A Homologous Series of Permanently Charged 1,4-Dihydropyridines : Novel Probes Designed To Localize Drug Binding Sites on Ion Channels[1]

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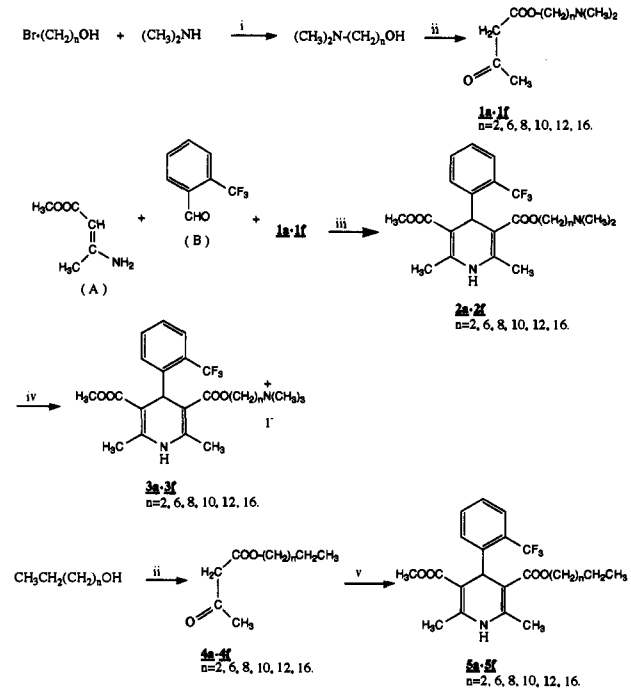
Voltage-gated ion channels are a family of integral membrane proteins that conduct ions (Na^+, K^+, Ca^{2+}) selectively and effectively across the cell membrane.[2] Their activity is regulated by activator and inhibitor drug molecules that bind to specific sites on the channel proteins.[2,3] Recent advances in molecular biology have permitted the cloning and sequencing of voltage-gated ion channels and the generation of putative organizational models.[4] However, the available biochemical techniques including photoaffinity labeling, site-directed mutagenesis, and antibody localization have permitted only a broad definition of the channel residues that may comprise drug binding sites.[5,6] Further localization of the drug binding sites is necessary both to define drug actions at the cellular level and to rationalize further the process of design of drugs active at voltage-gated ion channels.

Among the voltage-gated ion channels, one of the most widely studied is the L-type calcium channel which has separate binding sites for the three major chemical classes of clinically used calcium channel antagonist drugs: 1,4-dihydropyridines (nifedipine), phenylalkylamines (verapamil), and benzothiazepines (diltiazem).[7] The 1,4-dihydropyridine (1,4-DHP) binding site has been most extensively studied, and several structure-activity relationships (SAR) have been established at this site.[8] That the antagonist activities of the 1,4-DHPs are voltage-dependent has led to the suggestion that these drugs bind to an intramembrane site of the calcium channel sensitive to the electrical field across the membrane.[9] However, the precise location of the 1,4-DHP binding site still remains unknown owing to the limitations of the techniques and/or drugs/probes available. We now introduce a novel class of probes which has been designed to localize the position of the 1,4-DHP binding site in terms of its depth within the membrane bilayer.

These probes have a 1,4-DHP pharmacophore or moiety linked to a permanently charged group via a polymethylene chain of varying length. Unlike the lipophilic portions of these amphiphilic molecules such as the polymethylene chain and the 1,4-DHP moiety which should readily diffuse into the lipophilic membrane bilayer, the highly hydrophilic permanently charged group should be incapable of diffusing into the lipophilic membrane bilayer and therefore should remain restricted to the aqueous extracellular compartment.[10] The charged group should thus serve to virtually "anchor" the molecule at the extracellular surface of the membrane and allow only a limited access of the 1,4-DHP moiety, depending on the length of the polymethylene chain, to a putative intramembrane binding site. The binding affinity of this molecule would then depend on the access of the 1,4-DHP moiety to the binding site, which in turn would depend upon the length of the polymethylene chain. Thus the minimal chain length at which the binding affinity of the molecule is highest should reflect optimal access to the binding site. From such molecules, it should be possible to localize the position of the binding site within the membrane. We have applied this novel approach to the localization of the 1,4-dihydropyridine (1,4-DHP) binding site on the L-type calcium channel in rat heart cells.

