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# Extracellular Localization of the Benzothiazepine Binding Domain of L-Type $Ca^{2+}$ Channels

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**SUMMARY**

To determine which side of L-type $Ca^{2+}$ channels forms the benzothiazepine binding domain, we tested the effects of a membrane-impermeable, diltiazem-like, $Ca^{2+}$ antagonist, SQ32,428 [(*cis*)-1,3,4,5-tetrahydro-4-(4-methoxyphenyl)-3-methyl-6-(trifluoromethyl)-1-[2-trimethylammonio)ethyl]-2*H*-1-benzazepin-2-one], on $Ca^{2+}$ channels in smooth muscle-like cells (A7r5 cells) and skeletal muscle-like cells (differentiated $BC_3H1$ cells). This permanently charged, quaternary benzazepine bound to the benzothiazepine-selective domain of skeletal muscle $Ca^{2+}$ channels with a $K_i$ of 1.2 ± 0.1 μM. Extracellular application of SQ32,428 reversibly blocked whole-cell barium currents through L-type $Ca^{2+}$ channels in A7r5 and $BC_3H1$ cells with similar potencies (A7r5, $IC_{50}$ = 86 μM; $BC_3H1$, $IC_{50}$ = 50 μM). Block was fully reversible, was independent of stimulation frequency, and did not affect steady state inactivation of the channel in A7r5 cells. Intracellular dialysis of the cells with 100 μM SQ32,428 was without effect, but the same concentration of the quaternary phenylalkylamine D890 blocked channel activity from the cytoplasmic side. Our data demonstrate that the benzothiazepine binding domain of L-type $Ca^{2+}$ channels binds diltiazem-like benzazepine $Ca^{2+}$ antagonists and is formed by amino acid residues exposed to the extracellular channel surface.

Voltage-dependent $Ca^{2+}$ channels mediate the influx of $Ca^{2+}$ ions upon membrane depolarization in electrically excitable cells. At least four types (N-, P-, T-, and L-type) can be distinguished by pharmacological and biophysical criteria (1). L-type channels are modulated by different chemical classes of organic $Ca^{2+}$ channel blockers (e.g., dihydropyridines, phenylalkylamines, and benzothiazepines) that are widely used clinically to treat cardiovascular disorders. They bind to distinct, allosterically coupled, high affinity drug-binding domains on the $\alpha_1$ subunit of the channel (2). This subunit forms the voltage-sensitive ion-conducting pore and is structurally related to the pore-forming subunits of voltage-dependent $Na^+$ and $K^+$ channels (3). The regions participating in the formation of the binding domains for phenylalkylamines and dihydropyridines have recently been identified within the primary structure of the skeletal muscle $\alpha_1$ subunit, after photoaffinity labeling, using sequence-directed antibodies and defined proteolysis (4). Dihydropyridines bind close to the extracellular mouth of the channel pore, at the extracellular ends of transmembrane helices S6 in domains III and IV and the P-region in domain III (5, 6). In contrast, the phenylalkylamine binding domain is located at the intracellular side of helix S6 in domain IV, near the cytoplasmic opening of the channel (7). Earlier electrophysiological studies (8–11) elegantly demonstrated the extracellular and intracellular locations of the dihydropyridine and phenylalkylamine binding domains, respectively, by comparing the $Ca^{2+}$ channel-blocking properties of charged, membrane-impermeable, $Ca^{2+}$ antagonists after application to the intra- or extracellular surface of the plasma membrane in intact cells. The quaternary phenylalkylamine D890 blocked only from the cytoplasmic side (8, 9), whereas charged dihydropyridines (e.g., SDZ207-180, UK118, or amlodipine) were active only after extracellular application (9–11).

The location of the benzothiazepine binding domain within the $\alpha_1$ subunit primary structure is still unknown. As a first approach we studied its topology in functional experiments with novel tools. Here we demonstrate that the benzazepine $Ca^{2+}$ antagonists SQ32,910 and SQ32,428 bind to the benzothiazepine binding domain of L-type $Ca^{2+}$ channels. By studying the effects of the permanently charged derivative SQ32,428 on L-type $Ca^{2+}$ channels after extracellular and intracellular ap-

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**ABBREVIATIONS:** SQ32,910, (*cis*)-1-[2-(dimethylamino)ethyl]-1,3,4,5-tetrahydro-4-(4-methoxyphenyl)-3-methyl-6-(trifluoromethyl)-2*H*-1-benzazepin-2-one; SQ32,428, (*cis*)-1,3,4,5-tetrahydro-4-(4-methoxyphenyl)-3-methyl-6-(trifluoromethyl)-1-[2-trimethylammonio)ethyl]-2*H*-1-benzazepin-2-one; $V_{0.5}$, voltage where 50% of the $Ca^{2+}$ channels are available; $I_{Ba}$ and $I_{Na}$, inward currents carried by barium and sodium, respectively; $k_{-1}$, dissociation rate constant; FCS, fetal calf serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.



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plication to cells expressing smooth and skeletal muscle-like $Ca^{2+}$ channels, we provide evidence that the benzothiazepine binding domain of the channel is formed by amino acid residues on the extracellular channel surface. Preliminary data from this work have already been presented in abstract form (12).

## Experimental Procedures

**Materials.** (+)-[$^3$H]PN200–110, (−)-[$^3$H]desmethoxyverapamil, and (+)-(*cis*)-[$^3$H]diltiazem were obtained from New England Nuclear (Vienna, Austria), with specific activities of 70–80 Ci/mmol. (±)-PN200–110 was provided by Sandoz A.G. (Basel, Switzerland). Unlabeled phenylalkylamines were a gift from Knoll A.G. (Ludwigshafen, Germany). Unlabeled benzazepines were kindly provided by Dr. D. Floyd and Dr. S. D. Kimball, Bristol Myers Squibb (NJ). FCS was from GIBCO (Vienna, Austria). Penicillin and streptomycin were from Sigma (Deisenhofen, Germany). Permanent cell lines (A7r5 and $BC_3H1$ cells) were purchased from the American Type Culture Collection (Rockville, MD).

