Br. J. Pharmacol. (1992), **105**, 480–484

Interaction of pinaverium (a quaternary ammonium compound) with 1,4-dihydropyridine binding sites in rat ileum smooth muscle

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1 The interaction of pinaverium bromide, a quaternary ammonium compound, with binding sites for (L-type) calcium channel blockers was investigated in rat ileum smooth muscle.

2 Pinaverium inhibited [^{3}H]-(+)-PN200-110 ([^{3}H]-(+)-isradipine) specific binding to tissue homogenates incompletely (K_i 0.38 μM; maximal inhibition 80%). In contrast, binding to single cell preparations (obtained by collagenase treatment) and to saponin-treated homogenates was completely inhibited. These data are compatible with the view that, in untreated homogenates, 20% of [^{3}H]-(+)-isradipine binding sites are not accessible to pinaverium because it is associated with sealed inside-out vesicles.

3 Pinaverium bromide increased the apparent K_D of [^{3}H]-(+)-isradipine binding to saponin-treated homogenates but did not significantly affect the B_{max} value. Moreover, the dissociation rate constant of [^{3}H]-(+)-isradipine binding was not changed by pinaverium. These data suggest that pinaverium interacts with the dihydropyridine binding site in a competitive manner. However, in contrast to uncharged dihydropyridine calcium antagonists, pinaverium inhibited, rather than stimulated, [^{3}H]-diltiazem binding to rat brain membranes (at 30–37°C).

4 Although B_{max} values of [^{3}H]-(+)-isradipine were similar in homogenates prepared from tissue and cells (collagenase-treated), the K_D value was significantly higher in cell homogenates (166 vs 95 pM). Similarly, the K_i value of pinaverium was higher in cell preparations than in tissue homogenates (0.77 vs 0.38 μM). Thus, collagenase can significantly modify the dihydropyridine recognition site.

5 The competitive interaction of pinaverium, a permanently charged drug, with [^{3}H]-(+)-isradipine bound to intact cells and its absence of interaction with [^{3}H]-(+)-isradipine bound to sealed inside-out vesicles imply that the dihydropyridine receptor lies near the external surface of the plasma membrane.

Keywords: Pinaverium bromide; collagenase; dihydropyridine Ca^{2+} antagonists; ileum smooth muscle; diltiazem

Introduction

Pinaverium bromide, a quaternary ammonium compound, has been recommended for the treatment of hypermotility disorders of the intestine, especially irritable bowel syndrome (for review see Christen, 1990). Earlier studies on smooth muscle tissue suggest that pinaverium exerts its spasmolytic action mainly by blocking voltage-dependent Ca^{2+} channels and has little effect on receptor-operated channels and intracellular Ca^{2+} stores (Droogmans *et al.*, 1983; Baumgartner *et al.*, 1985; Beech *et al.*, 1990). Its selectivity for the intestinal tract is attributable to its low absorption by the gut and its marked hepatobiliary excretion (Jacquot *et al.*, 1989).

In this paper, we have characterized the interaction of pinaverium bromide with specific binding sites for L-type channel blockers (Godfraind *et al.*, 1986). We carried out competition experiments on homogenates of longitudinal smooth muscle from rat ileum and on dissociated 'intact' cells obtained after treatment with collagenase; we have also investigated the influence of collagenase on binding parameters. The results show that pinaverium bromide interacts with dihydropyridine binding in an apparently competitive manner at concentrations reported to be active on Ca^{2+} channel currents. Analysis of this interaction suggests that pinaverium bromide, a positively charged drug, has access to the dihydropyridine binding site from the outside of the cell.

