Photoaffinity labelling of Ca^{2+} channels with [^{3}H]azidopine

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A 1,4-dihydroypyridine arylazide photoaffinity ligand, [^{3}H]azidopine (50.6 Ci/mmol), has been synthesized. [^{3}H]Azidopine binds reversibly with a K_d of 350 pM to guinea-pig skeletal muscle membranes in the absence of ultraviolet light. The reversible [^{3}H]azidopine binding is inhibited stereoselectively by 1,4-dihydropyridines, phenylalkylamine Ca^{2+} channel blockers and La^{3+}. Covalent incorporation into membrane proteins after photolysis was investigated by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis. [^{3}H]Azidopine is photoincorporated specifically into a protein of M_r ~145000. The covalent labelling of the M_r ~145000 band is inhibited stereoselectively by drugs and cations which block the reversible [^{3}H]azidopine binding. It is suggested that [^{3}H]azidopine is photoincorporated into a subunit of the putative Ca^{2+} channel.

Calcium channel *Photoaffinity label* *Skeletal muscle* *[^{3}H]Azidopine* *1,4-Dihydropyridine*

1. INTRODUCTION

Ca^{2+} channels in the skeletal muscle t-tubulus are blocked by 1,4-dihydropyridines (e.g., nifedipine), phenylalkylamines (e.g., D-600) and divalent cations (e.g., Ni^{2+}) [1]. These Ca^{2+} channels can be identified in broken cell preparations by radiolabelled 1,4-dihydropyridines [2,3]. Target size analysis has revealed that the 1,4-dihydropyridine binding sites, associated with the skeletal muscle t-tubulus Ca^{2+} channels, are large structures of M_r 136000–210000 [4–6]. An oligomeric structure of the channel has been proposed from results with different 1,4-dihydropyridines and the effects of d-*cis*-diltiazem (an allosteric regulator) in modulating apparent channel size [4,6]. We report here the synthesis and properties of a novel 1,4-dihydropyridine arylazide ligand ([^{3}H]azidopine) which is photoincorporated specifically into skeletal muscle membrane proteins.

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2. MATERIALS AND METHODS

2.1. *Materials*

Sources of materials were as in [2,4,6,7]. Partially purified skeletal muscle t-tubule membranes from guinea-pig were prepared as in [2].

2.2. *Preparation of [^{3}H]azidopine*

All procedures were performed in close to total darkness. One mCi succinimidyl-4-azidobenzoate (50.6 Ci/mmol, NEN, Dreieich) was evaporated to dryness under a gentle stream of nitrogen. Two hundred nmol 1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridine carboxylic acid 2-(aminoethyl) ethyl ester in 20 μl absolute ethanol were added followed by 20 μl of 100 mM sodium borate buffer (pH 8.4). After 25 min on ice the reaction mixture was spotted on a 0.2 mm silica gel 60 thin-layer plate (Merck A.G., Darmstadt) and [^{3}H]azidopine purified by ascending chromatography (solvent system: acetic acid ethyl ester–diethyl ether, 30:70, v/v). [^{3}H]Azidopine had an R_f value of 0.54. The azidopine precursor remained on the origin and non-reacted succinimidyl-4-

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azidobenzoate had an R_f value of ~0.80. [^{3}H]Azidopine was eluted with absolute ethanol and stored for up to 6 weeks at −20°C with no radiochemical decay of the ligand. The reaction yield of [^{3}H]azidopine was ~30% of the starting radioactivity, the radiochemical purity ⩾95%.

2.3. *Binding experiments*

Characteristics of reversible [^{3}H]azidopine binding (in total darkness) were evaluated in 50 mM Tris–HCl buffer, supplemented with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (pH 7.4) (buffer A) in an assay volume of 0.25 ml at 25°C. Bound and free ligand were separated as in [3]. The nonspecific binding definition was with 1 μM nimodipine, the incubation time being 1 h unless stated otherwise. [^{3}H]Azidopine is racemic and the binding ability of the ligand is ~50% as reported in [2] for other racemic 1,4-[^{3}H]dihydropyridines. This was taken into account for the data calculation, except when stated otherwise.