**Radioligand binding studies.** Partially purified transverse-tubule membranes were prepared from rabbit skeletal muscle using previously published procedures (13). All binding studies were done in 50 mM Tris·HCl, pH 7.4, 0.1 mM phenylmethylsulfonyl fluoride, in a final assay volume of 0.505 ml. Bound and free ligand were separated by filtration over GF/C glass fiber filters after dilution in 4 ml of washing buffer (10%, w/v, polyethylene glycol 6000, 10 mM Tris, pH 7.4, 10 mM $MgCl_2$) as described (13). Incubation conditions for equilibrium binding studies were as follows: (+)-[$^3$H]PN200–110: 45 min at 37°, nonspecific binding defined in the presence of 1 μM (±)-PN200–110 or 1 μM (±)-nitrendipine; (−)-[$^3$H]desmethoxyverapamil: 60 min at 22°, nonspecific binding defined in the presence of 1 μM (±)-desmethoxyverapamil or 10 μM (±)-verapamil; (+)-(*cis*)-[$^3$H]diltiazem: 15 hr at 2° (or 4 hr at 10° followed by 60 min at 2°), nonspecific binding defined in the presence of 10 μM (+)-(*cis*)-diltiazem or 1 μM (+)-tetrandrine. To prevent adsorption of drugs to plastic surfaces upon serial dilution (14), drugs were diluted in dimethylsulfoxide in glass vials and added directly to the incubation mixture (or to solutions used in electrophysiological experiments; see below). Saturation studies with (+)-(*cis*)-[$^3$H]diltiazem were performed by decreasing the specific activity of the radioligand with unlabeled drug. All experiments were performed in duplicate. More experimental details and the procedures for kinetic studies are given in the figure legends. Dissociation rate constants were calculated from semilogarithmic plots of the dissociation data by linear regression analysis. Binding-inhibition data were fitted to the general dose-response equation (15) using nonlinear curve fitting. $K_i$ values for inhibition of (+)-(*cis*)-[$^3$H]diltiazem binding were calculated from the $IC_{50}$ values according to the method of Cheng and Prusoff (16).

**Patch-clamp experiments.** $I_{Ba}$ through L-type $Ca^{2+}$ channels were recorded at 22–25° using the whole-cell configuration of the patch-clamp technique (17). Patch pipettes with resistances of 1 to 4 MΩ were made from borosilicate glass (Clark Electromedical Instruments, Reading, UK) and filled with pipette solution containing (in mM) $CsCl_2$, 60; CsOH, 60; aspartic acid, 60; $MgCl_2$, 2; EGTA, 10; and HEPES, 10; adjusted to pH 7.25 with CsOH. $I_{Ba}$ were measured in high $Ba^{2+}$ external solution containing (in mM) $BaCl_2$, 20; *N*-methyl-D-glucamine, 93; HEPES, 10; glucose, 20; 4-aminopyridine, 10; tetraethylammonium chloride, 27; and $MgCl_2$, 3; buffered to pH 7.3 with methanesulfonic acid. $I_{Na}$ through L-type calcium channels were measured in $Ca^{2+}$-free external sodium solution containing (in mM) NaCl, 130; CsCl, 4.8; HEPES, 5; glucose, 5; EGTA, 2; and $MgCl_2$, 0.05; buffered to pH 7.3 with NaOH. Data were filtered at 3 kHz (four-pole Bessel filter), digitized, and sampled on-line to a computer hard disk. Leak currents were subtracted either digitally, using average values of scaled leakage currents elicited by a 10-mV hyperpolarizing pulse, or electronically, by means of an analog circuit.

For intracellular application drugs were diluted in the pipette solution and included in the patch pipette. The time constants for internal application were estimated according to the method of Pusch and Neher (18) from the molecular mass of the drugs, the access resistances, and the membrane capacitance. The membrane capacitance was 75 ± 37 pF (mean ± standard deviation, $n$ = 29) in differentiated $BC_3H1$ cells and 54 ± 25 pF (mean ± standard deviation, $n$ = 46) in A7r5 cells. Using these values, mean diffusion time constants for the quaternary phenylalkylamine D890 ($M_r$ 520) of 12 and 7 min were calculated for $BC_3H1$ and A7r5 cells, respectively.

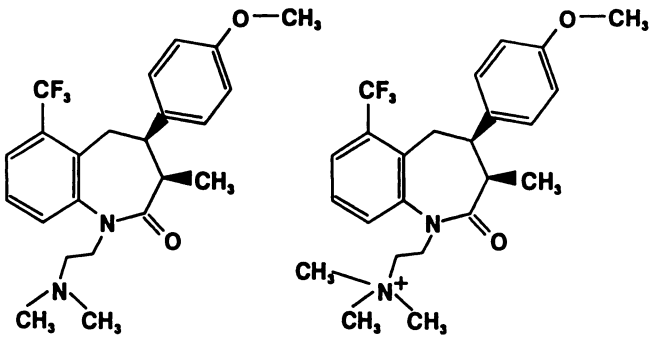
Steady state inactivation curves were analyzed by nonlinear least squares fitting of the experimental data to the Boltzmann equation.

**Rapid perfusion of single cells.** Drugs were applied by a modified fast perfusion system consisting of a six-barrel glass pipette, as described by Konnerth *et al.* (19). The individual tips of the perfusion barrels had a mean diameter of 20 ± 1.6 μm. The tip of the perfusion pipette was placed at a distance of 80–100 μm from an individual cell. A withdrawing pipette (tip diameter, 50–100 μm) was placed on the opposite side of the cell. When gentle suction was applied to the withdrawing pipette, a laminar perfusion of individual cells was achieved. This short distance between the application and withdrawal pipettes, as well as the short distance between the application pipette and the studied cell, allowed a change of test solutions within <100 msec (see Refs. 19 and 20).

