Bis(benzylisoquinoline) Analogs of Tetrandrine Block L-Type Calcium Channels: Evidence for Interaction at the Diltiazem-Binding Site

John P. Felix,[‡] V. Frank King,[†,‡] Judith L. Shevell,[‡] Maria L. Garcia,[‡] Gregory J. Kaczorowski,[‡] I. Ralph C. Bick,[§] and Robert S. Slaughter*[,‡]

Department of Membrane Biochemistry and Biophysics, Merck Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065-0900, and Department of Chemistry, University of Tasmania, Hobart, Tasmania, Australia

Received June 25, 1992; Revised Manuscript Received September 11, 1992

ABSTRACT: Bis(benzylisoquinoline) alkaloids block Ca^{2+} uptake through the L-type Ca^{2+} channel and modulate binding of ligands to four distinct sites (dihydropyridine, benzothiazepine, aralkylamine, and (diphenylbutyl)piperidine) in the Ca^{2+} entry blocker receptor complex of the channel. These alkaloids are structural analogs of tetrandrine, which has previously been demonstrated to block the L-type Ca^{2+} channel through interaction at the benzothiazepine (diltiazem) site (King et al., 1988). Different alkaloid conformational classes display either α–β, β–α, α–α, or β–β stereochemistry at the two chiral isoquinoline carbons. Compounds from all four classes were tested for their ability to interact with Ca^{2+} entry blocker ligands. All analogs completely inhibit diltiazem binding, but many only partially inhibit D-600 and fluspirilene binding. For dihydropyridine binding, the compounds show either stimulation or inhibition or exhibit no effect. This profile is quite different from the interaction displayed by diltiazem or tetrandrine. Scatchard analyses show effects predominantly on K_d for diltiazem, D-600, and PN200-110 binding. Representative conformers do not effect diltiazem dissociation rates but alter dissociation kinetics of ligands which bind to the other three sites. A correlation of the ability of these compounds to inhibit Ca^{2+} uptake through the L-type Ca^{2+} channel in GH_3 cells exists only with their inhibition of diltiazem binding but not with inhibition of binding of ligands representing other classes of Ca^{2+} entry blockers. These data, taken together, indicate that a variety of bis(benzylisoquinoline) congeners act to block the L-type Ca^{2+} channel by binding to the benzothiazepine site on the channel. The novel patterns exhibited by these agents in affecting allosteric coupling between the benzothiazepine site and the other receptors of the Ca^{2+} entry blocker receptor complex define a new class of Ca^{2+} entry blocker with binding properties distinct from diltiazem.

Several structurally dissimilar classes of molecules have been identified which potently block the voltage-dependent L-type Ca^{2+} channel in a variety of tissues, including cardiac and smooth muscle [for reviews, see Triggle and Janis (1987), Hosey and Lazdunski (1988), Schwartz (1989), and Glossman and Striessnig (1990)]. These compounds, known as Ca^{2+} entry blockers (CEB),[1] bind with high affinity to distinct sites in a CEB receptor complex that is functionally associated with the channel protein (Garcia et al., 1984, 1986; Ehrlich et al., 1986, Striessnig et al., 1990; Nakayama et al., 1991; Striessnig et al., 1991). At least four different receptors have been described in the complex present in cardiac sarcolemma. Three are sites for which therapeutically useful agents have been developed (the dihydropyridine, aralkylamine, and benzothiazepine chemical classes). More recently, an additional high-affinity site which binds fluspirilene, a substituted (diphenylbutyl)piperidine, has been characterized (King et al., 1989). Identification of the binding site of a particular compound in this complex can give important information about its mechanism of action. Compounds which act at any one of these four receptors have been described as exhibiting distinctive allosteric coupling patterns between sites, causing either stimulation or inhibition of ligand binding at other receptor sites in the CEB complex (Garcia et al., 1984, 1986; King et al., 1989). These profiles have been used diagnostically as an initial determinant for the site of action of novel L-type Ca^{2+} channel modulators (Garcia et al., 1987, 1990; Hwang et al., 1987; King et al., 1988; Siegl et al., 1988; Staudinger et al., 1991).

Extracts from certain plants are used in traditional Chinese medicine for treatment of angina and hypertension (Wang & Liu, 1985). Tetrandrine, extracted from *Stephania tetrandra*, is a dimer of benzylisoquinoline subunits condensed in a head-to-head, tail-to-tail fashion, with α–β stereochemistry at the two chiral isoquinoline carbons. This alkaloid exhibits the pharmacological profile of a CEB (Qian, et al., 1983). Tetrandrine was found to inhibit the L-type Ca^{2+} channel, and, through binding studies, to interact at the CEB receptor complex of the channel (King et al., 1988). Tetrandrine affects binding of [^{3}H]diltiazem, [^{3}H]D-600, and [^{3}H]nitrendipine to the CEB receptor complex in cardiac sarcolemmal membrane vesicles. Equilibrium and kinetic binding protocols show that tetrandrine produces the same allosteric coupling pattern as diltiazem (maximal stimulation of [^{3}H]nitrendipine binding at 37 °C, partial inhibition of [^{3}H]D-600 binding, and complete inhibiton of [^{3}H]diltiazem binding). This profile is consistent with a previously proposed model describing allosteric interactions between CEB receptors in heart (Garcia et al., 1986). These findings, coupled with other evidence, led to the conclusion that tetrandrine inhibits L-type Ca^{2+} channels by interacting at the benzothiazepine receptor and that this interaction most likely forms the basis for this compound's therapeutic effectiveness.

* To whom correspondence should be addressed.
‡ Merck Research Laboratories.
† Decreased December 31, 1991.
§ University of Tasmania.

[1] CEB, calcium entry blocker; D-600, methoxyverapamil; D-888, desmethoxyverapamil; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/92/0431-11793$03.00/0

Other bis(benzylisoquinoline) alkaloids have been reported which differ in substitutions about the basic ring structures and in stereochemistry at the two chiral centers [for a review, see Buck (1987)]. Representative compounds from four conformational classes of bis(benzylisoquinoline) analogs of tetrandrine with α–β, β–α, α–α, and β–β stereochemistry were characterized for inhibiton of $^{45}Ca^{2+}$ uptake through L-type Ca^{2+} channels in GH_3 cells and for their effects on the binding of ligands at each of the four sites in the CEB receptor complex present in purified porcine cardiac sarcolemmal membranes. All of these alkaloids block the L-type Ca^{2+} channel. However, binding experiments demonstrate that benzylisoquinoline analogs elicit different patterns of effects on interactions with dihydropyridines as compared to those seen with diltiazem or tetrandrine. Nonetheless, data from binding and $^{45}Ca^{2+}$ uptake studies localize the interaction of these bis(benzylisoquinoline) alkaloids to the benzothiazepine receptor. Thus, these compounds define an interesting new structural class of L-type Ca^{2+} channel modulator which can give rise to patterns of allosteric coupling among sites in the CEB receptor complex different from those found with traditional diltiazem-like agents. A preliminary report of these findings has been presented in abstract form (Kaczorowski et al., 1989).

