Subcellular Distribution of [3H]Nitrendipine Binding in Smooth Muscle*

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Distribution of specific binding sites for [3H]nitrendipine was studied in subcellular fractions isolated from rat gastric fundus smooth muscle and from rat myometrium. There was an excellent correlation between the distribution of [3H]nitrendipine binding determined at the nitrendipine concentrations of 0.138 and 1.38 nM, and the distribution of the plasma membrane markers K+-activated ouabain-sensitive p-nitrophenylphosphatase, 5' nucleotidase, phosphodiesterase I, and Mg-ATPase, but not between the mitochondrial markers cytochrome c, oxidase, succinate-dependent cytochrome c reductase, or rotenone-insensitive NADH-dependent cytochrome c reductase or the putative endoplasmic reticulum marker NADPH-dependent cytochrome c reductase. The binding occurred with high affinity and with a similar (0.097–0.146 nM) equilibrium dissociation constant to all the fractions, even though the density of binding sites varied and was highest in the plasma membrane marker-enriched fractions. The maximal binding in the plasma membrane-enriched fraction from the rat gastric fundus smooth muscle was 0.43 ± 0.04 pmol/mg, and in that from rat myometrium was 0.72 ± 0.09 pmol/mg. Thus in the two smooth muscles studied the plasma membrane fraction is the locus of the high affinity nitrendipine binding.

Nitrendipine and a number of other related dihydropyridines inhibit a number of Ca-mediated phenomena, including muscle contraction (review Ref. 1). The inhibition has been attributed to the action of these drugs as voltage-dependent Ca-channel blocking agents. In order to understand the biochemistry of the Ca-channel blocking event, several laboratories have carried out [3H]nitrendipine binding studies on membrane preparations from a number of tissues including smooth (2–7), cardiac (2–5, 7–11) and skeletal muscles (12, 13). In general, there is an excellent correlation between the potencies of various agents to inhibit the contractions in a given smooth or cardiac muscle and their ability to inhibit the high affinity specific saturable [3H]nitrendipine binding to the membranes prepared from the same tissue (2, 5, 6, 8). However, several of these studies have used subcellular par-

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EXPERIMENTAL PROCEDURES

Preparation of Subcellular Membrane Fractions—Rat myometrium subcellular fractions were obtained from estrogen dominated female Wistar rats weighing 200–250 g as described previously (14). Subcellular fractionation from the smooth muscle of gastric fundus of male Wistar rats weighing 450 g was carried out by the method of Kwan et al. (15) as shown in Scheme I.

Assay of Biochemical Markers—The assay procedures for 5' nucleotidase, phosphodiesterase I, Mg-ATPase, cytochrome c oxidase, succinate-dependent cytochrome c reductase, NADPH-dependent cytochrome c reductase, rotenone-insensitive NADH-dependent cytochrome c reductase, and K+-activated ouabain-sensitive FNPase (15, 16) have been described earlier. In order to overcome the problem of membrane sidedness in the K+-activated ouabain-sensitive FNPPase assay conditions resulting in hypotonic shock were used. The shock causes a release of the trapped [3H]sucrose (17) or [3H]ulin.

[3H]Nitrendipine Binding—[3H]Nitrendipine (7.25 Ci/mmol purchased from New England Nuclear) binding was carried out according to Bolger et al. (6). Briefly, the subcellular fractions (98 to 438 μg of protein) were incubated in glass tubes at 25 °C in total volumes of 5 ml of 50 mM Tris-HCl, pH 7.4, with the specified concentrations of [3H]nitrendipine for 60 min. The samples were then filtered through Whatman GF/B filters, and the filters washed and counted as described previously (6). The binding experiments were performed in subdued light (6). Nonspecific binding was defined as the [3H]nitrendipine binding in the presence of 100 nM unlabeled nitrendipine. Specific binding was defined as the total binding in the absence of unlabeled ligand minus the nonspecific binding. Since this involved the use of ethanolic solutions of unlabeled nitrendipine, an equivalent amount of ethanol, even though it did not influence the binding, was included in all the binding assays. In initial experiments, [3H]nitrendipine binding at 0.1–0.2 nM and at 1.3–1.5 nM [3H]nitrendipine was determined on fresh samples and those frozen in liquid nitrogen and stored at −70 °C. Freezing the samples and storing them up to 34 days (longer periods not checked) did not influence the specific binding significantly at either ligand concentration. Therefore, in all subsequent experiments, the subcellular fractions were frozen and stored as described above.