**Cell lines.** Electrophysiological studies were performed on A7r5 and $BC_3H1$ cells cultured in Dulbecco's modified medium supplemented with 10% FCS, 2 mM L-glutamine, 63 μg/ml penicillin, and 50 μg/ml streptomycin. Before use A7r5 cells were grown for 3–5 days in the presence of 3% FCS. To induce the expression of $Ca^{2+}$ channels, $BC_3H1$ cells were differentiated at 30% confluency by stepwise reduction of the concentration of FCS (21) to 5% (for 2 days) and 0.5% (for 12 days).

## Results

**Benzazepines bind to the benzothiazepine-selective domain of L-type $Ca^{2+}$ channels.** The chemical structure of the diltiazem analogues SQ32,428 and SQ32,910 is shown in




**SQ32,910** (tertiary) **SQ32,428** (quaternary)

**Fig. 1.** Chemical structure of the benzazepine $Ca^{2+}$ antagonists SQ32,428 and SQ32,910. These benzazepine are structurally related to diltiazem-like $Ca^{2+}$ antagonists but lack a sulfur atom in the heptagonyl ring. Both compounds are racemic mixtures of their *cis*-diastereoisomers. The structure-activity relationship has been extensively studied by Kimball *et al.* (22). As in the case of diltiazem, the (+)-*cis* form is the most active of the four possible diastereoisomers. The critical pharmacophores for drug binding are the basic amine (preferentially in ethyl linkage at N-1, p$K$ > 7.0) and the hydrogen bond acceptor at position 4′ on the 4-aryl ring. Appropriate ring substituents at positions C-6, C-7, and C-3 enhance the overall hydrophobicity and, provided they do not interfere with the binding of the pharmacophores, increase the affinity of these drugs. Introduction of an additional methyl group in the tertiary amino group of SQ32,910 yields SQ32,428, the quaternary, membrane-impermeable derivative.

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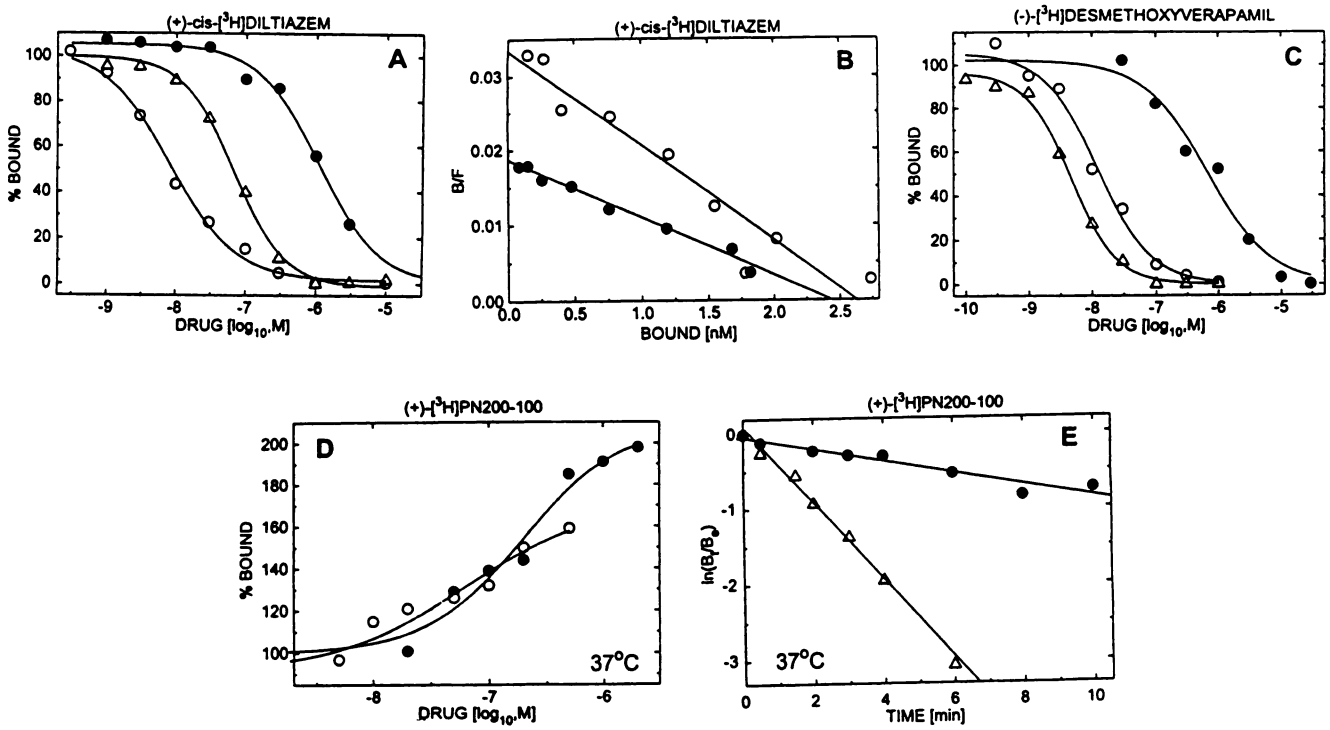