2.4. *Photoaffinity labelling*

Guinea-pig skeletal muscle membranes (protein 1–2 mg/ml) in buffer A were incubated with 2.5 nM [^{3}H]azidopine (total binding), or 2.5 nM [^{3}H]azidopine in the presence of 100 nM (+)-enantiomer of PN 200-110 (blank binding) or other drugs in a volume of 4 ml. After 60 min incubation at 25°C the assay mixture was centrifuged at 35000 × *g* for 10 min at 2°C. The first supernatant was discarded and the pellets resuspended in 4 ml ice-cold buffer A, supplemented with 0.25 mg/ml bovine serum albumin (fat free; Sigma, Munich). The centrifugation was repeated, the supernatant discarded and the pellets resuspended in 4 ml ice-cold buffer A. The suspensions were irradiated with a Philips TL 40 W/08 black-light lamp in an 8 cm diameter petri dish at a distance of 10 cm for 3 min on ice. The irradiated suspensions were spun down as above and the pellets resuspended in 50 μl buffer A. Twenty μl of a protease inhibitor mixture (containing 0.14 mg/ml leupeptin, 0.7 mM benzamidine, 0.7 mM PMSF, 2.8 mM EDTA, 0.02 mg/ml Trasylol® and 0.14 mg/ml pepstatin A) was added. After 20 min, 0.2 ml of boiling stop solution was added to each tube. The composition of the stop solution was 2.5 ml of 20% SDS, 0.5 ml β-mercaptoethanol, 1.0 ml glycerol, 1.0 ml of 0.625 M Tris–HCl, 0.1 mM PMSF (pH 7.5), with 1.8 g urea added to the mixture and bromphenol blue. After 10 min at 25°C the denatured membranes were applied to 11% homogeneous slab gels as in [7]. ^{14}C-labelled marker proteins were from NEN (Dreieich). Following electrophoresis the slab gels were either cut and counted for radioactivity or prepared for fluorography and dried. Gels which were counted for radioactivity were not fixed and cut into 3 mm slices. The slices were solubilized for 3 days in 3.5 ml of liquid scintillation cocktail (Lipoloma and Liposolve, Baker Chemicals, Deventer, The Netherlands). Gels for fluorography were first fixed for 30 min then impregnated with Enlightening (NEN) for 1 h. Gels were dried and then exposed to Kodak XO-mat film for 14 days at −70°C.

2.5. *Data analysis*

Binding inhibition data were fitted by non-linear methods to the general dose-response equation [8] and the K_i values determined as in [9]. Means were compared with Student's *t*-test, $p \leqslant 0.05$ being the level of accepted significance.

3. RESULTS

3.1. *Reversible binding of [^{3}H]azidopine*

[^{3}H]Azidopine bound with a K_d of 350 ± 50 pM (25°C) to guinea-pig skeletal muscle membranes. The B_{max} was 28 ± 4 pmol (n = 3) per mg membrane protein. The association rate constant, k_{+1} was 0.378 $nM^{-1} \cdot min^{-1}$, and the dissociation rate constant, k_{-1}, was 0.15 min^{-1} as determined by computer fitting of the association data. However, k_{-1}, when determined by blockade of the association reaction with unlabelled nimodipine (1 μM), was 0.016 min^{-1} (fig.1). This discrepancy is currently under investigation. Unlabelled 1,4-dihydropyridines displaced [^{3}H]azidopine binding in a stereoselective manner, the (+)-enantiomer of PN 200-110 being more potent than the (−)-enantiomer (fig.2). Phenylalkylamines were partially inhibitory under the assay conditions employed. (+)-Verapamil displaced 51% of specific [^{3}H]-azidopine binding whereas (−)-verapamil displaced only 24%. (−)-Verapamil is, however, more potent than (+)-verapamil. When (+)-verapamil at 10 μM together with 1 μM nimodipine was used to block the association reaction, the dissociation

