Br. J. Pharmac. (1985), **85**, 767–774

Characterization of [^{3}H]-nitrendipine binding to uterine smooth muscle plasma membrane and its relevance to inhibition of calcium entry

S. Batra

Department of Obstetrics and Gynaecology, University of Lund and Aktiebolaget Leo Research Laboratories[1], Box 941, S251 09 Helsingborg, Sweden

1 Specific, high affinity (K_D = 164 pM) binding of the Ca channel inhibitor [^{3}H]-nitrendipine was identified in plasma membrane-enriched fractions from the rat myometrium.

2 Although dihydropyridines effectively competed for [^{3}H]-nitrendipine binding sites, both verapamil and D600 were poor competitors. Diltiazem (10 μM) increased [^{3}H]-nitrendipine binding by about 40%, but had no effect on binding affinity. Among several other drugs tested, diethylstilboestrol (DES) caused a considerable inhibition of binding, with an IC_{50} value of 4 μM.

3 Both La^{3+} and EDTA (or EGTA) inhibited binding. The inhibition by the latter could be overcome by the addition of Ca^{2+} or Mg^{2+}.

4 A clear relationship was found between [^{3}H]-nitrendipine binding and 5'-nucleotidase activity in the various subcellular fractions.

5 Data on K^+-stimulated Ca^{2+} influx in the intact uterine strips showed a good agreement between the inhibition by both nitrendipine and DES of stimulated Ca influx and their inhibitory effect on [^{3}H]-nitrendipine binding to plasma membrane. This type of correlation was lacking in the case of D600.

6 These results suggest that Ca channels in the myometrial membrane possess multiple sites at which different drugs can act to block these channels.

Introduction

Smooth muscle contraction is initiated by depolarization-induced calcium entry into the myoplasm through voltage-dependent calcium channels. Various organic compounds with a considerable structural diversity are known to block these channels. Dihydropyridines represent the most potent class of calcium entry blockers. Recently, high affinity binding to various tissues, including heart and smooth muscle, has been described for one of the most potent dihydropyridine calcium entry blockers, namely [^{3}H]-nitrendipine (DePover *et al.*, 1982; Bolger *et al.*, 1982; Holck *et al.*, 1982; Triggle *et al.*, 1982; Williams & Tremble, 1982). In the present study, binding of this radioligand to isolated plasma membranes from uterine smooth muscle was used to characterize calcium channels in this tissue. To investigate the pharmacological relevance of the binding data, calcium influx, using ^{45}Ca, in intact uterine strips was also measured.

[1]Correspondence

Methods

Preparation of subcellular fractions

Female Sprague-Dawley rats weighing 150–200 g were ovariectomized and after about 2 weeks injected with polyoestradiol phosphate (Estradurin, Leo, Sweden), 2 mg kg^{-1} i.m. With this treatment, steady and not too high levels of oestradiol in the blood are maintained for at least 7 days (Batra *et al.*, 1978). The rats were killed 4 days after the injection and uterine horns removed. Generally, uteri from 12–15 rats were collected. After trimming excess fat and connective tissue the uterine horn was cut open and endometrium removed by thorough scraping.

The method for the isolation of various subcellular fractions was essentially similar to that described by Jaqua-Stewart *et al.* (1979), with some modifications. The tissue, after being weighed, was homogenized in about 4 volumes of sucrose-HEPES buffer with a Polytron homogenizer (PGA 10–35) for 2 s at half

speed. The homogenate was centrifuged at 200 *g* for 3 min. The supernant fluid was removed with a Pasteur pipette and saved. The residue was resuspended in the original volume of buffer and homogenized and centrifuged again as above. This process of residue homogenization and centrifugation was repeated six times and the supernatant fluid after each centrifugation was kept. The pooled supernantant was filtered through two layers of gauze and the filtrate centrifuged at 175,000 *g* for 20 min in a Beckman L-5-65 centrifuge. The crude membrane pellet (A), after discarding the supernatant, was suspended in 20 ml of buffer and subjected subsequently to centrifugation on a discontinuous sucrose gradient. Three sucrose solutions, 6 ml (1.5 M), 4 ml (1.22 M) and 2.5 ml (1.1 M) were layered successively in 6 (20 ml) Beckman cellulose nitrate tubes. Four and a half millilitres of the above crude membrane suspension were layered on top of the last sucrose layer and centrifuged at 106,000 *g* for 1.5 h in a Beckman L-5-65 centrifuge using a SW 25 rotor. The top band separating at the interface of the gradient and subcellular suspension was enriched in plasma membrane and was called PM. Fractions containing fragments of endoplasmic reticulum (ER) and mitochondria (M) were removed in the following bands, respectively. The residue at the bottom containing nuclei, myofibrils and cell debris was discarded. The PM, ER and M bands from each of the six gradient tubes were carefully aspirated with a Pasteur pipette, combined and suspended in sucrose-HEPES buffer. They were then centrifuged at 175,000 *g* for 30 min. The final pellet from each fraction was suspended in sucrose-HEPES buffer to give a protein concentration of approximately 1 mg ml^{-1} and stored at −70°C until used for nitrendipine binding experiments.