**Fig. 2.** Modulation of $Ca^{2+}$ antagonist binding to skeletal muscle $Ca^{2+}$ channels by benzazepines. A, Inhibition of (+)-(*cis*)-[$^3$H]diltiazem binding. Radioligand (1.4 nM) was incubated with 0.28 mg/ml partially purified skeletal muscle transverse-tubule membranes. Binding in the presence of drug was normalized with respect to control binding. The following binding parameters were obtained by nonlinear curve-fitting for the experiment shown. Values in parenthesis are means ± standard deviations for two to four independent experiments performed under similar experimental conditions: SQ32,910 (○), $K_i$ = 8.3 nM (8.9 ± 1.1), $n_H$ = 0.88 (0.81 ± 0.12); (+)-(*cis*)-diltiazem (△), $K_i$ = 69 nM (55 ± 19), $n_H$ = 1.24 (0.98 ± 0.17); SQ32,428 (●), $K_i$ = 1.12 μM (1.2 ± 0.1), $n_H$ = 1.04 (1.27 ± 0.19). B, Saturation analysis of (+)-(*cis*)-[$^3$H]diltiazem binding in the absence and presence of SQ32,910. Increasing concentrations of (+)-(*cis*)-[$^3$H]diltiazem [obtained by decreasing its specific activity with unlabeled (+)-(*cis*)-diltiazem] were incubated (15 hr, 2°) with 0.16 mg/ml membrane protein in the absence or presence of 6.5 nM SQ32,910. The following binding parameters were obtained by linear regression after Scatchard transformation of the specific binding data: control (○), $K_d$ = 78.6 nM, $B_{max}$ = 2.6 nM (20.8 pmol/mg of protein), $r$ = 0.96; 6.5 nM SQ32,910 present (●), $K_d$ = 129.9 nM, $B_{max}$ = 2.4 nM (19.2 pmol/mg of protein), $r$ = 0.98. In a second independent experiment the following results were obtained: control, $K_d$ = 44.5 nM, $B_{max}$ = 12.1 pmol/mg, $r$ = 0.98; 7 nM SQ32,910 present, $K_d$ = 129.4 nM, $B_{max}$ = 13.5 pmol/mg, $r$ = 0.92. C, Inhibition of (−)-[$^3$H]desmethoxyverapamil binding. Radioligand (1.0 nM) was incubated with 0.024 mg/ml membrane protein. Binding parameters are given as in A. (−)-Desmethoxyverapamil (△), $IC_{50}$ = 4.7 nM (3.3 ± 1.4), $n_H$ = 1.17 (0.90 ± 0.24); SQ32,910 (○), $IC_{50}$ = 12.0 nM (11.3 ± 3.9), $n_H$ = 1.03 (0.98 ± 0.12); SQ32,428 (●), $IC_{50}$ = 0.79 μM (3.6 ± 2.7), $n_H$ = 0.89 (1.14 ± 0.21). D, Modulation of (+)-[$^3$H]PN200–110 binding. Radioligand (0.2 nM) was incubated with 0.054 mg/ml membrane protein. At concentrations of >10 μM pronounced inhibition (not shown) of (+)-[$^3$H]PN200–110 binding was caused by both benzazepines. Because measured binding therefore reflects the net effect of the stimulatory minus the inhibitory component, no binding parameters for stimulation were calculated. In three experiments maximal stimulation was as follows (means ± standard deviation): SQ32,428 (●), 174 ± 23% (at 3 μM); SQ32,910 (○), 143 ± 14% (at 1 μM); (+)-(*cis*)-diltiazem (not shown), 171 ± 25% (at 100 μM). E, Effects of benzazepines on (+)-[$^3$H]PN200–110 dissociation. (+)-[$^3$H]PN200–110 (0.6 nM) was incubated with 0.02 mg/ml membrane protein (45 min, 37°) in the absence (control) or presence of 3 μM SQ32,428, in a total assay volume of 0.5 ml. After equilibrium was reached dissociation was induced by addition of 5 μl of (±)-PN200–110 to a final concentration of 1 μM. Specific (total minus nonspecific) binding was determined at the indicated times after induction of dissociation. Nonspecific binding was determined in parallel by addition of unlabeled (±)-PN200–110 to the membranes before the association reaction was started. Data were plotted as the natural logarithm of fractional binding versus time and $k_{-1}$ was determined as the negative slope of the regression line. Control (△), $k_{-1}$ = 0.51 min$^{-1}$; 3 μM SQ32,428 present (●), $k_{-1}$ = 0.08 min$^{-1}$. In independent experiments performed under similar experimental conditions 3 μM SQ32,428 and 1 μM SQ32,910 decreased $k_{-1}$ from 0.22 min$^{-1}$ to 0.09 min$^{-1}$ and from 0.40 min$^{-1}$ to 0.09 min$^{-1}$, respectively ($r$ > 0.96 in all experiments).

Fig. 1. Both compounds were used in racemic form (*cis* configuration). They represent a novel class of $Ca^{2+}$ antagonists that are structurally highly related to diltiazem-like benzothiazepines but lack a sulfur atom in the heptagonyl ring (benzazepines) (22). They possess the same stereochemical properties as benzothiazepines [the (+)-*cis*-diastereoisomers are most active] (23). Due to their structural similarity it has been postulated that benzazepines and benzothiazepines compete for the same site on the $\alpha_1$ subunit (22). To prove that the two benzazepines are specific tools for the benzothiazepine binding domain and to determine their affinities for the $Ca^{2+}$ channel *in vitro*, we investigated their reversible interaction with dihydropyridine-, phenylalkylamine-, and benzothiazepine-labeled skeletal muscle L-type $Ca^{2+}$ channels.

As shown in Fig. 2A both benzazepines monophasically inhibited (+)-(*cis*)-[$^3$H]diltiazem binding, with Hill slopes close to unity. The quaternary derivative SQ32,428 ($K_i$ = 1.2 ± 0.1 μM, mean ± standard deviation, three experiments) was about 135- and 20-fold less potent than the corresponding tertiary SQ32,910 ($K_i$ = 8.9 ± 1.1 nM, two experiments) and (+)-(*cis*)-diltiazem ($K_i$ = 55.0 ± 19 nM, four experiments), respectively. This indicates that introduction of a methyl group into the basic amine sterically interferes with drug binding directly or decreases the accessibility to the domain due to the introduction of a permanent positive charge. Because only one of the diastereoisomers in the racemic mixtures binds to the channel with high affinity (22), the respective $K_i$ values for the (+)-*cis*-compounds are approximately 5 (tertiary) and 600 nM (quater-

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nary). As shown for SQ32,910 in Fig. 2B, inhibition was caused by decreasing the apparent $K_d$ for (+)-(*cis*)-[$^3$H]diltiazem without significantly changing the $B_{max}$. These findings are compatible with a competitive type of interaction of these compounds with (+)-(*cis*)-[$^3$H]diltiazem binding.

