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INHIBITION BY SULOCTIDIL OF [^{3}H] NITRENDIPINE BINDING TO CEREBRAL CORTEX MEMBRANES

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Abstract—[^{3}H] Nitrendipine binds specifically with high affinity and high capacity ($K_A \simeq 0.20 \pm 0.01$ nM, $B_{max} = 4.4 \pm 0.3$ pM/g tissue, means ± S.E.M.; N = 4) to guinea-pig cerebral cortex membranes. Suloctidil fully inhibits [^{3}H] nitrendipine binding with a K_i value of 0.45 μM. The interaction between suloctidil and the putative Ca^{2+} channels is allosteric as shown by competition experiments performed in the presence of D 600 or diltiazem. Comparison of the activity of some close analogs of suloctidil provides evidence for the importance of the amino group and of the hydrophobic amino substituent in the interaction of suloctidil and the putative Ca^{2+} channel. It is suggested that part of the previously reported blockade of Ca^{2+} entry induced by suloctidil is due to a blockade of the Ca^{2+} channels.

Recently putative Ca^{2+} channels have been identified in various mammalian tissues using radiolabelled analogs of 1,4-dihydropyridines ([^{3}H] nitrendipine, [^{3}H] nimodipine) [1–8]. [^{3}H] Nitrendipine binding is regulated by cations, including Ca^{2+}; their effects correlate with their physiological actions on voltage-dependent Ca^{2+} channels [5, 6]. The different classes of Ca^{2+} antagonists interact with the putative Ca^{2+} channels labelled by [^{3}H] nitrendipine but in a complex manner suggesting allosteric interactions [4, 5, 7]. Furthermore, there is a good relationship between the potencies of the Ca^{2+} antagonists to alter binding and to depress contractions in different muscle preparations [7–9]. This observation leads to the conclusion that the binding site labelled by 1,4-dihydropyridine analogs at least partly mediates the pharmacological effects of these compounds.

Suloctidil has a complex pharmacological profile with two main properties, a vascular antispasmodic activity and an inhibitory action on platelet aggregation [10–13]. Its effect on vascular smooth muscle suggests that blockade of membrane mechanism responsible for Ca^{2+} entry regulation plays a central role [13, 14]. This conclusion was confirmed by the similarity of effects of suloctidil and verapamil on pancreatic islets functions [15] and on the translocation of Ca^{2+} in artificial models [16]. These data prompted us to study the interaction of suloctidil with the putative Ca^{2+} channels, by means of [^{3}H] nitrendipine binding.

MATERIALS AND METHODS

Membrane preparation. Isolated membranes were prepared essentially according to Ehlert *et al.* [4]. Briefly, male guinea-pigs (250–350 g) were killed by decapitation. All subsequent operations were conducted at 4°. The cerebral cortex was quickly removed and homogenized with a polytron in a 50 mM Tris–HCl, pH 7.4 buffer to a final concentration of 100 mg original weight/ml buffer. The homogenate was filtered through four layers of cheese cloth and washed four times by centrifugation at 46,000 *g* for 10 min followed by resuspension of the pellet in fresh 50 mM Tris–HCl, pH 7.4 buffer. The final pellet was resuspended to a concentration of 50 mg original wet tissue weight/ml buffer. The homogenate was stored in liquid nitrogen until use.

Binding assay. Aliquots (200 μl) of tissue homogenate were incubated in duplicate with the appropriate amount of [^{3}H] nitrendipine and other drugs in a final volume of 2 ml containing 50 mM Tris–HCl, pH 7.4. Incubations were carried out in the dark for 90 min at 25°. Membrane bound [^{3}H] nitrendipine was separated from free radioligand by rapid filtration over glass fiber filter (GF/C, Whatman, Maidstone, England). The filters were rinsed with two aliquots (8 ml) of 50 ml Tris–HCl, pH 7.4 buffer. Trapped radioactivity was measured by liquid scintillation spectrophotometry with 50% efficiency. Binding in the presence of 1 μM nifedipine was defined as non-specific and represented 15–25% of total binding. K_i values for interaction of the different drugs with the binding sites were calculated by the method of Cheng and Prusoff [17] using the equation:

$$K_i = \frac{IC_{50}}{1 + \frac{[A]}{K_A}}$$

where IC_{50} is the concentration of drug inhibiting [^{3}H] nitrendipine specific binding by 50%, A is the concentration of [^{3}H] nitrendipine and K_A is the affinity constant value for [^{3}H] nitrendipine determined from Scatchard analysis [18].