Methods

Single cell preparations

Single smooth muscle cells from rat ileum were prepared by the method of Momose & Gomi (1978) modified by Takayanagi *et al.* (1986). Wistar rats (250–350 g) were killed by decapitation and the ileum was quickly removed. The longitudinal muscle layers were peeled manually from the underlying circular muscle and incubated at 37°C for 90 min in a medium (mM: NaCl 137, KCl 2.7, $MgCl_2$ 1.0, $CaCl_2$ 0.18, glucose 5.6, HEPES 4.2; pH 7.4) bubbled with a gas mixture of 95% O_2: 5% CO_2. Tissue pieces were then incubated in a medium containing 0.1% purified collagenase (Sigma Ia) and 1% bovine serum albumin at 37°C for 30 min. The incubation was terminated by washing tissue fragments twice by centrifugation (500 *g* for 30 s). Pellets were resuspended in the incubation medium at 37°C and single cells could then be separated by gently pipetting the suspension through a wide-bore Pasteur pipette. The suspension of single cells was filtered through a nylon mesh (0.18 mm).

Membrane preparation

Freshly prepared tissue or single cells were suspended in about 10 volumes of an ice-cold solution containing 0.25 mM sucrose, 0.1 mM phenylmethylsulphonylfluoride and 5 mM Tris-HCl (pH 7.4), and homogenized with three strokes of the pestle rotating at 1500 rev min^{-1} in an all-glass Potter–Elvehjem-type grinder (Braun, Melsungen, Germany) kept at 2°C. Protein concentration was estimated according to Lowry *et al.* (1951).

In some experiments, tissue homogenates were treated by collagenase in the same way as were the muscle strips used for cell isolation. The incubation was then terminated by centrifugation at 200 000 *g* for 30 min and the pellet was resuspended with a Dounce homogenizer in ice-cold 0.25 mM sucrose buffered at pH 7.4 with 5 mM Tris-HCl.

Binding studies

The specific binding of [^{3}H]-(+)-isradipine was measured, as described previously (Wibo *et al.*, 1988), by incubating frac-

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Table 1 [^{3}H]-(+)-isradipine binding parameters in homogenates from rat ileum smooth muscle: effect of collagenase

	K_D (pM)	B_{max} (fmol mg^{-1} prot.)
Tissue homogenates (4)	95.2 ± 8.8	102.3 ± 15.5
Cells homogenates (3)	165.7 ± 6.4*	95.8 ± 6.8
Collagenase-treated tissue homogenates (3)	145.9 ± 3.0*	100.2 ± 10.2

Values are means ± s.e.mean from n (in parentheses) membrane preparations. [^{3}H]-(+)-isradipine binding parameters were estimated by linear regression (EBDA programme). *Significantly higher than the tissue homogenate value ($P < 0.01$, t test).

tions with the radioligand for 60 min at 37°C in 2.5 ml of a buffered salt solution of the following composition (mM): NaCl 145, KCl 5, $MgCl_2$ 1.25, $CaCl_2$ 2.5, Tris 20; pH 7.4 at 37°C. Non-specific binding was estimated in the presence of 1 μM nifedipine. After incubation, membranes collected on Whatman GF/F filters were washed twice with 10 ml of ice-cold 0.9% NaCl.

[^{3}H]-ouabain binding was measured in a 'Mg–Pi' medium (37°C, 60 min) as previously described (Noël & Godfraind, 1984). Non-specific binding was estimated in the presence of 1 mM ouabain. Membranes were collected on GF/F filters and washed twice with 10 ml of chilled 10 mM Tris-HCl (pH 7.4). In all binding assays, filters were immersed in Picofluor 15/ toluence (1/3, v/v) and radioactivity was counted with an efficiency of 40–45%.

For [^{3}H]-diltiazem binding studies, rat cerebral cortex membranes were prepared according to the method of Schoemaker *et al.* (1985). Membranes (50 μg of protein in a final volume of 0.5 ml) were incubated in 50 mM Tris-HCl buffer (pH 7.4) with 5 nM [^{3}H]-diltiazem for 60 min at 30° or 37°C. After incubation, membranes were harvested by centrifugation (10000 g for 10 min). The supernatant was removed by aspiration and the pellet was rapidly washed with 100 μl of ice-cold Tris-HCl buffer. The pellet was then solubilized in 100 μl of Soluene and its radioactivity was measured in the presence of 10 ml of Picofluor 15/toluene (1/3, v/v), with an efficiency of 40–45%.

