0026-895X/94/040660-07$03.00/0

MOLECULAR PHARMACOLOGY, 46:660–666, 1994.

L-Type Calcium Channels: Asymmetrical Intramembrane Binding Domain Revealed by Variable Length, Permanently Charged 1,4-Dihydropyridines

RAMESH BANGALORE, NANDKISHORE BAINDUR, ALETA RUTLEDGE, DAVID J. TRIGGLE, and ROBERT S. KASS

Department of Physiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642–8642 (R.B., R.S.K.), and Department of Biochemical Pharmacology, State University of New York at Buffalo, Buffalo, New York 14260 (N.B., A.R., D.J.T.)

Received March 28, 1994; Accepted July 14, 1994

SUMMARY

We have used an homologous series of dihydropyridine (DHP) derivatives to determine the location of the binding domain for DHPs on cardiac L-type calcium channels, relative to the extracellular and intracellular membrane surfaces. The series of test molecules consisted of DHP analogs in which the DHP moiety was linked to either a neutral ($-CH_2CH_3$) or permanently charged [$-^+N(CH_3)_3$] headgroup and the distance between the headgroup and the active moiety was systematically varied with alkyl spacer chains containing 2, 6, 8, 10, 12, or 16 methylene ($-CH_2$) groups. These compounds were previously shown, by radioligand binding experiments, to interact with the high affinity DHP binding site in intact neonatal rat heart cells. In the present experiments, access to the DHP binding site was assayed by inhibition of L-type calcium channel currents using whole-cell patch-clamp procedures in guinea pig ventricular myocytes. Intracellular application was achieved by dialysis via charged DHP-containing whole-cell patch pipettes, and cell dialysis was monitored by using a charged DHP labeled with a rhodamine fluorophore. Our results show that access of extracellularly applied charged, but not neutral, DHPs to the DHP binding domain depends markedly on the alkyl spacer chain, with the optimal length being near 10 methylene groups. Intracellular application failed to inhibit channel activity for spacer chain lengths up to 16 methylene groups. From our results, we conclude that the DHP binding domain of cardiac L-type calcium channels is not on the extracellular membrane surface but is probably within the lipid bilayer, approximately 11–14 Å from the extracellular surface.

Voltage-gated L-type calcium channels regulate calcium influx in diverse cell types and are of particular importance in the cardiovascular system, where calcium entry through them is closely linked to excitation of contraction and the modulation of intracellular second messengers (1). Calcium channel antagonists in general, and the DHPs in particular, constitute an important group of organic compounds that bind to the $\alpha 1$ subunit of the L-type calcium channel and control calcium influx by modulation of channel gating (2–5). Additionally, the calcium channel antagonists have served as primary therapeutic agents in the treatment of hypertension, angina, and certain forms of arrhythmias; more recently, their clinical use has been expanded to include congestive heart failure, cardiomyopathy, atherosclerosis, and cerebral and peripheral vascular disorders (6). Identification of the DHP binding domain on the L-type calcium channel $\alpha 1$ subunit at the molecular level is thus of great interest both for gaining insight into the control of L-type calcium channel gating and for designing and understanding the mode of action of this important group of therapeutic drugs.

The location of the DHP binding site within the primary structure of the L-type calcium channel has been probed using at least three molecular approaches. Regulla *et al.* (7) used photoaffinity labeling of purified skeletal muscle calcium channels and identified the major labeled peptide fragments of the $\alpha 1$ subunit, by proteolytic digestion, as those from the hydrophilic region of the cytoplasmic carboxyl-terminal loop. Based on these results, the proposed binding site location for DHPs is in the intracellular carboxyl-terminal region of the $\alpha 1$ subunit of the L-type calcium channel (7, 8). Striessnig *et al.* (9) and Nakayama *et al.* (10) identified the photolabeled peptides using site-specific antibodies and reported a binding site location for DHPs near the extracellular end of transmembrane segment 6 of the third and fourth membrane-spanning domains, and Kalasz *et al.* (11) have used photoaffinity labeling to localize the DHP binding site to the S5–S6 linker peptides of repeats I, III, and IV.

Most recently, Tang *et al.* (12) created chimeric calcium

This work was supported by USPHS award HL21922-16 and American Heart Association fellowship no. 93-217.

ABBREVIATIONS: DHP, dihydropyridine; RHD, rhodamine-labeled derivative; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

channels by using the cardiac $\alpha 1$ subunit as the parent protein and replacing targeted segments with the corresponding segments of the B1–2 calcium channel, a channel that is insensitive to DHPs (13, 14). The DHP sensitivity of the expressed chimeric channels indicated that the S5–S6 linker in motif IV of the L-type calcium channel is a critical site for DHP action (12). Functional studies of DHP modulation of recombinant cardiac and lung smooth muscle $\alpha 1$ channels also suggested a possible role for the S3 hydrophobic segment of motif IV of the L-type calcium channel in determining unique tissue-specific profiles of DHP modulation (15).

