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Subcellular localization of [^{3}H]-nitrendipine binding sites in guinea-pig ileal smooth muscle

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1 The binding of [^{3}H]-nitrendipine was studied in microsomal fractions isolated from guinea-pig ileal smooth muscle.

2 Only one class of specific binding sites was detected, with a K_D of 0.4 nM. For various dihydropyridine derivatives, including the stereoisomers of nimodipine and the 'Ca agonist' Bay K 8644, the potency for inhibition of [^{3}H]-nitrendipine binding correlated well with the reported pharmacological potency in smooth muscle preparations.

3 To establish the subcellular localization of [^{3}H]-nitrendipine binding sites, untreated and digitonin-treated microsomal fractions were subfractionated by isopycnic density gradient centrifugation. The density distribution of [^{3}H]-nitrendipine binding was markedly shifted by digitonin towards higher densities, as were the distributions of 5'-nucleotidase and [^{3}H]-ouabain binding, whereas the distributions of NADPH:cytochrome *c* reductase and NADH:cytochrome *c* reductase were hardly modified by digitonin.

4 It is concluded that most, if not all, [^{3}H]-nitrendipine binding sites in guinea-pig ileal smooth muscle are present in the plasma membrane, in agreement with the postulated mode of action of dihydropyridines as inhibitors of plasmalemmal Ca channels.

Introduction

Dihydropyridines are potent inhibitors of the influx of Ca evoked by various stimuli in smooth muscle and, to a lesser extent, in cardiac tissue (Godfraind, 1983: Janis & Scriabine, 1983). Tritiated dihydropyridines bind with high affinity to specific sites in membrane fractions from various tissues (reviewed by Janis & Scriabine, 1983; Miller & Freedman, 1984). In guinea-pig ileal smooth muscle, Bolger *et al.* (1983) found an excellent correlation between the potencies of nifedipine analogues for inhibition of the mechanical response to K^+-depolarization and for inhibition of [^{3}H]-nitrendipine binding to the microsomal fraction. These results provide strong evidence that the dihydropyridine binding sites are relevant for the action of these drugs as inhibitors of depolarization-stimulated contraction. However, up to now the subcellular distribution of these binding sites in guinea-pig ileal smooth muscle had not been investigated. In this respect, some fractionation studies conducted on cardiac tissue have suggested that, in addition to the sarcolemma, the sarcoplasmic reticulum, or at least a portion of it, might contain a high density of binding sites (Williams & Jones, 1983; De Pover *et al.*, 1983), while others have shown that in skeletal muscle the binding sites are concentrated in a specialized portion of the plasma membrane, i.e. transverse tubules (Fosset *et al.*, 1983; Glossmann *et al.*, 1983). Therefore, we thought it of interest to study, by analytical density gradient centrifugation techniques, the localization of [^{3}H]-nitrendipine binding sites in membrane fractions from guinea-pig ileal smooth muscle.

Methods

Tissue fractionation

Longitudinal smooth muscle (2 g) taken from female guinea-pig ileum was homogenized and fractionated by differential centrifugation as described previously (Wibo *et al.*, 1981a), except for the following modifications. The mitochondrial pellet was washed once (resuspension followed by centrifugation at 20,000 *g* for 30 min), then, in Method 1, the combined post-mitochondrial supernatants were spun at 100,000 *g* for

60 min in a W40/128 rotor (Heraeus-Christ). The microsomal pellet was suspended in 0.25 M sucrose buffered at pH 7.4 with 5 mM Tris-HCl. This suspension was loaded over Tris-buffered 31.5% (w/w) sucrose (density 1.14 at 0°C) in tubes of a S52/61 rotor, and then spun at 200,000 *g* for 2.5 h. The material banding at the interface was designated light microsomal fraction, and the pellet heavy microsomal fraction.