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]Diltiazem (74 Ci/mmol), [^{3}H]nitrendipine (84 Ci/mmol), [^{3}H]PN200-110 (70 Ci/mmol), [^{3}H]D-600 (methoxyverapamil, 50 Ci/mmol), [^{3}H]fluspirilene (21.3 Ci/mmol), and $^{45}CaCl_2$(30.4 Ci/mmol) were obtained from Du Pont–New England Nuclear. [^{3}H]D-888 (desmethoxyverapamil, 84 Ci/mmol) was purchased from Amersham. The chemical structures, properties, and preparation procedures of the various bis(benzylisoquinoline) alkaloids used in this study were reviewed by Buck (1987). Tetrandrine was purified from extracts of *S. tetrandra*, as previously outlined (King et al., 1988). Fluspirilene and Bay K-8644 were gifts of Janssen Pharmaceutica and Bayer Pharmaceuticals, respectively. All other reagents were purchased from commercial sources at the highest purity available.

Preparation of Purified Porcine Cardiac Sarcolemmal Membrane Vesicles. Sarcolemmal membranes were prepared from fresh porcine cardiac tissue by the method of Kuwayama and Kanazawa (1982), as modified by Slaughter et al., (1983). Briefly, cells were broken through a multiple-step homogenization procedure, and membranes were separated by differential and density gradient centrifugation, frozen in liquid nitrogen, and stored at −70 °C for periods of up to 1 year.

Binding Assays for L-Type Ca^{2+} Channel Modulators. The interactions of [^{3}H]diltiazem, [^{3}H]nitrendipine, [^{3}H]D-600, and [^{3}H]fluspirilene with cardiac sarcolemmal membranes were monitored as previously described (Garcia et al., 1984, 1986; Ehrlich et al., 1986; King et al., 1989). [^{3}H]D-888 binding assays (Ruth et al., 1985) were performed essentially in a fashion identical to the [^{3}H]D-600 assay. The binding assay for PN200-110 was done in the manner described for the interaction of this ligand with L-type Ca^{2+} channels in bovine aortic sarcolemmal membrane vesicles (Slaughter et al., 1989). At the end of the incubation period, reaction mixtures were quenched and filtered through Whatman GF/C filters using conditions appropriate for each ligand. Triplicate assays were routinely performed under each experimental condition, and the standard deviation of these results was typically less than 3%. Stock solutions of all alkaloids and drugs were prepared in dimethyl sulfoxide. The concentration of this solvent was never allowed to exceed 0.25%, except in the case of D-600 and D-888 binding where maximal levels of 0.05% were employed. Radioactivity trapped on filters was measured using liquid scintillation techniques.

Analysis of Binding Data. Results from saturation experiments were subjected to a Scatchard analysis, and linear regression was performed to determine the equilibrium dissociation constant (K_d) and maximum receptor concentration (B_{max}). The correlation coefficients for these determinations were usually greater than 0.95. Data from competition experiments were analyzed by the method of Cheng and Prusoff (1973) to calculate K_i values from IC_{50}'s determined with a two-parameter (IC_{50}, n_H) fit for complete inhibition. For partial inhibition, a three-parameter fit was used to determine the IC_{50}, n_H, and B_{min}. The kinetics of ligand loss from receptor were determined directly from a first-order plot of ligand dissociation versus time.

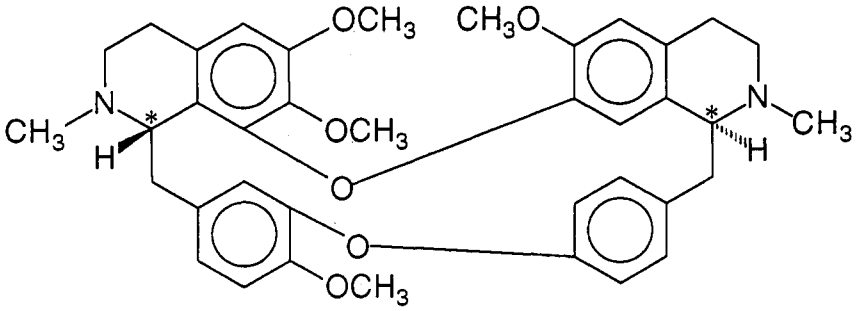
Calcium Uptake Assay Employing GH_3 Cells. $^{45}Ca^{2+}$ uptake through the L-type Ca^{2+} channel was monitored using GH_3 rat anterior pituitary cells in suspension as previously described (King et al., 1989). Briefly, GH_3 cells were resuspended in assay medium (4.6 mM KCl, 118 mM NaCl, 1 mM $MgCl_2$ 10 mM glucose, 0.05% methylcellulose, and 5 mM HEPES–NaOH, pH 7.4) at a density of 5×10^5 cells/mL and maintained at 37 °C in a shaking water bath. Uptake of $^{45}Ca^{2+}$ was initiated by the addition of cell-depolarizing medium (assay medium modified by increasing KCl to 50 mM, with a compensatory drop in NaCl concentration to 72.6 mM). Cells were allowed to remain in the depolarization medium for 2 min, at which time the reaction was terminated by addition of 4 mL of lanthanum quench (1 mM $LaCl_3$ in the original assay medium). Cells were collected on 2.5-cm Whatman GF/C glass fiber filters, followed by two 4-mL washes of the reaction vessel with lanthanum quench solution. Radioactivity in the samples was measured by liquid scintillation techniques. Controls were made in the original assay medium for basal uptake of $^{45}Ca^{2+}$ under membrane-polarized conditions, in La^{3+} for background, and with nitrendipine present in the normal or depolarizing assay medium as a measure of net $^{45}Ca^{2+}$ uptake through the L-type Ca^{2+} channel. Further confirmation of L-type Ca^{2+} channel activity was obtained in the original assay medium under polarized conditions by activating these channels with the dihydropyridine agonist Bay K-8644.

Protein Determination. Protein was determined by the amido blue–black method of Schaffner and Weissmann (1973), as modified by Newman et al., 1982. This method is optimized for the quantitative measurement of membrane protein with high sensitivity.

