Proc. Natl. Acad. Sci. USA
Vol. 78, No. 2, pp. 1237–1241, February 1981
Neurobiology

α-Adrenergic antagonists as possible calcium channel inhibitors

(α_1-adrenergic receptors/neuroblastoma–glioma hybrid cells/Ca^{2+} antagonists/verapamil)

DAPHNE ATLAS*†‡ AND MICHAEL ADLER*

*Laboratory of Biochemical Genetics, National Heart, Lung and Blood Institute, and †Laboratory of Bioorganic Chemistry, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205

Communicated by W. A. Hagins, September 12, 1980

ABSTRACT **The effects of various organic Ca^{2+} channel inhibitors were investigated on the binding of the α_1-antagonist ^{3}H-labeled 2-[(2′,6′-dimethoxyphenoxyethyl)aminomethyl]-1,4-benzodioxane ([^{3}H]WB-4101) to membranes from rat brain and neuroblastoma–glioma hybrid cells (NG108–15). As found by monitoring binding of [^{3}H]WB-4101, the Ca^{2+} channel inhibitors methoxyverapamil (D600), verapamil, and the nifedipine analogue YC-93 bind to two different sites in rat brain: a high-affinity site (dissociation constant K_d = 2.9 nM and binding capacity B = 360 fmol/mg of protein) and a low-affinity site (K_d = 260 nM and B = 2700 fmol/mg of protein). In NG108-15 cells, where no $\alpha_1$receptors were detected with [^{3}H]WB-4101, the Ca^{2+} antagonists were found to bind to nonadrenergic sites in the membrane with a capacity B = 976 fmol/mg of protein. The binding of Ca^{2+} antagonists to [^{3}H]WB-4101 sites led to the investigation of WB-4101 as a Ca^{2+} inhibitor by electrophysiological techniques. WB-4101 depressed the amplitude and reduced the rate of rise of the Ca^{2+} spike with an affinity slightly greater than that observed for D600. The concentration for 50% inhibition of the Ca^{2+} spike amplitude was 48 μM for WB-4101 and 80 μM for D600. The WB-4101-induced blockade of the Ca^{2+} spike was antagonized by high Ca^{2+} concentrations, indicating a common site for Ca^{2+} and the α-antagonist. D600 and WB-4101 also inhibited voltage-dependent Na^+ and K^+ conductances. The results suggest that Ca^{2+} channels can account for a fraction of the sites labeled with [^{3}H]WB-4101 in membrane preparations from brain and NG108-15 cells.**

The role of Na^+, K^+ and Ca^{2+} ions in physiological processes has been reviewed extensively (1, 2). The existence of specific antagonists enabled the differentiation and identification of the channels involved in the transport of these ions as separate entities. Relative to Na^+ channel blockers such as tetrodotoxin (TTX) and scorpion toxin (3, 4), the Ca^{2+} inhibitors display a rather low affinity and poor selectivity. Although compounds such as verapamil, methoxyverapamil (D600), and nifedipine are considered to be specific Ca^{2+} antagonists (1, 2) their actions are not confined to Ca^{2+} channels. In addition to blocking Ca^{2+} currents in smooth and cardiac muscle (1), D600 ($\leq$1 μM) has been reported to inhibit a slowly activating outward current in cardiac Purkinje cells that is carried principally by K^+ (5). At higher concentrations ($\geq$100 μM), D600 and verapamil are also known to block fast Na^+ currents (2). Moreover, these agents have been shown to inhibit uptake of dopamine, (−)-norepinephrine [(−)NE], serotonin, and choline in rat forebrain synaptosomes (6). During the preparation of this paper, Fairhurst *et al.* (7) demonstrated that D600 and verapamil display an affinity for the α-adrenergic receptors in rat cortex homogenates and for the muscarinic receptors in rat brain striatal homogenates.