To resolve the location of the binding site in physiological L-type calcium channels, molecular studies must be compared with data obtained from functional studies of native L-type calcium channels. Using the permanently charged DHP antagonist SDZ 207–180, Kass *et al.* (16) provided data consistent with a DHP binding site accessible from the extracellular, but not intracellular, side of the cell membrane. However, the use of a single permanently charged DHP antagonist (SDZ 207–180) in which the DHP moiety is separated from the ionized headgroup by a long hydrocarbon chain limits resolution of the binding site with respect to the membrane surface, and thus these data cannot be used to provide more detailed information needed to predict the precise binding domain. Here we report results of functional studies of native cardiac L-type calcium channels in which we have probed the DHP binding site location by measuring the sensitivity of whole-cell currents to external and internal application of a series of custom-synthesized DHP analogs, in which the active moiety was linked to either a neutral ($-CH_2CH_3$) or permanently charged [$-^+N(CH_3)_3$] headgroup and the distance between the headgroup and the active moiety was systematically varied by alkyl spacer chains containing 2, 6, 8, 10, 12, or 16 methylene ($-CH_2$) groups. Our results provide the first functional evidence for an intramembrane binding domain that may be located within 11–14 Å of the extracellular membrane surface.

Materials and Methods

Electrophysiology

Cell preparations, voltage protocols, and drug application. Single ventricular myocytes were isolated from either ventricle of adult guinea pigs using previously described methodology (17, 18). Solution changes were accomplished using a gravity-driven perfusion system containing a multibarreled ejection pipette that allows for local concentration changes in <1 sec. Control records were recorded under drug-free conditions, the external solution was changed to a drug-containing solution, and the same voltage protocol was immediately reapplied in the presence of a test drug concentration. Test drugs were first applied while the cell was held at −80 mV and were then maintained for onset and recovery protocols.

Drug onset was measured by using a depolarizing "train" protocol in which the holding potential was changed from −80 mV to −40 and test voltage pulses (+10 mV, 40 msec) were applied at 5-sec intervals for a total period of 75 or 150 sec. In this protocol, the first pulse was applied from −80 mV and subsquent pulses were applied from −40 mV. Current inhibited by drug during the first pulse reflected drug block that developed rapidly at −80 mV, which is referred to as tonic block. Recovery from block was measured using train protocols in which the holding potential was changed from −40 mV to −80 mV and test pulses (+10 mV, 40 msec) were again applied at 5-sec intervals for periods of 75 or 150 sec. Here, the first pulse was applied from −40 mV and subsquent pulses were applied from −80 mV.

Analysis of recovery kinetics was carried out after first promoting channel block by prolonged depolarization (30 sec) to 0 mV and then returning the membrane potential to −80 mV, from which voltage test pulses to +10 mV (40 msec, 2 Hz) were applied to assay for recovery from drug block. In the case of neutral drugs, the fraction of current that recovered with drug-induced slow kinetics was a reflection of drug-bound and inhibited channels. In the case of charged drugs, there was no drug-induced slow component to the kinetics of current recovery but the fraction of current that did not recover in the presence of charged drugs reflected drug-bound and blocked channels and could be used for comparison of relative potency of the charged compounds.

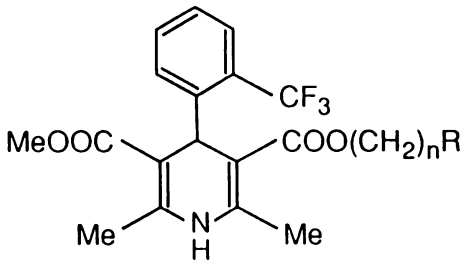
Solutions and drugs. The standard pipette solution contained 60 mM CsCl, 50 mM aspartic acid, 68 mM CsOH, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 5 mM K_2ATP, and 10 mM HEPES, pH 7.4. Cells were dialyzed with drug-free solution or solution containing appropriate concentrations of drugs throughout each experiment. The extracellular solution contained 130 mM *N*-methyl-D-glucamine, 4.8 mM CsCl, 5 mM $BaCl_2$, 5 mM glucose, and 5 mM HEPES, pH 7.4. The general structure of the family of DHP compounds used in this study is shown in Fig. 1. Methyl chains of various lengths separate the DHP moiety from the test headgroup (R). Spacer chain length is denoted by *n*, the number of methyl groups in the chain. Neutral headgroups are denoted as R^o and charged groups are denoted as R^+. DHP derivatives were dissolved in ethanol or dimethylsulfoxide to make concentrated (millimolar) stock solutions and were diluted in the bath solution to appropriate concentrations. The highest concentrations of solvents reached in the bath were included in controls to correct for any solvent effects.

Fluorescence Microscopy

Images were captured and analyzed via a Vision Plus AT frame-grabber board (Imaging Technology, Bedford, MA) interfaced into an IBM-compatible PC-386 computer (Dell Computer Corp., Austin, TX) under software control of OPTIMAS (Bioscan, Inc., Edmonds, WA). Optical measurements were made with a modified Olympus IMT-1 microscope (Olympus Corp., Lake Success, NY). Excitation light (560 nm) and emitted light (600 nm) were selected with custom-manufactured filters and dichroic reflector (Omega Optical Inc., Battleboro, VT). The cell was held at −40 mV throughout the experiment. Current-voltage relationships, generated by applying 100-msec test pulses at 1 Hz, were recorded at 10-min intervals from 0 to 30 min after establishment of the whole-cell configuration, at which times the fluorescent images were also captured to monitor diffusion of the RHD over the period of 30 min.

