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opening ROCs, leaving the question of the existence of ROCs open. The aim of the present work was to investigate the mode of interaction of the calcium antagonistic dihydropyridines nisoldipine and (+)-PN 200-110 ((+)-isradipine), with the putative ROCs and POCs in rat aorta. A comparative study of binding behaviour, inhibitory action on $^{45}\text{Ca}^{2+}$ influx and on contraction induced by noradrenaline and KCl-depolarization, allowed us to characterize the existence of voltage-dependent binding sites for dihydropyridine calcium antagonists and to show that noradrenaline-stimulated Ca^{2+} entry in rat aorta is partly carried out through POCs. The lower sensitivity to calcium antagonists of the action of noradrenaline compared to responses induced by KCl-depolarization may be accounted for by the sensitivity of dihydropyridines binding site to membrane potential.

Some of these results have been described previously (Godfraind *et al.*, 1989; Morel & Godfraind, 1990).

Methods

Wistar rats (weighing 250–300 g) were used. They were killed by decapitation, the thoracic aorta and the superior mesenteric artery were removed and immersed in physiological solution (mm: NaCl 122, KCl 5.9, NaHCO_3 15, MgCl_2 1.25, CaCl_2 1.25 and glucose 11) maintained at 37°C and aerated with a gas mixture of 95% O_2 , 5% CO_2 . Tissues were cleaned of adherent connective and fat tissue and cut in rings. When necessary endothelium was stripped off by mechanical rubbing.

Measurement of contractile responses

Aortic rings, about 2 mm wide, were suspended in 50 ml organ baths filled with physiological solution under a resting tension of 2 g. Rings were connected to an isometric level with two strain gauges as part of a balanced bridge. The output was fed into an analog/digital converter, which was connected to a microcomputer. The programme allowed input from up to 8 force transducers simultaneously. After suitable calibration, tension was displayed on the screen of the microcomputer, recorded on a printer, and stored on disk at predetermined time intervals. After mounting in organ chambers, rings of arteries were allowed to equilibrate for 90 min in physiological solution. Contraction was evoked either by changing the physiological solution in the bath to a K^+ -rich depolarizing solution containing (mm): NaCl 22, KCl 100, NaHCO_3 15, MgCl_2 1.25, CaCl_2 1.25 and glucose 11, or by addition of noradrenaline to the bath. The first stimulation was used to check the sensitivity of the preparation to the relaxing effect of acetylcholine (1 μM). After wash out for 30 min in physiological solution, a control stimulation was recorded for 35 min. After wash out and relaxation, the vessels were incubated for 90 min in physiological solution with or without various concentrations of nisoldipine. A third contraction was then induced in the continuous presence of the antagonist. Results obtained in the presence of the antagonist were compared to controls that were treated simultaneously as described above but without incubation with the antagonist.

Measurement of ^{45}Ca influx

Calcium influx was estimated by measuring changes in the specific activity of the calcium fraction resistant to displacement by lanthanum (Godfraind, 1976). Noradrenaline-dependent ^{45}Ca influx was estimated after an equilibration period of 60 min in physiological solution maintained at 37°C and aerated with a gas mixture of 95% O_2 and 5% CO_2 . After preincubation of 90 min in the same solution with or without nisoldipine, the artery segments were incubated for 5 min in physiological solution containing $^{45}\text{Ca}^{2+}$ and the antagonist and then for a further 2 min in the same solution with noradrenaline 10^{-5} M. A similar procedure was followed

with the $^{45}\text{Ca}^{2+}$ -K-depolarizing solution, except that prazosin (10^{-8} M) was added with nisoldipine (Godfraind, 1983). Thereafter the preparations were washed for 5 min in 500 ml of La^{3+} solution (mm: NaCl 122, KCl 5.9, MgCl_2 1.25, LaCl_3 50, glucose 11 and Tris-maleate buffer 15; pH 6.8) to remove extracellular Ca^{2+} . Arterial segments were then dried between two sheets of filter paper, weighed, dissolved in 0.1 ml of a mixture of perchloric acid: H_2O_2 (1:1). The radioactivity of the samples was counted in a liquid scintillation counter as usual with appropriate controls.

Measurement of [^3H]-(+)-isradipine binding

Binding studies were performed following the same protocol as described for the contraction experiments. Rings of mesenteric arteries or of aorta (1–2 mg wet weight) were incubated for 90 min with [^3H]-(+)-isradipine (New England Nuclear, 76 Ci mmol $^{-1}$) in physiological solution or for 60 min in physiological solution followed by 30 min in depolarizing solution containing different concentrations of KCl, maintained at 37°C and aerated with a mixture of 95% O_2 :5% CO_2 . When the effect of noradrenaline was tested, noradrenaline was added to the physiological solution during the last 30 min of incubation. [^3H]-(+)-isradipine concentration ranged from 40 to 100 pM. At the end of the incubation time, each ring was dried on filter paper, weighed and dissolved in 0.1 ml of a mixture of perchloric acid: H_2O_2 (1:1). Radioactivity of the tissue was counted by liquid scintillation with an efficiency of 42%. Non-specific binding was determined in the presence of 10^{-6} M nifedipine, and subtracted from the total binding to give the specific binding.

Membrane potential determination

The membrane permeant lipophilic cation [^3H]-tetraphenylphosphonium ([^3H]-TPP $^+$, Amersham, 26 Ci mmol $^{-1}$) was used to estimate the membrane potential in rat aortic segments. This compound has been shown to accumulate intracellularly as a function of membrane potential (Lichtstein *et al.*, 1979). Aortic rings were incubated for 60 min with [^3H]-TPP $^+$ (10^{-5} M). At the end of the incubation time, each ring was dried on filter paper, weighed and dissolved in 0.1 ml of a mixture of perchloric acid: H_2O_2 (1:1). Radioactivity of the tissue was counted by liquid scintillation.