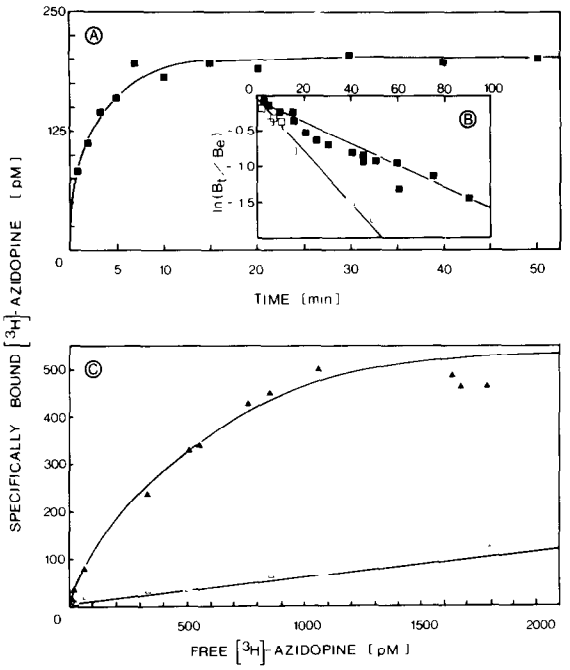


Fig.1. Kinetics and saturability of [^{3}H]azidopine binding. (A) Association reaction of [^{3}H]azidopine to guinea-pig skeletal muscle membranes. [^{3}H]Azidopine (339 pM) was incubated with 6.6 μg membrane protein for times between 1 and 50 min at 25°C. Blank binding was 22 pM, instantaneous, stable and was subtracted from the data points. (B) Dissociation of [^{3}H]azidopine. B_t is the specifically bound ligand at time t after addition of chasing drugs and B_e the equilibrium specific binding. Dissociation was induced by 1 μM nimodipine (■) resulting in a k_{+1} of 0.016 ± 0.01 min^{-1} (n = 3) or by 1 μM nimodipine together with 10 μM (+)-verapamil (□). The k_{-1} was 0.034 min^{-1}. (C) Saturation isotherm of [^{3}H]azidopine binding. [^{3}H]Azidopine (free concentration 13–1780 pM) was incubated with 5.4 μg membrane protein. Specific binding (▲) and nonspecific binding (△) are shown. Computerized data fitting gave a B_{max} of 543 ± 50 pM (B_{max} 25 ± 2.3 pmol/mg protein) and a K_d of 342 ± 40 pM.