Scheme I[a]



Chemistry. The design of the probes for the DHP binding site on calcium channels was based on the existing structure–activity relationship (SAR) of DHPs indicating considerable bulk tolerance for at least one of the ester groups in analogues of nifedipine (2,6-dimethyl-3,5-dicarbomethoxy-4-(2-nitrophenyl)-1,4-dihydropyridine).[8] Thus we synthesized a series of permanently charged 1,4-DHPs with a quaternary ammonium group attached to one of the ester groups via a polymethylene chain (Scheme I).[11] A second series of neutral molecules was also synthesized in which the quaternary ammonium group was replaced with an ethyl group to provide an approximate size equivalence (Scheme I).[11]

(Dimethylamino)alkanols were first prepared by alkylation of dimethylamine with ω-bromoalkanols and subsequently condensed with diketene to obtain ω-[(dimethylamino)alkyl]acetoacetates (**1a-f**).[12] Hantzsch condensation[13] of these acetoacetates with methyl 3-aminocrotonate and 2-(trifluoromethyl)benzaldehyde yielded the desired ω-(dimethylamino)alkyl ethyl-4-(2-(trifluoromethyl)phenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylates (**2a-f**). Quaternization with iodomethane then yielded the desired permanently charged 1,4-DHP methiodide salts (**3a-f**). The corresponding neutral analogues (**5a-f**) were synthesized in the usual manner by Hantzsch condensation of the appropriate alkylacetoacetates (**4a-f**) (obtained from the appropriate alkanol and diketene) with methyl 3-aminocrotonate and 2-(trifluoromethyl)benzaldehyde.

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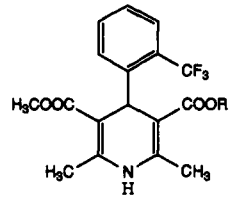
Table I. K_I Values of Charged and Neutral Compounds[a]



compound	R	K_I[b] (M ± SEM)
3a	$(CH_2)_2N^+(CH_3)_3$	$(3.32 \pm 0.95) \times 10^{-8}$
3b	$(CH_2)_6N^+(CH_3)_3$	$(2.34 \pm 0.78) \times 10^{-7}$
3c	$(CH_2)_8N^+(CH_3)_3$	$(1.73 \pm 0.33) \times 10^{-8}$
3d	$(CH_2)_{10}N^+(CH_3)_3$	$(1.17 \pm 0.35) \times 10^{-8}$
3e	$(CH_2)_{12}N^+(CH_3)_3$	$(1.74 \pm 0.62) \times 10^{-8}$
3f	$(CH_2)_{16}N^+(CH_3)_3$	$(6.36 \pm 2.25) \times 10^{-8}$
5a	$(CH_2)_2CH_2CH_3$	$(2.15 \pm 0.79) \times 10^{-9}$
5b	$(CH_2)_6CH_2CH_3$	$(2.40 \pm 0.40) \times 10^{-9}$
5c	$(CH_2)_8CH_2CH_3$	$(1.36 \pm 0.71) \times 10^{-8}$
5d	$(CH_2)_{10}CH_2CH_3$	$(1.67 \pm 0.45) \times 10^{-8}$
5e	$(CH_2)_{12}CH_2CH_3$	$(1.57 \pm 0.46) \times 10^{-8}$
5f	$(CH_2)_{16}CH_2CH_3$	$(8.67 \pm 2.38) \times 10^{-8}$
nifedipine		$(3.50 \pm 1.30) \times 10^{-9}$

[a] All compounds gave satisfactory ^1H NMR (300 MHz), mass spectra, and C, H, N microanalysis (±0.4%). [b] K_I values from the inhibition of [^{3}H]-(+)-PN200-110 binding in rat ventricular myocytes. Each K_I value is the mean of four to nine observations).