In the present study we describe the α-adrenergic properties of the organic Ca^{2+} inhibitors by monitoring their ability to displace the potent ^{3}H-labeled α_1-antagonist 2-[(2′,6′-dimethoxyphenoxyethyl)aminomethyl]-1,4-benzodioxane ([^{3}H]WB-4101) from binding sites in rat brain membranes. We also examine the Ca^{2+}-blocking effects of WB-4101 by electrophysiological techniques. WB-4101 inhibits both the Ca^{2+} spike and the mixed Na^+, Ca^{2+} spike in the neuroblastoma–glioma hybrid clone NG108-15. The blocking affinities of WB-4101 resemble closely those of the Ca^{2+} antagonist D600.

MATERIALS AND METHODS

Drugs. [4-^{3}H]Clonidine (22.2 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and [^{3}H]WB-4101 (24.4 Ci/mmol) were obtained from New England Nuclear. (+)-Norepinephrine [(+)NE] was purchased from Adams (Round Lake, IL). (−)-NE was from Sigma. D600 and verapamil were from Bayer (W. Germany). The following were generous gifts: WB-4101 from Ward Blenkinshop (England); phentolamine from Ciba–Geigy; and 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid 3-[2-(*N*-benzyl-*N*-methylamino)]ethyl, 5-methyl ester·HCl (YC-93) from D. J. Triggle. The other chemicals were of reagent quality.

Membrane Preparation. The preparation of rat brain P_2 pellet was carried out essentially as described (11). A similar procedure was used for obtaining membranes from the neuroblastoma–glioma hybrid NG108-15. The hybrid cells (subculture 17–22) were grown in Falcon flasks (75 cm^2 surface area) under standard growth conditions (9). Protein was determined by the method of Lowry *et al.* (10).

Binding of [^{3}H]WB-4101 to Membranes. Binding assays were carried out according to established procedures (11). Specific [^{3}H]WB-4101 binding to α-adrenergic receptors and other sites in the membrane was obtained by using 0.1 mM unlabeled WB-4101 or 0.1 mM D600 to determine nonspecific binding. Binding curves were analyzed with the aid of the SCATFIT program of V. B. Faden and D. Rodbard (12) on the IBM 370 computer (National Institutes of Health Computer Center). The binding of [^{3}H]clonidine to rat brain membranes was determined by described methods (11).

Electrophysiology. Experiments were routinely performed in Dulbecco's modified Eagle's medium in 35-mm tissue culture dishes containing 1–3 × 10^5 differentiated NG108-15 cells. The cells were induced to differentiate by addition of 1 mM dibutyryl cyclic AMP for ~1 week. Ca^{2+} spikes were recorded in a Na^+-free solution of the following composition (mM): KCl,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: D600, methoxyverapamil; WB-4101, 2-[(2′,6′-dimethoxyphenoxyethyl)aminomethyl]-1,4-benzodioxane; (−)NE, (−)-norepinephrine; (+)NE, (+)-norepinephrine; TTX, tetrodotoxin; YC-93, 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid 3-[2-(*N*-benzyl-*N*-methylamino)]ethyl ester, 5-methyl ester·HCl (nifedipine analogue).

‡ Permanent address: Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel.