Internal Dialysis

In dialysis experiments, cells were patch-clamped with pipettes containing either drug-free internal solution (control) or internal solution containing the appropriate concentration of charged DHPs, and depolarizing trains (15 pulses each, of 40-msec duration, from a holding



R = [CH_2CH_3] or [$^+N(CH_3)_3$]
n = 2, 6, 8, 10, 12 or 16

Fig. 1. General structure of all DHP derivatives used in this study. A neutral ($-CH_2CH_3$) or charged [$-^+N(CH_3)_3$] headgroup (R) is separated from the DHP moiety by alkyl chains of variable lengths (*n* = 2, 6, 8, 10, 12, or 16). *Me*, methyl.

potential of −40 mV to +10 mV at 0.2 Hz) were applied at 5-min intervals between 0 and 30 min. This train protocol was shown to promote block by 10-fold lower concentrations of each of the charged DHPs applied extracellularly. Because we measured no pulse-dependent block in these experiments, current amplitude was averaged within each train to determine mean current amplitude at each time point.

Curve Fitting

Curve fitting and statistical analysis were carried out using the Origin software package (Microcal, Inc., Northampton, MA).

Results

Externally applied charged and neutral drugs inhibit L-type calcium channel activity. Both neutral and permanently charged forms of the unique DHPs used in this study inhibited L-type calcium channel activity via extracellular application. Molecules with neutral headgroups (R^0) blocked currents in a voltage-dependent manner that closely resembled that of previously investigated neutral DHPs (19); depolarization promoted, and subsequent hyperpolarization relieved, block. Fig. 2 compares voltage-dependent onset and recovery for equal concentrations (500 nM) of two neutral drugs, with $n = 2$ and $n = 10$. Spacer chain length did not appear to affect the interaction of the neutral compounds with the DHP binding domain.

In contrast, block by molecules with charged headgroups (R^+) was strikingly affected by spacer chain length and was characterized by very slow depolarization-induced onset, little or no recovery upon subsequent hyperpolarization, and marked tonic block. Fig. 3 illustrates the effect of spacer chain length on charged DHP block and recovery and shows that, for charged drug molecules, a 20-fold higher drug concentration (10 μM) of a short-chain molecule ($n = 2$) was required to cause pharmacological effects comparable to those of a molecule with a longer spacer chain ($n = 10$).

Alkyl spacer chain length affects block by charged, but not neutral, drugs. To quantify differences in potency of the various DHPs, we focused on recovery kinetics after block was induced by long (30-sec) depolarizing pulses. For molecules with neutral headgroups, with all spacer chain lengths studied, there was a concentration-dependent increase in the fraction of current that recovered with slow kinetics (Fig. 4A). Interpreting the fraction of current recovering with drug-induced slow kinetics as a reflection of the proportion of channels that had become drug-bound during the conditioning depolarization (20), Fig. 4C shows that the same concentration (500 nM) of neutral drug inhibited approximately 70% of available channels under these conditions, irrespective of spacer chain length.

In contrast, the relative effectiveness of the charged DHPs showed a marked dependence on spacer chain length. Here, recovery kinetics remained rapid and unchanged from control, but the amplitude of recovering current decreased in a concentration-dependent manner (Fig. 4B). This is consistent with a concentration-dependent increase in drug-bound channels that do not recover from block upon hyperpolarization and a concentration-dependent decrease in unbound channels that recover with rapid kinetics consistent with removal of inactivation of drug-free channels at −80 mV. In this case, the steady state fraction of current that does not recover is a reflection of the proportion of channels that are inhibited by the charged DHPs. Using this analysis, we determined the relative potencies of the same concentration (500 nM) of five charged compounds (R^+) applied externally, R^+, $n = 2 \ll R^+$, $n = 6 \cong R^+$, $n = 12 < R^+$, $n = 8 \cong R^+$, $n = 16 < R^+$, $n = 10$. The relative potencies of these compounds are compared in Fig. 4C. When the DHP moiety is linked to a permanently charged headgroup, a spacer chain length of 10 methylene groups is optimal for access to the binding site from the extracellular surface of the membrane.

Internally applied charged DHPs do not inhibit L-type calcium channel activity. We next tested for a possible chain-length dependence of internal access to the DHP binding site by including high concentrations of each permanently charged compound in the patch pipettes. We first estimated diffusion times for charged DHPs by simultaneously measuring fluorescence of an ionized RHD (R^+, $n = 6$) and L-type calcium channel activity. Fig. 5, A–C, shows diffusion of RHD^+ out of a patch pipette containing 10 μM RHD^+ and throughout a