[^3H]-TPP $^+$ uptake due to the electrical potential across the membrane was estimated by subtracting the uptake measured in high KCl-solution (mm: KCl 130, NaCl 0, other constituents as in normal physiological solution), which was assumed to abolish the membrane potential. Intracellular concentration of TPP $^+$ ($[\text{TPP}^+]_{\text{in}}$) was calculated as shown in the following equation:

$$[\text{TPP}^+]_{\text{in}} = (\text{TPP}^+_{\text{total}} - \text{TPP}^+_{130 \text{ mM K}^+})/V_i$$

where $\text{TPP}^+_{\text{total}}$ is the total uptake of TPP $^+$ in physiological buffer, $\text{TPP}^+_{130 \text{ mM K}^+}$ is the total uptake in a solution containing 130 mM KCl, and V_i the intracellular volume (Kamp & Miller, 1987). Extracellular volume was determined by the diffusion space of [^{14}C]-inulin and was 375 ml kg $^{-1}$ wet weight. Intracellular volume, obtained by subtracting the extracellular volume from the total tissular volume (calculated to be equal to 910 ml kg $^{-1}$ on the basis of a density of 1.1), was 535 ml kg $^{-1}$. This intracellular volume is larger than the intracellular water volume and includes the volume of soluble and non-soluble material. It partially takes into account the dissolution of [^3H]-TPP $^+$ inside internal membranes. [^3H]-TPP $^+$ also accumulates inside intracellular organelles such as mitochondria that have a negative membrane potential. The uptake of [^3H]-TPP $^+$ in mitochondria is proportional to the cytoplasmic concentration of [^3H]-TPP $^+$, and will be higher in low external KCl concentration than in high external KCl concentration. To abolish the participation of mitochondrial uptake in the total uptake of [^3H]-TPP $^+$, uptake of [^3H]-TPP $^+$ was measured in physiological solution and in 130 mM

KCl-solution in the presence of carbonyl cyanide *m*-chlorophenylhydrazone ($0.3 \mu\text{M}$), an uncoupler of phosphorylation in mitochondria which has been reported to abolish the mitochondrial membrane potential without affecting the cytoplasmic membrane potential of intact synaptosomes (Scott & Nicholls, 1980).

Average membrane potential of the preparations was calculated from Nernst's equation:

$$E_{\text{TPP}^+} = RT/F \ln [\text{TPP}^+]_{\text{out}}/[\text{TPP}^+]_{\text{in}}$$

where $[\text{TPP}^+]_{\text{out}}$ is the concentration of TPP^+ in the medium and $[\text{TPP}^+]_{\text{in}}$ is the intracellular TPP^+ concentration measured in the presence of the mitochondrial uncoupler.

Drugs

(+)-PN 200-110 (isradipine; isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate) (Sandoz), nisoldipine and nifedipine (Bayer) were dissolved in ethanol as stock solutions at 10 mM and further diluted in water as required prior to use. $[\text{H}^3]$ -(+)-isradipine was obtained from New England Nuclear (specific activity 76 Ci mmol^{-1}); $[\text{H}^3]$ -tetraphenylphosphonium ($[\text{H}^3]$ - TPP^+) was obtained from Amersham (specific activity 26 Ci mmol^{-1}). All experiments with dihydropyridine were done under illumination of a sodium lamp.

Statistical analysis

Results of the experiments are expressed as means $\pm$ s.e.mean. Drug concentration producing 50% inhibition of the responses (IC_{50}) and their confidence limits ($p = 95\%$) were estimated by linear regression from log concentration-effect curves. In binding experiments, binding parameters were obtained by linear regression of Scatchard plot of the data. K_i values were obtained by the method of Cheng & Prusoff (1973). Tests of significance have been made using Student's *t* test, *P* values smaller than 0.05 being considered significant.

Results

Nisoldipine effect on contractile responses of rat aorta to noradrenaline and KCl

The presence of a functional endothelium is known to influence the contractile response of rat aorta to agonists (Eglème *et al.*, 1984). For this reason, the sensitivity to nisoldipine of the contractile response to noradrenaline was investigated in intact aorta and after removal of endothelial cells. The contraction produced by the application of noradrenaline ($10 \mu\text{M}$) was significantly higher in aortic rings without endothelium compared to intact aorta ($3376 \pm 100 \text{ mg}$ and $2717 \pm 109 \text{ mg}$ ($n = 16$) respectively, $P < 0.01$), in agreement with previous observations (Godfraind *et al.*, 1985). The presence of endothelium also affected the pattern of the response: contractions were more sustained after endothelium removal while tension tended to decrease slowly with time in intact preparations. Nisoldipine (30 pM – 300 nM) produced a concentration-dependent depression of the contraction evoked by 10^{-5} M noradrenaline in rat aorta with intact endothelium as well as after endothelium removal. An important part of the contraction was resistant to nisoldipine. In the presence of the endothelium, the nisoldipine-resistant fraction, measured in the presence of 100 nM nisoldipine, was equal to $60 \pm 2.8\%$ of the control contraction ($n = 14$). It was significantly higher after endothelium removal ($71 \pm 2\%$, $n = 14$). However, the presence of endothelium did not influence the IC_{50} value of nisoldipine, as shown in Figure 1a. In aortic rings with or without endothelium, nisoldipine potency increased with the duration of exposure of the tissue to noradrenaline, as indicated by a leftward shift of the dose-effect curves measured after 35 min exposure to noradrenaline; IC_{50} values were 570 pM and

Figure 1 Effect of nisoldipine on the contraction of aortic rings exposed to noradrenaline (10^{-5} M) (a) or to 100 mM KCl depolarizing solution (b). Aortic rings with intact endothelium (closed symbols) or without endothelium (open symbols) were preincubated for 90 min in the presence of different concentrations of nisoldipine. Responses were measured 2 min ($\square$, $\blacksquare$) or 35 min ($\triangle$, $\blacktriangle$) after commencement of the contraction and are expressed as a percentage of the contraction measured in the absence of nisoldipine. Nisoldipine-sensitive contraction of each aortic ring was obtained after subtraction of the nisoldipine-resistant contraction, measured in the same ring in the presence of 100 nM nisoldipine. Data are means from 4–9 determinations; vertical bars show s.e.mean.

170 pM in intact tissue and 645 pM and 181 pM in rubbed preparations, after 2 and 35 min exposure to noradrenaline, respectively (Table 1). As the endothelium did not appear to influence the IC_{50} value of nisoldipine as an inhibitor of noradrenaline-induced contractions, further experiments were performed in intact aortic rings.

