Br. J. Pharmacol. (1988), **95**, 252–258

Selective modulation by membrane potential of the interaction of some calcium entry blockers with calcium channels in rat mesenteric artery

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1 The effects of flunarizine, (+)-PN 200-110 and nifedipine on [^{3}H]-(+)-PN 200-110 specific binding were investigated in intact rat mesenteric arteries bathed in physiological solution or in KCl-depolarizing solution, and in a membrane fraction from rat mesenteric arteries.

2 Unlabelled dihydropyridines, (+)-PN 200-110 and nifedipine, inhibited [^{3}H]-(+)-PN 200-110 specific binding concentration-dependently in polarized as well as in depolarized intact arteries. The K_i value of (+)-PN 200-110 was decreased in arteries bathed in KCl-depolarizing solution compared to arteries bathed in physiological solution, while the K_i value of nifedipine was not significantly changed. K_i values measured in depolarized arteries were close to the IC_{50} values (concentrations inhibiting by 50% the KCl-contraction of rat mesenteric artery).

3 Flunarizine (10^{-6} M) was unable to displace the specific binding of [^{3}H]-(+)-PN 200-110 in intact arteries bathed in physiological solution. At 10^{-7} M–10^{-6} M, it inhibited the binding in depolarized arteries, suggesting that prolonged depolarization is required for the interaction of flunarizine with the dihydropyridine receptor.

4 In a membrane fraction isolated from rat mesenteric arteries, (+)-PN 200-110, nifedipine and flunarizine were all able to displace completely the specific binding of [^{3}H]-(+)-PN 200-110. Displacement curves were parallel and Hill coefficients were close to unity. K_i values were close to the values obtained in depolarized intact arteries.

5 These results revealed a good correlation between the data obtained from binding tests and from pharmacological studies for dihydropyridine calcium entry blocking drugs, taking into account the time-dependence associated with their action on KCl-contraction compared to their binding properties. There was an important discrepancy between the concentrations of flunarizine active in binding studies and those active in pharmacological studies, which could be accounted for by the existence of multiple binding sites for calcium entry blockers in calcium channels.

Introduction

Calcium entry blocking drugs are a group of chemically unrelated molecules that specifically block calcium entry during muscle activation, without interfering with calcium movements in resting preparations (Godfraind *et al.*, 1986). Despite this common mode of action, important differences have been shown to exist between these compounds that have led to the proposal that different subclasses of calcium entry blocking drugs exist on the basis of pharmacological effects, binding properties and clinical profiles (Spedding, 1984; Godfraind, 1986; 1987). Among the calcium entry blocking drugs, some compounds present a time-dependent inhibitory effect on KCl-induced contraction of smooth muscle. This is the case with dihydropyridine derivatives like (+)-PN 200-110, nisoldipine and (−)-nimodipine, and with phenylalkylamines like cinnarizine and flunarizine (Godfraind & Miller, 1983). However, the duration of the depolarization affects to a lesser extent the effect of nifedipine, (−)-PN 200-110, verapamil or diltiazem (Godfraind *et al.*, 1986). By analogy with the voltage-dependent effect of local anaesthetics on the Na channel in nerves and the heart, an influence of the state of the channel on drug inhibitory potency has been suggested to explain the enhanced blockade of Ca entry and contraction with depolarization (Godfraind *et al.*, 1986).

Interesting elements in the interpretation of time-

dependency have been found by directly studying the binding of calcium entry blocking drugs in vascular smooth muscles. Binding studies performed in intact mesenteric arteries with [^{3}H]-(+)-PN 200-110 revealed the existence of stereoselective specific binding sites for dihydropyridine compounds. Binding is voltage-dependent in such a way that depolarization increases binding affinity. Binding properties indicate the existence of high affinity binding of (+)-PN 200-110 to inactivated channels and low affinity binding to resting channels (Morel & Godfraind, 1987). In a recent study with rat aorta, Wibo *et al.* (1988) found a good correlation between the time course of inhibition of the KCl-induced contraction by (+)-PN 200-110 and the time course of its specific binding to isolated membranes. These observations suggest that the time-dependency of the (+)-PN 200-110 effect is caused by the very low binding of this inhibitor in vascular smooth muscle cells at rest and its preferential binding to inactivated calcium channels in depolarized preparations.