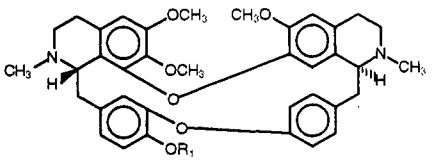
RESULTS

The tetrandrine analogs examined in this study can be grouped into four conformational classes. These alkaloids are head-to-head, tail-to-tail condensed dimers of benzylisoquinoline subunits, and the four classes are defined by the orientation of the hydrogen atom about the chiral center on each benzylisoquinoline moiety as illustrated by the structures in Table I. Compounds within each class vary by having different methoxy and hydroxyl substitutions about the benzyl and isoquinoline substituents and by having different attachment sites of ether linkages between the benzylisoquinoline monomers. Tetrandrine is an α–β conformer, where the chiral carbon in the upper left quadrant of the structure shown in Table I would have its proton oriented upward, while the chiral carbon in the upper right quadrant would have the proton

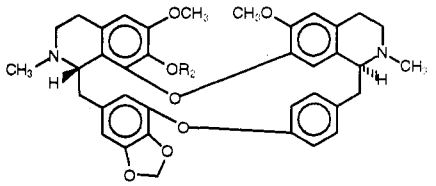
Table I: Structures of Bis(benzylisoquinoline) Alkaloids



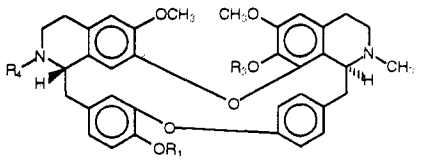
Tetrandrine



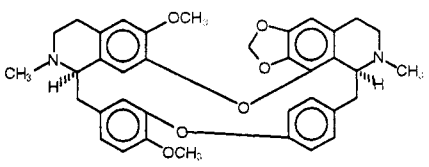
$R_1 = CH_3$: Tetrandrine (αβ)
Pheanthine (βα), Isotetrandrine (ββ)
$R_1 = H$: Berbamine (ββ)



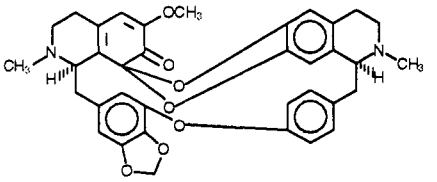
$R_2 = CH_3$:
(+)Tenuipine (αβ), (-)Tenuipine (βα)
$R_2 = H$:
(+)Nortenuipine (αβ), (-)Nortenuipine (βα)



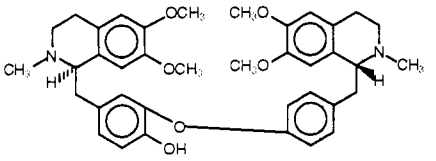
$R_1 = H$, R_3, $R_4 = CH_3$:
Repandine (αβ), Oxyacanthine (ββ)
$R_1 = CH_3$, R_3, $R_4 = CH_3$:
O-Methylrepandine (αβ)
R_1, $R_3 = H$:
$R_4 = H$: Daphnoline (ββ)
$R_4 = CH_2CH_3$: N-Ethyldaphnoline (ββ)
$R_4 = COCH_3$: N-Acetyldaphnoline (ββ)



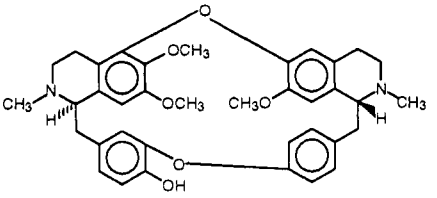
Cepharanthine (ββ)



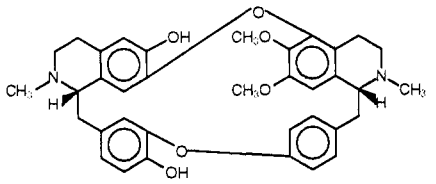
Repanduline (ββ)



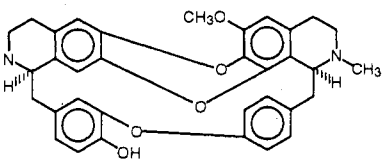
Dauricine (βα)



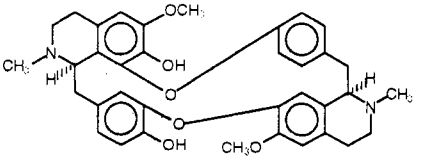
Nemuarine (βα)



Dryadodaphnine (αα)



Apateline (ββ), Micranthine (βα)



(-)Curine (ββ)

positioned downward. Using this convention, the other alkaloids fall into three additional conformational groups: β–α, β–β, and α–α (Table I). These compounds were tested for their ability to inhibit the L-type Ca^{2+} channel as well as to affect binding of CEB ligands.

The agents listed in Table I were all assayed for inhibitory activity against $^{45}Ca^{2+}$ uptake in depolarized GH_3 cells. This influx of $^{45}Ca^{2+}$ is through the L-type channel as demonstrated by three criteria (Figure 1): the dependence of flux on plasma membrane potential, as altered by changing external KCl

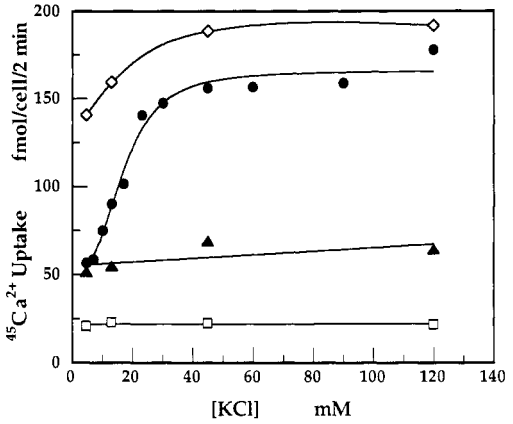


FIGURE 1: Ca^{2+} uptake through the L-type Ca^{2+} channel of GH_3 cells. Increasing amounts of KCl (replacing equal concentrations of NaCl) in 900 μL of reaction mixture containing 11 μM $^{45}Ca^{2+}$ were added to 100 μL of 5×10^5 GH_3 cells, and the reaction was terminated after 2 min (see Experimental Procedures). Net $^{45}Ca^{2+}$ uptake and Bay K-8644 stimulatable uptake through the L-type Ca^{2+} channel are determined as the difference between the total control uptake (●) or the total uptake in the presence of 500 nM Bay K-8644 (◇) and the uptake observed in the presence of 500 nM nitrendipine (▲). Non-nitrendipine-inhibitable uptake is the La^{3+} background uptake (□) subtracted from the nitrendipine-inhibitable uptake.

concentration; the ability of the dihydropyridine CEB, nitrendipine (500 nM), to inhibit KCl-stimulated $^{45}Ca^{2+}$ uptake; and the ability of the dihydropyridine L-type Ca^{2+} channel agonist Bay K-8644 (500 nM) to stimulate Ca^{2+} influx, especially at normal resting potential (low K^+_o, 4.6 mM). In addition, other CEB's such as diltiazem, verapamil, D-600, fluspirilene, and several structurally different dihydropyridine antagonists are able to inhibit $^{45}Ca^{2+}$ uptake in GH_3 cells to the same maximum level as that observed with nitrendipine (not shown). When tetrandrine analogs were tested in the KCl-stimulated $^{45}Ca^{2+}$ uptake protocol, all inhibit Ca^{2+} influx. The IC_{50} for each compound was determined by titration and comparison with the nitrendipine-inhibitable $^{45}Ca^{2+}$ uptake window as indicated in experimental procedures (Table II). Results from these experiments indicate that a variety of structurally and conformationally distinct bis(benzylisoquinoline) alkaloids possess functional CEB activity.