Contractions evoked by the 100 mM KCl-depolarizing solution are not affected by the presence of the endothelium (Godfraind, 1986). Their sensitivity to nisoldipine was investigated in intact preparations. The contractile response to 100 mM KCl was concentration-dependently inhibited by nisoldipine (10 pM – 10 nM); 10 nM nisoldipine completely abolished the response (Figure 1b). Contractions measured in the presence of nisoldipine were not sustained; the degree of inhibition markedly increased with the duration of depolarization, as already reported by Wibó *et al.* (1988). IC_{50} values measured 2 and 35 min after initiation of contraction were 374 and 49 pM , respectively (Table 1). As shown in Table 1, the potency of nisoldipine was significantly lower on noradrenaline-induced contraction than on KCl-induced contraction at 35 min, while the difference was not significant at 2 min.

In another series of experiments, the inhibition of the contractile response to a short test pulse of KCl or of noradrenaline was tested after incubation of aortic rings in the presence of a fixed concentration of nisoldipine (100 pM) for 60 min in

Table 1 Effect of nisoldipine on contraction, $^{45}\text{Ca}^{2+}$ influx (IC_{50}) and $[^3\text{H}]$ -(+)-isradipine binding (K_i) in aorta and mesenteric artery exposed to 100 mM KCl depolarizing solution or to noradrenaline (NA, 10 μM)

			Contraction IC ₅₀ (pM)	⁴⁵ Ca ²⁺ influx IC ₅₀ (pM)	K _i (pM)
<i>Aorta</i>					
Endothelium present	KCl	2 min	374 (332–421)	743 (560–986)	67 (57–78)
		35 min	49 (44–56)	42 (32–55)	
	NA	2 min	570 (488–821)	617 (464–821)	160 (131–196)
		35 min	170 (124–233)		
Endothelium removed	NA	2 min	645 (566–735)		263 (204–339)
		35 min	181 (149–220)		
<i>Mesenteric artery</i>					
	Physiol solution				
	KCl	2 min	287 (244–237)		58 (45–75)
35 min		49 (45–53)			

IC_{50} values and their 95% confidence limits (in parentheses) in the aorta were calculated from the data shown in Figure 1. K_i values in aortic segments were obtained by the method of Cheng & Prusoff (1973) from IC_{50} values calculated from data shown in Figure 8.

normal physiological solution followed by 30 min either in normal physiological (KCl, 5.9 mM) solution or in 40 mM KCl-depolarizing solution. The duration of the 100 mM KCl test pulse was short (1 min) to ensure that the effect of this high concentration of KCl could be neglected. Ca^{2+} was omitted from all bathing solutions during the last 30 min of incubation with nisoldipine to avoid the development of tension in the presence of high KCl concentration. Figure 2 shows that the effectiveness of nisoldipine as an inhibitor of the contraction induced by KCl 100 mM solution or by exposure to noradrenaline (10 μM) was similarly enhanced after preincubation of the tissue in 40 mM KCl depolarizing solution (run 2). A third run was then performed to test the reversibility of the increase of inhibition induced by depolarization. In this experiment, after the second run, arteries were further incubated in the presence of nisoldipine (100 pM) in normal, Ca-free physiological solution. Determination of the contrac-

tile responses to noradrenaline (10 μM) and to KCl (100 mM) clearly indicated that the increased inhibition observed after preincubation in high KCl solution was reversed when arteries were further equilibrated in normal physiological solution. The nisoldipine-resistant fraction of the tension evoked by noradrenaline, measured in the presence of nisoldipine 100 nM, was not significantly modified by the preincubation conditions (not shown).

Nisoldipine effect on $^{45}\text{Ca}^{2+}$ influx stimulated by noradrenaline and KCl in rat aorta

Exposure of aortic segments to noradrenaline (10 μM) for 2 min increased the $^{45}\text{Ca}^{2+}$ influx in the lanthanum-resistant compartment by $77.7 \pm 3.08 \mu\text{mol Ca}^{2+} \text{ kg}^{-1}$ wet weight ($n = 15$). Noradrenaline-stimulated $^{45}\text{Ca}^{2+}$ influx was only partly blocked by nisoldipine; 38% of the noradrenaline stimulated $^{45}\text{Ca}^{2+}$ influx was unaffected by nisoldipine ($29.8 \pm 3.2 \mu\text{mol Ca}^{2+} \text{ kg}^{-1}$) (Figure 3a). Figure 3b shows the concentration-effect curve of the inhibition by nisoldipine (30 pM–300 nM) of the $^{45}\text{Ca}^{2+}$ influx stimulated by 2 min exposure to 10^{-5} M noradrenaline. The IC_{50} value for nisoldipine of the nisoldipine-sensitive part of the influx correlated well with the inhibition of the contraction, measured at the same time (Table 1).

Figure 3 also illustrates the effect of nisoldipine on the $^{45}\text{Ca}^{2+}$ uptake stimulated by 100 mM KCl. K^{+} -stimulated $^{45}\text{Ca}^{2+}$ influx was dose-dependently depressed by nisoldipine (30 pM–100 nM) and completely abolished by 100 nM of the antagonist. The degree of inhibition of the KCl-stimulated $^{45}\text{Ca}^{2+}$ influx was enhanced when the muscles were preincubated in the presence of nisoldipine in the depolarizing solution for 33 min before exposure to the KCl (100 mM)-depolarizing solution containing $^{45}\text{Ca}^{2+}$ for 2 min, so that dose-effect curves were shifted to the left after a total of 35 min of depolarization compared to 2 min of depolarization (Figure 3b). IC_{50} values measured after 2 and 35 min in the depolarizing solution were 743 pM and 42 pM, respectively. These values correlated well with the inhibition of the contraction induced by 100 mM KCl, measured at the same time intervals (Table 1).