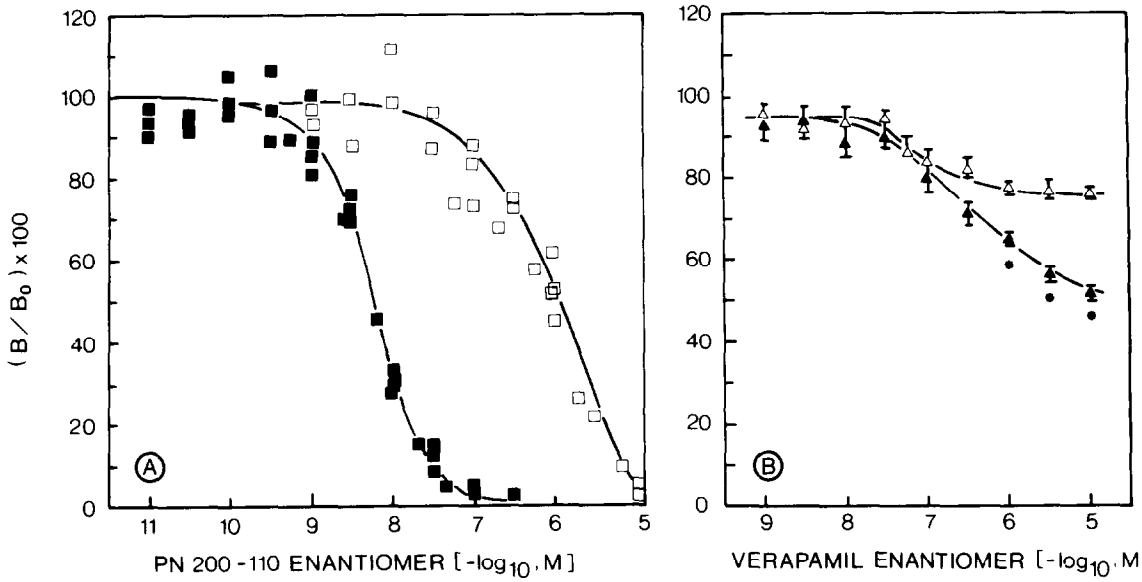


Fig.2. Pharmacological profile of [^{3}H]azidopine. [^{3}H]Azidopine (0.4–0.6 nM) was incubated with 10–12 μg skeletal muscle membrane protein. The total bound radioactivity was 10000–12000 dpm and blank binding 5–600 dpm. Data were fitted with the BMDP general curve fitting program to the dose-response equation [8]. Values are given ± asymptotic SD. (A) Stereoselective inhibition of [^{3}H]azidopine binding by the (+)-enantiomer (■) and (−)-enantiomer (□) of PN 200-110. Each point is the mean of a duplicate determination, data points having been pooled from 3 independent experiments. For (+)-PN 200-110 an IC_{50} of 3.6 ± 0.5 nM and a slope factor of 1.4 ± 0.15 gave the best fit. The K_i of (+)-PN 200-110 was 0.3 nM. For (−)-PN 200-110 the IC_{50} was 1800 ± 970 nM and the slope factor 0.76 ± 0.17. The K_i of (−)-PN 200-110 is 343 nM. (B) Stereoselective inhibition of specific [^{3}H]azidopine binding by (+)-verapamil (▲) and (−)-verapamil (△). Data points are means of duplicate determinations from 3 independent experiments ± SE. For (+)-verapamil the IC_{50} was 350 ± 100 nM, slope factor 0.74 ± 0.15 and the maximal inhibition 51 ± 4% of specific binding. For (−)-verapamil the IC_{50} was 44 ± 36 nM, the slope factor 0.62 ± 0.35 and maximal inhibition 24 ± 3%. * $p < 0.01$ for (+)-verapamil *vs* (−)-verapamil.

was accelerated 2-fold (k = 0.034 min^{-1}). This demonstrates the negative allosteric nature of phenylalkylamine inhibition of [^{3}H]azidopine binding. As previously reported for [^{3}H]PN 200-110 binding [6] d-*cis*-diltiazem caused a very small stimulation of [^{3}H]azidopine binding (not shown). In addition, [^{3}H]azidopine binding was inhibited by the inorganic Ca^{2+} channel blocker La^{3+} with an IC_{50} of 100 μM whereas Ca^{2+} up to 10 mM and Na^+ (all as chloride salts) up to 100 mM were completely inactive.

3.2. *Photoaffinity labelling with [^{3}H]azidopine*

The fate of [^{3}H]azidopine in photoaffinity experiments was monitored. With the above protocol (means from 3 balance experiments ± SE) 26 ± 4% of the added racemic [^{3}H]azidopine was specifically bound; 27 ± 5% of the total [^{3}H]azidopine was recovered in the first supernatant and an additional 34 ± 6% was found in the subsequent washing fluids. Following ultraviolet light exposure, specific binding (assessed by filtration) was reduced by ~50% and approx. 400000 dpm were added to a single lane of the slab gel for electrophoresis. The efficiency of photoincorporation of [^{3}H]azidopine (defined as the ratio of dpm found in specific bands in the slab gels relative to the equilibrium level of specific binding prior to ultraviolet light exposure) was 0.6 ± 0.1% (n = 5). The small percentage of covalent incorporation is common to most arylazide photoaffinity ligands (e.g., [10]) because the nitrene radicals formed can react with water [11]. Preliminary experiments without the use of the protease inhibitor mixture yielded very little specific covalent incorporation of [^{3}H]azidopine in skeletal muscle membranes after gel electrophoresis. In guinea-pig heart membranes only nonspecific photoincorporation into a