The purpose of the present study was to compare the influence of depolarization on the effects of different calcium antagonistic compounds in rat mesenteric artery. Flunarizine, (+)-PN 200-110 and nifedipine were used because they present different degrees of time-dependency, as indicated by the ratio of the IC_{50} concentrations for the inhibition of KCl-contraction measured 2 and 30 min after the beginning of the depolarization. The interaction of these compounds with the dihydropyridine-specific binding site of the calcium channel, labelled by [^{3}H]-(+)-PN 200-110, has been measured in polarized and in depolarized rat mesenteric arteries and compared to their inhibitory potency on depolarization-induced contraction, to determine whether differential effects observed in pharmacological tests are related to different modes of interaction with the voltage-dependent calcium channel.

Methods

[^{3}H]-(+)-PN 200-110 binding in mesenteric artery segments

Wistar rats (weighing 250–300 g) were used. They were killed by decapitation and the superior mesenteric artery was removed and cleaned. Segments (1–2 mg wet weight) were cut and immersed in physiological solution (mM: NaCl 122, KCl 5.9, $NaHCO_3$ 15, $MgCl_2$ 1.25, $CaCl_2$ 1.25, glucose 11) maintained at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2. After a recuperation period of two hours, groups of three segments were incubated either for 90 min in physiological solution (polarized arteries) or for 60 min in physiological solution followed by 30 min in KCl-depolarizing solution (mM: NaCl 22, KCl 100, $NaHCO_3$ 15, $MgCl_2$ 1.25, $CaCl_2$ 1.25, glucose 11) containing [^{3}H]-(+)-PN 200-110 at 100 pM (polarized arteries) or 40 pM (depolarized arteries) and various concentrations of unlabelled drugs in a total volume of 25 ml. These concentrations of tritiated ligand were chosen to label approximately the same proportion of sites in polarized and depolarized arteries (Morel & Godfraind, 1987). 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 37%. Non-specific binding was determined in the presence of 10^{-6} M nifedipine, and subtracted from the total binding to give the specific binding.

Preparation of a membrane fraction from mesenteric artery

Mesenteric arteries taken from 50–100 rats were homogenized in 25 ml of ice-cold 0.25 M sucrose buffered at pH 7.4 with Tris-HCl 5 mM. Homogenate was spun at 1000 *g* for 10 min. The pellet was washed twice and the combined supernatants were spun at 100,000 *g* for 60 min in a W40/128 rotor (Heraeus-Christ). The pellet was suspended in 3 ml of sucrose buffer. Protein was assayed according to Lowry *et al.* (1951).

[^{3}H]-(+)-PN 200-110 binding in the membrane fraction

To measure [^{3}H]-(+)-PN 200-110 binding in membrane preparation from mesenteric artery, membrane protein (70 μg ml^{-1}) was incubated in glass tubes in 1 ml of a salt buffer containing (mM): NaCl 137, KCl 5.9, $CaCl_2$ 1.25, $MgCl_2$ 1.25, glucose 11 and maleic acid 10, buffered at pH 7.4 with Tris; with [^{3}H]-(+)-PN 200–110 and various drugs as indicated. Incubation was carried out at 37°C for 60 min. At the end of the incubation time, samples were diluted with 4 ml ice-cold 0.9% w/v NaCl solution, and filtered on Whatman GF/F filters. Filters were washed with two 10 ml portions of 0.9% NaCl.

Specific binding was defined as the binding displaced by 10^{-6} M nifedipine. The radioactivity retained on the filters was counted in 10 ml Picofluor/toluene (1/4) by liquid scintillation spectrophotometry with an efficiency of 43%.