$[^3\text{H}]$ -(+)-isradipine binding in segments of arteries exposed to different KCl concentrations

In aorta bathed in physiological solution (KCl 5.9 mM), the level of specific binding of $[^3\text{H}]$ -(+)-isradipine was very low, and reached only 10–15% of the total binding. When aortic segments were incubated in 100 mM KCl depolarizing solution, $[^3\text{H}]$ -(+)-isradipine specific binding increased with the duration of incubation. At 100 pM $[^3\text{H}]$ -(+)-isradipine binding reached a steady state after 45 min (not shown). At this ligand concentration, specific binding was about 60% of the total binding. When tissues were thereafter transferred

Figure 2 Inhibition produced by nisoldipine on noradrenaline-induced contraction (hatched columns) and on KCl-induced contraction (solid columns) after preincubation in physiological solution or in 40 mM KCl depolarizing solution. After recording of control contractions, aortic rings were preincubated for 60 min in the presence of nisoldipine (0.1 nM) in physiological solution. They were then incubated for 30 min in zero Ca^{2+} physiological solution with nisoldipine (0.1 nM) before contraction was elicited by exposure to 10^{-5} M noradrenaline or to 100 mM KCl-solution for 1 min, in the continuous presence of nisoldipine (run 1). Aortic rings were thereafter incubated for 30 min in zero Ca^{2+} , 40 mM KCl-depolarizing solution containing the same concentration of nisoldipine (0.1 nM) before a second run of contraction was recorded. At the end of this contraction, aortic rings were further incubated in zero Ca^{2+} physiological solution with nisoldipine (0.1 nM) and a third run of contraction was recorded. Nisoldipine-resistant contraction was determined in the presence of nisoldipine 100 nM in the same conditions and subtracted from the total response to give the nisoldipine-sensitive contraction. All contractions were corrected for control responses recorded in the same conditions. Data are means from 4 determinations with s.e.mean shown by vertical bars.

Figure 3 Effect of nisoldipine on $^{45}\text{Ca}^{2+}$ influx. (a) ^{45}Ca influx was measured after 7 min incubation in ^{45}Ca -containing physiological solution (unstimulated arteries open columns) or after 5 min incubation in ^{45}Ca -containing physiological solution followed by 2 min incubation in ^{45}Ca -containing 100 mM KCl solution (hatched columns) or ^{45}Ca -containing physiological solution with $10\ \mu\text{M}$ of noradrenaline (solid columns). Aortic rings were preincubated for 90 min in physiological solution with (Nis) or without (control) nisoldipine (100 nM). Data are means from n determinations; s.e.mean shown by vertical bars. * indicates significant difference. (b) Nisoldipine dose-effect curves on ^{45}Ca influx stimulated by KCl or noradrenaline. KCl-stimulated ^{45}Ca influx was measured in aortic rings incubated for 2 min in ^{45}Ca -containing 100 mM KCl solution either after 90 min preincubation in physiological solution (■) or after 60 min preincubation in physiological solution and 30 min in 100 mM KCl solution (□) with different concentrations of nisoldipine. Noradrenaline-stimulated ^{45}Ca influx (◆) was measured after 7 min incubation in ^{45}Ca -containing physiological solution, noradrenaline being added to the solution during the last 2 min. Aorta rings were preincubated for 90 min in the presence of different concentrations of nisoldipine. Control ^{45}Ca influx was measured in aortic rings incubated for 7 min in ^{45}Ca -containing physiological solution; this value was subtracted from total ^{45}Ca content of stimulated aortic rings. Data are means from 6–12 determinations; vertical bars show s.e.mean.

from the 100 mM KCl solution into physiological solution, the level of specific binding decreased and after 45 min in the physiological solution it was similar to that measured in arteries exposed to the physiological solution for 90 min (Table 2).

To test the effect of the KCl concentration on [^3H]-(+)-isradipine binding, specific binding was determined in the presence of a fixed concentration of [^3H]-(+)-isradipine in solutions containing various concentrations of KCl. The NaCl concentration was correspondingly modified. As illustrated in Figure 4, binding at 100 pM [^3H]-(+)-isradipine increased when KCl concentration was enhanced from 5.9 mM (physiological solution) to 100 mM.

Saturation studies of the specific binding of [^3H]-(+)-isradipine were determined at equilibrium in solutions containing

Table 2 Influence of KCl concentration on [^3H]-(+)-isradipine specific binding in rat aorta

Incubation conditions	[^3H]-(+)-isradipine specific binding (fmol mg^{-1} wet wt.)	
KCl 5.9 mM 45 min	0.58 ± 0.14	$P < 0.01$
KCl 100 mM 45 min	2.42 ± 0.26	
KCl 100 mM 45 min + KCl 5.9 mM 45 min	0.39 ± 0.22	NS
KCl 5.9 mM 90 min	0.42 ± 0.25	

All aortic segments were first incubated with [^3H]-(+)-isradipine (50 pM) for 60 min. Incubation was further carried out as indicated, in the presence of [^3H]-(+)-isradipine (50 pM). Data are means $\pm$ s.e.mean from 6 determinations.

15, 20 or 100 mM KCl. Figure 5 shows the results of these experiments. Scatchard plots of saturation curves were linear; binding parameters calculated from these data are given in Table 3. The B_{max} value was not significantly affected by the concentration of KCl in the medium. On the contrary, K_D values decreased when arteries were exposed to increasing concentrations of KCl. K_D values determined from saturation studies in aortic segments bathed in 100 mM KCl were close to K_D values measured in rat aortic microsomes (0.085 (0.065–0.112) nM; Wibó *et al.*, 1988).

Figure 4 Influence of the KCl concentration on [^3H]-(+)-isradipine specific binding. (a) [^3H]-(+)-isradipine specific binding was measured after 60 min of incubation in physiological solution containing [^3H]-(+)-isradipine at 50 pM followed by 30 min incubation in solutions containing different concentrations of KCl and the same concentration and specific activity of [^3H]-(+)-isradipine. Data are means from 5–20 determinations; s.e.mean shown by vertical bars. (b) Influence of the KCl concentration on the value of K_{app} , the apparent dissociation constant of [^3H]-(+)-isradipine. K_{app} value was calculated from the data of Figure 4a according to Michaelis-Menten's equation, $K_{\text{app}} = L(B_{\text{max}} - b)/b$ with $L = [^3\text{H}]\text{-}(+)\text{-isradipine concentration (50 pM)}$, b the specific binding of [^3H]-(+)-isradipine and a B_{max} value of $5.5\ \text{fmol}\ \text{mg}^{-1}$ wet wt.