The tetrandrine analogs were next evaluated for their ability to modulate binding of the benzothiazepine, diltiazem, the aralkylamine, D-600, the dihydropyridine, nitrendipine, and the substituted (diphenylbutyl)piperidine, fluspirilene in porcine cardiac sarcolemmal membrane vesicles. All four sites in the CEB receptor complex are affected by the alkaloids. For diltiazem, all analogs cause complete inhibition of binding and corresponding Hill coefficients are unitary. The large differential in potency observed between 25 and 37 °C with tetrandrine (King et al., 1988) is usually not seen with other analogs but is most pronounced with members of the α–β series (Table II).

The experiments monitoring the interaction with dihydropyridines demonstrate that certain of the compounds are able to stimulate nitrendipine binding at 37 °C while some have no effect and others inhibit the binding reaction (Table II). Because bis(benzylisoquinoline) alkaloids cause a wide range of effects in modulating nitrendipine binding, compounds from each of the three conformational classes, along with the sole α–α representative, were chosen for illustration of interactions with the dihydropyridine receptor (Figure 2). The titration curves with (+)tenuipine (α–β), cepharanthine (β–β), oxyacanthine (β–β), and dryadodaphnine (α–α) illustrate typical stimulation profiles. Conversely, three β–α alkaloids tested,

Table II: Determination of Bis(benzylisoquinoline) Inhibition of $^{45}Ca^{2+}$ Uptake and Potency of Interaction at Calcium-Entry Blocker Sites[a]

	K_i's (μM)					
		diltiazem				
compound	$^{45}Ca^{2+}$ uptake	25 °C	37 °C	D-600	flusp	nitrendipine
α–β						
(+)tetrandrine	5.4	0.9	0.10	8.7	0.87†	stimulate
(+)tenuipine	6.6	2.0	0.44	3.6	1.5†	stimulate
(+)nortenuipine	6.2	0.9	0.52	3.5	NE	4.8
repandine	9.6	2.1	0.82	9.2	7.7	stimulate
O-methylrepandine	9.4	3.3	1.2*	4.2	6.1	NE
β–α						
phaeanthine	17	1.5	1.6	44*	ND	WI
(–)nortenuipine	12	3.3	1.2	28	130	8.6
dauricine	19	1.3	1.3	6.5*	ND	41†
(–)tenuipine	6.8	4.5	0.63	14*	ND	11
nemuarine	7.6	1.4	0.43	2.1*	8.1†	47†
micranthine	5.3	3.1	9.6	29*	ND	40
β–β						
cepharanthine	7.1	ND	0.36	0.85†	2.4†	stimulate
berbamine	9.3	ND	1.2	5.8*	0.50	NE
oxyacanthine	13	3.5	0.98	1.6	ND	stimulate
isotetrandrine	15	0.3	1.2	19	240*	WI
apateline	11	3.5	2.5	NE	ND	WI
daphnoline	40*	12	0.84	84*	ND	stimulate
N-acetyldaphnoline	21	29	2.4	1.8*	ND	NE
N-ethyldaphnoline	620*	ND	6.0*	ND	ND	ND
repanduline	19	9.1	2.3	100	110	21
dryadodaphnine (α–α)	12	0.2	1.1	1.0	40†	stimulate
(–)curine	15	0.3	0.26	ND	ND	WI
diltiazem	4.6	0.1	0.13	ND	ND	stimulate

[a] Abbreviations and symbols: flusp, fluspirilene; *, Hill coefficient of less than 0.6; †, partial inhibition; ND, not determined; NE, no effect; WI, weak inhibition profile, no determinable K_i.

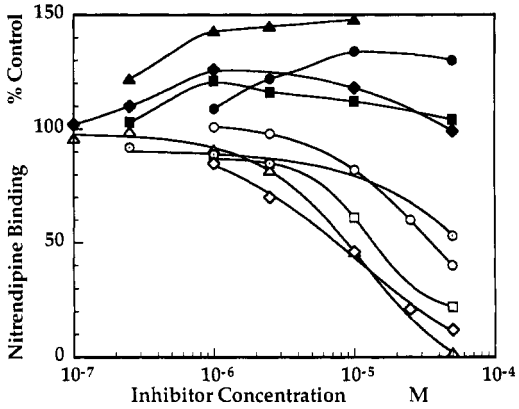


FIGURE 2: Effect of bis(benzylisoquinoline) analogs of tetrandrine on the binding of nitrendipine to purified porcine cardiac sarcolemmal membranes. [^{3}H]nitrendipine was incubated with porcine cardiac sarcolemmal membranes in the presence of varying concentrations of bis(benzylisoquinolines). Binding of nitrendipine to the membranes was determined in a filtration assay as described in Experimental Procedures. Nitrendipine, was tested at 0.25 nM, with increasing concentrations of the α–β's (+)nortenuipine (▲) and (+)tenuipine (▲), the β–α's nemuarine (⊙), (–)nortenuipine (□), and micranthine (◇), the β–β's cepharanthine (◆), oxyacanthine (●), and repanduline (○), and the α–α dryadodaphnine (■).

(–)nortenuipine, nemuarine, and micranthine, the α–β, (+)-nortenuipine, and the β–β conformer, repanduline, all inhibit nitrendipine binding. From the data in Table II, α–β and β–β conformers have mixed activities, including no effect, while the β–α conformers are all inhibitory. Thus, except for the β–α conformers, no clear pattern emerges linking the effects at the dihydropyridine site with the conformation of the bis-(benzylisoquinoline) tested. Stimulation of nitrendipine

binding at 37 °C would be expected for compounds which interact at the benzothiazepine receptor (Garcia et al., 1986). Therefore, compounds producing inhibition of nitrendipine binding do not follow the diltiazem- or tetrandrine-like profile previously observed (Garcia et al., 1986; King et al., 1988).

These compounds also block D-600 and fluspirilene binding, but some are partial inhibitors while others exhibit weak interactions spread over a larger concentration range (Hill coefficient <0.6). For example, most of the β–α and half of the β–β analogs exhibit a gradual concentration dependence of inhibition when titrated against [^{3}H]D-600 binding (n_H < 0.6) while the β–β analog cepharanthine exhibits partial inhibition. On the other hand, all of the α–β analogs and many of the alkaloids from the other three classes listed in Table II do inhibit D-600 binding to completion. Five of the compounds, including tetrandrine, that were titrated against fluspirilene binding at the (diphenylbutyl)piperidine site exhibit partial inhibition. Five others block fluspirilene binding totally. Although those compounds which exhibit broad or incomplete inhibition of D-600 and fluspirilene binding would not be expected to bind at the aralkylamine or (diphenylbutyl)-piperidine site in the CEB receptor complex, these data do not define at which sites the bis(benzylisoquinoline) alkaloids do interact.