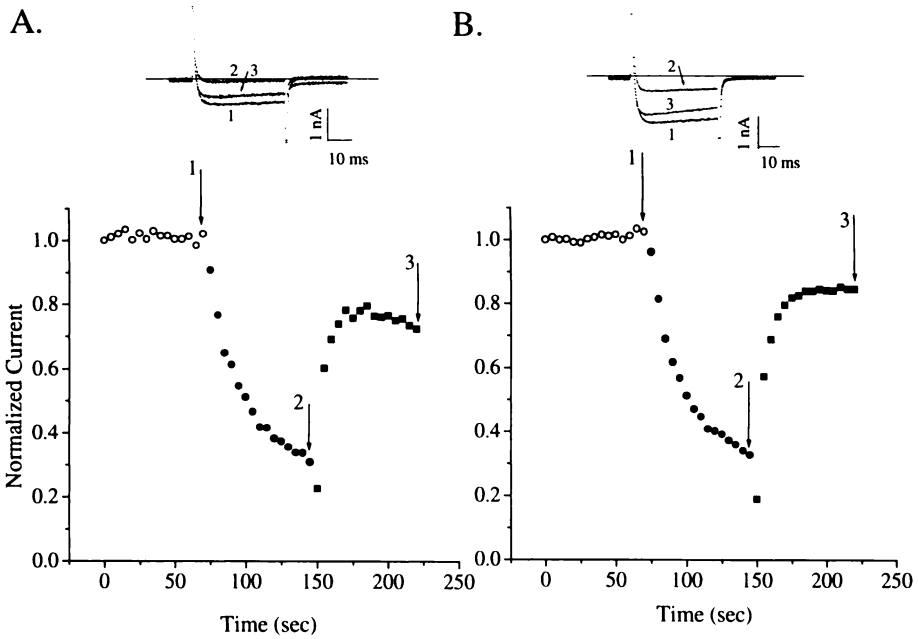


Fig. 2. Modulation of heart L-type calcium channel currents by externally applied neutral DHPs. Representative plots show onset of and recovery from block of L-type calcium channels. Normalized peak current values (*ordinates*) are plotted versus time of voltage train protocol (*abscissae*), in the absence (○) or presence (●, ■) of short chain ($n = 2$; 500 nM) (A) or long chain ($n = 10$; 500 nM) (B) neutral DHPs. *Traces*, currents recorded at the indicated times of the trains (*arrows* and *labels*). Test voltages were +10 mV. Train protocols were as described in Materials and Methods. Holding potentials were changed from −80 mV to −40 mV (onset) (○, ●) and from −40 mV to −80 mV (recovery) (■).

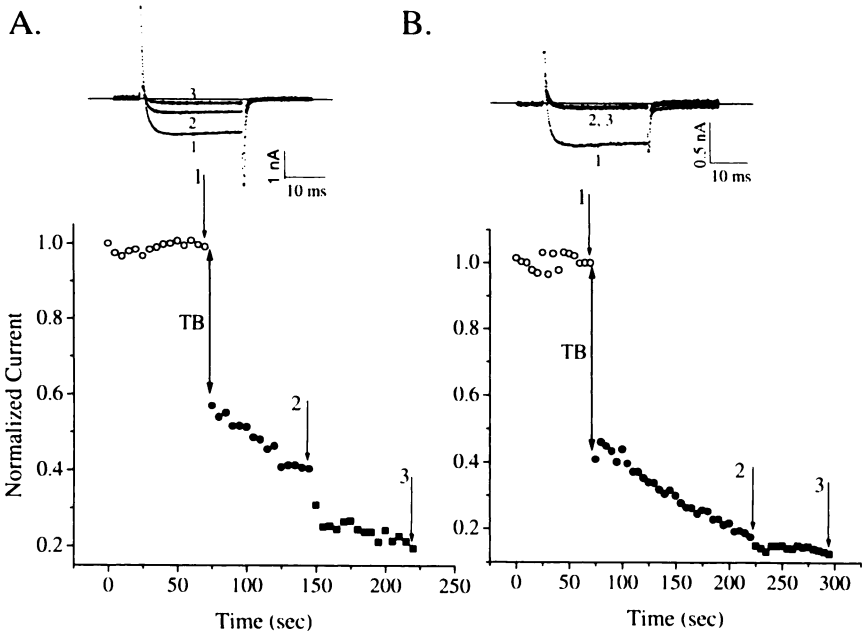


Fig. 3. Inhibition of L-type calcium channel currents by externally applied charged DHPs. Representative plots show onset of and tests for recovery from inhibition of L-type calcium channel current. Normalized peak current (*ordinates*) is plotted versus time of voltage train protocol (*abscissae*) in the absence (○) or presence (●, ■) of short chain (n = 2; 10 μM) (A) or long chain (n = 10; 500 nM) (B) charged DHPs. *Traces*, currents recorded at the indicated times of the trains (*arrows* and *labels*). Test voltages were +10 mV, and the train protocols were as described in Materials and Methods. Holding potentials were changed from −80 mV to −40 mV (onset protocol) (○, ●) and from −40 mV to −80 mV (recovery protocol) (■). *TB*, tonic block of current measured during the first test pulse in the onset train. Note that current does not recover from inhibition at the −80 mV conditioning voltage of the recovery protocol.