When a total microsomal fraction, isolated according to Method 1, was loaded over a continuous sucrose density gradient (instead of 31.5% sucrose), and thereafter centrifuged as above, a band of gel-like material appeared at densities higher than 1.19. This band was cumbersome when subfractions had to be recovered from centrifugation tubes after bottom puncturing. Thus, for analytical density gradient centrifugation, microsomal samples were prepared as described below (Method 2). The combined post-mitochondrial supernatants were loaded over 43% (w/w) sucrose (density 1.19) in tubes of the W40/128 rotor, and spun at 100,000 *g* for 90 min. The material banding at the interface was diluted with 3 mM imidazole-HCl (pH 7.4) to a final sucrose concentration of about 7%. Membranes were then pelleted by centrifugation at 100,000 *g* for 60 min and resuspended in imidazole-buffered 0.25 M sucrose.

Before analytical density gradient centrifugation, a portion of the microsomal fraction, prepared according to Method 2, was supplemented with digitonin (0.3 mg mg^{-1} protein; final concentration 0.7 mg ml^{-1}) as described previously (Morel *et al.*, 1981). Untreated and digitonin-treated microsomal samples were subfractionated by isopycnic density gradient centrifugation essentially as described by Morel *et al.* (1981), except that gradients were centrifuged at 200,000 *g* for 2.5 h. Densities (at 0°C) of subfractions were determined by refractometry. Density frequency histograms and median densities were obtained as described by Beaufay & Amar-Costesec (1976). Density limits were assumed to be 1.0 and 1.25.

Binding and enzyme assays

To measure [^{3}H]-nitrendipine binding, tissue samples (50 µl) were first diluted in glass tubes with 0.4 ml of a buffered salt solution (BSS) of the following composition (mM): NaCl 145, KCl 5. $MgCl_2$ 1.3, $CaCl_2$ 1.2 and Tris 20, pH 7.5. The samples were then incubated at 30°C with [^{3}H]-nitrendipine (New England Nuclear, 70–80 Ci mmol^{-1}), usually at a final concentration of about 0.3 nM. Protein concentration (Lowry *et al.*, 1951) did not exceed 100 µg ml^{-1}. After 45 min, incubated suspensions were diluted with 3 ml of ice-cold BSS and filtered on Whatman GF/F filters. Tubes were washed with 3 ml of ice-cold BSS and filters were further washed with two 10 ml portions of BSS. Non-specific binding was determined from samples incubated in the presence of 1 µM nitrendipine. The radioactivity retained on filters was counted in 10 ml of Picofluor (Packard)/toluene (1/4, v/v), with an efficiency of 40%.

To measure [^{3}H]-ouabain binding, tissue samples were preincubated 5 min at 37°C with saponin (0.5 mg ml^{-1}). This treatment increased the maximal number of binding sites without affecting the K_D (0.15 µM), presumably by disrupting the membrane barrier in inside-out plasma membrane vesicles (Wibo *et al.*, 1982). Samples (50 µl) were then further incubated for 40 min at 37°C after addition of a solution (0.4 ml) containing 30 nM [^{3}H]-ouabain (New England Nuclear; 18 Ci mmol^{-1}), 3 mM $MgCl_2$, 3 mM phosphate, 1 mM EGTA and 20 mM maleate, adjusted to pH 7.4 (37°C) with Tris (Noël & Godfraind, 1984). Bound radioactivity was determined as described for [^{3}H]-nitrendipine, except that the stopping and washing solution was ice-cold 10 mM maleate-Tris (pH 7.4). Non-specific binding, as determined in the presence of 0.6 mM ouabain, was very low and practically independent of the amount of protein filtered.

5'-Nucleotidase, NADPH:cytochrome *c* reductase, NADH:cytochrome *c* reductase and cytochrome *c* oxidase were assayed as described previously (Beaufay *et al.*, 1974; Wibo *et al.*, 1980), except that 5'-nucleotidase activity was measured on samples preincubated with saponin (see above). Enzyme activities presented in the legend to Figure 3 are expressed in units, one unit corresponding to 1 µmol of product formed per min.