Figure 5 Scatchard plot of the [^3H]-(+)-isradipine specific binding as a function of [^3H]-(+)-isradipine concentration in aortic segments incubated in various KCl concentrations (a) or exposed to noradrenaline (10^{-5} M) (b). Aortic segments were incubated for 60 min in physiological solution containing different concentrations of [^3H]-(+)-isradipine and further incubated for 30 min in solutions containing KCl at 15 mM ($\square$), 20 mM ($\blacklozenge$) or 100 mM ($\triangle$) or in physiological solution containing noradrenaline $10\text{ }\mu\text{M}$ ($\blacktriangle$) containing the same concentrations and specific activities of [^3H]-(+)-isradipine. Data are means from 10–12 determinations.

On the basis of the observation that varying the KCl concentration changed only the apparent dissociation constant of [^3H]-(+)-isradipine (K_{app}), we calculated the value of K_{app} at different KCl concentrations from Michaelis-Menten's equation applied to the binding data shown in Figure 4a, with unique value of B_{max} of 5.5 fmol mg^{-1} wet wt. These values are shown in Figure 4b: a small variation of the KCl concentration between 5.9 and 20 mM induced a large modification of the K_{app} value. A further increase of the KCl concentration only slightly decreased the value of K_{app} .

Table 3 Binding parameters of [^3H]-(+)-isradipine in aortic segments incubated in different KCl concentrations or exposed to noradrenaline $10\text{ }\mu\text{M}$ (NA)

	K_D (pM)	B_{max} (fmol mg^{-1} wet wt.)
KCl 15 mM	250	5.8 ± 0.8
KCl 20 mM	125	6.3 ± 0.7
KCl 100 mM	68	5.4 ± 0.3
KCl 5.9 mM + NA 10^{-5} M	126	5.8 ± 1.6

Binding parameters of [^3H]-(+)-isradipine were calculated by linear regression of data shown in Figure 5.

Figure 6 Time-course of [^3H]-(+)-isradipine dissociation in aortic segments. Aortic segments were first incubated with [^3H]-(+)-isradipine (80 pM) in KCl-depolarizing solution for 60 min. Dissociation was induced by transferring the aortic segments to various solutions containing $1\text{ }\mu\text{M}$ nifedipine: physiological solution (KCl, 5.9 mM) ($\blacksquare$); 100 mM KCl-solution ($\blacktriangle$). Semi-logarithmic plot demonstrating the existence of a single process of dissociation. Data are means from 3–6 experiments performed in triplicate; vertical bars show s.e.mean.

Electrophysiological and binding studies in cardiac cells have shown that dissociation kinetics of dihydropyridine calcium antagonists are markedly influenced by the membrane potential of the cells (Triggle *et al.*, 1989). To examine whether this could be applied to vascular smooth muscle, dissociation was studied either in physiological solution, or in 100 mM KCl solution. Dissociation was produced by the addition of an excess of unlabelled competitor ((+)-isradipine or nifedipine 10^{-6} M), after the tissue had been equilibrated with [^3H]-(+)-isradipine in 100 mM KCl solution. The tritiated ligand concentration was maintained unchanged during the dissociation process so that non specific binding was unchanged. Figure 6 shows that membrane potential influenced the rate of dissociation of [^3H]-(+)-isradipine. A monophasic dissociation curve indicated the existence of a single process of dissociation. The dissociation rate measured in physiological solution gave a value of k_{-1} equal to $0.050 \pm 0.007\text{ min}^{-1}$ ($n = 6$). The dissociation rate was lower in 100 mM KCl-solution ($k_{-1} 0.012 \pm 0.002\text{ min}^{-1}$, $n = 6$). The value of k_{-1} in depolarized aortic segments was close to the value measured by Triggle *et al.* (1989) in depolarized cardiac myocytes ($0.018 \pm 0.0012\text{ min}^{-1}$) but not in physiological solution since a markedly higher k_{-1} value was found by those authors in cardiac myocytes in physiological solution ($0.53 \pm 0.06\text{ min}^{-1}$).

Binding of [^3H]-(+)-isradipine in tissues exposed to noradrenaline

[^3H]-(+)-isradipine binding was determined in aorta bathed in physiological solution containing different concentrations of noradrenaline. In the presence of noradrenaline (10^{-7} – 10^{-5} M) the specific binding of 80 pM [^3H]-(+)-isradipine was significantly increased without change of the non-specific binding (Figure 7).

Equilibrium binding studies in the presence of 10^{-5} M noradrenaline indicated that this agonist did not affect the linear pattern of the Scatchard plot of binding data (Figure 5b). Maximum binding capacity was similar to that measured in other conditions, while the K_D value was close to the value measured in 20 mM KCl solution (Table 3).

Nisoldipine effect on [^3H]-isradipine binding

The effect of nisoldipine on [^3H]-(+)-isradipine specific binding was determined in rat aorta bathed in KCl (25 and

Figure 7 Effect of noradrenaline on [³H]-(+)-isradipine specific binding measured in aortic segments incubated for 60 min in physiological solution containing [³H]-(+)-isradipine at 80 pM, and further incubated for 30 min in solutions containing 5.9 mM (physiological solution), 20 mM or 100 mM of KCl or in physiological solution containing different concentrations of noradrenaline, in the presence of the same concentration and specific activity of [³H]-(+)-isradipine. Data are means of *n* determinations; vertical bars show s.e.mean.

100 mM) solutions (Figure 8), and in rat mesenteric artery bathed in KCl (100 mM) solution or in normal physiological solution (KCl 5.9 mM), as [³H]-(+)-isradipine binding can be determined in the latter tissue in resting conditions (Morel & Godfraind, 1987). Nisoldipine was able to displace completely [³H]-(+)-isradipine specific binding in both tissues and in all cases, nisoldipine potency was higher in 100 mM KCl solution. *K_i* values obtained in KCl 100 mM solution were in good agreement with *IC₅₀* values on KCl (100 mM)-induced contraction and ⁴⁵Ca influx measured after 35 min exposure of the tissues to the depolarizing solution (Table 1). On the other hand, the *K_i* value in the mesenteric artery bathed in physiological solution was close to the *IC₅₀* value on the KCl contraction of the same tissue exposed for only 2 min to the depolarizing solution.