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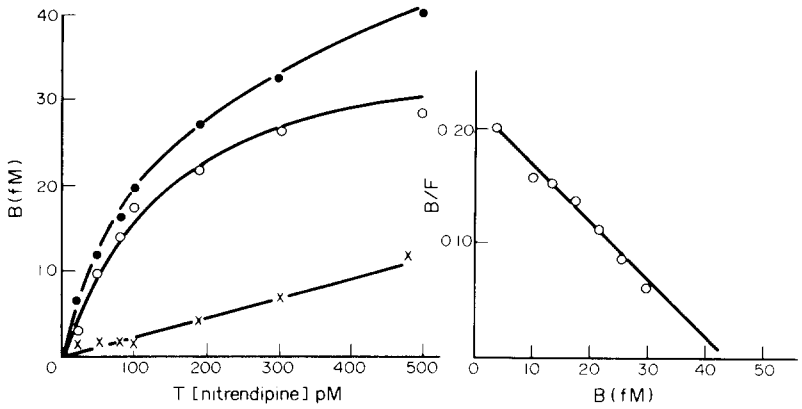


Fig. 1. Isotherm of absorption (left panel) and Scatchard plot of [^{3}H] nitrendipine specific binding (right panel) to guinea-pig cortex membrane. In the left panel, the symbols (●, ×, ○) represent the total, non-specific and specific binding respectively; in the right panel, B represents the specific binding. Cortex homogenates were incubated with different concentrations of [^{3}H] nitrendipine (0.02–0.5 nM) for 90 min at 25°. T, B and F refer to total, bound and free [^{3}H] nitdrendipine respectively. Each value is the mean of 4 experiments. Variation was less than 10%.

Chemicals. [^{3}H] Nitrendipine (specific activity 85.4 Ci/mmole) was purchased by New England Nuclear (Dreieich, F.R.G.). Suloctidil [1-(4-isopropylthiophenyl)-2-*n*-octylamino-1-propranol], CP 894 S [1-(4-isopropylthiophenyl)-2*n*-octylthio-1-propanol], CP 555 S/B [1-(4-isopropylthiophenyl)-2-*n*-butylamino-1-propanol] and CP 1136 S [1-(4-isopropylthiophenyl)-2-amino-1-propanol] were synthetized by the Department of Organic Chemistry of Continental Pharma. The other compounds were from the following sources: Bayer AG (nifedipine), Tanabe Seiyaku (diltiazem) and Knoll AG (D 600).

Suloctidil stock solution was a glucuronate salt. Stock solution of the other compounds were made in ethanol at such a concentration that the alcohol concentration neither exceed 0.5% in the assay. Dilution was performed with distilled water. Neither glucuronate nor ethanol interferred with the tracer binding in the range of concentration used.

RESULTS

(a) *Characteristics of* [^{3}H] *nitrendipine binding to guinea-pig cortex membranes.* Incubation of increasing amounts of [^{3}H] nitrendipine with guinea-pig cortex membrane in absence and in presence of 1 μM nifedipine resulted in a specific binding of 75–85% of the total binding (Fig. 1, left panel). The binding was maximal at 0.6–0.7 nM. Scatchard analysis of the binding isotherm indicated that the radioactive ligand binds to a single class of high affinity receptors

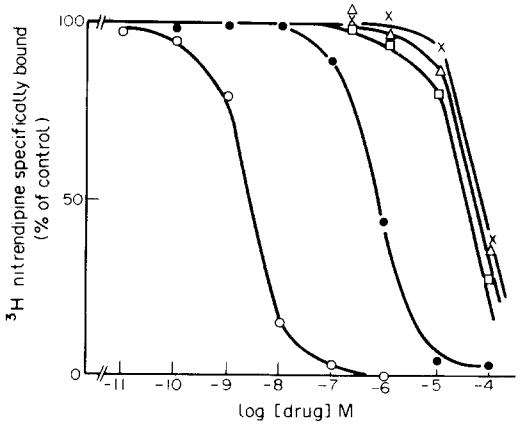
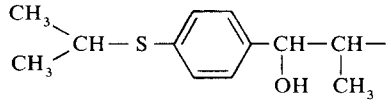