Drugs

Dihydropyridines (Bayer AG) were dissolved in ethanol, at a concentration of 1–2 mM, and used under yellow light. Flunarizine (Janssen Pharmaceutica) was dissolved in boiling 0.25% tartaric acid, at a concentration of 1 mM.

Results

In a first series of experiments, we characterized dihydropyridine binding in the light and heavy microsomal fractions. Saturation experiments (Figure 1) revealed only one class of [^{3}H]-nitrendipine binding sites in both fractions, with a K_D of about 0.4 nM. This value is somewhat higher than that found by Bolger *et al.* (1983), which amounts to 0.16 ± 0.06 nM, possibly owing to a higher incubation temperature in our experiments (see Janis & Scriabine, 1983). The maximal number of binding sites (B_{max}) amounted to 2.33 and 0.29 pmol mg^{-1} protein, or 3.84 and 1.49 pmol g^{-1} tissue (wet weight), in the light and heavy fractions, respectively.

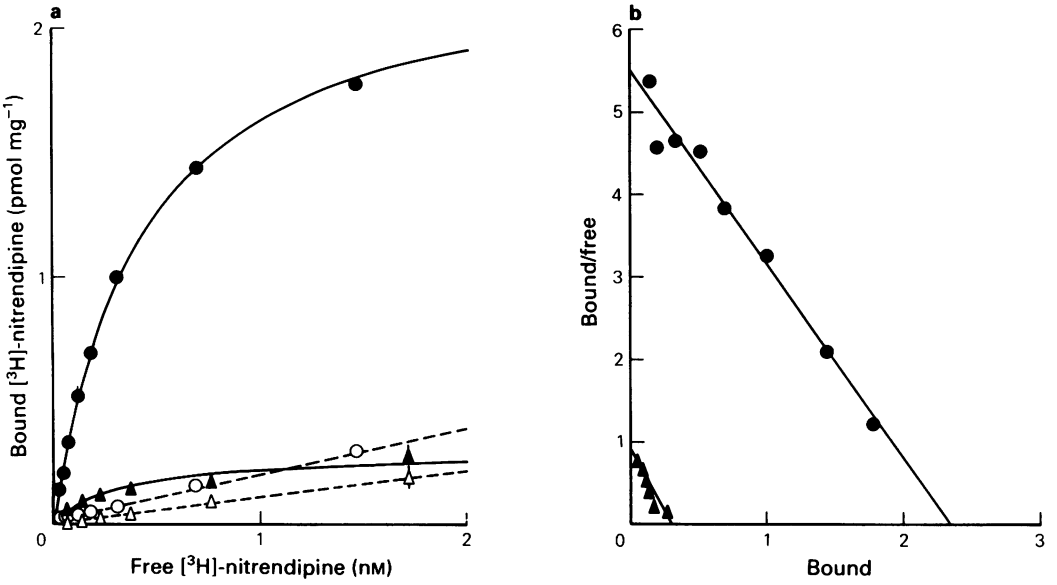

Figure 1 (a) [^{3}H]-nitrendipine binding as a function of increasing concentrations of radioligand. Samples from a light microsomal fraction (○, ●) and from a heavy microsomal fraction (△, ▲) were incubated as described under Methods. Closed and open symbols refer to, respectively, specific and non-specific binding. Each value is the mean from triplicate determinations; vertical lines indicate s.e.means when they exceed the size of the symbols. (b) Scatchard plots of the specific binding. From a linear regression analysis, the following parameters were obtained: K_D 0.43 nM (light fraction) and 0.33 nM (heavy fraction); B_{max} 2.33 pmol mg^{-1} protein (light fraction) and 0.29 pmol mg^{-1} (heavy fraction).