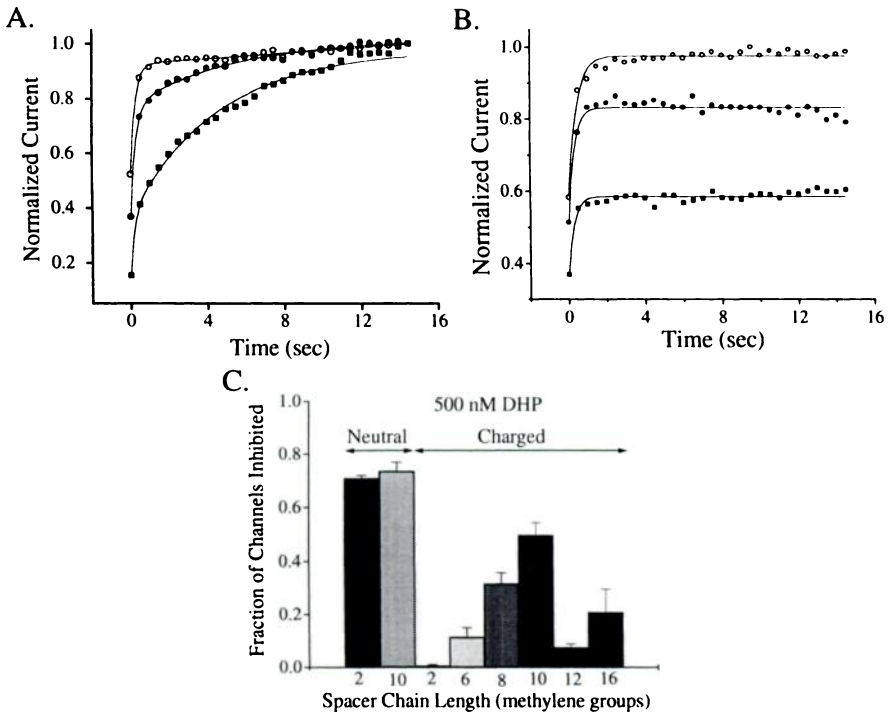


Fig. 4. Influence of charge and spacer chain length on L-type calcium channel recovery from, and relative potency of, DHP block. A and B, Protocols to measure recovery of current were applied at −80 mV after channel block had been induced by depolarization (0 mV, 30 sec). Peak current is plotted as a function of time after returning to −80 mV in the absence (○) and presence of short chain (n = 2) neutral (A) (●, 100 nM; ■, 500 nM) and charged (B) (●, 1 μM; ■, 10 μM) DHPs. *Smooth curves in A*, fits to a biexponential equation (τ_1 = 0.4 sec; τ_2 = 7.8 sec); there is a concentration-dependent increase in the fraction of current recovering slowly. *Smooth curves in B*, fits to an equation with a single time constant (τ = 0.4 sec), because there is no slow component of recovery in the presence of drug as with the neutral DHPs. C, Comparative block of L-type calcium channel current by 500 nM levels each of neutral [n = 2 (three experiments) and n = 10 (three experiments)] and charged [n = 2 (five experiments), n = 6 (eight experiments), n = 8 (seven experiments), n = 10 (four experiments), n = 12 (10 experiments), and n = 16 (eight experiments)] DHPs is shown. The fraction of channels inhibited was determined from recovery kinetics as described in the text. Mean ± standard error values of the fraction of channels blocked by each drug at 500 nM are shown.

myocyte during a 30-min dialysis period. The labeled DHP clearly diffuses throughout the cell but does not block L-type calcium channel activity. From the profile of the fluorescence signal along the cell (data not shown) we were able to estimate the average intracellular RHD^+ concentrations at different times. After 20 min, we estimated that the average intracellular RHD^+ concentration was 2.7 μM and the RHD^+ concentration in >70% of the cell was ≥1 μM, values that are consistent with theoretical predictions for this experimental arrangement (21). Because control experiments showed that 1 μM extracellularly applied RHD^+ completely blocked channel activity using depolarization protocols, a 20-min dialysis period was sufficiently long for intracellular drug concentrations to reach levels where block of channel activity should be apparent. However, even after an additional 10-min dialysis period, channel activity was not inhibited by RHD^+ (Fig. 5, D-G). Because RHD^+ (M_r 990) was the largest charged molecule used, 20 min could be used as an upper limit for dialysis periods for the other charged derivatives in this study (21).

We then tested L-type calcium channel sensitivity to intracellular application of three charged DHPs (n = 2, n = 10, and n = 16) by dialyzing cells for 30 min with pipette solutions containing maximal concentrations of the charged DHPs, as determined by extracellular application. We found no statistically significant decrease in current amplitude between control currents recorded with drug-free pipettes and currents measured with pipettes containing charged derivatives with short or long spacer chains (Fig. 6). In the case of the charged compound