Nisoldipine also displaced [³H]-(+)-isradipine binding in aorta exposed to 10⁻⁵ M noradrenaline, in a dose-dependent manner and the displacement curve was parallel to that obtained in aortic segments exposed to the depolarizing solution (KCl 100 mM) and to KCl (25 mM) solution (Figure 8).

Figure 8 Effect of nisoldipine on [³H]-(+)-isradipine specific binding in aortic segments: binding was measured in aortic segments incubated in the presence of different concentrations of nisoldipine for 60 min in physiological solution containing [³H]-(+)-isradipine (40–60 pM) and further incubated for 30 min either in physiological solution with noradrenaline (10⁻⁵ M) (●), in 25 mM KCl solution (Δ), or in 100 mM KCl solution (□), containing [³H]-(+)-isradipine at the same concentration. Data are means from 10 determinations; vertical bars show s.e.mean.

Figure 9 Time-course of the uptake of [³H]-tetraphenylphosphonium bromide ([³H]-TPP⁺) in aortic segments. Aortic rings were incubated for different times in physiological solution (□) or in solution containing 130 mM KCl (◆) in the presence of [³H]-TPP⁺ (10 μM). Data are means from 4 determinations, s.e.means were smaller than symbols.

The *IC₅₀* value of nisoldipine in the aorta exposed to noradrenaline (10⁻⁵ M) was close to the value obtained in the aorta bathed in KCl (25 mM) solution. The *K_i* value in the presence of noradrenaline (10⁻⁵ M) corresponded to the *IC₅₀* value of nisoldipine on contraction evoked by 10⁻⁵ M noradrenaline when measured after 35 min of stimulation (Table 1).

[³H]-tetraphenylphosphonium bromide uptake

[³H]-TPP⁺ uptake was measured in aortic segments with intact endothelium to estimate the membrane potential of the preparations. The uptake of [³H]-TPP⁺ increased with the duration of the incubation and reached a steady state level after 45 min (Figure 9). Increasing the KCl concentration in the bathing solution from 5.9 mM to 130 mM (with simultaneous decrease of the NaCl concentration) dose-dependently decreased [³H]-TPP⁺ uptake. At equilibrium, the [³H]-TPP⁺ content of the tissues bathed in 130 mM KCl solution was 51% of the [³H]-TPP⁺ content of arteries bathed in physiological solution. Further increase of the KCl concentration up to 150 mM did not significantly modify the uptake of [³H]-TPP⁺ (not shown). At these high KCl concentrations, [³H]-TPP⁺ uptake presumably represents KCl-insensitive accumulation in membranes and inside internally negative compartments, as membrane potential may be assumed to be abolished. This value was subtracted from [³H]-TPP⁺ uptake measured in other conditions to evaluate the value of the KCl-sensitive component of [³H]-TPP⁺ uptake. The participation of mitochondria in the [³H]-TPP⁺ uptake was estimated by the use of a mitochondrial uncoupler (carbonyl cyanide *m*-chlorophenylhydrazone) (Scott & Nicholls, 1980). In the presence of 0.3 μM of the mitochondrial uncoupler, [³H]-TPP⁺ uptake was reduced by 53 ± 2.1% (*n* = 38); the degree of inhibition was unaffected by the composition of the incubation medium. When [³H]-TPP⁺ uptake corrected for the mitochondrial uptake, was inserted into the Nernst's equation, a membrane potential value of -63.3 ± 0.67 mV (*n* = 40) was obtained for aorta incubated in physiological solution.

The influence of increasing concentrations of KCl in the bath on the K⁺-sensitive [³H]-TPP⁺ uptake is represented in Figure 10, where it is compared to the effect of noradrenaline. Exposure of the aortic segments to noradrenaline (10⁻⁸ M–10⁻⁵ M) in physiological solution decreased the KCl-sensitive [³H]-TPP⁺ uptake without changing the KCl-insensitive uptake; the effect of the agonist was dose-dependent (Figure 10b). Reduction of [³H]-TPP⁺ uptake plateaued at noradrenaline concentrations higher than 10⁻⁶ M; [³H]-TPP⁺

Figure 10 Effect of KCl and noradrenaline on the KCl-sensitive $[^3\text{H}]\text{-tetraphenylphosphonium bromide } ([^3\text{H}]\text{-TPP}^+)$ uptake in aortic segments: uptake was measured in aortic segments incubated with $[^3\text{H}]\text{-TPP}^+$ ($10 \mu\text{M}$) for 60 min in solutions containing different concentrations of KCl (a) or in physiological solution containing different concentrations of noradrenaline (b). KCl-sensitive $[^3\text{H}]\text{-TPP}^+$ uptake was obtained after subtraction of the uptake of $[^3\text{H}]\text{-TPP}^+$ in a solution containing 130 mM KCl and the same concentration of $[^3\text{H}]\text{-TPP}^+$. Data are means from at least 12 determinations; s.e.mean shown by vertical bars.

content of tissues exposed to noradrenaline 10^{-6} – 10^{-5} M was close to the values measured in aorta incubated in solution containing KCl concentration near 25 mM. This effect of noradrenaline was not affected by nisoldipine (10^{-7} M) but was inhibited by the α -adrenoceptor antagonists phentolamine

(10^{-6} M) and prazosin (10^{-6} M) (Table 4). The uptake of $[^3\text{H}]\text{-TPP}^+$ measured in the presence of $0.3 \mu\text{M}$ of the mitochondrial uncoupler in aorta exposed to noradrenaline (10^{-5} M) or to 25 mM KCl solution is shown in Table 4; membrane potential values estimated by applying the Nernst equation to these data are -55.4 mV and -56 mV, in aortic segments exposed to noradrenaline 10^{-5} M and KCl 25 mM, respectively.

Discussion

By use of segments of intact vessels, this study shows the existence of voltage-dependent specific binding sites for calcium antagonistic dihydropyridines in rat aorta. Voltage-dependence of the binding was related to a modification of binding affinity without change of maximum binding capacity. These results are in agreement with previous reports describing voltage-sensitive binding of dihydropyridines in cardiac cells (Kokubun *et al.*, 1986; Wei *et al.*, 1989), in mesenteric artery (Morel & Godfraind, 1987), coronary artery (Sumimoto *et al.*, 1988) and cerebral microvessels (Morel & Godfraind, 1989).