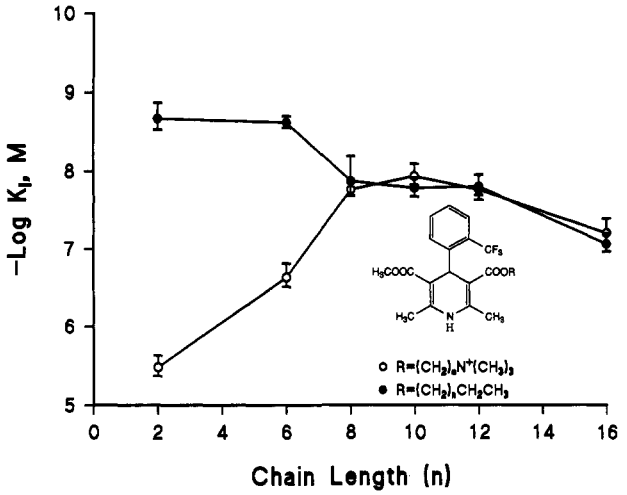


Figure 1. The relationship between binding affinity and chain length for charged (O) and neutral (b) 1,4-dihydropyridines.

Pharmacology. The affinities of these probes for the 1,4-DHP binding site of the L-type calcium channel were determined by radioligand binding experiments using intact cells and the obtained K_I values are listed in Table I.[14] The relationship between binding affinity and chain length is depicted in Figure 1. Compounds in the charged series show an initial dramatic increase in activity with increase in chain length (from $n = 2$ to $n = 6$ and from $n = 6$ to $n = 8$) followed by a broad plateau (no significant increase in activity with further increase in chain length from $n = 8$ to $n = 16$).[15] In contrast, compounds in the neutral series show only a slow decrease in activity with increasing chain length. These data suggest that the observed initial dramatic increase in activity with an increase in chain length from $n = 2$ to $n = 8$ in the charged series results from an increase in access of the compounds to their intramembrane binding site rather than from an increase in their lipophilicity. The data support the hypothesis that the charged group anchors the molecule at the extracellular surface of the membrane bilayer permitting only a limited access of the 1,4-DHP moiety to the binding site. At a chain length of $n = 2$, the externally anchored charged group presumably permits only minimal access of the 1,4-DHP moiety to the binding site and therefore the binding affinity of the molecule is low. With increases in chain length to $n = 6$ and then again to $n = 8$, the binding affinity of the molecule increases presumably as the access of the 1,4-DHP moiety to the binding site is improved. Since further increases in chain length to $n = 10$ and then to $n = 12$ are not accompanied by significant changes in the binding affinities of the molecules, it is likely that the binding site is completely accessed at a minimal chain length of $n = 8$. That the ratio of activities of the charged and neutral compounds is approximately unity from $n \geq 8$ further illustrates this point. When the binding site is accessed at this minimal chain length, the binding of the molecule should feature the polymethylene chain stretched out from the extracellular surface of the membrane (where the charged group of the molecule is anchored) to the intramembrane binding site (where the 1,4-DHP moiety of the molecule is bound). In such a scenario, the length of the polymethylene chain should reflect the distance from the membrane surface to the binding site, i.e., the depth of the binding site within the membrane. Thus the optimization of the binding affinity of the charged compounds and hence of their access to the binding site at a minimal chain length of $n = 8$ (compound **3c**) suggest the following:

(i) The 1,4-DHP binding site is located in the extracellular face of the membrane bilayer. This is so since the $n = 8$ chain length in the anchored molecule only permits access of the 1,4-DHP moiety into this half of the membrane bilayer.

(ii) This chain length reflects the depth of the binding site within the membrane. Molecular modeling showed that in the minimum energy conformer of **3c** with the polymethylene chain optimally stretched out, the distance from the quaternary nitrogen of the charged group to the ester oxygen of the 1,4-DHP moiety is approximately 11 Å.[16] This distance presumably approximates the distance from the membrane surface to the binding site, i.e., the depth of the binding site within the membrane. Thus the DHP binding site of the L-type calcium channel in rat heart cells appears to be located in the extracellular face of the membrane bilayer[17] and at a depth of approximately 11 Å from the membrane surface.