Drugs

[^{3}H]-(+)-PN200-110 ([^{3}H]-(+)-isradipine, isopropyl 4-(2,1,3-benzoxadiozol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate; 85.9 Ci mmol^{-1}), [^{3}H]-diltiazem (77 Ci mmol^{-1}) and [^{3}H]-ouabain (15 Ci mmol^{-1}) were obtained from NEN Research Products (Boston, MA, U.S.A.). Pinaverium bromide (4-(6-bromoveratryl)-4-[2-{2-(10 norpinan-2-yl)ethoxy}ethyl] morpholinium bromide) was provided by Latema Sarbach (Suresnes, France). Nifedipine and nitrendipine were gifts from Bayer AG (Leverkusen, Germany) and (+)-*cis*-diltiazem from Synthelabo (Paris, France). Ouabain was from Merck (Darmstadt, Germany), and collagenase (type Ia) from Sigma Chemical Co (St Louis, MO, USA). Other chemicals used were of analytical grade. All the experiments with 1,4-dihydropyridines were carried out under yellow light to prevent their degradation.

Statistical analysis

Results of the experiments are expressed as means ± s.e.mean. Tests of significance have been made by Student's t test, P values smaller than 0.05 being considered significant.

In binding experiments, saturation isotherms were analysed by Scatchard and Hill plots. The dissociation constant (K_D) and B_{max} were calculated by linear regression. In competitive experiments, displacement curves were analysed by a sigmoid curve fitting programme (Munson & Rodbard, 1980). An iterative technique gave estimate of IC_{50} (concentration of displacer inhibiting 50% of specific binding) and the K_i value was calculated according to Cheng & Prusoff (1973).

Results

The specific binding of [^{3}H]-(+)-isradipine to tissue homogenates was saturable in the concentration range of 25–250 pM. As shown in Table 1, the values of K_D and B_{max} calculated from the Scatchard plots ($n = 4$) were 95.2 ± 8.8 pM and 102.3 ± 15.5 fmol mg^{-1} of protein, respectively. B_{max} values were similar in homogenates prepared from intact tissue and dissociated cells, unlike K_D values which were increased by 75% in cell homogenates. Since cell preparations had been submitted to the action of collagenase, we treated tissue homogenates with collagenase to study its eventual influence on [^{3}H]-(+)-isradipine binding. The results showed an increase of the K_D value by 50% and no significant variation of the B_{max} value (Table 1).

Pinaverium bromide inhibited [^{3}H]-(+)-isradipine specific binding to tissue homogenates incompletely (80% inhibition at 10–100 μM, Figure 1). Slopes of Hill plots (not shown) were close to 1. The K_i value derived from the concentration for half-maximal inhibition (IC_{50}) according to Cheng & Prusoff (1973) was 0.38 ± 0.10 μM (Table 2). The incomplete inhibition of [^{3}H]-(+)-isradipine binding by pinaverium bromide might

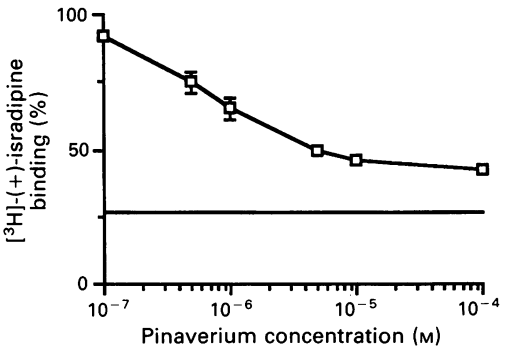

Figure 1 Inhibition by pinaverium of [^{3}H]-(+)-isradipine binding to homogenates of ileum smooth muscle. Competition experiments were performed with [^{3}H]-(+)-isradipine at a concentration near the K_D, 0.1 nM, and increasing concentrations of pinaverium. Total binding (specific + nonspecific) is expressed as a percentage of the value obtained in the absence of pinaverium. Data are means from 6 determinations (2 different preparations); vertical bars show s.e.mean when they are larger than symbols. Non-specific binding (horizontal line) was determined in the presence of 1 μM nifedipine and was significantly lower ($P < 0.01$; *t test*) than the value of total binding at each pinaverium concentration.