As the light fraction contained 70–80% of the microsomal binding sites and offered the advantage of a comparatively lower non-specific binding (see Figure 1), competition experiments were carried out on that preparation. Displacement curves obtained with several dihydropyridine derivatives and with flunarizine are shown in Figure 2. The curves are parallel and all compounds, including flunarizine, are able to displace completely [^{3}H]-nitrendipine from its binding site. IC_{50} values were obtained from Hill plots (not shown) and converted into inhibition constants (K_i) according to Linden (1982). The average pseudo-Hill coefficient was 1.11 ± 0.04 (n = 7). K_i values were (nM): nisoldipine 0.14, nitrendipine 0.33, (−)-nimodipine 0.47, nifedipine 1.36, (+)-nimodipine 2.29, Bay K 8644 9.8 and flunarizine 102.

Density distributions obtained from a total microsomal fraction after gradient centrifugation are shown in Figure 3. In the untreated preparation (thin line), [^{3}H]-nitrendipine and [^{3}H]-ouabain binding sites were identically distributed around a peak at a density of 1.12. The distribution of 5′-nucleotidase was similar, but more flattened. The two reductases showed rather broad distributions, which overlapped those of the other constituents, but extended towards higher densities. The effect of digitonin allowed two

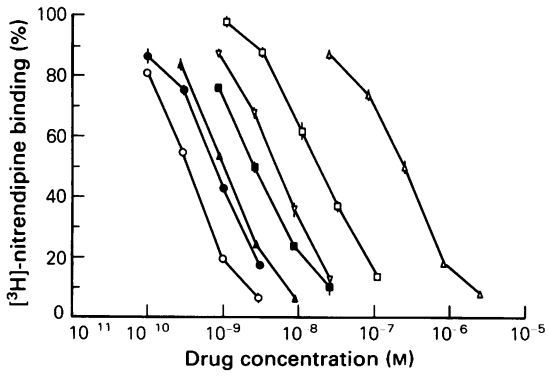

Figure 2 Displacement of specifically bound [^{3}H]-nitrendipine by various drugs. The radioligand was incubated at a concentration of 0.35 nM with increasing concentrations of unlabelled drugs. Each value is the mean from 3 determinations and vertical lines indicate s.e.means. Two light microsomal fractions were used. In the first experiment, displacement curves were obtained with nifedipine (■), (−)-nimodipine (▲), (+)-nimodipine (▽) and flunarizine (△). In the second experiment, nisoldipine (○), nitrendipine (●), Bay K 8644 (□) and nifedipine (not shown) were used. The K_i values for nifedipine in the two experiments were very similar (1.41 and 1.32 nM).