To determine the mechanism by which tetrandrine analogs inhibit CEB binding, saturation experiments for diltiazem, D-600, D-888, and PN200-110, in the absence or presence of various tetrandrine analogs, were performed. When [^{3}H]D-600 became no longer available, D-888 was substituted for testing (+)tenuipine. As shown by the data in Figure 3, each of these ligands binds to a single class of sites in cardiac sarcolemmal membranes with respective K_d values of 90 nM, 28 nM, 4.8 nM, and 0.024 nM and B_{max} values that display a stoichiometry of 1:1:1. These results are virtually identical to those obtained previously with this membrane vesicle system (Garcia et al., 1986; King et al., 1989).

While other analogs were subjected to Scatchard analysis, in the interest of continuity and clarity, only those compounds tested against the three binding sites in all of the Scatchards and in all of the subsequently illustrated dissociation experiments are shown. As is apparent from the data in Figure 3, most of the bis(benzylisoquinoline) alkaloids tested have no significant effect on the site density of the three receptors. This result is the same as the pattern produced by tetrandrine (King et al., 1988). When diltiazem binding was examined with selected alkaloids (Figure 3A), the α–β analog (+)-tenuipine, the β–α conformer, (–)nortenuipine, and the β–β representative cepharanthine all give rise to a decrease in affinity of the ^{3}H-labeled ligand. Similar K_d effects were observed, but not shown, with the α–β (+)nortenuipine, the β–β isotetrandrine, and the α–α dryadodaphnine. Scatchard analyses of bis(benzylisoquinoline) effects on D-600 (Figure 3C) and D-888 (Figure 3D) binding to the aralkylamine site produce data similar to that found with diltiazem: increases in the K_d of ligand. Isotetrandrine and dryadodaphnine also inhibited D-600 binding, showing only K_d effects as well (not shown).

PN200-110 was used as a probe of the dihydropyridine site because nonspecific binding of this ligand is quite low and it is technically easier to generate data for a Scatchard analysis under conditions of inhibition with this agent. In Figure 3B, two alkaloids which stimulate dihydropyridine binding, the α–β conformer (+)tenuipine and β–β conformer cepharanthine increase the affinity of PN200-110, a profile identical to that noted with tetrandrine and diltiazem, while the β–α analog

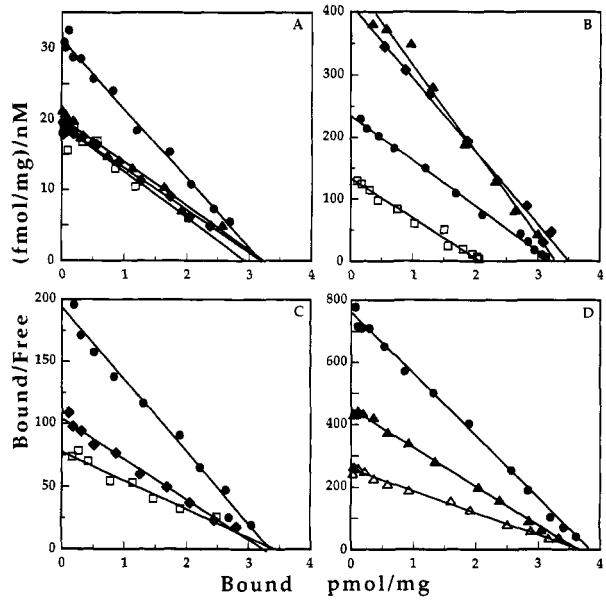


FIGURE 3: Scatchard analyses of the effects of bis(benzylisoquinolines) on the binding of calcium entry blockers to the L-type Ca^{2+} channel of cardiac sarcolemmal membranes. Tritiated ligands were incubated to equilibrium with cardiac sarcolemmal membranes in the presence of the indicated concentrations of bis(benzylisoquinolines). Filtration assays to determine the binding of ligands were performed as described in Experimental Procedures. (A) Diltiazem binding was analyzed for type of inhibition as the control (●) or in the presnce of 0.3 μM of the α–β (+)tenuipine (▲), the β–α (–)-nortenuipine (□), or the β–β cepharanthine (◆). This diltiazem figure is a composite of three different experiments with only one of the controls shown. The control diltiazem binding parameters were within 7% for the three experiments. (B) Scatchard analysis was performed on PN200-110 binding, as the control (●) or in the presence of the α–β (+)tenuipine (▲) at 8 μM, the β–α (–)nortenuipine (□) at 4 μM, or the β–β cepharanthine (◆) at 11 μM. This figure is a composite of two experiments. (C) D-600 binding to the aralkylamine site was tested under control conditions (●) or in the presence of 60 μM β–α (–)nortenuipine (□) or 5 μM β–β cepharanthine (◆). (D) D-888 binding to the aralkylamine binding site was tested as the control (●) or in the presence of 1.25 μM (▲) or 3.25 μM (△) α–β (+)tenuipine.

(–)nortenuipine decreases site density. Nemuarine, another β–α inhibitor, increases the K_d of PN200-110 without an effect on B_{max} (not shown). Because of limited sample availability, it was not possible to test dryadodaphnine, a representative of the α–α class. The results from equilibrium analyses, then, would be consistent with tetrandrine analogs interacting directly at either the benzothiazepine or aralkylamine site of the CEB receptor complex.

A technique more diagnostic for the interaction of an agent at a particular site is the examination of dissociation kinetics of a ligand for that site in the presence of test compound. After incubating membranes with [^{3}H]diltiazem, [^{3}H]D-600, [^{3}H]PN200-110, or [^{3}H]fluspirilene until equilibrium was achieved, either unlabeled ligand to give the control dissociation rate or ligand plus test compound was added, and loss of radiolabeled compound from its receptor was followed with time (Figure 4). As is illustrated in Figure 4A, the dissociation rate of diltiazem is unaffected by the representative set of bis(benzylisoquinoline) alkaloids shown or by several others tested: (+)nortenuipine, isotetrandrine, and daphnoline (not shown). It should be noted that curine, a non-tetrandrine analog, consisting of a head-to-tail, head-to-tail condensed dimer, causes a slight increase in diltiazem dissociation rate. The lack of effect of the benzylisoquinolines as a group is in marked contrast to the enhanced dissociation of diltiazem caused by D-600.