Radioligand binding studies

The standard binding assay was performed in KCl (100 mM) HEPES (20 mM) medium, pH 7.2 in a total volume of 0.5 ml for each assay tube. The protein concentration in each assay tube was approximately 50 μg and the [^{3}H]-nitrendipine concentration in various assays varied between 0.05 and 1 nM. In order to measure non-specific binding a second set of tubes containing additionally 0.25 μM nitrendipine was run. Non-specific binding accounted for 15–20% of the total binding. The binding reaction was initiated by addition of membrane protein and incubation was allowed to proceed unless otherwise stated for 30 min at 25°C. The reaction was terminated by filtration of 0.4 ml of the incubation mixture through Whatman GF/F glass fibre filters. The filters were washed three times with 4 ml of buffer and then dropped in a counting vial containing 10 ml Instagel and 1 ml 5% HCl in methanol. The radioactivity was counted in a Packard 4640 Liquid Scintillation Spectrophotometer. When needed, identical experiments were done using ER and M fractions. The protein concentration was determined by the method of Lowry *et al.* (1951).

Determination of 5'-nucleotidase activity

The assay mixture for the determination of 5'-nucleotidase contained 10 mM each of adenosine 5'-monophosphate and $MgCl_2$ in Tris-HCl buffer (100 mM pH 7.5) and about 100 μg of membrane protein in a total volume of 0.2 ml. After incubation at 37°C for 20 min, the reaction was terminated by the addition of 3.8 ml ice-cold trichloroacetic acid. The liberated phosphate was measured after extraction with butyl acetate according to the method of Sanui (1974).

Measurement of ^{45}Ca influx

The net increase in the influx of calcium into the uterine smooth muscle following K^+-depolarization was estimated by measuring the increase in ^{45}Ca content of the uterine strip before and after depolarization. Lanthanum, which has been shown to displace extracellular calcium while having no effect on intracellular calcium (van Breemen *et al.*, 1972; Godfraind, 1974), was used to remove ^{45}Ca in the extracellular space. The washing of uterine tissue in ice-cold $LaCl_3$-containing solution ensured removal of extracellular calcium as well as inhibition of the active extrusion of intracellular calcium (Batra, 1982).

The uterine horns were cut open longitudinally and endometrium removed. Strips weighing about 8–10 mg were equilibrated for 45 min in a physiological Na-HEPES solution (composition, mM: NaCl 135, KCl 4.6, $MgCl_2$ 1.2, $CaCl_2$ 1.5, glucose 11, HEPES 10) maintained at 37°C and bubbled with 100% O_2. After preincubation for 15 min in 5 ml of the above solution with or without test substance, the uterine strips were incubated for 2 min in Na-HEPES solution containing ^{45}Ca to allow exchange of extracellular calcium with the tracer. Thereafter the strips were incubated additionally for 2 min in ^{45}Ca-containing Na-HEPES solution (unstimulated ^{45}Ca uptake) or depolarizing K-HEPES solution (composition, mM: NaCl 4.6, KCl 135, $MgCl_2$ 1.2, $CaCl_2$ 1.5, glucose 11, HEPES 10). This short period of exposure to ^{45}Ca and K^+ stimulation ensured that essentially the net calcium influx was being measured. The tissues were then taken out and rinsed quickly (about 5 s) in a large volume (150 ml) of La-HEPES solution (composition, mM: NaCl 125, KCl 4.6, MgCl 1.2, $LaCl_3$ 10, glucose 10, HEPES 10) maintained at 2°C. Uterine strips were then washed for 45 min in 5 ml of ice-cold (2°C) La-HEPES solution. After washing, the tissues

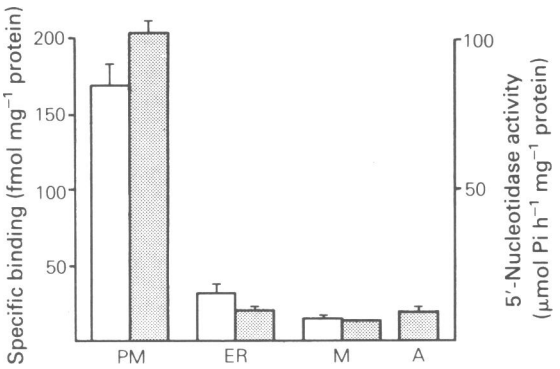

Figure 1 Specific binding of [^{3}H]-nitrendipine (open columns) and 5'-nucleotidase activity (stippled columns) in plasma membrane (PM), endoplasmic reticulum (ER) and mitochondrial (M) fractions of the rat myometrium. For nitrendipine binding subcellular fractions were incubated with 0.5 nM [^{3}H]-nitrendipine for 30 min at 25°C. Non-specific binding was measured by the addition of 0.25 μM nitrendipine. 5'-Nucleotidase was also measured in crude membrane pellet (A) for comparison. Each column represents the means and vertical lines s.e.mean of three or four separate determinations.

were lightly blotted with a filter paper, placed in scintillation vials and weighed. To each vial, 1 ml of Soluene-350 was added and the vials placed in a shaking water bath at 37°C for 3–4 h; this resulted in a complete digestion of the tissue. After the addition of 10 ml Instagel to the vial, radioactivity of the samples was counted in a liquid scintillation counter. The radioactivity was related to the apparent tissue content of calcium (μmol g^{-1} wet weight).