Fig. 2. Inhibition of [^{3}H] nitrendipine specific binding by nifedipine (○), suloctidil (●), CP 555 S/B (□), CP 894 S (△) and CP 1136 S (×) in guinea-pig cortex membranes. Tissue homogenates were incubated with 0.2 nM [^{3}H] nitrendipine for 90 min at 25°. Values are expressed in percent of the control [^{3}H] nitrendipine binding in absence of competing drug. Each value is the mean of 3–5 experiments. Variation was less than 10%.

Table 1. Chemical structure and constant of inhibition of specific binding (K_i) by suloctidil and related compounds to Ca^{2+} channels in guinea-pig cerebral cortex membranes

Name	Structure	K_i (μM)*
Suloctidil	 —NH—$(CH_2)_7$—CH_3	0.45 ± 0.04
CP 894 S	—S—$(CH_2)_7$—CH_3	30 ± 0.3
CP 555 S/B	—NH—$(CH_2)_3$—CH_3	20 ± 4
CP 1136 S	—NH_2	40 ± 4

* K_i values were calculated using the equation [17] $K_i = \frac{IC_{50}}{1 + \frac{[A]}{K_a}}$ where IC_{50} is the concentration of drug inhibiting [^{3}H] nitrendipine binding by 50% determined in Fig. 2; A is the concentration of [^{3}H] nitrendipine (0.2 nM) and K_A is the affinity constant value for [^{3}H] nitrendipine determined from Scatchard analysis (Fig. 1). Results are means ± S.E.M. of 3–5 experiments.

(Fig. 1, right panel) with an average dissociation constant (K_D) of 0.20 ± 0.01 nM (mean ± S.E.M., N = 4) and a capacity of 4.4 ± 0.5 pM/g tissue (mean ± S.E.M., N = 4). Specific binding is reversible (not shown).

(b) *Effects of suloctidil on* [3H] *nitrendipine binding*. As shown in Fig. 2, [3H] nitrendipine specific binding was fully inhibited by suloctidil with an IC_{50} value of 0.9 μM. Three compounds closely related to suloctidil (Table 1) interfered also with [3H] nitrendipine specific binding but at a higher concentration. At 100 μM, the highest concentration tested, the [3H] nitrendipine specific binding is not fully displaced. IC_{50} values can be tentatively determined and are of 40, 60 and 80 μM respectively for CP 555 S/B, CP 894 S and CP 1136 S. K_i value for the three compounds are 44 to 89 times higher than the K_i value for suloctidil (Table 1). Inhibition of [3H] nitrendipine specific binding by nifedipine yielded an IC_{50} of 2.0 nM (K_i = 1.0 nM) in agreement with published data [2, 6, 7]. Hill coefficients for the competition curves of nifedipine and suloctidil are 1.10 and 1.08 respectively.

(c) *Allosteric interaction of suloctidil with* [3H] *nitrendipine binding site*. To test whether suloctidil interacted allosterically with [3H] nitrendipine binding, its effect was tested in the presence of D 600 or diltiazem. In a first series of experiments, competition curves of [3H] nitrendipine binding by suloctidil were performed in the presence of increasing concentrations of D 600 (Fig. 3). This compound, which maximally inhibited [3H] nitrendipine binding by 25% (Fig. 4, right panel) produced rightwards shifts in the concentration response curves of [3H] nitrendipine by suloctidil. IC_{50} values of suloctidil were reduced by a factor of 2–60, this factor increasing with the concentration of D 600. These data indicate that suloctidil interacts allosterically with the [3H] nitrendipine binding sites at the level of the Ca^{2+} channels.