Drugs

[^{3}H]-(+)-PN 200-110 (isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridine carboxylate) was purchased

from New England Nuclear (76 Ci mmol^{-1}). Nifedipine (Bayer) and (+)-PN 200-110 (Sandoz) were dissolved in ethanol as stock solutions at 10 mM and further diluted in distilled water as required before use. Flunarizine (Janssen) was dissolved in an aqueous solution of 100 mM tartaric acid to a concentration of 1 mM and further diluted with distilled water as required. All experiments were done under illumination only by means of a sodium lamp.

Data analysis

Data are presented as means ± s.e.mean. IC_{50} values were estimated from displacement curve by linear regression. K_i values were calculated from IC_{50} values according to the method of Cheng & Prusoff (1973). Significance of the difference between means was assessed by Student's t test; P values smaller than 0.05 were considered to be significant.

Results

Binding of [^{3}H]-(+)-PN 200-110 to mesenteric artery segments

Figure 1 compares the effects of nifedipine, (+)-PN 200-110 and flunarizine on [^{3}H]-(+)-PN 200-110 specific binding in intact mesenteric arteries. Segments of mesenteric arteries were incubated either for 90 min in physiological solution in the presence of 100 pM [^{3}H]-(+)-PN 200-110 or for 60 min in physiological solution followed by 30 min in KCl-depolarizing solution both containing 40 pM [^{3}H]-(+)-PN 200-110. Those concentrations of radioligand labelled about 30 and 50% of the total number of binding sites respectively in polarized and in depolarized arteries, as calculated from the binding equation: $B = B_{max} \cdot L/(L + K_D)$, with B representing the amount of drug specifically bound in the presence of a ligand concentration L, using K_D (dissociation constant) and B_{max} (maximum binding capacity) values previously obtained (Morel & Godfraind, 1987). Non-specific binding amounted to about 65% and 25% of the total binding, respectively, in polarized and depolarized arteries, under the experimental conditions described above.

Nifedipine and (+)-PN 200-110 inhibited the specific binding of [^{3}H]-(+)-PN 200-110 dose-dependently in polarized as well as in depolarized arteries. In arteries incubated in KCl medium, the curve describing the displacement of the tritiated ligand by unlabelled (+)-PN 200-110 was shifted to the left compared to that obtained in NaCl-medium. This is in agreement with saturation studies, which showed a significant increase of [^{3}H]-(+)-PN 200-110 binding affinity in depolarized arteries com-

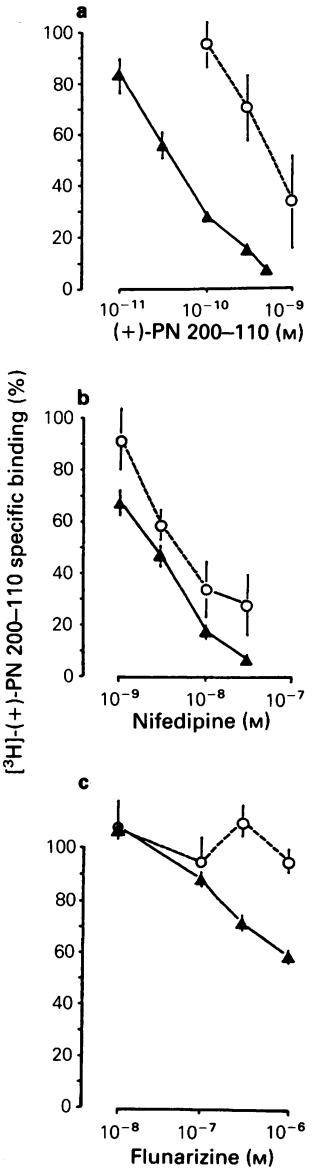

Figure 1 Displacement of [^{3}H]-(+)-PN 200-110 specific binding by (a) unlabelled (+)-PN 200-110, (b) nifedipine and (c) flunarizine in mesenteric artery segments bathed in physiological solution (○) or in KCl-depolarizing solution (▲). Mesenteric artery segments were incubated for 90 min in physiological solution containing 100 pM [^{3}H]-(+)-PN 200-110 and various concentrations of competitors or for 60 min in physiological solution followed by 30 min in KCl-depolarizing solution containing 40 pM [^{3}H]-(+)-PN 200-110 and various concentrations of competitors. Each value is the mean of 6 to 18 determinations. Vertical lines indicate s.e.means.