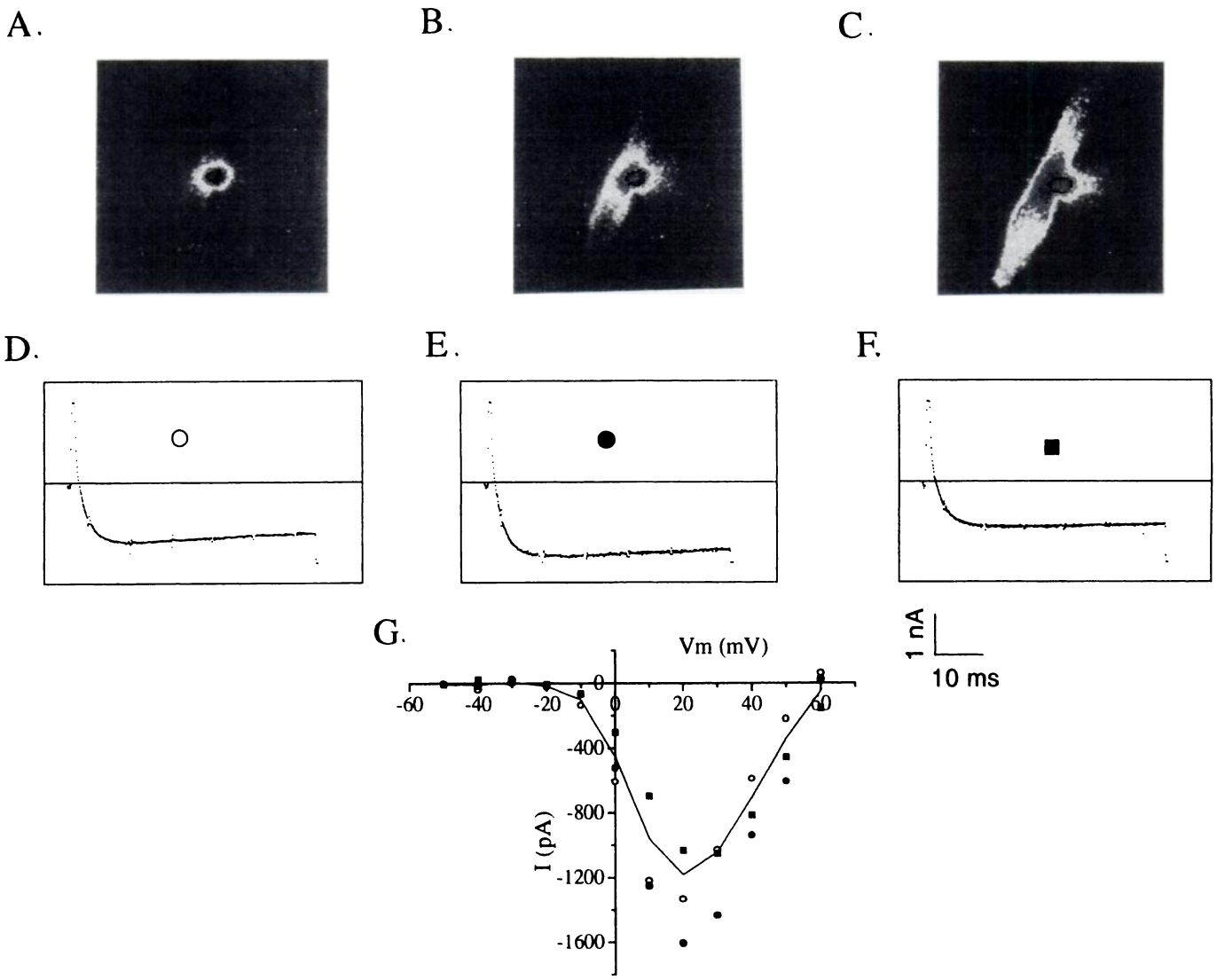


Fig. 5. Test for internal dialysis and effect on L-type calcium channel current of a charged fluorescent DHP. A–C, Time course of diffusion of RHD^+ (n = 6, M_r 990) from the patch pipette into a patch-clamped myocyte. RHD^+ (10 μM) was included in the patch pipette and, after establishment of the whole-cell configuration, fluorescence and L-type calcium channel currents were monitored over a period of 30 min, at 10-min intervals. A–F, Fluorescent images and current traces recorded at +10 mV immediately after establishment of the whole-cell configuration (A and D), 10 min later (B and E), and 30 min later (C and F). G, Current-voltage plots at 0 min (○), 10 min (●), and 30 min (■) after establishment of the whole-cell configuration. *Line*, nonlinear curve fitted to the mean of control and 30-min data, intended for reference purposes only. At 10 min there is a slight increase, and at the end of 30 min an approximately 18% decrease consistent with channel rundown, in current amplitude. The holding potential was −40 mV throughout this experiment.

with n = 16, there was a trend for inhibition of current, compared with control, after 30 min, but this trend was not statistically significant at the p = 0.05 level (control, seven experiments; R^+, n = 16, four experiments). The trend for inhibition by this charged derivative with a long spacer chain suggests, however, that at this chain length the drug may be approaching the lower limit for access to the DHP binding site from the intracellular surface.

Discussion

Resolution of the DHP binding domain. Our results extend previous functional data supporting external but not internal access to the DHP binding site for ionized DHP derivatives (16) and radioligand binding data demonstrating a chain-length dependence of binding affinity for the charged but not neutral compounds (22). In the experiments of Baindur *et al.* (22), displacement of (+)-[^{3}H]PN200–110 binding was measured directly in intact neonatal rat ventricular myocytes, and the binding affinities obtained shared the same chain-length dependence that we have determined from measurement of L-type calcium channel inhibition. Thus, both neutral and charged DHPs inhibit L-type calcium channel activity by interacting with the high affinity DHP binding site, and differences in relative potency and voltage dependence of block are most likely due to restrictions placed on access to the DHP binding domain by drug structure.

Our data are consistent with, but do not prove the validity of, previous models of the partitioning of the ionized form of amlodipine into lipid bilayers (23, 24), in which the ionized amino group is extracellular and the DHP moiety interacts