To ascertain the allosteric character of the interaction, competition curves were performed for diltiazem or D 600, alone and in the presence of increasing concentrations of suloctidil. As expected [6, 7], diltiazem stimulated binding when tested alone (Fig. 4, left panel). The stimulation increased gradually from 0.1 to 10 μM where it was maximal and amounted to ≈30% of the [3H] nitrendipine binding in absence of drug. Co-incubation of diltiazem and suloctidil reversed the inhibition of tracer binding by suloctidil. Contrasting with diltiazem, D 600 did inhibit the [3H] nitrendipine binding with an IC_{50} value which can be estimated around 0.1 μM.

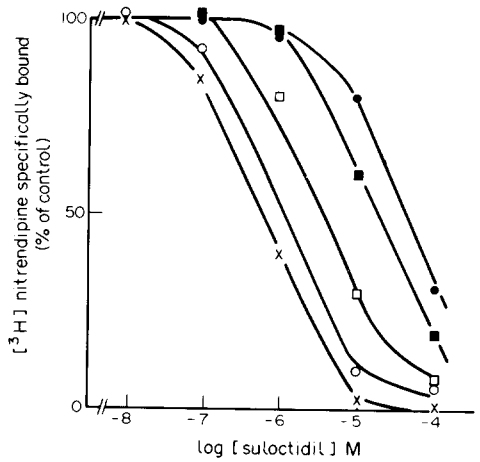


Fig. 3. Inhibition of [3H] nitrendipine specific binding by suloctidil alone (×) and in the presence of increasing concentrations of D 600, respectively 0.03 μM (○), 0.3 μM (□), 3 μM (■), 30 μM (●), in guinea-pig cortex membranes. Cortex homogenates were incubated with 0.2 nM [3H] nitrendipine for 90 min at 25°. Values are expressed in percent of the corresponding control [3H] nitrendipine binding. Each value is the mean of 3 experiments. Variation was less than 10%.

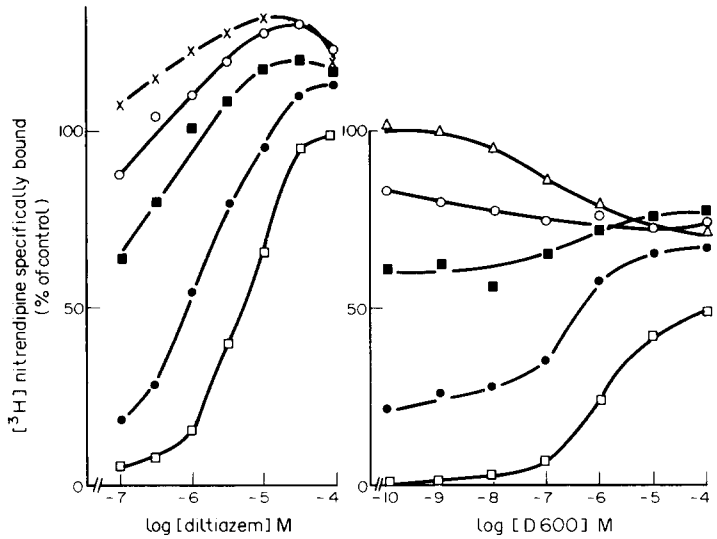


Fig. 4. Effect of diltiazem (left panel), and D 600 (right panel) on the inhibition of [3H] nitrendipine specific binding by suloctidil. Suloctidil concentrations were 0.3 μM (○), 1.0 μM (■), 3.0 μM (●) and 10.0 μM (□). The effects of diltiazem (X) and D 600 (△) alone are also represented. Cortex homogenates were incubated with 0.2 nM [3H] nitrendipine. Each value is the mean of 3–5 experiments. Variation was less than 10%.

These properties were in agreement with published data [4, 6, 7]. Simultaneous incubation of suloctidil and D 600 overcame the inhibition of [^{3}H] nitrendipine binding produced by suloctidil in a dose-dependent manner. Up to 3 μM suloctidil the tracer binding was restored to the level observed with the highest concentrations of D 600 alone.