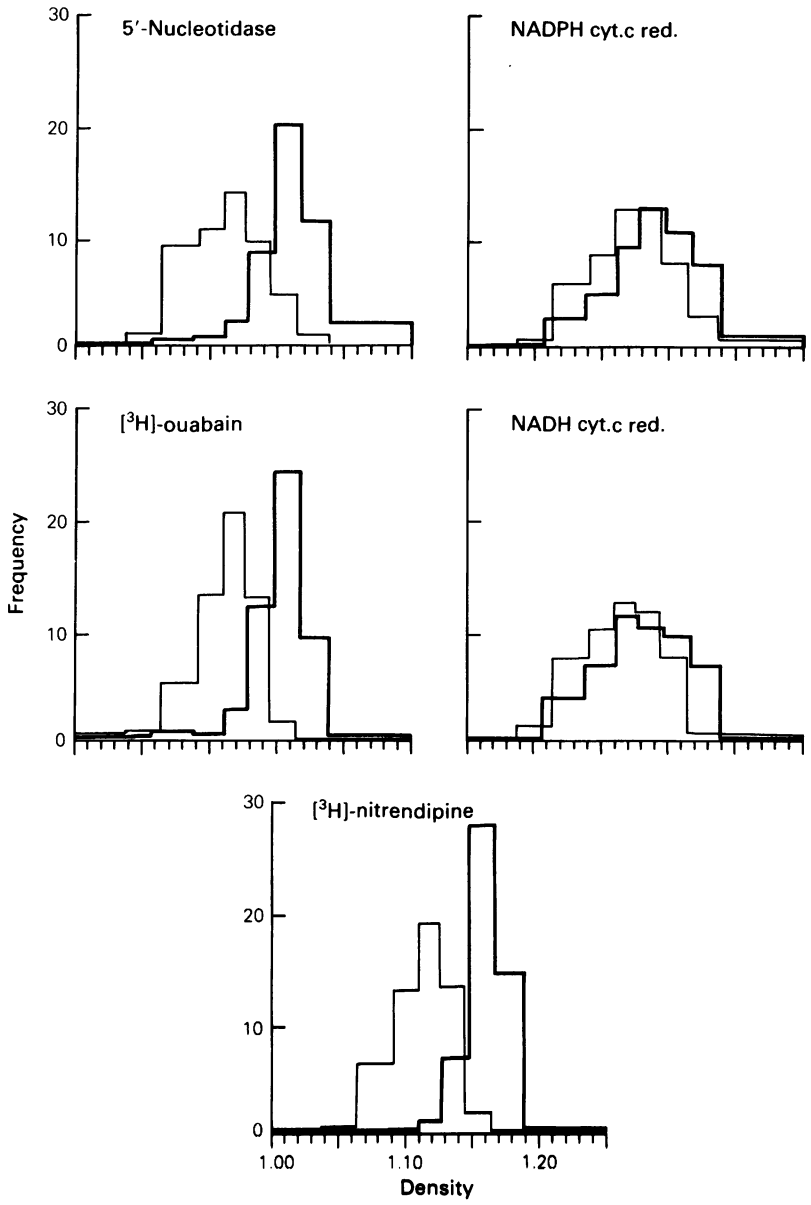

Figure 3 Density distribution histograms obtained from an untreated microsomal fraction (thin line) and from a microsomal fraction that had been treated with digitonin (thick line). Specific binding or enzyme activities in the total microsomal fraction, related to 1 g of tissue wet weight, were: 5'-nucleotidase 2.08 units; NADPH:cytochrome *c* reductase (cyt. c red.) 0.083 units; [^{3}H]-ouabain 17 pmol (radioligand concentration 30 nM); NADH:cytochrome *c* reductase 4.5 units; [^{3}H]-nitrendipine 1.38 pmol (radioligand concentration 0.25 nM); protein 4.08 mg. Specific binding or enzyme activities recovered in gradient subfractions, expressed as % of the total microsomal fraction, were: 5'-nucleotidase 100 (untreated sample) and 95 (digitonin-teated sample): NADPH:cytochrome *c* reductase 87 and 105; [^{3}H]-ouabain 85 and 64; NADH:cytochrome *c* reductase 81 and 92; [^{3}H]-nitrendipine 84 and 67. The frequency is the fractional amount of specific binding, or of enzyme activity, recovered in a given subfraction divided by the density increment across this subfraction. The abscissa of each panel reproduces the density scale indicated in the bottom panel.

groups of constituents to be distinguished more clearly. Indeed, in the digitonin-treated preparation (thick line) the distributions of 5'-nucleotidase, [^{3}H]-ouabain and [^{3}H]-nitrendipine were markedly shifted towards higher densities (peaks at 1.16) with respect to the untreated preparation, whereas the distribution profiles of the two reductases were little modified.

The close resemblance between the behaviours of [^{3}H]-nitrendipine binding, [^{3}H]-ouabain binding and 5'-nucleotidase was confirmed in other density gradient experiments. With 4 untreated and digitonin-treated microsomal fractions, median equilibrium densities were, respectively, 1.106 ± 0.003 (s.e.mean) and 1.143 ± 0.007 for [^{3}H]-nitrendipine binding; 1.108 ± 0.003 and 1.141 ± 0.006 for [^{3}H]-ouabain binding; 1.094 ± 0.006 and 1.137 ± 0.010 for 5'-nucleotidase.