1 The abbreviations used are: PNPase, p-nitrophenylphosphatase; EGTA, ethylene glycol bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid.

2 A. K. Grover, C.-Y. Kwan, E. Luchowski, E. E. Daniel, and D. J. Triggle, unpublished results.

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against the activity of membrane markers in these fractions. An oxidase, and F3 in the putative endoplasmic reticulum marker activity but not with NADPH:cytochrome c reductase, and rotenone-insensitive cytochrome c reductase. Confirms of this result was obtained in several experiments on subcellular fractionation of rat myometrium as well. A summary of results from both tissues is presented in the form of linear correlation coefficients (r) between the specific [H]nitrendipine binding and the various biochemical markers (Table I). The distribution of the specific binding at 0.138 and 1.38 nM correlated extremely well (r > 0.95) with those of the four plasma membrane markers, namely, K+ activated ouabain-sensitive PNPPase, 5'-nucleotidase, phosphodiesterase I, and Mg-ATPase. However, such correlations with the mitochondrial markers cytochrome c oxidase, succinate-dependent cytochrome c reductase, and rotenone-insensitive NADH-dependent cytochrome c reductase were poor. Similarly, the [H]nitrendipine binding showed poor correlation with the putative endoplasmic reticulum marker NADPH-dependent cytochrome c reductase as well.

Total protein, enzyme marker, and [H]nitrendipine binding recoveries were also compared in all these experiments (see legend to Fig. 1). The average per cent recoveries in both the tissues for the specific binding at 0.138 or 1.38 nM nitrendipine concentration, and for the protein were in the range of 70-90% during the subcellular fractionation of the postnuclear supernatants from the two smooth muscles.

Since specific [H]nitrendipine binding was observed in various subcellular fractions, the binding was examined as a function of nitrendipine concentration using mitochondrial, microsomal, and plasma membrane-enriched fractions to ascertain whether there were differences in the binding affinity in these fractions. Representative data from one experiment on rat gastric fundus are shown in the form of Scatchard plots in Fig. 3. The three plots exhibited similar slopes but differed.

**RESULTS**

Fig. 1 shows the distribution of three membrane markers and that of specific [H]nitrendipine binding at [H]nitrendipine concentrations of 0.138 nM and 1.38 nM in the subcellular fractions isolated from the smooth muscle of rat gastric fundus. These results are pooled from four to five preparations for the markers and two to three preparations for the binding data. The distribution of the membrane markers is similar to that reported previously (15). The fraction F2 (see Scheme I for nomenclature of subcellular fractions) is maximally enriched in the plasma membrane marker 5'-nucleotidase, MIT in the inner mitochondrial marker cytochrome oxidase, and F3 in the putative endoplasmic reticulum marker NADPH:cytochrome c reductase (Fig. 1). The distribution of specific [H]nitrendipine binding at 0.138, as well as, 1.38 nM of [H]nitrendipine parallels fairly well with that of 5'-nucleotidase but not with the other two markers.