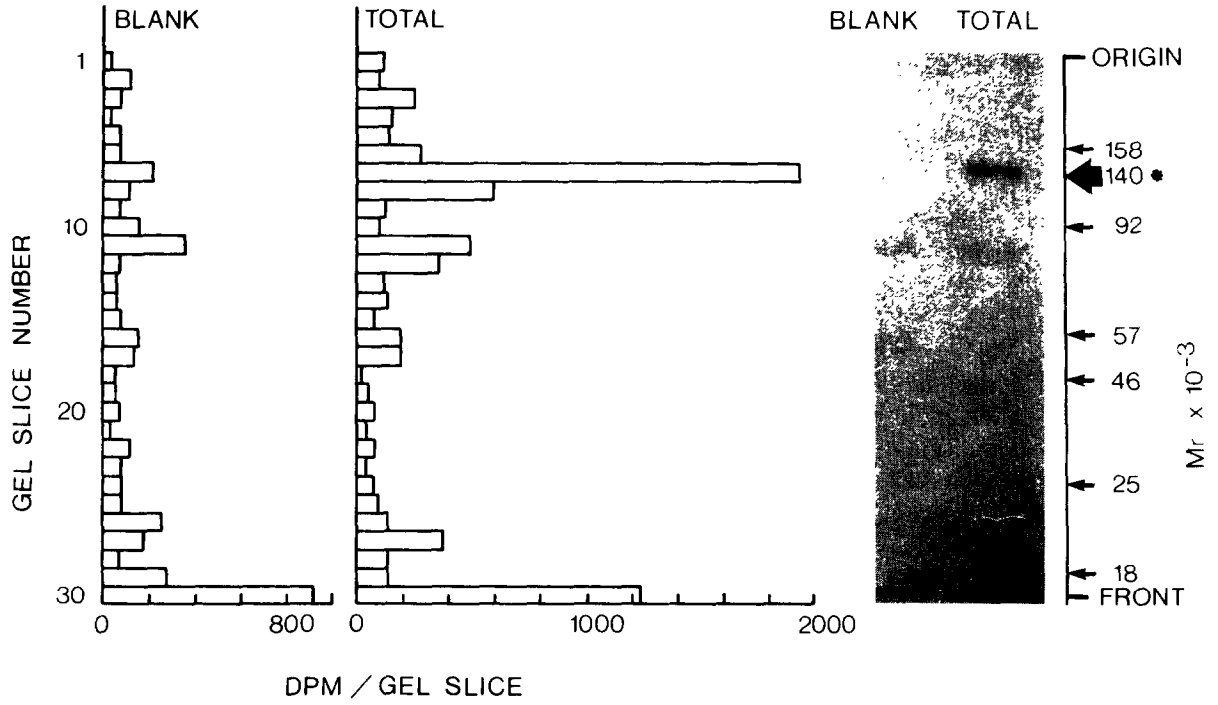
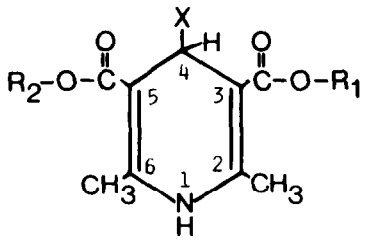


Fig.3. Results of a typical photoaffinity experiment. Guinea-pig skeletal muscle membrane protein (1.5 mg/ml) was incubated with 2.5 nM [^{3}H]azidopine (total binding) or 2.5 nM [^{3}H]azidopine and 100 nM (+)-PN 200-110 (blank binding) for 1 h at 25°C. The membranes were then washed, photolyzed, denatured and electrophoresed as described. The left side shows the dpm/3 mm gel slice for the blank as well as the corresponding total binding. In gel slices 7 and 8 of the total binding, 2332 dpm was incorporated specifically. The fluorography of the total and blank binding lanes from the same slab gel is shown on the right. M_r standards are shown (non-reduced gamma globulin, 158000; phosphorylase *b*, 92500; bovine serum albumin, 67000; ovalbumin, 46000; β-lactoglobulin, 18000; all reduced). The arrow (*) indicates the position of the specifically labelled band and the apparent M_r in these experiments.

band of M_r 41000 was seen in the presence of PMSF only or even with other protease inhibitors present. With the above protocol 2000–4000 dpm were consistently incorporated into a band of M_r 145000 ± 5000 (n = 5) in skeletal muscle membranes. Photoincorporation into this band was completely blocked by 100 nM (+)-PN 200-110 (see fig.3) but not at all by 100 nM (−)-PN 200-110, and only partially by saturating concentrations of the verapamil enantiomers (not shown). This demonstrates that the pharmacological specificity of the irreversible [^{3}H]azidopine binding is similar to that observed in reversible binding studies.