Table 2 Comparison of pinaverium binding parameters in tissue homogenates and 'intact' cells from rat ileum smooth muscle

	IC_{50} (μM)	K_i (μM)	*Hill coeff.*
Tissue homogenates (3)	0.68 ± 0.19	0.38 ± 0.10	0.96 ± 0.07
'Intact' cells (3)	1.33 ± 0.02*	0.77 ± 0.01*	0.98 ± 0.09

Values are means ± s.e.mean from n (in parentheses) preparations. IC_{50} values were estimated from displacement curves by a sigmoid curve-fitting iterative technique (EBDA programme). K_i values were calculated according to Cheng & Prusoff (1973).
* Significantly higher than the tissue homogenate values ($0.01 < P < 0.05$, t test).

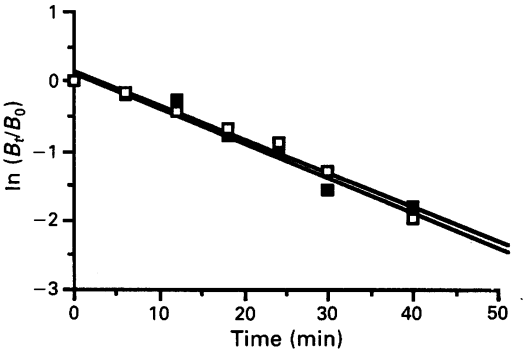

Figure 2 Effect of pinaverium bromide on the rate of dissociation of [^{3}H]-(+)-isradipine binding. After incubation with 0.1 nM [^{3}H]-(+)-isradipine for 60 min at 37°C, dissociation was induced by addition of 1 μM nifedipine in the presence (■) or absence (□) of 10 μM pinaverium. B_0 and B_t are specific [^{3}H]-(+)-isradipine bound at time 0 and time t, as indicated on the abscissa scale. Data are means from 6 determinations; s.e.means were smaller than symbols.

suggest that this drug allosterically interacts with the dihydropyridine recognition site, as previously found for other drugs (Murphy *et al.*, 1983; Glossmann *et al.*, 1985). However, in dissociation experiments (Figure 2), we did not observe any significant effect of pinaverium bromide (10 μM), the dissociation rate constant (k_{-1}) of [^{3}H]-(+)-isradipine being equal to 0.049 ± 0.009 min^{-1} and 0.048 ± 0.006 min^{-1} in the presence or absence of pinaverium, respectively. Moreover, in saturation experiments pinaverium at concentrations up to 10 μM did not significantly affect the B_{max} of [^{3}H]-(+)-isradipine binding (Figure 3).

The incomplete inhibition of [^{3}H]-(+)-isradipine binding by pinaverium bromide in tissue homogenates, might be explained by the presence of sealed vesicles, in which dihydropyridine binding sites are inaccessible to pinaverium, a positively charged drug. Indeed, in samples preincubated with saponin (0.5–1.0 mg mg^{-1} protein), the specific binding of [^{3}H]-(+)-isradipine was completely inhibited by pinaverium bromide (Table 3). Additional experiments showed that the addition of saponin did not alter [^{3}H]-(+)-isradipine binding up to a saponin/protein concentration ratio of 1 (results not shown). The existence of sealed vesicles in tissue homogenates was supported by the results of binding experiments with [^{3}H]-ouabain (Figure 4). In the presence of saponin (0.5 mg mg^{-1} protein), the K_D of [^{3}H]-ouabain binding was not modified (98 vs 96.5 nM), whereas the B_{max} increased from 452 to 557 fmol mg^{-1} protein.