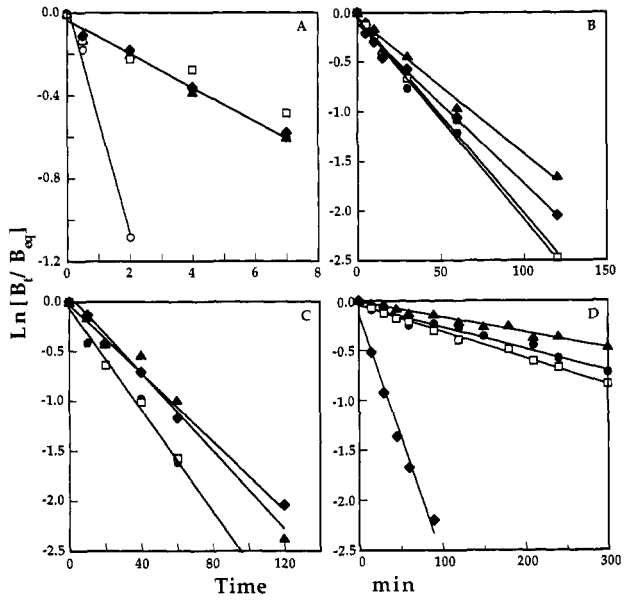


FIGURE 4: Effect of bis(benzylisoquinoline) alkaloids on the dissociation of bound calcium entry blockers from cardiac sarcolemmal membranes. (A) [^{3}H]Diltiazem (25 nM) was allowed to bind for 1.5 h. Then the amount of [^{3}H]diltiazem remaining bound to the membranes at various times was determined in the presence of 10 μM unlabeled diltiazem (●) or unlabeled diltiazem plus 100 μM of either the α–β (+)tenuipine (▲), the β–α (–)nortenuipine (□), or the β–β cepharanthine (◆) or 5 μM aralkylamine D-600 (○). (B) [^{3}H]-PN200-110 (40 pM) was incubated with the membranes for 1.5 h. PN200-110 remaining was measured in the presence of 1 μM unlabeled PN200-110 (●) or of unlabeled PN200-110 plus 57 μM of either the α–β (+)tenuipine (▲), the β–α (–)nortenuipine (□), or the β–β cepharanthine (◆). (C) After binding of 10 nM [^{3}H]D-600 for 4.5 h, dissociation rates were determined in the presence of either 5 μM unlabeled D-600 (●) or unlabeled D-600 plus 100 μM of either the α–β (+)tenuipine (▲), the β–α (–)nortenuipine (□), or the β–β cepharanthine (◆). (D) [^{3}H]Fluspirilene (100 pM) was bound to the membranes overnight. After this time, dissociation was determined in the presence of either 1 μM unlabeled fluspirilene (●) or unlabeled fluspirilene in the presence of 120 μM of either the α–β (+)tenuipine (▲), the β–α (–)nortenuipine (□), or the β–β cepharanthine (◆).

Such results contrast with those obtained by investigating dissociation kinetics of ligands at other sites in the CEB receptor complex. The representative compounds from two of the bis(benzylisoquinoline) conformational classes (the α–β (+)tenuipine and the β–β cepharanthine) inhibit rates of D-600 dissociation to various extents (Figure 4C) as does the β–α nemuarine (not shown), while the β–α (–)nortenuipine is without marked effect. (+)Tenuipine and cepharanthine both decreased the dissociation rates of PN200-110 (Figure 4B), while (–)nortenuipine again is without effect on ligand dissociation. Although tetrandrine itself exhibited no significant effect on fluspirilene dissociation kinetics, another α–β representative, (+)tenuipine, was slightly inhibitory (Figure 4D). The β–β cepharanthine stimulated fluspirilene dissocaition markedly, while the β–α (–)nortenuipine had no significant effect. Another β–α, nemuarine, and the α–α dryadodaphnine also produced no effect on fluspirilene dissociation, while another β–β, berbamine, stimulated dissociation (not shown). These data, then, are consistent with the hypothesis that all tetrandrine analogs examined compete directly at the benzothiazepine receptor.

As a final means of determining the site of action of the tetrandrine analogs shown in Table I, possible correlations were sought between the CEB activity of these compounds and their ability to modulate binding of the different classes of CEB ligands. When the rank order of potency of bis-

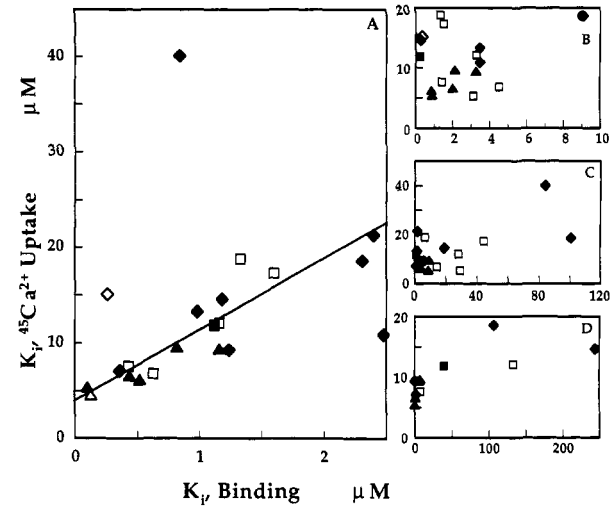


FIGURE 5: Comparison of effects of bis(benzylisoquinolines) on binding of CEB ligands to cardiac sarcolemmal membranes with effects on $^{45}Ca^{2+}$ uptake through the L-type Ca^{2+} channel. Assays were performed as described in Figures 1, 2, and 3. The α–β's (▲), β–α's (□), β–β's (◆), the α–α (■), diltiazem (△), and the head-to-tail-condensed benzylisoquinoline curine (◇) are shown. (A) A linear correlation is described by comparing the K_i's of the benzylisoquinolines for blocking $^{45}Ca^{2+}$ uptake at 37 °C as compared to the K_i's for inhibiting [^{3}H]diltiazem binding at 37 °C. No such correlation exists for the comparison of effects on $^{45}Ca^{2+}$ uptake with effects on (B) diltiazem binding at 25 °C, (C) D-600 binding at 37 °C, or (D) fluspirilene binding at 37 °C.

(benzylisoquinoline) alkaloids is compared at 37 °C for block of $^{45}Ca^{2+}$ uptake in GH_3 cells and inhibition of diltiazem binding to cardiac sarcolemmal membranes, a linear correlation is observed (Figure 5A) for 17 of the 22 compounds tested. As would be expected, diltiazem itself falls directly on the line (Figure 5). The compounds are more potent at inhibiting diltiazem binding than in blocking $^{45}Ca^{2+}$ uptake by a factor of 7. This difference in absolute potencies may reflect differences in assay conditions (e.g., time or ionic strength variations) or be due to dissimilarity in the state of the channel in depolarized cells versus isolated membrane vesicles. Of these alkaloids, the α–β conformers are the most potent as a class, while the β–α and the β–β analogs are less potent but do overlap the range of the α–β compounds. The sole α–α representative, dryadodaphnine, falls about midway on the line. Four of the five compounds which are not methylated at the upper left quadrant isoquinoline nitrogen deviate significantly from the linear relationship: micranthine (β–α) and its enantiomer apateline (β–β) and daphnoline and its *N*-ethyl form. Micranthine is much more potent in blocking $^{45}Ca^{2+}$ uptake than in inhibiting diltiazem binding and is not shown in the figure, but it would be below the line and far to the right (cf. Table II), while apateline deviates less but in the same direction. Daphnoline, which inhibits diltiazem binding well but is very weak at blocking $^{45}Ca^{2+}$ uptake, is shown well above the line. *N*-Ethyldaphnoline is another 15-fold weaker against $^{45}Ca^{2+}$ uptake and 8-fold weaker than daphnoline against diltiazem binding, and it is also found above the line (not included, cf. Table II). Interestingly, the *N*-acetyl form of daphnoline falls back on the correlation line. Also, as expected, curine, a bis(benzylisoquinoline) dimer in which the subunits are linked together in a head-to-tail ring configuration, and which is believed to interact at the aralkylamine site of the CEB receptor complex in heart (King et al., 1988), does not fit the relationship displayed in Figure 5. The lack of correlation by curine is consistent with its action in modestly stimulating diltiazem dissociation rates (see above).