4. DISCUSSION

The putative Ca^{2+} channels from skeletal muscle and brain, labelled with tritiated 1,4-dihydropyridines, have been solubilized and partially purified with lectin affinity chromatography and sucrose gradient centrifugation [12,13]. In skeletal muscle the Ca^{2+} channel 1,4-dihydropyridine binding site has an $s_{20,w}$ value of ~13 S [12]. Target size analysis has been employed to estimate the M_r of the 1,4-dihydropyridine binding domain. An M_r of 185000 ± 10000 [4] was found for the guinea-pig skeletal muscle [^{3}H]nimodipine labelled site, whereas an M_r of 210000 ± 20000 was reported for the rabbit t-tubule [^{3}H]nitrendipine labelled binding site [5]. When [^{3}H]PN 200-110 (a benzoxadiazol-1,4-dihydropyridine) was employed as a Ca^{2+} channel label, a somewhat smaller M_r of 136000 ± 12000 was found for the skeletal muscle 1,4-dihydropyridine receptor [6]. [^{3}H]PN 200-110 has been classified as a fully 'antagonistic' 1,4-dihydropyridine derivative, because of its efficacy in stabilizing the high-affinity state of the Ca^{2+} channel 1,4-dihydropyridine receptor [14]. [^{3}H]Nimodipine and [^{3}H]nitrendipine label considerably less high-affinity sites than [^{3}H]PN 200-110 at 37°C and have been suggested to be partially agonistic 1,4-dihydropyridines [14]. We have performed binding studies with the 'agonistic' 1,4-dihydropyridine [^{3}H]Bay K 8644 (83 Ci/mmol) and find that it stabilizes only $\leqslant$1% of the skeletal muscle receptors in the high-affinity state, when compared with [^{3}H]PN 200-110 or [^{3}H]azidopine. This gives further support to the theory that the antagonistic nature of 1,4-dihydropyridines is related to their ability to stabilize the channel in the high-affinity state for this class of drugs. To explain the difference in M_r values of the [^{3}H]nimodipine and [^{3}H]PN 200-110 labelled channel it was proposed that 1,4-dihydropyridines bind directly to a channel element (α), which, when binding to fully antagonistic 1,4-dihydropyridines, is coupled to a smaller element than when binding a partially agonistic 1,4-dihydropyridine. From the calculated M_r values the 1,4-dihydropyridine binding element (α) was suggested to have an M_r of <75000 [6]. However, we found that [^{3}H]azidopine, a 1,4-dihydropyridine photoaffinity ligand, was covalently incorporated into a band of M_r 145000, which is twice the size of the proposed 1,4-dihydropyridine binding element. Several possibilities may explain this apparent paradox. In the target size analysis experiments [4,6] an underestimate of the M_r by 100% may have been made. This possibility seems remote as the irradiation experiments with the Ca^{2+} channel were performed according to [16] where the authors determined the M_r of recombinant DNA interferon to within 5% of the M_r known from the amino acid sequence. The proposed 'elements' in our model to explain the target size data may be equivalent to what has been recently termed 'radiation sensitive domains' in the case of ($Na^+ + K^+$)-ATPase [17]. We cannot exclude at the present time that the element (or radiation sensitive domain) is in fact smaller than the receptor carrying peptide chain. Another possibility is that [^{3}H]azidopine is photoincorporated into a channel protein other than the 1,4-dihydropyridine binding subunit. This suggestion is not unlikely especially when one calculates the molecular dimensions of [^{3}H]azidopine and considers the structure-activity relationship of several 1,4-dihydropyridines (fig.4). The least active 1,4-dihydropyridine in this series is M 5579, with a K_i of 1.8 μM. At the C3 position of the dihydropyridine ring M 5579 has a carboxylic acid residue. Substitution with a methyl group (length ~2 Å) at this position leads to nifedipine with nanomolar K_d values in ligand binding experiments. Substitution with 2-(N-benzyl-N-methylaminoethyl) group (length ~9.8 Å) as in nicardipine results in a 1,4-dihydropyridine with EC_{50} values in functional tests of 1–7 nM [18] and IC_{50} values in the same range in ligand binding experiments [19,20]. For [^{3}H]azidopine the addition of a bulky 4-(azido-