Competition experiments were also carried out on single cells preparations that had not been homogenized. As shown

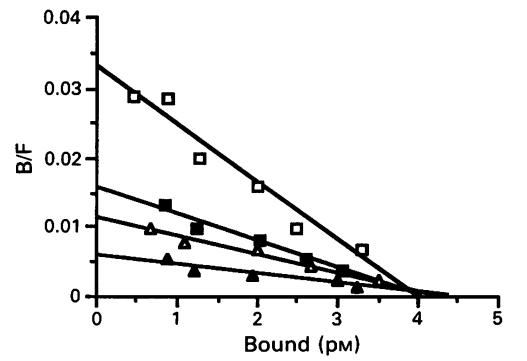

Figure 3 Effect of pinaverium on saturation curves of [^{3}H]-(+)-isradipine binding to homogenates of ileum smooth muscle. Saturation experiments were performed with pinaverium bromide at concentrations of 0.3 μM (■), 1 μM (Δ) and 10 μM (▲), or without drug (□). Results are shown as Scatchard plots. Samples were preincubated with saponin (0.5 mg mg^{-1} prot.) for 20 min at room temperature before incubation with [^{3}H]-(+)-isradipine. Each value is the mean of 3–6 determinations; s.e.means were smaller than symbols.

Table 3 Relationship between saponin concentration and inhibition of [^{3}H]-(+)-isradipine binding by 100 μM pinaverium

Saponin/protein ratio (w/w)	*Inhibition* (%)
No saponin	80.1 ± 0.8
1/3	88.7 ± 1.5
1/2	98.9 ± 0.1
1/1	99.0 ± 0.1

Tissue homogenates (750 μg protein ml^{-1}) were preincubated with various concentrations of saponin, as indicated (20 min at room temperature). Samples (200 μl) were then further incubated at 37°C for 60 min with 0.1 nM [^{3}H]-(+)-isradipine, in a final volume of 2.5 ml (see Methods). Data are means ± s.e.mean from 3 different preparations.

in Figure 5, pinaverium completely inhibited [^{3}H]-(+)-isradipine specific binding to intact cells in the absence of saponin treatment. Pinaverium IC_{50} and K_i values in single cells preparations were about twice as high as those found in tissue homogenates (Table 2).

It is well known that diltiazem, a benzothiazepine inhibitor of L-type Ca^{2+} channels, stimulates [^{3}H]-dihydropyridine binding at 30°–37°C, and that conversely, some dihydropyridines enhance the binding of [^{3}H]-diltiazem (Schoemaker & Langer, 1985). To gain further insight into the interaction of pinaverium with the dihydropyridine binding site, we compared its effect on [^{3}H]-diltiazem binding with that of nitrendipine. This experiment was carried out on a membrane

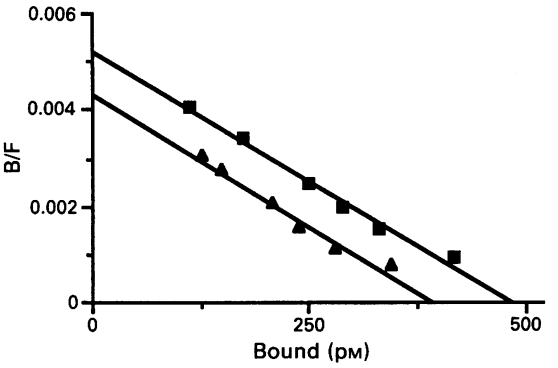

Figure 4 Effect of saponin on saturation curve of [^{3}H]-ouabain binding to homogenates of ileum smooth muscle. After 20 min preincubation at room temperature in the presence (■) or absence (▲) of saponin (0.5 mg mg^{-1} prot.), membranes were incubated at 37°C for 60 min with 100 nM [^{3}H]-ouabain and increasing concentrations of cold ouabain. Non-specific binding was determined in the presence of 1 mM ouabain. Results are shown as Scatchard plots; each value is the mean of 6 determinations.

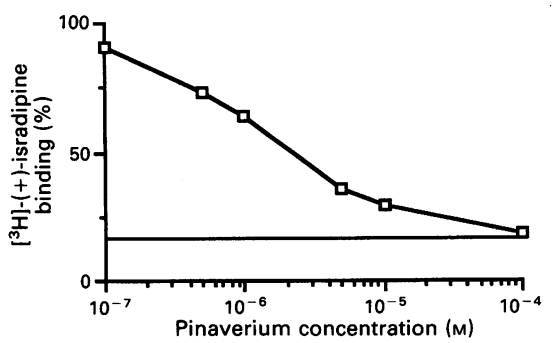

Figure 5 Inhibition by pinaverium of [^{3}H]-(+)-isradipine binding to cells isolated from ileum smooth muscle. Competition experiments were performed as described in legend of Figure 1, except that isolated cells were used instead of tissue homogenates. Data are means from 6 determinations (2 different preparations); s.e.means were smaller than symbols. Non-specific binding (horizontal line) was not significantly different from total binding at a pinaverium concentration of 100 μM.