The amount of specific [H]nitrendipine binding at 0.138 nM or 1.38 nM in the various subcellular fractions was plotted against the activity of membrane markers in these fractions. Fig. 2 shows that in such a plot the specific [H]nitrendipine binding correlated extremely well with the 5'-nucleotidase activity but not with NADPH:cytochrome c reductase. Confirmation of this result was obtained in several experiments on subcellular fractionation of rat myometrium as well. A summary of results from both tissues is presented in the form of linear correlation coefficients (r) between the specific [H]nitrendipine binding and the various biochemical markers (Table I). The distribution of the specific binding at 0.138 and 1.38 nM correlated extremely well (r > 0.95) with those of the four plasma membrane markers, namely, K+ activated ouabain-sensitive PNPPase, 5'-nucleotidase, phosphodiesterase I, and Mg-ATPase. However, such correlations with the mitochondrial markers cytochrome c oxidase, succinate-dependent cytochrome c reductase, and rotenone-insensitive NADH-dependent cytochrome c reductase were poor. Similarly, the [H]nitrendipine binding showed poor correlation with the putative endoplasmic reticulum marker NADPH-dependent cytochrome c reductase as well.

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Nitrendipine Binding Localization in Smooth Muscle

FIG. 2. Correlation between specific [3H]nitrendipine binding and membrane markers. The data for specific [3H]nitrendipine binding and for 5'-nucleotidase and NADPH:cytochrome c reductase activities are taken from Fig. 1 to show a correlation between the binding and the enzyme activities. [3H]Nitrendipine concentration and the linear correlation coefficients (r) are given for each panel.

TABLE I
Correlation between nitrendipine binding and biochemical markers in subcellular fractions

| Biochemical marker | Rat fundus | Rat myometrium |
|--------------------|------------|----------------|
| 0.138 nM nitrendipine | 0.138 nM nitrendipine |
| 1.38 nM nitrendipine | 1.38 nM nitrendipine |
| Linear correlation coefficient (r) | 0.9729 | 0.9897 |

For subcellular fractions (n = 9), for p < 0.05, the value of r is 0.965 and for rat myometrium (n = 8), for p < 0.05, r = 0.700.

in their intercepts. These results were transformed into the dissociation constant (Kd) and the maximum number of binding sites, and several identical experiments were conducted using membrane preparations from rat myometrium as well as rat gastric fundus smooth muscle. The results are shown in Table II. The maximum number of binding sites/mg of protein (Bmax) varied in the various fractions, being highest in the plasma membrane-enriched fractions as expected from the data of Fig. 1. However, the Kd values did not differ significantly from one fraction to another, but ranged from 0.097 to 0.146 nM in the various fractions obtained from the two smooth muscles (Table II).

TABLE II
Nitrendipine binding parameters for some subcellular fractions
The values are mean ± S.E. (n preparations).

| Subcellular fraction | Bmax (pmol/mg) | Kd (nM) |
|----------------------|----------------|---------|
| Rat fundus (MIT II)  | 0.07 + 0.097   | 0.145   |
| Mitochondrial (MIC)  | 0.14 ± 0.03    | 0.138 ± 0.03 |
| Plasma membrane (P2)| 0.128          | 0.128   |
| Mitochondrial (MIT) | 0.14 ± 0.03    | 0.138 ± 0.03 |
| Microsomal (MIC)    | 0.128          | 0.128   |
| Plasma membrane (N1)| 0.14 ± 0.03    | 0.145 ± 0.03 |

DISCUSSION
The results show that there is an excellent correlation between the high affinity specific [3H]nitrendipine binding sites in the subcellular fractions obtained from rat myometrium and rat gastric fundus smooth muscles and the plasma membrane markers and not to other membrane markers. The binding in all the subcellular fractions occurs with similar affinity suggesting that a single type of nitrendipine binding sites are distributed in these fractions, and the amount of binding relates to their plasma membrane content. Thus, these data unequivocally establish the occurrence of the binding sites for this Ca-channel blocking agent in the sarcolemma of the smooth muscle. In this discussion, these results will be examined further in terms of reliability of the markers employed, membrane orientation of the isolated vesicles, validity of the binding study, and also compared to the results in the literature. However, since this is the first detailed report on such a distribution in any smooth muscle, a comparison can only be made with studies on other tissues.