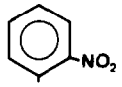
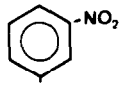
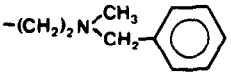
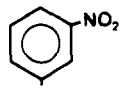
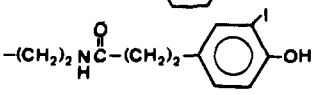
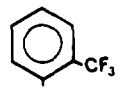
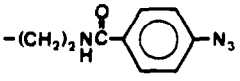
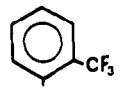
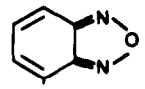
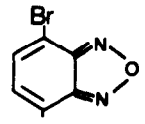
DRUG	R_1	R_2	X	K_D (nM)	Ref.
NIFEDIPINE	$-CH_3$	$-CH_3$		4	14
M 5579	$-H$	$-CH_3$		1860	18
NICARDIPINE		$-CH_3$		7	18
IODIPINE		$-C_2H_5$		0.4	18
AZIDOPINE		$-C_2H_5$		0.4	-
PN 200-110		$-CH_3$		1.5	14
VO 2605		$-CH_3$		320	18

Fig.4. Structures of 1,4-dihydropyridines. Structural formulas and potencies of 1,4-dihydropyridines in binding to skeletal muscle Ca^{2+} channels in membrane fragments. Dreiding scale models were made, and measured to determine approximate molecule dimensions. For nifedipine the crystal structure is known [22]. For the other 1,4-dihydropyridines the molecules were constructed with extended chain forms at the C3 and C5 ester substituents of the pyridine ring. The molecules were then measured across the C3, C5 axis of the pyridine ring. Total width of the molecules and lengths of R_1 (in Å): nifedipine, 10.5, 2; M 5579, 10.1, 1.3; nicardipine, 18, 9.8; iodipine, 23.5, 13,2; azidopine, 22, 11.8; PN 200-110 and Vo 2605, 12.2, 3.3.

phenyl)oxoaminoethyl group (length ~11.8 Å) at the C3 position of the dihydropyridine ring results in a ligand with a K_d value in the picomolar range. Even addition of a 2-[1-(3-iodo-4-hydroxyphenyl)-3-oxopropyl]aminoethyl group does not significantly alter ligand affinity. However, introduction of a single Br atom at the *para* position of the 4-phenyl ring causes a 200-fold loss in PN 200-110 affinity [19]. Substitutions at the *para* position of the 4-phenyl ring residue are known to be highly detrimental to 1,4-dihydropyridine Ca^{2+} channel blocking potency [21,22]. Thus, substituents at the

C3 ester group of the pyridine ring may not be in close contact with the surface of the 1,4-dihydropyridine binding protein of the channel because of the wide range of potentially sterically hindering substituents which are compatible with high-affinity binding.

A spherical protein of M_r 75000 would have a radius of ~29 Å. This is close to the total length of [^{3}H]azidopine (~22 Å). When the arylazido group is activated with ultraviolet light and forms active nitrene radicals, this reactive center is ~12 Å from the 1,4-dihydropyridine ring, and could possibly become incorporated into a closely neighboring channel protein. The development of 1,4-dihydropyridines with photoactivatable groups on the pyridine nucleus will allow this hypothesis to be examined experimentally. A potential 1,4-dihydropyridine affinity ligand with an isothiocyanate group at the *ortho* position of the 4-phenyl ring has been reported to be incorporated into a protein of M_r 45000 in guinea-pig heart and ileum membranes using 50 mM Tris buffer [23]. However, only PMSF was used to block proteolysis, the 'specifically' incorporated radioactivity was very low (peak radioactivity ~160 cpm) and no fluorograph was shown. So far we have not succeeded in covalently incorporating this 1,4-[^{3}H]dihydropyridine into guinea-pig skeletal muscle or heart membranes specifically even in amine-free buffers. Obviously further characterisation and purification of the channel components are required to resolve these discrepancies. The development of photoaffinity labels for the other drug receptor sites of the channel may help to answer the questions which are raised by the apparently divergent results obtained by target size analysis and covalent labelling with [^{3}H]azidopine.

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