DISCUSSION

The present data indicate that suloctidil interacts with the putative Ca^{2+} channels labelled with [^{3}H] nitrendipine. Compared to published data obtained in the same experimental conditions with various diphenylalkylamines and phenylalkylamines [7], suloctidil has a similar IC_{50} value (0.9 μM). Indeed, it can be calculated from the concentration–response curves obtained in the same experimental conditions [7], that IC_{50} values for prenylamine, flunarizine, lidoflazine and tiapamil ranged between 0.3 and 0.8 μM. Furthermore, based on the two following effects: (1) the rightward shift of the displacement curve of [^{3}H] nitrendipine binding by suloctidil induced by D 600; (2) the reversal by D 600 and diltiazem of the inhibition of [^{3}H] nitrendipine binding by suloctidil, it is apparent that suloctidil interacts allosterically with the putative Ca^{2+} channels. Based on the present binding data, suloctidil can thus be classified as a Ca^{2+} channel antagonist. Whether the site of action of suloctidil is common to that of all the Ca^{2+} channel antagonists [7] or can be subclassified [6] remains to be determined. It should be noted that suloctidil completely displaces [^{3}H] nitrendipine from its binding site. However, this site is clearly different from the 1,4-dihydropyridines binding site.

Although limited, the comparison of the inhibition of [^{3}H] nitrendipine binding by suloctidil and its three analogs leads to the following conclusions. The three analogs are able to displace [^{3}H] nitrendipine. As the 1-(4-isopropylthiophenyl) propanol moiety is common to the four structures, it appears determinant in the interaction with the putative Ca^{2+} channel. However they are 44–89 times less potent that suloctidil. Additional features thus ensure the lower IC_{50} value of suloctidil. Substitutions of the amino group of suloctidil by a thio group (CP 894 S) increases drastically the IC_{50} value, pointing out the importance of the amino group in the interaction with the binding site. Shortening or elimination of the alkyl chain substituting the amine (CP 555 S/B, CP 1136 S) increases also the IC_{50} values. This suggests the importance of an alkyl substituent on the amino group with an adequate hydrophobic content.

Suloctidil is a highly lipophilic compound with a measured partition coefficient (log P) between buffer and neutral phospholipids of 2 [19]. It might therefore interfere with the ^{3}H-nitrendipine binding indirectly via an interaction with the lipidic component of the membrane. Nevertheless, a non-specific displacement of [^{3}H] nitrendipine must be excluded. Indeed as shown in Fig. 2, suloctidil displaces the same amount of radioactivity as nifedipine. At its IC_{50} value ($\simeq$ 0.9 μM), suloctidil has only a marginal effect on membrane fluidity as measured by fluorescence depolarization of diphenylhexatriene in artificial [19] as well as natural membranes, synaptosomes [20] or platelets [21]. By contrast, at the same concentration, CP 894 S has an effect on membrane fluidity which is twice that of suloctidil [19, 20]. Although, CP 894 S fluidifies the lipid matrix to a greater extent than suloctidil, it has no effect on [^{3}H] nitrendipine binding in the range of concentrations where suloctidil displaces the tracer. At its IC_{50} value ($\simeq$ 60 μM), CP 894 S has a large effect on membrane fluidity. However, even in this case, a non-specific interaction of the compound with the lipid matrix may not be the cause of [^{3}H] nitrendipine displacement since a compound like CP 1136 S which has no hydrophobic substituent and has no effect on membrane fluidity [19] inhibits [^{3}H] nitrendipine with the same potency than CP 894 S.

Suloctidil displays various activities *in vivo* [13] and *in vitro* in pharmacological studies [14, 15] and in physico-chemical systems [16] which lead to the suggestion that the effect of the compound could be attributed to a blockade of the membrane mechanism of Ca^{2+} intake. Reported IC_{50} values are of 2 μM for the inhibition of the rat aorta contraction induced by Ca^{2+} [14], and of 1.25 μM for the inhibition of insulin release in pancreatic islets [15]. They are close to the IC_{50} values of [^{3}H] nitrendipine binding obtained in this study. It might thus be concluded that at least part of the blockade by suloctidil of the membrane mechanism for Ca^{2+} intake is due to a specific interaction with Ca^{2+} channels.

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