The mitochondrial fraction bound about the same amount (per g tissue) of [^{3}H]-nitrendipine as did the microsomal fraction. Upon subfractionation of a mitochondrial fraction by density gradient centrifugation (not shown), the behaviour of [^{3}H]-nitrendipine binding, in particular its marked digitonin shift, was again similar to those of 5'-nucleotidase and [^{3}H]-ouabain binding, but markedly differed from that of NADPH:cytochrome *c* reductase. Moreover, in the untreated mitochondrial fraction, the distribution of the binding sites was almost completely resolved from that of the mitochondrial marker cytochrome *c* oxidase.

Discussion

As shown previously in extensive studies dealing with liver subcellular fractions (Amar-Costesec *et al.*, 1974; Wibo *et al.*, 1981b), a marked density shift after treatment with digitonin is a strong argument in favour of a plasma membrane localization. Digitonin greatly increases the equilibrium density of typical plasma membrane enzymes, such as 5'-nucleotidase, without affecting enzymes located entirely (NADPH: cytochrome *c* reductase) or mainly (NADH:cytochrome *c* reductase) in the endoplasmic reticulum. Digitonin reacts with cholesterol to form an insoluble, equimolecular complex, and the particular sensitivity of plasma membranes to digitonin is probably related to their high cholesterol content (Amar-Costesec *et al.*, 1974). We took advantage of this selective effect of digitonin in previous studies on smooth muscle subcellular fractions (Wibo *et al.*, 1980; 1981a; Morel *et al.*, 1981). The validity of this approach is supported by the marked digitonin shift of ouabain-specific binding sites, which in all likelihood may be identified with the plasmalemmal Na pump in guinea-pig smooth muscle as in other tissues (Gerthoffer & Allen, 1981).

Thus, our results clearly indicate that the dihydropyridine-specific binding sites recovered in microsomal and mitochondrial fractions from guinea-pig ileal smooth muscle are mostly, if not exclusively, associated with plasma membranes. In their recent fractionation study, Grover *et al.* (1984) also concluded that the plasma membrane is the locus of the high-affinity nitrendipine binding in rat gastric fundus smooth muscle and in rat myometrium. We feel, however, that the digitonin method, as applied in our work, is more discriminative than conventional cell fractionation and allows more definitive conclusions to be drawn.

The plasma membrane localization of [^{3}H]-nitrendipine binding sites is in full agreement with the view that dihydropyridines inhibit smooth muscle contraction by interfering with plasma membrane Ca channels (Bolger *et al.*, 1983; Godfraind, 1983). Bolger *et al.* (1983) found a good correlation between inhibition of [^{3}H]-nitrendipine binding by nifedipine analogues and inhibition of mechanical response to K^+-depolarization in guinea-pig ileal smooth muscle. The competition experiments shown in Figure 2 confirm and extend the binding data of Bolger *et al.* (1983). We found in particular that (−)-nimodipine was 5 fold more potent than (+)-nimodipine at displacing [^{3}H]-nitrendipine. Interestingly, a similar potency ratio (4–7) was observed by Towart *et al.* (1982) on K^+-induced contractions of vascular smooth muscle preparations. We also found that the affinity of the 'Ca agonist' Bay K 8644 for the dihydropyridine binding site of ileal smooth muscle ($K_i = 9.8$ nM) correlates with its potency as a contracting agent on a partly depolarized rabbit aortic strip ($EC_{50} \approx 3$ nM; Schramm *et al.*, 1983) or on guinea-pig taenia coli ($EC_{50} = 14$ nM; N. Morel & T. Godfraind, unpublished results). Finally, the K_i of flunarizine (102 nM) is in fair agreement with its potency as inhibitor of the response of guinea-pig ileal smooth muscle to various stimulant agonists (Van Nueten & Janssen, 1973), which suggests that this compound could inhibit Ca entry by interacting with the dihydropyridine binding site.

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