Drugs and chemicals

Tritiated nitrendipine with a specific activity of 72 Ci $mmol^{-1}$ was purchased from New England Nuclear Corporation. Authentic dihydropyridines were a gift from Bayer (Sweden). The radiochemical purity of [^{3}H]-nitrendipine was checked by h.p.l.c. Over 95% of the radioactivity was obtained in a single peak. Nitrendipine was protected from light during use and storage and it was checked periodically for purity. Verapamil and D600 (methoxyverapamil) were gifts from Knoll (West Germany) and diltiazem from Ferrosan (Sweden).

Results

Binding of nitrendipine in subcellular fractions

The data on specific binding of nitrendipine (Figure 1) to plasma membrane (PM) endoplasmic reticulum (ER) and mitochondrial (M) fractions clearly show that the binding in PM was 6 and 16 times higher than that in ER and M fraction, respectively. That PM fraction was indeed enriched in fragments derived from the cell membrane, as indicated by the distribution of 5'-nucleotidase which was used as a marker for PM (Figure 1). These data show that PM content of the fraction was about 10 times greater than that in the original crude membrane preparation (column A) or ER fraction and 20 times higher than that in the M fraction.

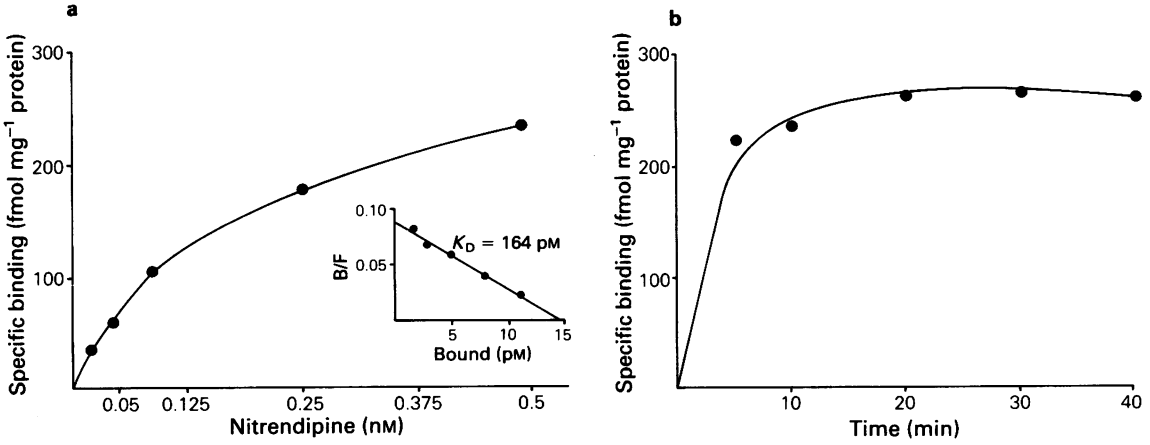

Figure 2 (a) A typical experiment of saturation and Scatchard analyses of specific binding of [^{3}H]-nitrendipine to the plasma membrane (PM) fraction of the rat myometrium; PM was incubated for 30 min at 25°C with various concentrations of nitrendipine. (b) Time-course of association of [^{3}H]-nitrendipine binding to PM fraction; PM was incubated with 0.5 nM [^{3}H]-nitrendipine at 25°C for various time intervals as described in Methods. Each point is the mean of four determinations.

Characteristics of nitrendipine binding to plasma membrane

Figure 2a shows the specific [^{3}H]-nitrendipine binding which is the difference between the binding obtained in the absence (total) and presence of 0.25 μM nitrendipine (non-specific binding). Non-specific binding was 10–15% of the total binding. A Scatchard analysis of these data (inset) indicated a single population of binding sites with a very high affinity (K_D = 164 pM). The density of binding sites in the myometrial PM was 315 fmol mg^{-1} protein.

At 25°C half maximum binding occurred at approximately 4 min with complete saturation achieved within 30 min (Figure 2b). Preliminary data showed that at 37°C nitrendipine binding was considerably reduced.