Previous studies have analyzed the location of the 1,4-DHPs nimodipine [2,6-dimethyl-3-[(2-methoxyethoxy)-carbonyl]-5-(ethoxycarbonyl)-4-(3-nitrophenyl)-1,4-dihydropyridine] and amlodipine [2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-3-(ethoxycarbonyl)-5-(methoxycarbonyl)-6-methyl-1,4-dihydropyridine] in phospholipid and cardiac sarcoplasmic reticulum membranes.[18,19] Amlodipine is positively charged at physiological pH by virtue of its (2-aminoethoxy)methyl side chain and its localization determined by X-ray crystallography, and deuterium magnetic resonance spectroscopy suggested a specific interaction between the protonated amino group and the negatively charged phospholipid region of the membrane. These data support the thesis that a charged group in the 1,4-DHPs can anchor the molecule at the surface of the membrane. However, the structural data refer only to the bulk disposition of the 1,4-DHPs in the membrane bilayer whereas the data presented here infer the localization of the probe specifically bound at the channel protein.

In conclusion, a homologous series of permanently

charged 1,4-dihydropyridine derivatives has been designed and developed as novel probes with which to locate the position of the intramembrane 1,4-DHP binding site on the L-type calcium channel in rat heart cells. These probes may also be useful in the homologous voltage-gated ion channel family since DHPs are known to be active, although less potently, at other types of calcium channel and at sodium and potassium channels.[20,21] Moreover, this concept has general utility since it should also be possible to use this approach to design and develop a similar homologous series of permanently charged probes for localizing the putative intramembrane drug binding sites on the various G-protein-coupled receptors (adrenergic, muscarinic, dopaminergic, and serotonergic) and the various ligand-gated ion channels (nicotinic, excitatory amino acid).

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(12) Since 2-(dimethylamino)ethanol is commercially available, it was used directly for the preparation of the corresponding acetoacetate (**1a**). Since 16-bromohexadecanol is not available commercially, hexadecane-1,16-diol was converted to the monotosylate which on treatment with dimethylamine gave the desired 16-(dimethylamino)hexadecanol. This was then condensed with diketene to obtain the required 16-(dimethylamino)hexadecyl acetoacetate (**1f**).

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(14) Binding experiments were carried out by measuring the inhibition of [^{3}H]-(+)-PN200–110 (isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-5-(methoxycarbonyl)-2,6-dimethyl-1,4-dihydropyridine-3-carboxylate) binding in intact neonatal rat ventricular myocytes (Wei, X.-Y.; Rutledge, A.; Triggle, D. *J. Mol. Pharmacol.* 1989, *35*, 541–552). High-affinity binding is shown in depolarizing conditions. This protocol is believed to measure plasma membrane 1,4-DHP binding sites.

(15) Though **3d** (charged $n = 10$) is slightly more active than **3c** (charged $n = 8$), the difference in activity between the two compounds is not statistically significant as determined by the *t*-test. Hence we consider the optimization of the activity to occur at $n = 8$ (**3c**) followed by a broad plateau of activity through $n = 10$ (**3d**) and $n = 12$ (**3e**).

(16) Molecular Modeling was carried out using the Alchemy III Molecular Modeling program (Tripos Associates, 1992).

(17) This has also been suggested from electrophysiologic observations using amlodipine (bearing a $CH_2OCH_2CH_2NH_2$ group at the C-2 position of the DHP ring) and SDZ 207–180 (similar in structure to **3d** except for the benzoxadiazole group at the 4-position of the DHP ring in place of the *o*-trifluorotolyl group) which were both found to be inactive when applied intracellularly but active when applied from the extracellular surface of cardiac cells. (a) Kass, R. S.; Arena, J. P. Influence of pHo on calcium channel block by amlodipine, a charged DHP compound. Implications for location of the DHP receptor. *J. Gen. Physiol.* 1989, *93*, 1109–1127. (b) Kass, R. S.; Arena, J. P.; Chin, S. Block of L-type calcium channels by charged DHPs. Sensitivity to side of application and calcium. *J. Gen. Physiol.* 1991, *98*, 63–75.

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