Similarly to other $Ca^{2+}$ antagonists selectively binding to the benzothiazepine binding domain [e.g., (+)-tetrandrine (24) or (+)-(*cis*)-diltiazem (2)], both compounds inhibited binding at the (−)-[$^3$H]desmethoxyverapamil-labeled binding domain (Fig. 2C) but stimulated (+)-[$^3$H]PN200–110 binding at 37° (Fig. 2D). The binding stimulation is due to the known positive heterotropic allosteric interaction between the dihydropyridine and benzothiazepine binding domains (2). This is also evident in kinetic experiments. Both benzazepines (Fig. 2E and legend to Fig. 2E) increased the dissociation half-life of the (+)-[$^3$H]PN200–110-$\alpha_1$ subunit complex. Taken together, these data clearly demonstrate that benzazepines competitively inhibit (+)-(*cis*)-[$^3$H]diltiazem binding to L-type $Ca^{2+}$ channels. Despite a decrease in affinity due to quaternarization, SQ32,428 should therefore provide a suitable tool to determine the sidedness of the benzothiazepine binding domain.

**Extracellular application of SQ32,428 blocks L-type $Ca^{2+}$ channel currents.** To investigate from which side of $Ca^{2+}$ channels diltiazem-like $Ca^{2+}$ antagonists act, we studied the effects of SQ32,428 on muscle L-type $Ca^{2+}$ channels in intact cells after extra- and intracellular application. This membrane-impermeable compound is expected to block the channel only if it has direct access to its binding site via a hydrophilic pathway. $I_{Ba}$ through L-type $Ca^{2+}$ channels were measured using the whole-cell configuration of the patch-clamp technique in $BC_3H1$ and A7r5 cells. A7r5 cells express smooth muscle-like L-type $Ca^{2+}$ channels, whereas differentiated $BC_3H1$ cells express skeletal muscle-type $\alpha_1$, $\beta$, and $\alpha_2$ channel subunits (25, 26), giving rise to skeletal muscle-like, slowly activating and inactivating, $Ca^{2+}$ currents (21).

Drugs were applied by means of a rapid perfusion system in which the cell was constantly superfused with drug-containing or control solution. This technique enables rapid application and wash-out of drugs, thus minimizing an overestimation of the drug effect as the result of the decrease of $I_{Ba}$ due to $Ca^{2+}$ channel current run-down. Run-down of $I_{Ba}$ was typically about 20% of the initial current within the first 20 min (see also Fig. 6). $I_{Ba}$ was elicited by 100-msec depolarizations from a holding potential of −70 mV to +30 mV.

Fig. 3 shows the effect of increasing concentrations of extracellularly applied SQ32,428. Superfusion of the cell with extracellular solution containing 30 μM or 50 μM SQ32,428 inhibited about 30 or 40% of $I_{Ba}$, respectively, within about 30 sec. Block by SQ32,428 was reversible and $I_{Ba}$ returned to control values within 2–3 min after switching to drug-free solution. No evidence for a use- or frequency-dependent inhibition was found for the quaternary analogue SQ32,428 under our experimental conditions. Fig. 3, A and C, shows that increasing the stimulation frequency from 0.1 to 0.5 Hz did not induce additional block of calcium channels by SQ32,428. SQ32,428 did not change channel kinetics (Fig. 3, B and C) and did not shift steady state inactivation in A7r5 cells (Fig. 3D). The $V_{0.5}$ of the steady inactivation curve was −15.2 ± 4.8 mV under control conditions and −17.9 ± 7.2 mV (means ± standard errors, four experiments) in the presence of 50 μM SQ32,428.