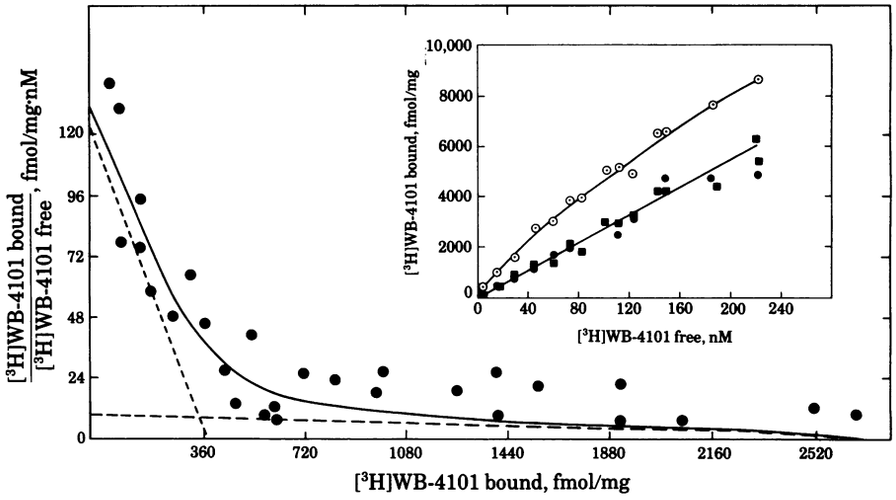

FIG. 1. Binding of [^{3}H]WB-4101 to rat brain membrane preparations in the absence and in the presence of WB-4101 (unlabeled) and D600. Reaction mixtures contained 114 μg of membrane protein and [^{3}H]WB-4101 in increasing concentrations. The buffer used was 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$. Incubations were carried out at 25°C for 40 min, in the absence (○) or presence of 0.1 mM WB-4101 (■) or 0.1 mM D600 (●). (*Inset*) Total binding and nonspecific binding of [^{3}H]WB-4101. The outer figure is a Scatchard analysis of specific [^{3}H]WB-4101 binding to rat brain membrane preparations. Computer fits are shown in broken lines. The best fit was a two-site model with dissociation constants K_{d_1} = 2.9 nM and K_{d_2} = 260 nM. The corresponding binding capacities were B_1 = 360 fmol/mg of protein and B_2 = 2700 fmol/mg of protein, respectively.

5.4; $MgCl_2$, 0.81; $CaCl_2$, 1.8; glucose, 25; and Tris, 170. The 80 mM Ca^{2+} solution was prepared by substitution of Ca^{2+} for Tris. The Ca^{2+} solutions were adjusted to pH 7.4 with HCl and were approximately isosmotic with Dulbecco's modified Eagle's medium (330–340 milliosmoles/kg). The temperature of the bathing medium was maintained at 37 ± 0.5°C. The cells were visualized with the aid of phase-contrast optics (Zeiss) and impaled by 3 M KCl-filled microelectrodes (20–40 MΩ). Stimulation was achieved by passing current through the recording electrode via the bridge circuit of a W-P Instruments M-707 amplifier.

The maximal rates of rise and fall of the action potential were obtained by electronic differentiation using an operational amplifier (time constant, 100 μsec). Unless stated otherwise, the spikes were elicited from a hyperpolarized level of −90 mV to ensure optimal and uniform electrical responses. Under these conditions the majority of spikes in both the modified Eagle's medium and 1.8 mM Ca^{2+} saline exhibited all-or-none characteristics. The spikes become graded in the presence of high concentrations of the Ca^{2+} and α-adrenergic antagonists. The maximal amplitude and rate of rise of such graded responses were then used for construction of the dose–response relationships.

Drug solutions were prepared from frozen stock solutions dissolved in distilled water. The cells were washed two to three times with 2-ml samples of the desired drug solution prior to recording, and a similar washing procedure was employed when switching from Dulbecco's modified Eagle's medium to Ca^{2+} saline. A 15- to 30-min period was allowed for equilibration of cells to all solution changes.

RESULTS

Binding of [^{3}H]WB-4101 to Rat Brain Membranes. Binding of [^{3}H]WB-4101 to rat brain α-receptors and to other sites is depicted in Fig. 1. The Scatchard analysis suggests two distinct populations. The data were fitted to a model with two types of binding sites: a class of high-affinity sites with K_d = 2.9 nM and binding capacity of 360 fmol/mg of protein, and a class of low-affinity sites with K_d = 260 nM and a binding capacity of 2700 fmol/mg protein. The high-affinity sites most probably represent the α_1-receptors. Some heterogeneity in the high-affinity α_1-receptors was described (13–16).