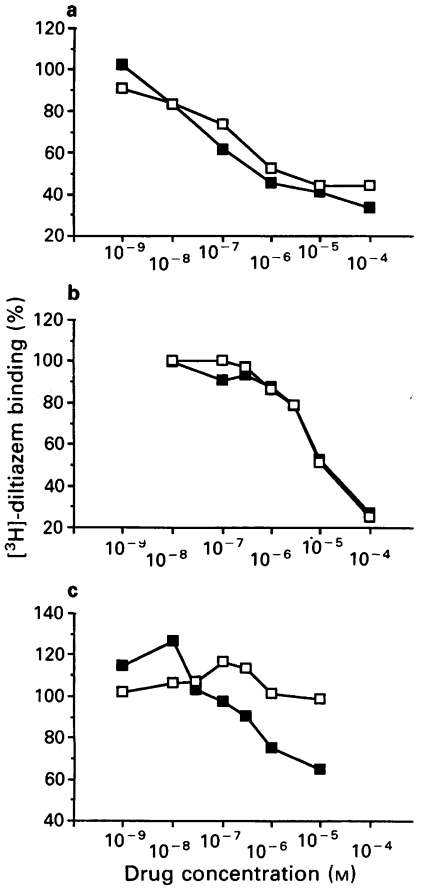

Figure 6 Effects of (+)-*cis*-diltiazem, pinaverium and nitrendipine on the binding of [^{3}H]-diltiazem to rat cerebral cortex membranes. Binding was measured at 30°C (■) or 37°C (□) as described in Methods, with 5 nM [^{3}H]-diltiazem and increasing concentrations of competitor: (a) (+)-*cis*-diltiazem; (b) pinaverium; (c) nitrendipine. Total binding (without non-specific binding subtracted) is expressed as a percentage of the value obtained in the absence of unlabelled ligand. Data are means of 3–6 determinations. The s.e.means (not shown) do not exceed 5% of the mean values.

fraction from rat brain, because we met with difficulties when using membranes from intestinal muscle (data not shown). As shown in Figure 6, (+)-*cis*-diltiazem inhibited [^{3}H]-diltiazem binding with an IC_{50} of 128 nM at 37°C and 42 nM at 30°C (Hill coefficient 0.6–0.7), in agreement with previous findings (Schoemaker & Langer, 1985). At 37°C, nitrendipine stimulated [^{3}H]-diltiazem binding at concentrations of 10–300 nM, whereas, at 30°C, it stimulated binding at 1–10 nM and inhibited binding at concentrations higher than 100 nM. Pinaverium had no detectable stimulatory effect, but inhibited binding at concentrations of 1–100 μM, and the curves at 30°C and 37°C were superimposable. The IC_{50} estimated from these curves by nonlinear regression was 5–6 μM, a value distinctly higher than that measured in [^{3}H]-(+)-isradipine displacement experiments (Table 2).