We also tested the effect of extracellular application of the

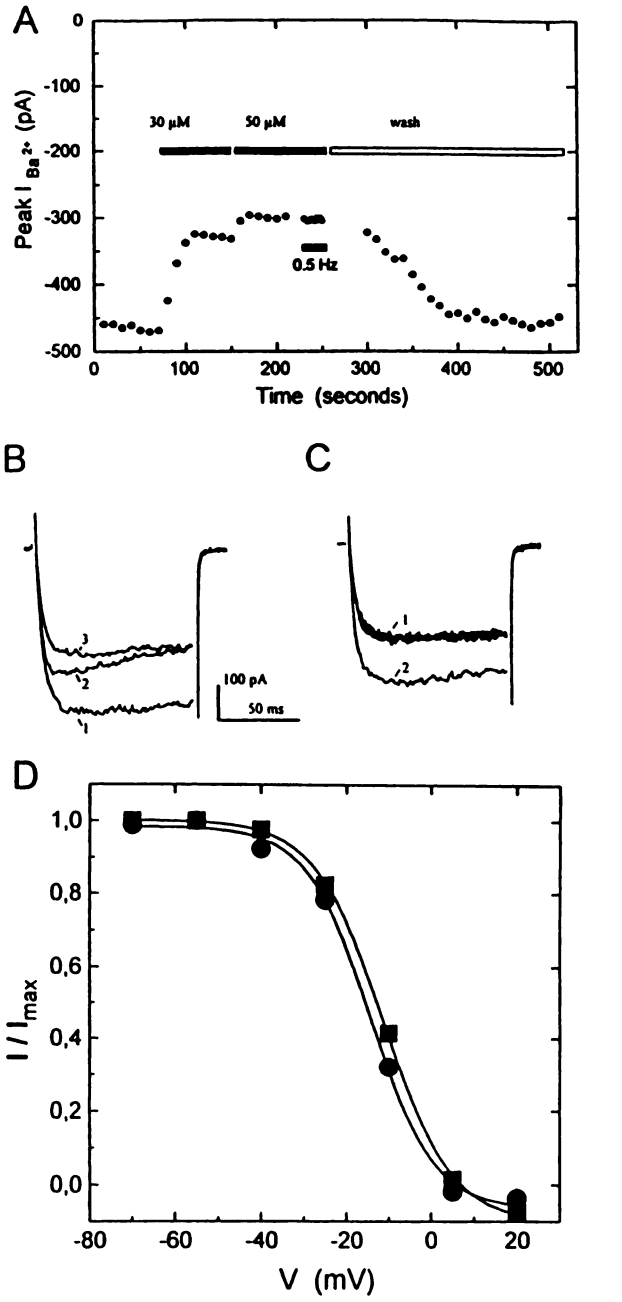


**Fig. 3.** Reversible inhibition of $I_{Ba}$ in an A7r5 cell by external application of SQ32,428. A, $I_{Ba}$ was elicited by 100-msec depolarizations from a holding potential of −70 mV to +30 mV (0.1 Hz). Peak current amplitudes are plotted against time. Current at time zero corresponds to the first recording (10–20 sec after the whole-cell configuration was established). SQ32,428 (30 and 50 μM) was applied by fast perfusion. In the presence of 50 μM SQ32,428 a train of 10 test pulses at 0.5 Hz was applied (see C, *trace 2*). The drug effect was reversed by perfusion of the cell with drug-free solution. B, Current traces from the same experiment recorded before (*trace 1*) and after application of 30 μM (*trace 2*) and 50 μM (*trace 3*) SQ32,428. C, $I_{Ba}$ during a 0.5-Hz pulse train in the presence of 50 μM SQ32,428 (*trace 1*) and after wash with control solution (*trace 2*). D, Effect of 50 μM SQ32,428 on the steady state inactivation of $I_{Ba}$ in A7r5 cells. Peak $I_{Ba}$ was measured in the absence of SQ32,428 with 100-msec test pulses (−70 to +30 mV) after application of conditioning prepulses (10 sec) to the indicated potentials (interpulse interval was 3 msec). The cell was then perfused with 50 μM SQ32,428 and steady state inactivation was measured again. The *solid lines* represent the best fits to the Boltzmann equation, with $V_{0.5}$ = −11.8 mV before (■) and $V_{0.5}$ = −15.9 mV after (●) application of SQ32,428 (with corresponding slope factors of 7.6 mV and 6.9 mV, respectively).

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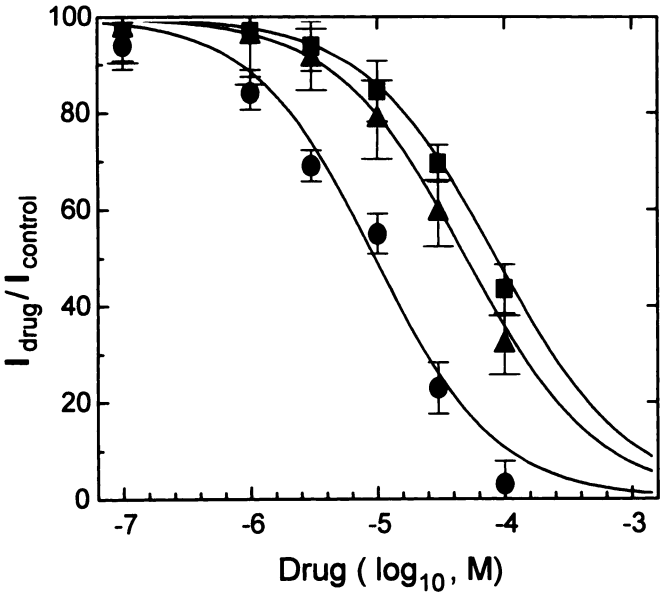


**Fig. 4.** Dose-dependent inhibition of $I_{Ba}$ and $I_{Na}$ by extracellular application of SQ32,428 in A7r5 and $BC_3H1$ cells. Currents were elicited by depolarizations (100-msec pulse) from a holding potential of −70 mV to +30 mV at 0.1 Hz. $I_{drug}$, peak current measured after induction of block with the indicated concentration of SQ32,428; $I_{control}$, maximal peak current observed before drug application. Data points were obtained from a total of 13 cells (for $I_{Na}$ in A7r5 cells) (●), nine cells (for $I_{Ba}$ in A7r5 cells) (■), and five cells (for $I_{Ba}$ in $BC_3H1$ cells) (▲) and are given as means ± standard deviations (at least three determinations). The *lines* represent the best fit to the general dose-response equation (14), yielding the following $IC_{50}$ values: ●, $IC_{50}$ = 9.4 μM; ■, $IC_{50}$ = 86 μM; ▲, $IC_{50}$ = 50 μM. Inhibition of $I_{Ba}$ and $I_{Na}$ was observed over the whole voltage range of current activation (data not shown).