Inhibition of nitrendipine binding by dihydropyridines and other calcium entry blockers

Nitrendipine was most effective at competing with [^{3}H]-nitrendipine, and was followed closely by nimodipine and nifedipine, for binding to PM (Figure

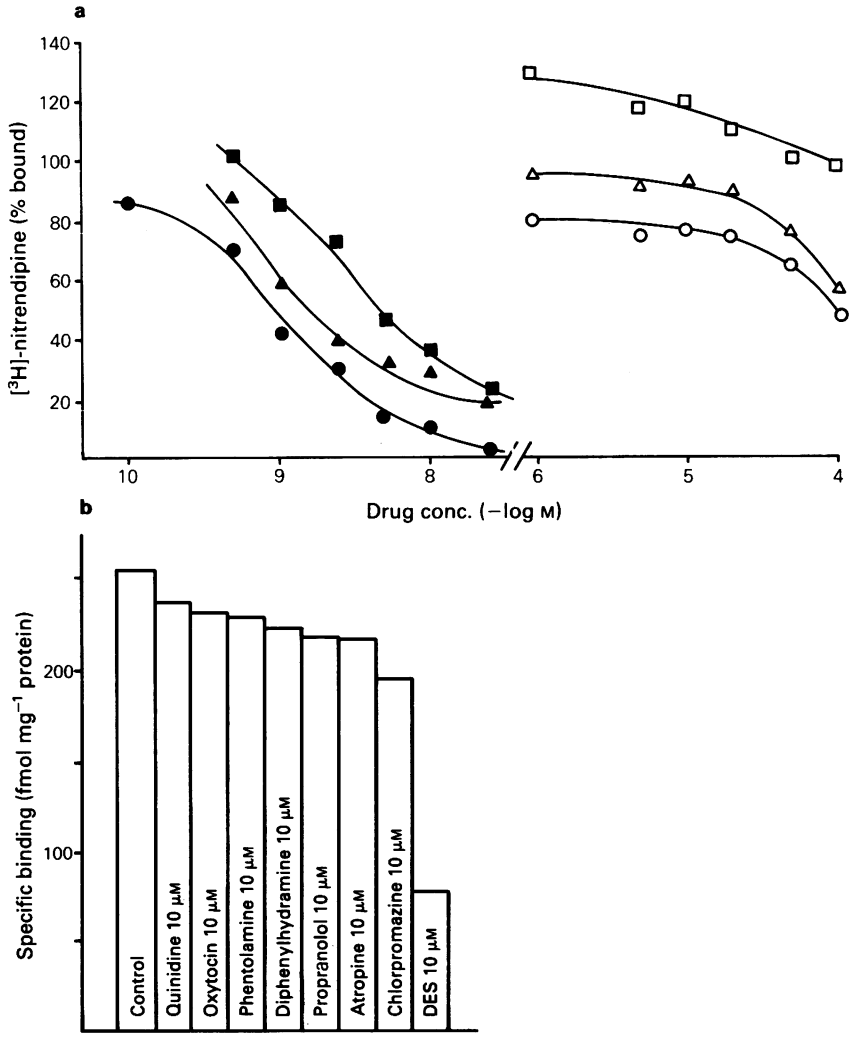

Figure 3 Inhibition of [^{3}H]-nitrendipine binding to plasma membrane fraction (a) by nitrendipine (●), nimodipine (▲), nifedipine (■), verapamil (○), D600 (△), diltiazem (□) and (b) by various other drugs (DES = diethylstilboestrol). The IC_{50} values for nitrendipine, nimodipine and nifedipine were 0.9, 1.6 and 4.5 nM respectively. Incubations were carried out at 25°C for 30 min. Medium [^{3}H]-nitrendipine concentration was 0.5 nM. Values are means of duplicate determinations on two separate preparations.

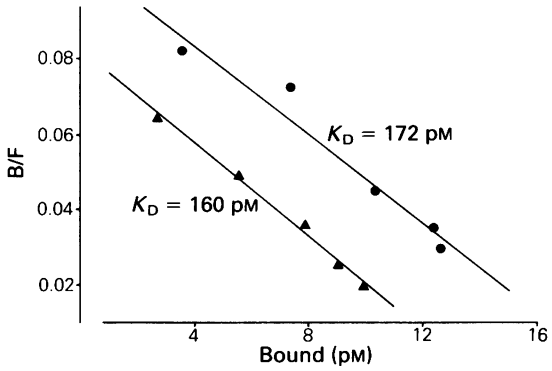

Figure 4 Scatchard analysis of the effect of diltiazem on [^{3}H]-nitrendipine binding to the plasma membrane (PM) fractions. Specific binding of [^{3}H]-nitrendipine was measured as described in Figure 2 in the absence (▲) and presence (●) of 10 μM diltiazem.

3a). The K_i values for these dihydropyridines, which were calculated from the IC_{50} values (Cheng & Prusoff, 1973) were 0.35 nM, 0.61 nM and 1.7 nM for nitrendipine, nimodipine and nifedipine, respectively. Both verapamil and its methoxy derivative D600 were poor competitors for [^{3}H]-nitrendipine binding sites. In fact diltiazem increased binding and the maximum increase was obtained with a concentration of 1–10 μM (Figures 3a and 4). With lower concentrations of diltiazem (1–10 nM), there was little or no increase in nitrendipine binding (data not shown).