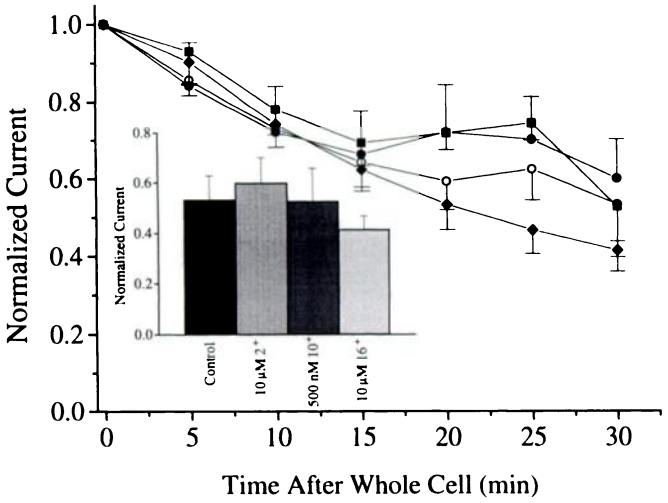


Fig. 6. Lack of inhibition of L channel currents by internally applied charged DHPs. The plot shows peak normalized current values (mean ± standard error) recorded as functions of time after establishment of the whole-cell configuration, with patch pipettes containing drug-free internal solution (○) or internal solution containing 10 μM short chain (n = 2, M_r 568) (●), 500 nM intermediate chain (n = 10, M_r 680) (■), or 10 μM long chain (n = 16, M_r 764) (♦) charged DHPs. The concentration chosen for each DHP was ≥10-fold higher than that required for external block of channel activity. There is no significant difference at any time point between current amplitude in the absence and presence of each of the three drugs by Student t test, at $p < 0.05$ (three to 12 experiments). *Inset*, mean current amplitude remaining at the end of 30 min with drug-free internal solution and internal solution containing the three charged DHPs at the concentrations shown. There is no significant difference in current amplitude between the absence (seven experiments) and presence (three to five experiments) of any of the charged drugs in the patch pipette. The holding potential was −40 mV in these experiments.

with a binding site within the membrane. If the homologous series of charged compounds behave similarly, then this work and previous radioligand binding data (22) suggest a DHP binding domain located eight to 10 methylene groups, or approximately 11–14 Å, from the external membrane surface. If the binding domain were extracellular, then a chain-length dependence for channel inhibition by the charged compounds would not be likely. The failure of internally applied charged derivatives, with spacer chains of up to 16 methylene groups in length, to inhibit channel activity provides additional evidence that the DHP binding domain is located closer to the extracellular than the intracellular membrane surface in cardiac L-type calcium channels. Additionally, although it was not statistically significant, we did observe a consistent decrease in currents after 17 min of dialysis with pipettes containing the longest chain (R^+, n = 16) DHP that we used. This suggests the possibility that charged DHPs with longer spacer chains may be able to interact with the DHP binding site from the cytoplasmic side of the cell membrane.

Our data can be used to complement recent experiments probing the molecular locus of the DHP binding domain. Because we find that ionized, but not neutral, drugs are limited in access to the DHP binding site by the distance between the charged headgroup and the DHP moiety, our data strongly suggest that the binding site is not extracellular but must reside within the membrane or channel pore. Molecular candidates for the DHP binding site that are consistent with our functional results have been proposed based on photoaffinity labeling and antipeptide antibody mapping (9, 10). Additionally, based on the sensitivity of chimeric L-type calcium channels to DHP modulation, Tang *et al.* (12) provided strong evidence that the S5–S6 linker in motif IV of the α1 subunit is critical for DHP modulation. Data comparing DHP-dependent modulation of cardiac and smooth muscle recombinant α1 channels have also suggested that the S3 segment of motif IV may play an important role in the voltage-dependent properties of DHPs (15). Because we find that access of the charged compounds to the DHP binding site, as assayed by channel inhibition, is asymmetrical and optimal for a spacer chain length near 10 methylene groups, we can estimate the location and suggest molecular regions that may serve as candidates for future mutagenesis experiments.

Influence of the charged group on the voltage dependence of block. Changing from a neutral to a charged headgroup consistently affected not only the relative potency of the DHP analogs studied but also the voltage dependence of channel block and unblock. Two striking characteristics of L-type calcium channel modulation by ionized DHPs emerged from this work, i.e., 1) charged drugs promote greater tonic block (block at −80 mV without depolarizing test pulses) and 2) channels blocked by charged forms of the DHP analogs do not recover from block at negative potentials. The fact that charged drugs promote tonic block in the absence of voltage pulses that promote channel openings suggests that both tonic block and failure to recover blocked channels at negative voltages are probably not caused by trapping of the charged headgroup within the channel pore but instead result from a charge-induced stabilization of the drug-bound and unavailable state of the channel. However, distinction between these two possibilities awaits future experimental tests.