The relationship between the apparent dissociation constant (K_{app}) of $[^3\text{H}]\text{-}(+)\text{-isradipine}$, and KCl concentration in the bath indicates a modulation of binding affinity as a function of the membrane potential imposed to the cell. These results may be interpreted according to the modulated receptor model: binding sites coexist in two different conformations, one with high affinity, associated with inactivated Ca^{2+} channels in depolarized preparations, and the other with low affinity for the ligand, associated with resting Ca^{2+} channels. K_{app} has been estimated for various KCl concentrations which clamped smooth muscle membrane potential at various levels. Assuming that K_{app} is a function of the proportion of high and low affinity sites, its value can be calculated from the equation:

$$1/K_{\text{app}} = L/K_L + (1 - L)/K_H(1)$$

with L representing the proportion of low affinity binding sites, K_L the low affinity dissociation constant and K_H the high affinity dissociation constant. At KCl concentrations higher than 30 mM, the value of K_{app} of $[^3\text{H}]\text{-}(+)\text{-isradipine}$ in rat aorta was not markedly affected by variations in the KCl concentration. The K_{app} value at 100 mM KCl may be assumed to correspond to the K_D value for the high affinity binding sites. The high affinity dissociation constant measured in intact depolarized tissue is close to the K_D value found in membrane preparation (Wibo *et al.*, 1988) indicating that prolonged depolarization and tissue homogenization favoured a similar conformation of calcium channels, which is most likely, according to the modulated receptor model, the inactivated form of the calcium channel (see Bean, 1984; Sanguinetti & Kass, 1984). At lower KCl concentrations, the K_{app} value was very sensitive to small changes of KCl concentration and the absolute value of the K_D for the low affinity binding site could be much higher than that measured in

Table 4 $[^3\text{H}]\text{-Tetraphenylphosphonium bromide } ([^3\text{H}]\text{-TPP}^+)$ distribution in segments of rat aorta

Drug added	KCl (5.9 mM)	K^+ -sensitive $[^3\text{H}]\text{-TPP}^+$ content (nmol mg^{-1} wet wt.)		
		NA (10^{-5} M)	KCl (20 mM)	KCl (25 mM)
None	138.7 ± 4.8 (25)	98.7 ± 3.7 (25) ^a	100.3 ± 4.9 (16) ^a	95.8 ± 6.1 (5)
Nisoldipine (10^{-7} M)	146.6 ± 3.8 (6)	97.1 ± 7.4 (6)		
Phentolamine (10^{-6} M)	137.8 ± 13 (5)	120.1 ± 9.6 (5) ^b		
Prazosin (10^{-6} M)	136.5 ± 4.4 (6)	125.6 ± 6.2 (6) ^b		
Carbonyl cyanide <i>m</i> -chloro phenylhydrazone (5×10^{-7} M)	64.4 ± 2.8 (10) ^b	44.9 ± 1.9 (6) ^{a, b}		44.5 ± 2.3 (6) ^{a, b}

$[^3\text{H}]\text{-TPP}^+$ content of rat aortic segments was measured after 60 min incubation in the indicated solution. Aortic segments were preincubated for 30 min in the presence of phentolamine or prazosin or for 60 min in the presence of nisoldipine. Data are means from (n) determinations $\pm$ s.e.mean. ^aSignificantly different from corresponding control in KCl 5.9 mM; ^bsignificantly different from $[^3\text{H}]\text{-TPP}^+$ content in segments incubated in the same solution without any addition.

physiological solution. This marked sensitivity of binding affinity to small variations of KCl concentration implies that any situation causing a small variation of the membrane potential near the resting level may induce important change in the affinity and in the potency of (+)-isradipine and other voltage-sensitive dihydropyridines such as nisoldipine.

As a consequence of the modulated receptor model, and because those drugs are characterized by slow association kinetics, inhibition of KCl-induced contractile responses by voltage-sensitive calcium antagonists is markedly dependent on the duration of contact of the drug with the depolarized tissue (Wibo *et al.*, 1988). The IC_{50} value of nisoldipine on KCl-induced contraction measured after a short depolarization was close to the value of K_i obtained in aorta incubated in physiological solution and reflected the binding to a low proportion of high affinity sites in resting tissue. The potency of nisoldipine as an inhibitor of KCl-induced contraction and ^{45}Ca entry was increased after prolonged depolarization. This resulted either in a time-dependent effect on 100 mM KCl-induced contraction or in an increase of the inhibition of short contractions after preincubation of the tissue with the antagonist in depolarizing solution. In agreement with our previous observations (Morel & Godfraind, 1987), Nelson *et al.* (1988) using rabbit mesenteric artery, and Triggle *et al.* (1988), using guinea-pig longitudinal smooth muscle, have shown that the potency of nitrendipine and related 1,4-dihydropyridines as inhibitors of KCl-induced tension is a function of membrane potential. Nisoldipine IC_{50} values on KCl-stimulated $^{45}Ca^{2+}$ entry or contraction measured after 35 min of exposure of the tissue to the high KCl-depolarizing solution were very close to the K_i value measured in 100 mM KCl solution, which is consistent with equation 1.

In many tissues, calcium entry blockers have been reported to be less potent inhibitors of the contraction induced by agonist stimulation than by depolarization (Cauvin *et al.*, 1983; Marriott, 1988; Godfraind *et al.*, 1986). This observation led to the hypothesis of the existence of two different types of calcium channels, one controlled by the occupation of specific receptors and the other controlled by membrane potential (Cauvin & van Breemen, 1985). However, analysis of the effect of nisoldipine in rat aorta shows that although agonist- and depolarization-induced responses have different sensitivities to calcium entry blocking drugs, this does not necessarily mean that responses are mediated by different Ca^{2+} channels. It is worth noting that, while the potency of nisoldipine was higher against the 100 mM KCl-induced contraction than against the noradrenaline-induced contraction when determination of the inhibition was performed after prolonged contraction, there was no significant difference between IC_{50} values of nisoldipine on $^{45}Ca^{2+}$ entry or contraction induced by 100 mM KCl-solution or by noradrenaline, when the responses were measured after a short duration of stimulation (2 min). The inhibitory potency of nisoldipine on the response evoked by a short application of noradrenaline could be increased by preincubation of the tissue with the antagonist in depolarizing solution, just as the contractile response induced by short exposure to KCl-depolarizing solution was, suggesting that noradrenaline-evoked contraction depends at least partly on the same nisoldipine-sensitive Ca^{2+} entry pathway as depolarization-induced contraction. Modulation of the voltage-dependent binding sites of dihydropyridines by noradrenaline was confirmed by binding studies. Noradrenaline produced an increase in the affinity of both nisoldipine and (+)-isradipine, which can be attributed to an increase in the proportion of high affinity binding sites for dihydropyridines. As this effect of noradrenaline was comparable to that of 20 mM KCl, it may be suggested that it is the result of a small depolarization produced by noradrenaline.