Several other drugs were tested for their inhibition of [^{3}H]-nitrendipine binding using 10 μM of each drug and the results are shown in Figure 3b. The most interesting result was the inhibition caused by diethylstilboestrol (DES), which amounted to 70% and the IC_{50} value for DES inhibition was found to be 4 μM (see Figure 5b). Inhibition by other drugs was relatively weak, the highest being 25% caused by chlorpromazine.

The effects of diltiazem on the kinetics of nitrendipine binding were further examined by saturation and Scatchard analysis of the data on [^{3}H]-nitrendipine binding to PM obtained in the presence of 10 μM diltiazem. Data shown in figure 4 clearly indicate that while diltiazem increases nitrendipine binding, it has no effect on the affinity of binding since almost parallel lines were obtained in a Scatchard plot.

Effect of cations and chelating agents

Up to 1 mM calcium or magnesium seemed to have no effect on nitrendipine binding. However, addition of lanthanum, EDTA (or EGTA, not shown) considerably inhibited binding (Figure 5a) and this loss in binding induced by EDTA could be restored by the addition of calcium or magnesium. Results of the inhibition by lanthanum and EDTA (Figure 5b) indicated that the concentrations causing 50% inhibition (IC_{50}) for these agents were 110 and 30 μM, respectively. The inhibition of binding by different concentrations of DES is also shown in Figure 5b.

Calcium influx in uterine strips

These were performed in order to check the pharmacological relevance of the data obtained in ligand binding studies. Using appropriate concentrations, the effect of nitrendipine (Figure 6a), DES and D600

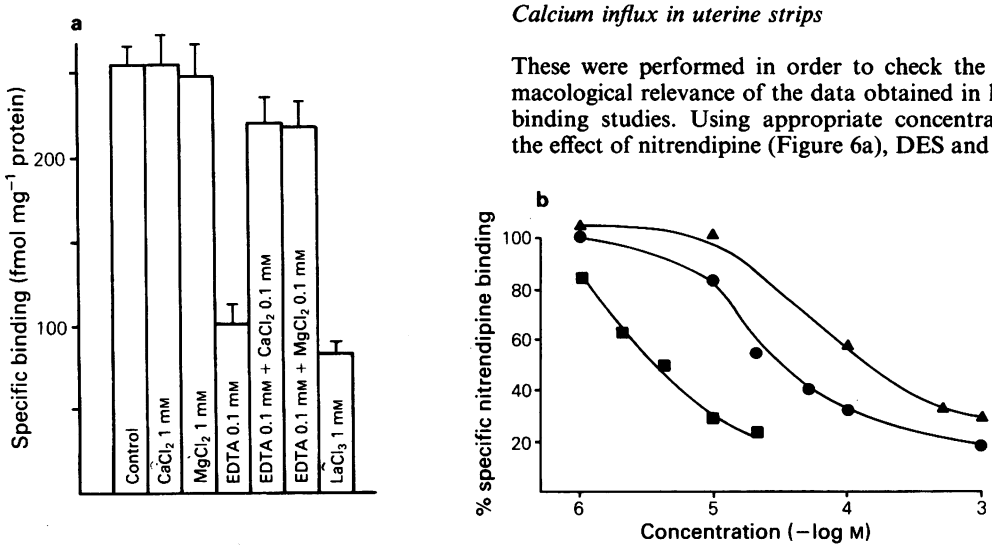

Figure 5 (a) Effect of cations and chelating agents on [^{3}H]-nitrendipine binding to the plasma membrane fraction and (b) inhibition of this binding by different concentrations of diethylstilboestrol (DES; ■), EDTA (●) and lanthanum (▲). The IC_{50} values for DES, EDTA and lanthanum were 4, 30 and 110 μM, respectively. Incubations were carried out as described in Figure 4. Values are means, and vertical lines show s.e.means, of three separate determinations.