When the same comparison is made between block of $^{45}Ca^{2+}$ uptake and inhibition of D-600 or fluspirilene binding at 37 °C or inhibition of diltiazem binding at 25 °C for these tetrandrine analogs, no linear correlation is observed (parts C, D, and B of Figure 5, respectively). Such a comparison is irrelevant with respect to the dihydropyridine site because many of the bis(benzylisoquinoline) alkaloids stimulate binding and, therefore, must interact allosterically from another site. Since a linear correlation is only observed between CEB activity and inhibition of diltiazem binding at 37 °C, this is strong evidence that all of the various conformational classes of tetrandrine analogs investigated in this study interact directly at the benzothiazepine receptor on the L-type Ca^{2+} channel.

DISCUSSION

The results presented in this paper demonstrate that a family of structurally and conformationally related bis(benzylisoquinoline) analogs of tetrandrine inhibit the L-type Ca^{2+} channel by binding directly at the benzothiazepine site of the CEB receptor complex. There are four lines of evidence supporting this claim: these compounds all completely block binding of diltiazem; Scatchard analyses of diltiazem binding always demonstrate K_d effects consistent with competitive interactions; kinetics of diltiazem dissociation are unaffected by the alkaloids; there is a linear correlation between the ability of these compounds to inhibit diltiazem binding to cardiac sarcolemmal vesicles and to block $^{45}Ca^{2+}$ uptake into GH_3 cells. Since bis(benzylisoquinoline) compounds are chemically distinct from the benzothiazepine family, they comprise a new class of CEB. Tetrandrine is the most potent member of this new class as a CEB and is comparable in binding affinity to that of diltiazem. This is only the second structural group of molecules that has been shown to interact at the benzothiazepine receptor.

The evidence leading to the localization of the binding of the bis(benzylisoquinolines) to the benzothiazepine site is a combination of positive correlations with diltiazem binding coupled with negative indicators for the other sites in the CEB receptor complex. While inhibition of diltiazem binding by the alkaloids is complete, many of the compounds exhibit only partial inhibition or an $n_H < 1$ for inhibition of D-600 and fluspirilene binding and show stimulation of dihydropyridine binding. For diltiazem binding, these alkaloids always display a Hill coefficient of 1, indicative of a single class of binding sites, and exhibit K_d effects in saturation experiments. The observations of complete inhibition of binding of a particular ligand and K_d effects in saturation experiments are necessary but not sufficient criteria for the establishment of a competitive interaction. Interference with rates of ligand dissociation in the presence of a test compound is most likely through an allosteric interaction. For competitive ligands, only multiple interacting sites on a target protein could result in a change in dissociation rates. Diltiazem is the only CEB ligand whose dissociation rate is unaffected by all of the bis(benzylisoquinolines) tested.

The final evidence that bis(benzylisoquinoline) alkaloids as a group compete with diltiazem for a common site is the correlation between the potencies of block of $^{45}Ca^{2+}$ uptake through the L-type Ca^{2+} channel with inhibition of diltiazem binding to the benzothiazepine site. The linear relationship described in Figure 5 is observed only when inhibition of diltiazem binding is considered but not when that of D-600 or fluspirilene binding is analyzed. The range of the K_i's for diltiazem binding is not large (about 30-fold), and compounds from all four conformational classes show a remarkable consistency in their action.

Out of 22 different alkaloids tested, only five do not fall on the correlation line. Four of the compounds which deviate have the similarity that they are not methylated at the upper left quadrant isoquinoline nitrogen. Acetylation of daphnoline at this position restores the correlation fit. These data imply an important role for substitution at this nitrogen in the interaction of benzylisoquinolines at the benzothiazepine site. On the other hand, curine, a bis(benzylisoquinoline) alkaloid distinct from the other tetrandrine analogs tested in that it is a head-to-tail condensed dimer, also lacks the correlation between binding and flux data and increases the diltiazem dissociation rate. It has previously been suggested that this structural type interacts at the aralkylamine receptor (King et al., 1988), and therefore, results obtained with such an agent would not be expected to support the relationship with diltiazem binding presented in Figure 5.

Diltiazem exhibits a characteristic pattern of interaction with the dihydropyridine receptor which has traditionally been used to determine whether or not a CEB with an unknown mechanism of action interacts at the benzothiazepine site. The ability to stimulate dihydropyridine binding is regarded as the keystone of a diltiazem-like agent (Garcia et al., 1986; Triggle & Janis, 1987; Hosey & Lazdunski, 1988; Schwartz, 1989; Glossman & Striessnig, 1990). While some α–β and β–β conformers plus the α–α dryadodaphnine follow this profile, those bis(benzylisoquinolines) which inhibit or have no effect on dihydropyridine binding contradict this paradigm as being diagnostic for the benzothiazepine site. However, the latter compounds have been established as being competitive with diltiazem on the basis of the other criteria described above. Therefore, these data provide the first evidence that binding at the benzothiazepine site can produce either positive, negative, or no heterotropic coupling with the dihydropyridine receptor. Furthermore, the converse is also true. The ability to stimulate dihydropyridine binding does not necessarily mean that a compound must bind at the benzothiazepine receptor, since molecules have been identified (e.g., MDL 12330A, KB 944, BM20.1140) which cause such stimulation but interact at unique sites in the CEB receptor complex (Garcia et al., 1987; Staudinger et al., 1991). Thus, simple models concerning the allosteric coupling between a limited number of sites in the CEB receptor complex (Garcia et al., 1986) must be revised and extended.

Several reports have provided contradictory evidence as to whether the receptor site for benzothiazepines is distinct from the aralkylamine site. An early study investigating skeletal muscle t-tubule receptors has concluded that those sites at which aralkylamine and benzothiazepine structures interact must be identical because respective compounds inhibit each other's binding by producing K_d effects in Scatchard analyses, an apparently competitive profile (Galizzi et al., 1986). Another group has claimed that these sites overlap in the CEB receptor complex present in brain (Murphy et al., 1983). However, this laboratory has previously provided evidence that the two receptors are distinct because dissociation rates of diltiazem are markedly enhanced in the presence of D-600 (Garcia et al., 1986). Recent data obtained using photoaffinity probes based on aralkylamine and dihydropyridine structures have localized the binding domains for these CEB classes on the α1 subunit of the L-type Ca^{2+} channel isolated from skeletal muscle (Striessnig et al., 1990; Nakayama et al., 1991; Striessnig et al., 1991). These two sites are distinct. However, it is not known where the benzothiazepine receptor is located on this same protein. Nevertheless, data obtained with tetrandrine have been used to argue for the individuality of

the benzothiazepine and aralkylamine receptor types (King et al., 1988). The results presented in the current investigation confirm and extend the conclusions from the other studies (Garcia et al., 1986; King et al., 1988) that suggest that the aralkylamine and benzothiazepine sites are distinct. A number of the alkaloids shown in Table I produce incomplete inhibition of D-600 binding, arguing against a strictly competitive interaction at the aralkylamine site. Moreover, these agents modify rates of D-600 dissociation from membranes, ruling out the possibility that tetrandrine analogs and D-600 interact in a mutually exclusive fashion at a common site. Therefore, these data clearly demonstrate that unique receptors must exist in the CEB receptor complex for aralkylamines and agents acting at the common benzothiazepine/bis(benzylisoquinoline) site.