Table 1 Pharmacological and radioligand binding data for (+)PN 200-110, nifedipine and flunarizine in rat mesenteric artery

a *Concentrations producing 50% inhibition of the contractile response of rat mesenteric artery to 100 mM KCl-solution measured after 2 and 30 min contraction (IC_{50})*

	2 min	*30 min*
(+)-PN 200-110	270 ± 65 pM	33 ± 3.4 pM
Nifedipine	4.0 ± 0.6 nM	1.9 ± 0.2 nM
Flunarizine	37 ± 8 nM	2.0 ± 0.3 nM

b *Inhibition constants calculated from displacement of [^{3}H]-(+)-PN 200-110 specific binding in rat mesenteric artery* (K_i)

	Arterial segments		
	Physiological solution	*KCl-rich solution*	*Membrane preparation*
(+)-PN 200-110	410 ± 120 pM	25 ± 3 pM	55 ± 5 pM
Nifedipine	3.1 ± 0.9 nM	1.2 ± 0.3 nM	3 ± 0.5 nM
Flunarizine	no effect	900 ± 420 nM	500 ± 10 nM

Values are means ± s.e.means. K_i values were calculated from data shown in Figures 1 and 2, taking into account [^{3}H]-(+)-PN 200-110 K_D values of 200 pM for arterial segments bathed in physiological solution and 40 pM for arterial segments bathed in KCl solution (Morel & Godfraind, 1987). K_D was 59 pM in the membrane preparation. IC_{50} values were taken from: ((+)-PN 200-110) Morel & Godfraind, 1987; (nifedipine) Godfraind, 1983; (flunarizine) Godfraind & Dieu, 1981.

pared to arteries bathed in normal physiological solution (Morel & Godfraind, 1987). K_i values calculated from IC_{50} values according to Cheng & Prusoff are presented in Table 1. They are in good agreement with apparent K_D values estimated from saturation studies (Morel & Godfraind, 1987). In contrast to that observed with (+)-PN 200-110, the effectiveness of nifedipine was not significantly modified by prolonged depolarization and K_i values calculated from the displacement curves obtained in polarized and in depolarized arteries were not significantly different (Table 1). With both compounds the K_i values were close to the concentrations inhibiting by 50% the K^+-induced contraction of the rat mesenteric artery (Table 1).

Flunarizine up to 10^{-6} M was unable to displace [^{3}H]-(+)-PN 200-110 specific binding in arteries bathed in physiological solution, but it inhibited the binding in depolarized arteries. Higher concentrations of flunarizine induced non-reproductible perturbations of binding data (with modification of non-specific binding), probably due to the lipophilic nature of this compound. Fifty % inhibition was obtained in the presence of about 10^{-6} M flunarizine. This concentration is much higher than the concentration required to block the K^+-contraction in the same tissue (Table 1—Godfraind & Dieu, 1981).

Binding of [^{3}H]-(+)-PN 200-110 in membrane preparations from mesenteric artery

Competition experiments were carried out on the membrane fraction of the mesenteric artery incubated in NaCl-buffer in the presence of a fixed concentration of [^{3}H]-(+)-PN 200-110 (50–80 pM). Figure 2 shows the displacement curves obtained with unlabelled (+)-PN 200-110, nifedipine and flunarizine. All the compounds, including flunarizine, were able to displace the specific binding of [^{3}H]-(+)-PN 200-110. Curves were parallel and Hill coefficients were in all cases close to unity. K_i values calculated from IC_{50} values are presented in Table 1.