References

1. Tsien, R. W., P. T. Ellinor, and W. A. Horne. Molecular diversity of voltage-dependent Ca channels. *Trends Pharmacol. Sci.* **12**:349–354 (1991).
2. Glossmann, H., D. R. Ferry, A. Goll, J. Striessnig, and G. Zernig. Calcium channels: introduction into their molecular pharmacology, in *Cardiovascular Effects of Dihydropyridine-Type Calcium Antagonists and Agonists* (A. Fleckenstein, C. Van Breemen, R. Gross, and F. Hoffmeister, eds.). Springer-Verlag, Heidelberg, 113–139 (1984).
3. Galizzi, J. P., M. Borsotto, J. Barhanin, M. Fosset, and M. Lazdunski. Characterization and photoaffinity labeling of receptor sites for Ca channel inhibitors D-*cis*-diltiazem, bepridil, desmethoxyverapamil, and (+)-PN 200–110 in skeletal muscle transverse tubule membranes. *J. Biol. Chem.* **144**:211–215 (1986).
4. Hess, P., J. B. Lansman, and R. W. Tsien. Different modes of gating behaviour favoured by dihydropyridine agonists and antagonists. *Nature (Lond.)* **311**:538–544 (1984).
5. Sieber, M., W. Nastainczyk, V. Zubor, W. Wernet, and F. Hofmann. The 165-kDa peptide of the purified skeletal muscle dihydropyridine receptor contains the known regulatory sites of the calcium channel. *Eur. J. Biochem.* **176**:117–122 (1987).
6. Janis, R. A., and D. J. Triggle. *The Calcium Channel: Its Properties Function, Regulation and Clinical Relevance.* CRC Press, Cleveland (1990).
7. Regulla, S., T. Schneider, W. Nastainczyk, H. W. Meer, and F. Hofmann. Identification of the site of interaction of the dihydropyridine channel blockers nitrendipine and azidopine with the calcium-channel α1 subunit. *EMBO J.* **10**:45–49 (1991).
8. Hess, P., and R. W. Tsien. Mechanism of ion permeation through calcium channels. *Nature (Lond.)* **311**:538–544 (1984).
9. Striessnig, J., B. J. Murphy, and W. A. Catterall. Dihydropyridine receptor of L-type calcium chanels: identification of binding domains for ^{3}H-PN200–110 and ^{3}H-azidopine within the *alpha*1 subunit. *Proc. Natl. Acad. Sci. USA* **88**:10769–10773 (1991).
10. Nakayama, H., M. Taki, J. Striessnig, H. Glossmann, W. A. Catterall, and Y. Kanaoka. Identification of 1,4-dihydropyridine binding regions within the *alpha*1 subunit of skeletal muscle Ca^{2+} channels by photoaffinity labeling with diazepine. *Proc. Natl. Acad. Sci. USA* **88**:9203–9207 (1991).
11. Kalasz, H., T. Watanabe, H. Yabana, K. Itagaki, K. Naito, H. Nakayama, A. Schwartz, and P. L. Vaghy. Identification of 1,4-dihydroyridine binding domains within the primary structure of the α1 subunit of the skeletal muscle L-type calcium channel. *FEBS Lett.* **331**:177–181 (1993).
12. Tang, S., A. Yatani, A. Bahinski, Y. Mori, and A. Schwartz. Molecular

localization of regions in the L-type calcium channel critical for dihydropyridine action. *Neuron* **11**:1013–1021 (1993).
13. Williams, M. E., P. F. Brust, D. H. Feldman, S. Patthi, S. Simmerson, A. Maroufi, A. F. McCue, G. Velicelebi, S. B. Ellis, and M. M. Harpold. Structure and functional expression of an ω-conotoxin-sensitive human N-type calcium channel. *Science (Washington D. C.)* **257**:389–395 (1992).
14. Mori, Y., T. Friedrich, M.-S. Kim, A. Mikami, J. Nakai, P. Ruth, E. Bosse, F. Hofmann, V. Flockerzi, T. Furuichi, K. Nikoshiba, K. Imoto, T. Tanabe, and S. Numa. Primary structure and functional expression from complementary DNA of a brain calcium channel. *Nature (Lond.)* **350**:398–402 (1991).
15. Welling, A., Y. W. Kwan, E. Bosse, V. Flockerzi, F. Hofmann, and R. S. Kass. Subunit-dependent modulation of recombinant L-type calcium channels: molecular basis for dihydropyridine tissue selectivity. *Circ. Res.* **73**:974–980 (1993).
16. Kass, R. S., J. P. Arena, and S. Chin. Block of L-type calcium channels by charged dihydroyridines: sensitivity to side of application and calcium. *J. Gen. Physiol.* **98**:63–75 (1991).
17. Mitra, R., and M. Morad. A uniform enzymatic method for dissociation of myocytes from hearts and stomachs of vertebrates. *Am. J. Physiol.* **249**:H1056–H1060 (1985).
18. Arena, J. P., and R. S. Kass. Block of heart potassium channels by clofilium and its tertiary analogs: relationship between drug structure and type of channel blocked. *Mol. Pharmacol.* **34**:60–66 (1988).
19. Kass, R. S., and J. P. Arena. Influence of pH_o on calcium channel block by amlodipine, a charged dihydropyridine compound: implications for location of the dihydropyridine receptor. *J. Gen. Physiol.* **93**:1109–1127 (1989).
20. Hille, B. The pH-dependent rate of action of local anesthetics on the node of Ranvier. *J. Gen. Physiol.* **69**:475–496 (1977).
21. Pusch, M., and E. Neher. Rates of diffusional exchange between small cells and a measuring patch pipette. *Pfluegers Arch.* **411**:204–211 (1988).
22. Baindur, N., A. Rutledge, and D. J. Triggle. A homologous series of permanently charged 1,4-dihydropyridines: novel probes designed to localize drug binding sites on ion channels. *J. Med. Chem.* **36**:3743–3745 (1993).
23. Mason, R. P., S. F. Campbell, S. D. Wang, and L. G. Herbette. Comparison of location and binding for the positively charged 1,4-dihydropyridine calcium channel antagonist amlodipine with uncharged drugs of this class in cardiac membranes. *Mol. Pharmacol.* **36**:634–640 (1989).
24. Bauerle, H. D., and J. Seelig. Interaction of charged and uncharged calcium channel antagonists with phospholipid membranes: binding equilibrium, binding enthalpy, and membrane location. *Biochemistry* **30**:7203–7211 (1991).

Send reprint requests to: Robert S. Kass, Department of Physiology, University of Rochester School of Medicine and Dentistry, 601 Elmwood Ave., Rochester, NY 14642–8642.