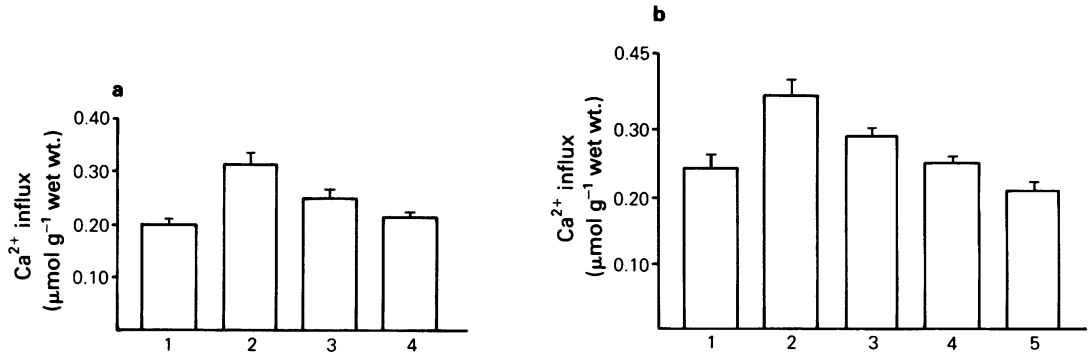

Figure 6 Inhibition by (a) nitrendipine (b) diethylstilboestrol (DES) and D600 of K^+-stimulated Ca^{2+} influx in oestrogen treated rat myometrium. In both (a) and (b) control (unstimulated) Ca^{2+} influx is shown by column 1 and column 2 represents K^+-stimulated Ca^{2+} influx in the absence of inhibitors. In (a) columns 3 and 4 represent K^+-stimulated Ca^{2+} influx in the presence of nitrendipine, 1 and 10 nM respectively. In (b) columns 3, 4 and 5 represent K^+-stimulated Ca^{2+} influx in the presence of DES 5 and 10 μM and D600 10 μM, respectively. Each column represents the mean and vertical lines s.e.mean of 4 separate determinations.

(Figure 6b) were studied on K^+-stimulated ^{45}Ca influx into the uterine cells. With 1 nM nitrendipine, which is close to its IC_{50} value for inhibition of binding (Figure 3a), almost 50% inhibition of ^{45}Ca influx was obtained (Figure 6a); nearly complete inhibition was observed with a concentration of 10 nM. Likewise 5 μM DES, which caused about 60% inhibition ($IC_{50} = 4$ μM) of [^{3}H]-nitrendipine binding (Figure 5b), resulted in 50% inhibition of ^{45}Ca influx (Figure 6b). Again in agreement with the inhibition of binding, 10 μM DES caused an almost complete inhibition of K^+-stimulated ^{45}Ca influx. D600 10 μM also completely inhibited Ca influx, but this concentration was several orders of magnitude lower than that which inhibited nitrendipine binding by about 50% (Figure 3a).

Discussion

The data presented here described saturable high affinity binding of [^{3}H]-nitrendipine to components of uterine plasma membranes. Since binding to ER and M fractions was considerably lower than that in the PM and since it corresponded well with 5'-nucleotidase activity in these fractions, it may be concluded that nitrendipine binding is probably a sole feature of plasma membrane and that the small amount of binding observed in other fractions represents contamination of these with PM. These findings thus indicate that [^{3}H]-nitrendipine can be used with confidence for identification and determination of recovery of plasma membrane of the myometrium. The affinity of nitrendipine for the binding sites in PM (K_D value) is similar to that found for other smooth muscles (Bolger *et al.*, 1982; Williams & Tremble, 1982; Grover *et al.*, 1984). A comparison of the density of the binding sites with the data reported by others is not justified since this parameter, which is usually expressed on the basis of protein concentration, will be dependent on the degree of enrichment of the membrane fractions. In the various published results (DePover *et al.*, 1982; Bolger *et el.*, 1982; Holck *et al.*, 1982; Triggle *et al.*, 1982; Williams & Tremble, 1982), both tissue homogenates and microsomal fractions have been used.

Working with a preparation of rat myometrium which was highly enriched with plasma membrane, Grover *et al.* (1984) recently presented data on [^{3}H]-nitrendipine binding. The density of binding sites found by these authors was about 2 fold that observed in this study. However, we have previously shown that oestrogen treatment increases calium entry in the uterine smooth muscle (Batra & Sjögren, 1983), and have recently found that it also increases the calcium channel density (Batra, 1984). Therefore, a difference in the degree of oestrogenization may in part be responsible for these quantitative differences in binding density observed.

The pharmacological evidence suggesting that dihydropyridines have different sites of action from drugs such as verapamil and diltiazem is supported by the present data showing relatively weak activity of these latter drugs at [^{3}H]-nitrendipine binding sites. Holck *et al.* (1983), who recently published data on [^{3}H]-nifendipine binding to myocardial membrane, found IC_{50} values for both verapamil and D600 in the sub-micromolar range. However, the present data indicate that these drugs are extremely poor competitors for [^{3}H]-nitrendipine binding sites in uterine membranes, which is in general agreement with the results of Williams & Tremble (1982), Bellemann *et al.* (1981) and Toll (1982), who studied [^{3}H]-nitrendipine binding in aortic smooth muscle, heart and PC 12 cells, respectively. In fact, the inhibition induced by

verapamil or D600 was no greater than that caused by a number of other drugs such as atropine, diphenhydramine or propranolol, which are not strictly considered as calcium entry blockers. The concept of different sites of action, albeit within the same so-called calcium channel, for dihydropyridines and D600 or verapamil is supported by the present data showing a potent inhibitory effect of D600 on Ca influx and its near lack of competition for nitrendipine binding sites. These data also indicate that binding of nitrendipine occurs at a pharmacologically relevant site since a good agreement was obtained between the binding of nitrendipine to plasma membrane fractions and inhibition by it of Ca influx in the intact tissue.

DES, which we have previously shown to be an inhibitor of contractile activity as well as calcium uptake in uterine smooth muscle (Batra & Bengtsson, 1978), caused considerable inhibition of nitrendipine binding. The concentrations of DES required for both 50 and 100% inhibition of Ca influx agreed closely with those causing equivalent inhibition of nitrendipine binding. Therefore, DES appears to block Ca entry by acting at a site within the membrane which is also shared by dihydropyridines. This relationship obviously does not extend to D600. Taken together, these observations suggest that the structural requirements for inhibition of calcium entry and binding to [^{3}H]-nitrendipine sites are different.