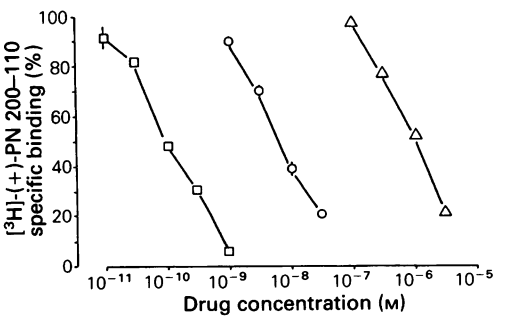

Figure 2 Displacement of [^{3}H]-(+)-PN 200-110 specific binding by unlabelled (+)-PN 200-110 (□), nifedipine (○) and flunarizine (△) in a membrane preparation from mesenteric artery. Membranes were incubated with [^{3}H]-(+)-PN 200-110 at 50 pM in the presence of increasing concentrations of unlabelled competitors. Each value is the mean of 2 separate determinations carried out in duplicate. Vertical lines indicate s.e.means.

Discussion

By performing binding experiments in the intact mesenteric artery this study allows the correlation of the binding and pharmacological properties of calcium entry blocking drugs. The results revealed a good correlation between the existence of time-dependency in the inhibition of KCl-induced contraction and the sensitivity of drug binding to depolarization. For all three compounds, binding data obtained with the membrane preparation from mesenteric arteries were similar to the data obtained with depolarized intact arteries.

Depolarization of vascular smooth muscle is known to increase the cell membrane permeability to calcium by opening membrane potential-dependent calcium channels (Bolton, 1979). Recently it has been found that different subtypes of potential-dependent calcium channels coexist in cells of the rat mesenteric artery (Bean *et al.*, 1986), as has already been demonstrated in cardiac and nerve cells (Carbone & Lux, 1984; Bean, 1985; Nilius *et al.*, 1985; Nowycky *et al.*, 1985). They present distinct patterns of voltage- and time-dependence: the first type of calcium channel is activated by a small depolarization and inactivates rapidly, the other requires stronger depolarization and inactivates slowly. Only this second type of calcium channel has been found to be sensitive to dihydropyridines (Bean *et al.*, 1986). Considering the kinetics and pharmacological properties of the two subtypes of calcium channel, it seems that the slowly inactivating channel mainly contributes to the tonic contraction induced by 100 mM KCl-depolarizing solution.

It has been shown that the inhibitory effect of (+)-PN 200-110 on KCl-contraction is markedly time-dependent (Morel & Godfraind, 1987; Wibo *et al.*, 1988). Binding studies have indicated that depolarization increases the binding affinity of arteries for this compound (Morel & Godfraind, 1987). Voltage-dependence of the binding of (+)-PN 200-110 was indeed confirmed by the shift of the displacement curves observed in depolarized arteries compared to polarized arteries. A similar depolarization-induced increase in the binding affinity for (+)-PN 200-110 has been obtained in cardiac cells (Kokubun *et al.*, 1986). In contrast to (+)-PN 200-110, depolarization did not significantly affect the potency of nifedipine in displacement studies. There was, however, a good correspondence between K_i and IC_{50} values of nifedipine, so that the weak influence of depolarization on nifedipine binding was in agreement with contractility studies, which showed that the inhibition produced by nifedipine on the KCl-contraction is affected to a lesser degree by the duration of the depolarization (the ratio of the IC_{50} of nifedipine measured at 2 and 30 min of depolarization is equal to 2, while it reaches 8 for (+)-PN 200-110—see Table 1).

These binding data indicate that, at pharmacologically relevant concentrations, (+)-PN 200-110 binding to resting mesenteric arteries is very low while nifedipine is able to bind significantly to the calcium channel at rest. The low binding of (+)-PN 200-110 in arteries incubated in physiological solution explains why inhibition of KCl-contraction is very low during the first minutes of depolarization, despite a long period of equilibration of the tissue with the drug. Thus, this is in agreement with the observation by Wibo *et al.* (1988) that the time-course of the binding of (+)-PN 200-110 to isolated membranes can account for the typical time-dependent pattern of the inhibitory effect of this compound.

On the other hand, the absence of a marked time-dependence in the effect of nifedipine can be accounted for by some interaction of nifedipine with calcium channels in resting tissues. Binding of nifedipine to resting calcium channels has also been observed in atrial cells, where nifedipine exhibits tonic block of calcium current at holding potentials negative enough to ensure that calcium channels were not inactivated (Uehara & Hume, 1985).