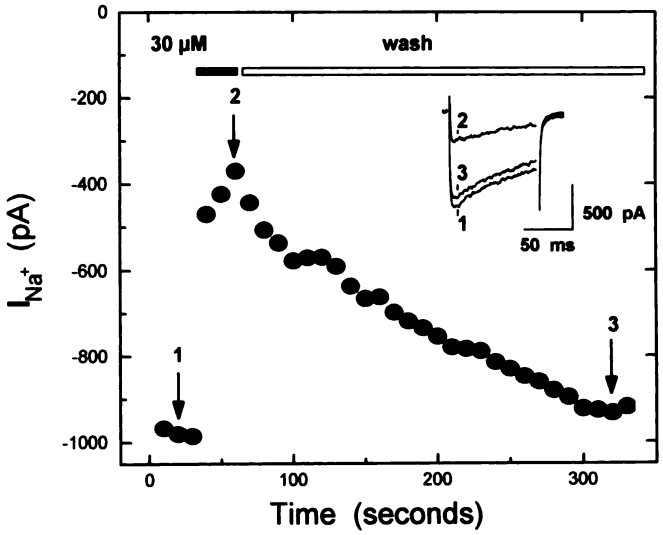


**Fig. 5.** Reversible inhibition of $I_{Na}$ through $Ca^{2+}$ channels in an A7r5 cell by external application of SQ32,428. Currents were elicited by 100-msec depolarizations from a holding potential of −70 mV to +30 mV (0.1 Hz). Peak current amplitudes are plotted against time. SQ32,428 (30 μM) was applied by fast perfusion. The drug effect was reversed by perfusion of the cell with control solution. *Inset*, current traces from the same experiment recorded before (*trace 1*) and after (*trace 2*) application of 30 μM SQ32,428 and after washout (*trace 3*).

quaternary phenylalkylamines D575 and D890, which display affinities for the (−)-[$^3$H]desmethoxyverapamil-labeled phenylalkylamine binding domain (D575, $K_i$ = 3.9 ± 1.1 μM, three experiments; D890, $K_i$ = 13.2 ± 2.2 μM, five experiments; means ± standard deviations) similar to that of SQ32,428 for the benzothiazepine binding domain. Neither drug affected $I_{Ba}$ at concentrations up to 100 μM (<5% decrease of $I_{Ba}$ within the first 5 min after application in A7r5 cells, four experiments). This finding is compatible with previous reports demonstrating that the phenylalkylamine binding domain is not accessible to quaternary drugs from the extracellular side in cardiac and neuroendocrine cells (8, 9).

Dose-response curves for the inhibition of $I_{Ba}$ by extracellular SQ32,428 in A7r5 and $BC_3H1$ cells are shown in Fig. 4. The calculated $IC_{50}$ values of 86 μM and 50 μM, respectively, were higher than the affinity of SQ32,428 for the benzothiazepine binding domain of the channel in skeletal muscle ($K_i$ = 1.2 μM; Fig. 2). This difference in potency could be due to the fact that low millimolar concentrations of divalent cations inhibit drug binding to the benzothiazepine binding domain (27–29). We therefore also measured L-type $Ca^{2+}$ channel block by extracellular SQ32,428 after chelation of extracellular divalent cations by EGTA, using $Na^+$ as the charge carrier (9). The experiment in Fig. 5 shows that 30 μM SQ32,428 blocks >50% of $I_{Na}$. The $IC_{50}$ calculated from the resulting dose-response curve (Fig. 4) was 9.4 μM, which is in good agreement with the affinity of SQ32,428 determined in binding experiments (Fig. 2) performed in the absence of added divalent cations.

Because quaternary, permanently charged, $Ca^{2+}$ antagonists are considered membrane impermeable, these experiments indicate that SQ32,428 approaches its binding domain from the extracellular side, suggesting that amino acid residues exposed to the extracellular side of L-type $Ca^{2+}$ channels form the benzothiazepine binding domain.

**SQ32,428 does not block $Ca^{2+}$ channels from the cytoplasmic side.** To further test this hypothesis and to investigate the possibility that SQ32,428 blocks the channel by interacting with binding domains accessible from the cytoplasm (like, for example, the phenylalkylamine binding domain) (8, 9), SQ32,428 was applied to the intracellular surface of the membrane by inclusion in the pipette solution. As shown in Fig. 6A, intracellular dialysis of A7r5 cells with 100 μM SQ32,428 did not block whole-cell inward currents. Decay of peak $I_{Ba}$ with 100 μM SQ32,428 included in the pipette solution did not differ significantly from channel run-down in the absence of drug (Fig. 6B). However, the time-dependent decay of current was significantly faster when 100 μM levels of the permanently charged phenylalkylamine D890 were included in the pipette. This finding is compatible with the proposed intracellular localization of the phenylalkylamine binding domain in these cells (8, 9, 30). It also suggests that insufficient drug delivery into the cell is unlikely to account for the lack of an effect of SQ32,428 in our experiments (see Experimental Procedures).

## Discussion

Based on electrophysiological (8–11) and biochemical evidence (5–7), current models of the drug binding domains of L-type $Ca^{2+}$ channels propose that the dihydropyridine binding domain is formed mainly by the extracellular ends of transmembrane helices IIIS6 and IVS6 and the P-region in domain III, whereas the phenylalkylamine binding domain is formed by the intracellular end of IVS6 and adjacent intracellular

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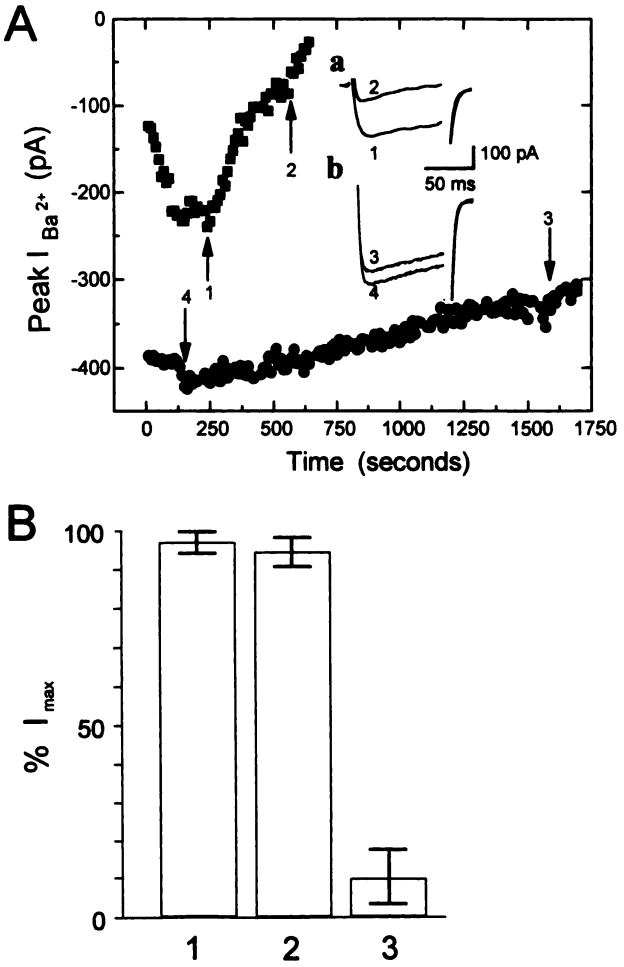