Discussion

As shown recently by Beech *et al.* (1990), pinaverium bromide is able to block voltage-dependent, L-type Ca^{2+} channels in rabbit intestinal smooth muscle cells. In binding experiments, pinaverium bromide appears to interact competitively at the dihydropyridine receptor, and the K_i value that we measured with 'intact' cells (0.77 μM) is in good agreement with the inhibitory potency in patch-clamp experiments (IC_{50} 1.5 μM). That the inhibition of dihydropyridine binding is competitive is supported by the following arguments. (1) [^{3}H]-(+)-isradipine specific binding could be completely inhibited by pinaverium bromide in 'intact' cells and saponin-treated homogenates and the Hill coefficients were close to 1. (2) The effect of pinaverium bromide on equilibrium binding parameters was compatible with a competitive inhibition, since, at concentrations up to 10 μM, this drug increased the apparent K_D of [^{3}H]-(+)-isradipine binding but did not significantly affect B_{max} (Figure 3). The average K_i calculated from the apparent K_D ($K_{D,app}$) values at 4 different concentrations of pinaverium by the equation: $K_{D,app} = K_D(1 + [\text{pinaverium}]/K_i)$, is 0.69 μM, in good agreement with the values deduced from the analysis of displacement curves (Table 2). (3) The dissociation rate constant of [^{3}H]-(+)-isradipine binding was not changed by 10 μM pinaverium bromide (Figure 2). Nevertheless, some subtle differences may be noted between the effects of pinaverium and dihydropyridines, a finding which is not unexpected in view of the different chemical structures involved and the presence of a positively charged group in pinaverium. In contrast to most dihydropyridine calcium antagonists, which stimulate [^{3}H]-diltiazem binding in a temperature-dependent manner, pinaverium inhibited [^{3}H]-diltiazem binding and its effect was identical at 30°C and 37°C. Interestingly, amlodipine, a predominantly charged dihydropyridine, enhances [^{3}H]-diltiazem binding (at 37°C) over a narrow concentration range and inhibits binding at concentrations higher than 1 μM (Burges *et al.*, 1989). Its behaviour is thus intermediary between those of pinaverium and uncharged dihydropyridines. Moreover, calcium channel blockade by pinaverium is not appreciably voltage-dependent, in contrast to the blockade evoked by dihydropyridine calcium antagonists (Beech *et al.*, 1990).

The complete inhibition of [^{3}H]-(+)-isradipine binding by pinaverium bromide in intact cells suggests that this permanently charged drug has access to the dihydropyridine binding site from the outside of the cell. The incomplete inhibition of [^{3}H]-(+)-isradipine binding in tissue homogenates is probably due to the presence of sealed inside-out vesicles, in which dihydropyridine binding sites are accessible to [^{3}H]-(+)-isradipine (uncharged) but not to pinaverium (positively charged). Saponin removed a permeability barrier in inside-out vesicles, which accounted for some 20% of [^{3}H]-(+)-isradipine binding sites in homogenates. This interpretation is confirmed by the effect of saponin on [^{3}H]-ouabain binding (Figure 3). It is well-known that ouabain binding sites are exposed on the outside of the plasma membrane and are, therefore, inaccessible to ouabain in sealed inside-out vesicles. The percentage of sealed inside-out vesicles as determined in this manner (18.8%), is in good agreement with the proportion of the [^{3}H]-(+)-isradipine binding sites that appear to be inaccessible to pinaverium bromide (20%). Thus, our findings support the view that the site of the calcium channel that interacts with dihydropyridines lies near the external surface of the plasma membrane, as previously proposed by Kass *et al.* (1989) from their study on the effect of external pH on the interaction of amlodipine (pK_a 8.6) with the calcium channel, and more recently, from the finding that a permanently charged (quaternary ammonium) dihydropyridine derivative inhibits L-channel current when applied extra-, but not intracellularly (Kass *et al.*, 1991). However, a recent study that used photoaffinity labelling to locate the dihydropyridine binding site in the Ca channel α_1 subunit of skeletal muscle claimed that this site has a cytosolic localization (Regulla *et al.*, 1991).

An additional conclusion that can be drawn from our results is that collagenase might be deleterious for the voltage-dependent Ca^{2+} channels. Indeed, the K_D of [^{3}H]-(+)-isradipine and the K_i of pinaverium bromide were both appreciably increased in cells obtained by collagenase digestion and in tissue homogenates treated with collagenase. These observations support the view, expressed by Rusch & Hermsmeyer (1988), that 'dissociating methods for cell dispersion cannot be regarded as innocent of influencing calcium channel viability'.

The authors wish to thank M. C. Hamaide for her technical assistance. This work was supported by the Fonds de la Recherche Scientifique Médicale (grant FRSM No. 3.9006.87) and by the Ministère de l'Education et de la Recherche Scientifique (grant Action Concertée No. 89/95-135).

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(Received April 16, 1991
Revised September 20, 1991
Accepted October 18, 1991)