There was an important difference in the behaviour of flunarizine in arteries bathed in physiological- and KCl-depolarizing medium. It is unlikely that the inefficacy of flunarizine observed in physiological medium was due to an overestimation of the free concentration of this highly lipophilic compound. Indeed, flunarizine was active in the same tissue bathed in KCl-depolarizing solution and displaced [^{3}H]-(+)-PN 200-110 bound to the membrane fraction isolated from mesenteric arteries. This latter result is in agreement with previous data showing that flunarizine completely displaces dihydropyridine specific binding in membrane preparations from various tissues (intestinal smooth muscle, heart, brain) (Bellemann *et al.*, 1983; Bolger *et al.*, 1983; Gould *et al.*, 1984; Godfraind & Wibo, 1985). Furthermore, the absence of effect of flunarizine (10^{-6} M) on [^{3}H]-(+)-PN 200-110 specific binding in arteries bathed in physiological solution indicates that, at those concentrations, flunarizine did not cause non-specific disturbance of membrane structures that could affect the binding of dihydropyridine compounds. The potency of flunarizine was the same in depolarized arteries and in membrane fragments, since at 10^{-6} M it evoked 50% displacement of [^{3}H]-(+)-PN 200-110 binding. This selective effect of flunarizine on depolarized isolated tissues and on membranes thus indicates that flunarizine was able to interact with the dihydropyridine receptor only when membranes were depolarized by incubation of the tissue in KCl-rich solution, or

following tissue disruption. When binding data are compared to contractility data it appears that, like (+)-PN 200-110, flunarizine also exhibits a marked time-dependent inhibition of the KCl-contraction (Godfraind & Dieu, 1981). The influence of membrane potential on the effect of flunarizine could then reflect a dependence on calcium channel state, so that flunarizine does not have access to or has a very low affinity for resting channels, in a manner similar to that described for (+)-PN 200-110. It is interesting to compare the present results with Spedding's observation that increasing the negative surface charge increased the inhibitory effectiveness of flunarizine on the KCl-contraction of taenia while nifedipine potency was unchanged (Spedding, 1984). These effects have been ascribed to an increased accumulation of lipophilic compounds like flunarizine in the cell membrane. Alternatively, this author suggests that the increase of negative surface charge decreases the proportion of inactivated calcium channels, which could be responsible for the increase of flunarizine inhibitory potency. It is, however, difficult to fit the latter hypothesis with our observation that depolarization, which is known to inactivate channels, increased flunarizine potency. The low potency of flunarizine in displacement studies and the large degree of non-specific binding of this compound (Godfraind & Morel, 1981) do not allow a definite conclusion to be reached until the present observation can be confirmed, using other calcium channel ligands. It is worth pointing out that there is a good correlation between binding and pharmacological data for both nifedipine and (+)-PN 200-110, which confirms that the binding site specifically labelled by [^{3}H]-(+)-PN 200-110 in rat mesenteric artery is responsible for the inhibitory action of dihydropyridines on calcium entry. However, the interaction of flunarizine with the dihydropyridine binding site in the mesenteric artery occurs at concentrations of flunarizine higher than those required to inhibit the KCl-contraction of the same tissue, suggesting that the site of action of flunarizine is different from the specific binding site of dihydropyridine compounds. The existence of different sites of action for calcium antagonists in the calcium channel has been described in other tissue preparations. Binding studies indicate that the dihydropyridine binding site differs from that of verapamil or diltiazem (Murphy *et al.*, 1983; Glossmann *et al.*, 1985). It has been proposed that diphenylpiperazine compounds like flunarizine interact with the verapamil/diltiazem recognition site (Murphy *et al.*, 1983).

These results are compatible with the observation of different behaviours of calcium antagonists in functional tests (Spedding & Berg, 1984). They also support the classification of calcium antagonists into different sub-groups (Godfraind, 1987) and indicate that a selective interaction with inactivated calcium channels could be a major determinant of such a classification.

The authors thank N. Bussy for technical assistance. This work has been supported by grant F.R.S.M. No. 3-9006-87.

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(Received January 27, 1988
Revised April 7, 1988
Accepted April 15, 1988)