Despite the fact that data concerning the effect of noradrenaline on membrane potential are quite variable, it generally appears that high concentrations of noradrenaline produce a small depolarization while low concentrations may induce a contractile response without change of the mem-

brane potential (see Bolton & Large, 1986). $[^3H]$ -TPP⁺ was used in the present work to estimate and compare the membrane potential of aorta exposed to KCl and noradrenaline. TPP⁺ is a membrane permeant lipophilic cation that is distributed across the membranes as a function of membrane potential (Lichtshtein *et al.*, 1979). It has been successfully used in different cell lines to measure membrane potential (Tan & Tashjian, 1984; Greenberg *et al.*, 1986; Wei *et al.*, 1989). The membrane potential value estimated from the TPP⁺ distribution is an average value of the potential of all the cells of the preparation and is then directly comparable with any other data like those from $[^3H]$ -(+)-isradipine binding. The main disadvantages of the TPP⁺ method are that (1) it is an indirect method and transformation of uptake data in potential values requires the estimation of a cytosolic concentration of TPP⁺, which depends on the value of the intracellular volume, (2) it does not allow detection of rapid or transient changes of potential, and (3) the existence of an uptake of TPP⁺ in internally negative organelles like mitochondria may give overestimated values of potential. The uptake of TPP⁺ into mitochondria can be suppressed by a mitochondrial uncoupler, which has been reported to depolarize selectively the mitochondrial membrane without affecting the cellular membrane potential in intact synaptosomes (Scott & Nicholls, 1980). In rat aortic segments, $[^3H]$ -TPP⁺ uptake was dose-dependently reduced when KCl concentration was increased up to 130 mM and remained unchanged when KCl concentration was further increased to 150 mM. A concentration of KCl of 130 mM was assumed to abolish the membrane potential and the tissue content of TPP⁺ measured in these conditions was considered to represent KCl-insensitive uptake. When $[^3H]$ -TPP⁺ uptake data, corrected for KCl-insensitive uptake and for mitochondrial uptake, were translated into membrane potential value, the membrane potential value that was obtained (−63.3 mV) was similar to the data obtained with the classical microelectrode technique in rat aorta (−53.8 mV, Chen & Suzuki, 1989; −57 mV, Taylor *et al.*, 1988) and in other vascular tissues (−63.3 mV in rabbit ear artery, Droogmans *et al.*, 1977; −59.2 mV in rat small mesenteric arteries, Mulvany *et al.*, 1982; −70 mV in guinea-pig mesenteric artery, Kuriyama & Suzuki, 1981), indicating that the $[^3H]$ -TPP⁺ distribution may be a fairly good indicator of membrane potential. Noradrenaline produced a dose-dependent decrease of the KCl-sensitive $[^3H]$ -TPP⁺ uptake, with a maximum effect close to that of 30 mM KCl. In rat aorta exposed to 0.1 μ M of noradrenaline, Chen & Suzuki (1989) measured a depolarization of 7 mV. For this noradrenaline concentration, the uptake of $[^3H]$ -TPP⁺ gave a value between the values measured in aorta bathed in 10 and 15 mM KCl, corresponding to a mean value of depolarization of about 5 mV which corresponds with Chen & Suzuki's result. This effect of noradrenaline also corresponds to observations reported in other vessels (Casteels *et al.*, 1977; Haeusler, 1983; Mulvany *et al.*, 1982). The mechanisms underlying the depolarizing effect of noradrenaline are still conflicting. Recently however, evidence has been provided, which suggests that the noradrenaline-produced depolarization is mediated by an increase in chloride and cation membrane conductance (Amédée & Large, 1989; Pacaud *et al.*, 1989).

The effects of noradrenaline on $[^3H]$ -TPP⁺ distribution and on $[^3H]$ -(+)-isradipine do correspond very well, and are in agreement with the hypothesis that the effect of noradrenaline on $[^3H]$ -(+)-isradipine and nisoldipine binding is related to the depolarization produced by the agonist. These data suggest that the noradrenaline-evoked contraction is caused by the opening of voltage-dependent Ca channels as a result of the depolarization produced by noradrenaline. Comparison of noradrenaline and KCl effects on membrane potential shows that depolarization produced by higher concentrations of noradrenaline is large enough to cause Ca^{2+} entry and contraction, while the depolarization produced by lower concentrations of the agonist ($<10^{-7}$ M) is probably too small to open voltage-dependent Ca^{2+} channels. We postulate an

additional effect of noradrenaline on the activation of the Ca^{2+} channel. Noradrenaline at a concentration that does not depolarize cells has been shown to increase the open-state probability of Ca^{2+} channels in rabbit mesenteric artery (Nelson *et al.*, 1988). An increase of the L-type Ca^{2+} current mediated by an adrenoceptor different from the α - or β -adrenoceptor has been reported in rabbit ear artery (Benham & Tsien, 1988). The occurrence of this type of adrenoceptor in rat aorta is less probable, as all responses to noradrenaline were abolished by pre-treatment with phentolamine or prazosin (Alosachie & Godfraind, 1988).

Our observations indicate that opening of voltage-operated calcium channels as a result of noradrenaline-induced depolarization was apparently not the only mechanism of increase

of intracellular calcium leading to contraction. Beside the component of the contraction that is known to be related to the release of intracellular Ca^{2+} (Godfraind & Kaba, 1969), a fraction of noradrenaline-stimulated $^{45}\text{Ca}^{2+}$ entry was resistant to nisoldipine blockade, suggesting that another Ca^{2+} entry pathway was opened by the agonist. Further experiments are required in order to characterize the nature of this pathway.

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