In fact, diltiazem was found to stimulate [^{3}H]-nitrendipine binding to uterine PM which is somewhat paradoxical but confirms recent observations made on cardiac (DePover *et al.*, 1982), skeletal (Ferry *et al.*, 1983) and smooth muscle (Bolger *et al.*, 1983) preparations. Diltiazem (10 μM), although it increased specific binding by about 40%, did not influence the affinity of binding sites for nitrendipine. This is in complete agreement with the recent data of Bolger *et al.* (1983) on intestinal smooth muscle. In membrane preparations from brain, an increase in the affinity of nitrendipine binding by diltiazem has been described (Murphy *et al.*, 1983; Boles *et al.*, 1984). However, Murphy *et al.* (1983), in contrast to Boles *et al.* (1984), reached the conclusion that diltiazem allosterically affects [^{3}H]-nitrendipine at a single site. Apparently, interactions of nitrendipine and diltiazem in their binding to calcium channel sites are highly dependent on temperature and are complex in nature, as shown by the studies of Glossman *et al.* (1983a). Recently a unitary model for drug modulation of calcium channels was proposed in order to explain the lack of relation between the calcium blocking action of verapamil or diltiazem and their competition for dihydropyridine binding sites (Murphy *et al.*, 1983). It is also possible that some of the differences in the above mentioned studies may be tissue- or species-dependent.

Also of considerable interest in the present study is the finding that whereas calcium or magnesium (1 mM) did not affect specific [^{3}H]-nitrendipine binding, the inorganic calcium blocker lanthanum inhibited binding with an IC_{50} of 0.11 mM. IC_{50} values for both lanthanum and EDTA are comparable to those found recently by Glossman *et al.* (1983b) who studied [^{3}H]-nimodipine binding to guinea-pig brain membranes. Since the loss of binding induced by EDTA could be restored by the addition of calcium or magnesium, a requirement for a tightly bound divalent metal at nitrendipine binding sites is indicated, which supports the conclusion reached by Glossman *et al.* (1983b). Apparent differences in cation dependence between calcium antagonist binding sites of membrane from different tissue have been observed (Murphy *et al.*, 1983; Glossman *et al.*, 1983b).

Published data on the effect of various calcium channel blockers seem to suggest that the blocking of calcium channels by these agents is use-dependent. Although the state of activation or inactivation in the broken cell membranes as used in this study cannot be known, the present data on the inhibition by nitrendipine of cellular calcium influx in the intact uterine strip indicate a good agreement between the inhibition of calcium influx and [^{3}H]-nitrendipine binding to membranes. Attempts to measure specific [^{3}H]-nitrendipine binding in the intact uterine tissue strips by us have not been successful, primarily because of very high non-specific binding in the intact tissue.

I thank Bodil Nilsson and Bengt Nordgren for technical assistance and Inger Ericsson for preparing the typescript. This work was supported by the Swedish Medical Research Council (Project No. 04X-07150-01A).

References

BATRA, S. (1982). Uptake and energy-dependent extrusion of calcium in the rat uterus. *Acta physiol. scand.*, **114**, 447–452.

BATRA, S. (1984). Calcium entry and calcium channel density in uterine smooth muscle. *International Symposium C.N.R.S. - I.N.S.E.R.M. on Calcium Regulation in Smooth Muscle*. Abstract p. 44.

BATRA, S. & BENGTSSON, B. (1978). Effects of diethylstilbestrol and ovarian steroids on the contractile responses and calcium movements in rat uterine smooth muscle. *J. Physiol.*, **276**, 329–342.

BATRA, S. & SJÖGREN, C. (1983). Effect of estrogen treatment on calcium uptake by the rat uterine smooth muscle. *Life Sci.*, **32**, 315–319.

BATRA, S., SJÖBERG, N.O. & THORBERT, G. (1978). Estrogen and progesterone interactions in the rabbit uterus *in vivo* after steroid administration. *Endocrinology*, **102**, 268–273.

BELLEMAN, P., FERRY, D., LUBBECKE, F. & GLOSSMANN, H. (1981). [^{3}H]-nitrendipine, a potent calcium antagonist, binds with high affinity to cardiac membranes. *Arzneim. Forsch.*, **31**, 2064–1066.

BOLES, R.G., YAMAMURA, H.I., SHOEMAKER, H. & ROESKE, W.R. (1984). Temperature-dependent modulation of [^{3}H]-nitrendipine binding by calcium channel antagonists verapamil and diltiazem in rat brain synaptosomes. *J. Pharmac. exp. Ther.*, **229**, 333–339.

BOLGER, G.T., GENGO, P.J., LUCHOWSKI, E.M., SIEGEL, H., TRIGGLE, D.J. & JANIS, R.A. (1982). High affinity binding of a calcium channel antagonist to smooth and cardiac muscle. *Biochem. biophys. Res. Commun.*, **104**, 1604–1609.