The basis for the therapeutic efficacy of tetrandrine in treatment of various cardiovascular disorders like hypertension, as well as that of structurally similar bis(benzylisoquinoline) alkaloids employed in traditional Chinese folk medicine, may lie in their ability to inhibit the L-type Ca^{2+} channel. Indeed, tetrandrine displays a pharmacological profile in vivo similar to that of diltiazem (Zeng et al., 1985). Perhaps some of the other pharmacological activities of tetrandrine, such as the antiinflammatory effects produced by this compound in pulmonary silicosis (Ferrante et al., 1990) or its immunosuppressive activity (Seow et al., 1988) are also linked to modulation of ion channel activities. Localizing the site of action of bis(benzylisoquinoline) alkaloids to the benzothiazepine site in the CEB receptor complex adds to our understanding of the molecular parameters which define binding at this locus. Given the structural uniqueness of this class of compounds, it will be interesting to determine whether these alkaloids provide novel probes for elucidating structure–function relationships with the L-type Ca^{2+} channel.

ACKNOWLEDGMENT

We dedicate this paper to the memory of Mr. V. Frank King, whose dedication and scientific contributions were vital to the projects of this laboratory. His friendship as well as his scientific presence will be missed.

REFERENCES

Buck, K. T. (1987) *Alkaloids 30*, 1–222.

Cheng, Y., & Prusoff, W. H. (1973) *Biochem. Pharmacol. 22*, 3099–3108.

Ehrlich, B. E., Schen, C. R., Garcia, M. L., & Kaczorowski, G. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 193–197.

Ferrante, A., Seow, W. K., Rowan-Kelley, B., & Thong, Y. H. (1990) *Clin. Exp. Immunol. 80*, 232–235.

Galizzi, J.-P., Borsotto, M., Barhanin, J., Fosset, M., & Lazdunski, M. (1986) *J. Biol. Chem. 261*, 1393–1397.

Garcia, M. L., Trumble, M. J., Reuben, J. P., & Kaczorowski, G. J. (1984) *J. Biol. Chem. 259*, 15013–15016.

Garcia, M. L., King, V. F., Siegl, P. K. S., Reuben, J. P., & Kaczorowski, G. J. (1986) *J. Biol. Chem. 261*, 8146–8157.

Garcia, M. L., King, V. F., & Kaczorowski, G. J. (1987) *Fed. Proc. 46*, 345 (Abstract).

Garcia, M. L., King, V. F., Shevell, J. L., Slaughter, R. S., Suarez-Kurtz, G., Winquist, R. J., & Kaczorowski, G. J. (1990) *J. Biol. Chem. 265*, 3763–3771.

Glossman, H., & Striessnig, J. (1990) *Physiol. Biochem. Pharmacol. 114*, 1–105.

Hosey, M. M., & Lazdunski, M. (1988) *J. Membr. Biol. 104*, 81–105.

Hwang, S. B., Chang, M. N., Garcia, M. L., Han, Q. Q., Huang, L., King, V. F., Kaczorowski, G. J., & Winquist, R. J. (1987) *Eur. J. Pharmacol. 141*, 269–281.

Kaczorowski, G. J., King, V. F., Shevell, J. L., Felix, J. P., Slaughter, R. S., & Garcia, M. L. (1989) *Eur. J. Clin. Pharmacol. 36*, A21.

King, V. F., Garcia, M. L., Himmel, D., Reuben, J. P., Pan, J.-X., Lam, Y.-K., & Kaczorowski, G. J. (1988) *J. Biol. Chem. 263*, 2238–2244.

King, V. F., Garcia, M. L., Shevell, J. L., Slaughter, R. S., & Kaczorowski, G. J. (1989) *J. Biol. Chem. 264*, 5633–5641.

Kuwayama, H., & Kanazawa, T. (1982) *J. Biochem. 91*, 1419–1426.

Murphy, K. M. M., Gould, R. J., Largent, B. L. & Snyder, S. H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 860–864.

Nakayama, H., Taki, M., Striessnig, J., Glossman, H., Catterall, W. A., & Kanaoka, Y. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9203–9207.

Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1982) *J. Biol. Chem. 256*, 11804–11808.

Qian, J. Q., Thoolen, M. J. M. C., vanMeel, J. C. A. Timmermanns, P. B. M. W. M., & vanZwieten, P. A. (1983) *Pharmacology 27*, 187–197.

Ruth, P., Flockerzi, V., Nettelbladt, E. v., Oeken, J., & Hofmann, F. (1985) *Eur. J. Biochem. 150*, 313–322.

Schaffner, W., & Weissmann, C. (1973) *Anal. Biochem. 56*, 502–514.

Schwartz, A. (1989) *Am. J. Cardiol. 64*, 3I–9I.

Seow, W. K., Gerrante, A., Goh, D. B. H., Chalmers, A. H., Li, S. Y., & Thong, Y. H. (1988) *Int. Arch. Allergy Appl. Immunol. 85*, 410–416.

Siegl, P. K. S., Garcia, M. L., King, V. F., Scott, A. L., Morgan, G., & Kaczorowski, G. J. (1988) *Naunyn-Schmiedeberg's Arch. Pharmacol. 338*, 684–691.

Slaughter, R. S., Sutko, J. L., & Reeves, J. P. (1983) *J. Biol. Chem. 258*, 3183–3190.

Slaughter, R. S., Shevell, J. L., Felix, J. P., Garcia, M. L., & Kaczorowski, G. J. (1989) *Biochemistry 28*, 3995–4002.

Staudinger, R., Kaus, H. G., & Glossman, H. (1991) *J. Biol. Chem. 266*, 10787–10795.

Striessnig, J., Glossman, H., & Catterall, W. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9108–9112.

Striessnig, J., Murphy, B. J., & Catterall, W. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10769–10773.

Triggle, D. J., & Janis, R. A. (1987) *Annu. Rev. Pharmacol. Toxicol. 28*, 347–369.

Wang, Z. G., & Liu, G. J. (1985) *Trends Pharmacol. Sci. 6*, 423–426.

Zeng, D., Shaw, D. H., Jr., & Ogilvie, R. I. (1985) *J. Cardiovasc. Pharmacol. 7*, 1034–1039.