**Fig. 6.** Intracellular application of the quaternary phenylalkylamine D890 and SQ32,428 in A7r5 cells. $I_{Ba}$ was elicited by 100-msec depolarizing pulses from −70 to +30 mV at 0.1 Hz. A, Peak inward currents were recorded with either 100 μM D890 (■) or 100 μM SQ32,428 (●) included in the patch pipette. Currents were recorded immediately after the whole-cell configuration was established. Increase in $I_{Ba}$ during the first minute is due to inhibition of remaining $K^+$ outward currents during internal perfusion of the cell. *Insets*, selected current traces (see *arrows*) during internal perfusion with D890 (*inset a*) and during internal perfusion with SQ32,428 (*inset b*). B, Comparison of the effects of internal perfusion of A7r5 cells with pipette solution containing no drug (*1*), 100 μM SQ32,428 (*2*), or 100 μM D890 (*3*). $I_{Ba}$ was determined 10 min after the maximal current ($I_{max}$) was measured (usually within 2–3 min after start of perfusion) and is expressed as percentage of $I_{max}$. Means ± standard deviations are given for 16 (*1*), six (*2*), or five cells (*3*).

amino acid residues (4). According to current folding models of voltage-gated ion channels (31), these regions must be located close to the extra- and intracellular mouths, respectively, of the ion-conducting pathway of the $\alpha_1$ subunit.

Our studies clearly demonstrate that diltiazem-like $Ca^{2+}$ antagonists bind to the extracellular side of the channel. This conclusion is based on the finding that the membrane-impermeable, permanently charged, diltiazem-like benzazepine SQ32,428 blocked the channels in A7r5 and $BC_3H1$ cells with equal potencies after application from the extracellular side but had no detectable effect after intracellular application. Extracellular block occurred in a dose-dependent manner, was independent of membrane potential, and developed without any evidence for use dependence. This is in contrast to the action of the tertiary derivative SQ32,910, for which potency increased with increasing pulse length and stimulation frequency (data not shown).

The potency of extracellularly applied SQ32,428 for L-type channel block was decreased by divalent cations in the extracellular solution. In the presence of divalent cations (3 mM $MgCl_2$, 20 mM $Ba^{2+}$), i.e., with $Ba^{2+}$ as the charge carrier, the $IC_{50}$ for channel block was about 1 order of magnitude higher than in the absence of extracellular divalent cations, using $Na^+$ as the charge carrier. This finding is compatible with the results of radioligand binding studies, which demonstrated that divalent cations decrease the affinity of drugs for the benzothiazepine binding domain [$IC_{50}$ values for inhibition of reversible (+)-(*cis*)-[$^3$H]diltiazem binding were 0.1–1 mM for $Mg^{2+}$ and $Ca^{2+}$] (29). Therefore, differences in the ion composition of the extracellular solutions may account for the slight differences between the potency of SQ32,428 for block of $I_{Na}$ in A7r5 cells ($IC_{50}$ = 9.4 μM; Fig. 4), its potency for the inhibition of KCl-induced smooth muscle contraction ($IC_{50}$ = 6.2 μM) (22), and its affinity for skeletal muscle $Ca^{2+}$ channels ($K_i$ = 1.2 μM; Fig. 2). A 6–10-fold difference between the measured binding affinity (measured in isolated membrane fragments in the absence of added divalent cations) and the $IC_{50}$ for inhibition of smooth muscle contraction (measured in physiological buffers) can therefore not be taken as evidence for a sidedness of drug action, as claimed in an earlier study (22).

The $Ca^{2+}$ antagonistic potency of benzazepines (as well as benzothiazepines) is critically dependent on the presence of two pharmacophores, i.e., the basic amino group in ethyl linkage at position N-1 and a hydrogen bond acceptor (preferentially a methoxy group) at position 4′ on the 4-aryl ring (21, 22). SQ32,428 carries its permanent charge within one of the pharmacophores that interact directly with the channel. Therefore, at least one hydrophilic (most likely acidic) group must be accessible to the basic nitrogen within the binding domain. The affinity of benzazepines also increases with their hydrophobicity (provided that hydrophobic substituents do not interfere with the binding of the two pharmacophores; see Ref. 22), indicating partitioning into a hydrophobic environment. This suggests that the benzothiazepine binding domain consists of a hydrophobic pocket that accomodates the hydrophobic substituted benzazepine (or benzothiazepine) ring (see legend to Fig. 1) next to a polar region that interacts with the basic amine. Based on our present results and the fact that benzothiazepines specifically photoaffinity label exclusively the $\alpha_1$ subunit of the channel complex (32, 33), we propose that residues on the extracellular surface of the $\alpha_1$ subunit participate in the formation of this binding pocket. Photoaffinity ligands carrying photoreactive groups in different positions of the benzazepine or benzothiazepine structure should be suitable tools to label these regions and allow their localization within the $\alpha_1$ subunit by biochemical means (e.g., using sequence-directed antibody mapping). Such biochemical studies should provide another step towards a more detailed understanding of the molecular pharmacology of voltage-dependent L-type $Ca^{2+}$ channels.

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