BOLGER, G.T., GENGO, P., KLOCKOWSKI, R., LUCHOWSKI, E., SIEGEL, H., JANIS, R.A., TRIGGLE, A.M. & TRIGGLE, D.J. (1983). Characterization of binding of the Ca^{++} channel antagonist, [^{3}H]-Nitrendipine, to guinea-pig ileal smooth muscle. *J. Pharmac. exp. Ther.*, **225**, 291–309.

CHENG, Y.C. & PRUSOFF, N.H. (1973). Relationship between the inhibition constant (K) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmac.*, **22**, 3099–3108.

DEPOVER, A., MATLIB, M.A., LEE, S.W., DUBE. G.P., GRUPP, I.L., GRUPP, G. & SCHWARTZ, A. (1982). Specific binding of ^{3}H-nitrendipine to membranes from coronary arteries and heart in relation to pharmacological effects. Paradoxical stimulation by diltiazem. *Biochem. biophys. Res. Commun.*, **108**, 110–117.

FERRY, D.R., GOLL, A. & GLOSSMANN, H. (1983). Differential labelling of putative skeletal muscle calcium channels by ^{3}H-nifedipine, ^{3}H-nitrendipine, ^{3}H-nimodipine and ^{3}H-PN 200 110. *Naunyn-Schmiedebergs Arch. Pharmac.*, **323**, 276–277.

GLOSSMANN, H., LINN, T., ROMBUSCH, M. & FERRY, D.R. (1983a). Temperature-dependent regulation of d-cis-^{3}H-diltiazem binding to Ca^{2+} channels by 1,4-dihydropyridine channel agonists and antagonists. *FEBS Letts.*, **160**, 226–232.

GLOSSMAN, H., FERRY, D.R., LUBBECKE, F., MEWES, R. & HOFMANN, F. (1983b). Identification of voltage operated calcium channels by binding studies: Differentiation of subclasses of calcium antagonist drugs with ^{3}H-nimodipine radioligand binding. *J. Receptor Res.*, **3**, 177–190.

GODFRAIND, T. (1974). The action of cinnarizine and of phentolamine on the noradrenaline dependent calcium influx in vascular smooth muscle. *Br. J. Pharmac.*, **52**, 120P.

GROVER, A.K., KWAN, C.-Y., LUCHOWSKI, E., DANIEL, E.E. & TRIGGLE, D.J. (1984). Subcellular distribution of ^{3}H-nitrendipine binding in smooth muscle. *J. biol. Chem.*, **259**, 223–2226.

HOLCK, M., THORENS, S. & HAEUSLER, G. (1982). Characterization of ^{3}H-nifedipine binding sites in rabbit myocardium. *Eur. J. Pharmac.*, **85**, 305–315.

HOLCK, M., THORENS, S. & HAEUSLER, G. (1983). Does ^{3}H-nifedipine label the calcium channel in rabbit myocardium? *J. Receptor Res.*, **3**, 191–198.

JAWUA-STEWEART, M.L., READ, W.O. & STEFFEN, R.P. (1979). Isolation of pure myocardial subcellular organelles. *Anal. Biochem.*, **96**, 293–297.

LOWRY, O.H., ROSEBOROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin-phenol reagent. *J. biol. Chem.*, **193**, 165–275.

MURPHY, K.M.M., GOULD, R.J., LARGENT, B.L. & SNYDER, S.H. (1983). A unitary mechanism of calcium antagonist drug action. *Proc. natn. Acad. Sci. U.S.A.*, **80**, 860–864.

SANUI, H. (1974). Measurement of inorganic orthophosphate in biological materials: Extraction properties of butyl acetate. *Anal. Biochem.*, **60**, 489–504.

TOLL, L. (1982). High-affinity binding and inhibition of calcium transport in a clonal cell line. *J. biol. Chem.*, **257**, 13189–13192.

TRIGGLE, C.R., AGRAWAL, D.K., BOLGER, G.T., DANIEL, E.E., KWAN, C.Y., LUCHOWSKI, E.M. & TRIGGLE, D.J. (1982). Calcium-channel antagonist binding to isolated vascular smooth muscle membranes. *Can. J. Physiol. Pharmac.*, **60**, 1738–1741.

VAN BREEMEN, C., FARINAS, B.R., GERBA, P. & MCNAUGHTON, E.D. (1972). Excitation contraction coupling in rabbit aorta studied by the lanthanum method for measuring cellular Ca^{++} influx. *Circulation Res.*, **30**, 44–53.

WILLIAMS, L.T. & TREMBLE, P. (1982). Binding of a calcium antagonist, ^{3}H-nitrendipine, to high affinity sites in bovine aortic smooth muscle and canine cardiac membranes. *J. clin. Invest.*, **70**, 209–212.

(Received September 11, 1984.
Revised March 19, 1985.
Accepted April 10, 1985.)