The validity of the conclusion that nitrendipine binding is localized mainly in the plasma membrane depends on the
reliability of the markers employed. Among the markers employed, K'-activated ouabain-sensitive PNPase has been established to represent a partial reaction of Na pump in rat myometrium (18, 19). Both the K'-activated ouabain-sensitive PNPase and the membrane-bound 5'-nucleotidase have been shown to parallel the distributions of the lectin and the oxytocin receptors (16, review Ref. 20). The membrane bound 5'-nucleotidase, phosphodiesterase I, and Mg-ATPase parallel together during the differential centrifugation as well as during isopycnic centrifugation (15). Digitonin treatment of microsomes isolated from various smooth muscles results in a shift in the distribution of the plasma membrane markers 5'-nucleotidase, Mg-ATPase, as well as phosphodiesterase I to higher densities. This shift has been associated with the high cholesterol content of the plasma membranes (21, 22). Thus, the four plasma membrane markers employed here are extremely reliable. Similarly, the validity of the two inner mitochondrial markers, namely, cytochrome c oxidase and succinate-dependent cytochrome c reductase is also unquestionable (review Ref. 20). The outer mitochondrial marker, rotenone-insensitive NADH-dependent cytochrome c reductase, and the endoplasmic reticulum marker NADPH-dependent 5'-nucleotidase, Mg-ATPase, as well as phosphodiesterase I and Mg-ATPase parallel together during the differential centrifugation as well as during isopycnic centrifugation (15). It can be argued that the distribution of the markers as well as of the specific [3H]nitrendipine binding is hampered by the problem of membrane orientation since the isolation procedure employed here results in vesicles of mixed sidedness (17). However, all the assays were conducted in media devoid of sucrose, whereas the membrane vesicles were prepared in media containing 250 mM sucrose (14, 15). In such experiments the membrane vesicles undergo a hypotonic shock resulting in (a) release of trapped [3H]inulin or [3H]sucrose (17), (b) maximally enhanced K'-activated ouabain-sensitive PNPase activity (17), (c) similarly enhanced accessibility of plasma membrane-bound galactoside residues to galactose oxidase (17), and (d) accessibility of EGTA to intravesicular sites (17). Thus, the possibility that the differential activities or binding to the low molecular weight lipophilic ligand like [3H]nitrendipine observed in these experiments results from differential membrane orientation appears unlikely.

In parallel contractility and binding experiments, the high affinity specific [3H]nitrendipine binding has been shown to represent binding to Ca-channel blocking sites in vascular and nonvascular smooth muscles and cardiac muscle (2, 5, 8). Therefore, only the subcellular distribution will be discussed further. The overall per cent recovery during the subcellular fractionation was quantitative, i.e. comparable to the total per cent recovery of protein. This ruled out any substantial activation or inactivation of the observed property during the isolation procedure. It is important to measure the yields since a change in the yield during a multistep purification process can artifically affect the conclusions on distribution as we have recently shown for the oxalate-stimulated ATP-dependent Ca uptake in these two smooth muscles (23, 24). It is also important to show that affinity of the ligand is similar for the various subcellular subfractions in such a distribution study. This was accomplished here by (a) studying the [3H]nitrendipine binding at two concentrations; namely, 0.138 nM which is near the Kd value for the binding and at 1.38 nM which gives near saturation, and (b) by carrying out Scatchard plots on several membrane fractions to show that the Kd values are similar in all the instances. Thus, after all the considerations, it is concluded that the specific [3H]nitrendipine binding is localized in the plasma membrane in the two smooth muscles. In comparison, the distribution of [3H]nitrendipine binding in cardiac muscle is in dispute. In one study the specific binding of [3H]nitrendipine in canine cardiac ventricle subcellular fractions was reported to be solely in sarcosome and not in sarcoplastic reticulum or mitochondria (10), while in another study the binding was highest in the ryanodine-sensitive sarcoplastic reticulum (9). It is pointed out that the total per cent recovery was not emphasized in either study. In skeletal muscle, however, the specific nitrendipine binding has been reported to be associated with heavy sarcoplastic reticulum (12) or T tubules (13). Thus, the reported distribution of [3H]nitrendipine binding varies in different studies and only more detailed studies can resolve as to which differences are due to the techniques involved and which ones are due to the different tissues.

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