Displacement of [^{3}H]WB-4101 by D600 and Verapamil. D600 and verapamil compete effectively with [^{3}H]WB-4101 for sites in rat brain membranes (Fig. 2). The concentration of [^{3}H]WB-4101 used in this experiment was 15 nM at which [^{3}H]WB-4101 binds to α_1-receptors plus additional sites (see Fig. 1). Indeed phentolamine, a well-defined α_1-antagonist, displaces only a fraction of the [^{3}H]WB-4101 bound (Fig. 2). Like phentolamine, D600 and verapamil bind specifically to α-receptors (the concentration inhibiting binding by 50%, IC_{50}, ± SD, = 2.0 ± 0.9 μM), but, in contrast to phentolamine, the Ca^{2+} antagonists bind also to the low-affinity fraction of the [^{3}H]WB-4101 binding sites. These observations suggest that WB-4101, D600, and verapamil bind with high affinity to α_1-receptors and to "other" sites with lower affinity. The "other" sites may be involved with Ca^{2+} transport, because both verapamil and D600 have distinct effects on Ca^{2+} entry.

α-Adrenergic and "Other" Sites Labeled with [^{3}H]WB-4101. In an effort to define the two main classes of binding sites monitored by [^{3}H]WB-4101, we studied the displacement of

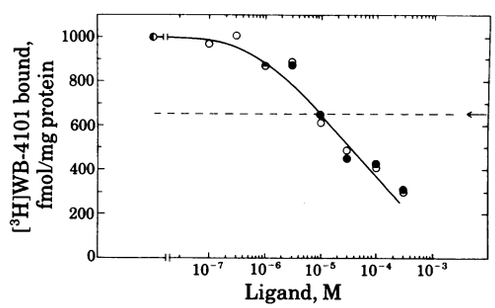

FIG. 2. Displacement of [^{3}H]WB-4101 by D600 and verapamil from rat brain membrane preparations. The binding assays were carried out in the buffer described in Fig. 1, containing 123 μg of rat membrane protein, 15 nM [^{3}H]WB-4101, and the unlabeled Ca^{2+} antagonists [D600 (●) or verapamil (○)] in the appropriate concentrations. Incubations were carried out for 40 min at 25°C. The amount bound in the presence of 10 μM phentolamine is shown by the broken line (660 fmol/mg of protein). Total binding was 1000 fmol of [^{3}H]WB-4101 per mg of protein.

bound [^{3}H]WB-4101 by α-receptor-directed ligands and Ca^{2+} channel antagonists at different [^{3}H]WB-4101 occupancies. When 4.3 nM [^{3}H]WB-4101 was used, (−)NE and the 1/100th as effective (+)NE displaced a fraction of [^{3}H]WB-4101 bound (121 fmol/mg of protein). The amount of [^{3}H]WB-4101 displaced by 10 μM phentolamine was used as the criterion for monitoring specific binding to α_1-receptors (151 fmol/mg of protein) (Fig. 3A). Both unlabeled WB-4101 and higher concentrations of phentolamine displaced [^{3}H]WB-4101 further, below the value obtained with 10 μM phentolamine. Neither (−)NE nor (+)NE showed an affinity for sites other than those defined as α-adrenergic receptors. When the concentration of [^{3}H]WB-4101 in the binding assay was increased, the fraction displaced by (−)NE was progressively decreased (Fig. 3B). (−)NE (100 μM) displaced a maximum of 9% of the total binding when 24 nM [^{3}H]WB-4101 was used (Fig. 3A); however, phentolamine displaced up to 25% (at 10 μM) and 30% (at 100 μM) of the [^{3}H]WB-4101 bound (Fig. 3B). Similarly, the Ca^{2+} channel inhibitors D600 and YC-93 displaced not only the [^{3}H]WB-4101 bound to α-receptors but also the [^{3}H]WB-4101 bound to "other" sites.

α_1-Adrenergic Properties of D600, YC-93, and Verapamil. In displacement of 1.1 nM [^{3}H]WB-4101 by D600, YC-93, and verapamil, conditions in which [^{3}H]WB-4101 labels α_1-adrenergic sites almost exclusively, the IC_{50} values obtained were 1.4 μM for verapamil and 4 μM for D600 and YC-93 (data not shown). The dissociation constants calculated for these compounds are 0.31 ± 0.5 μM and 0.93 ± 0.9 μM, respectively.

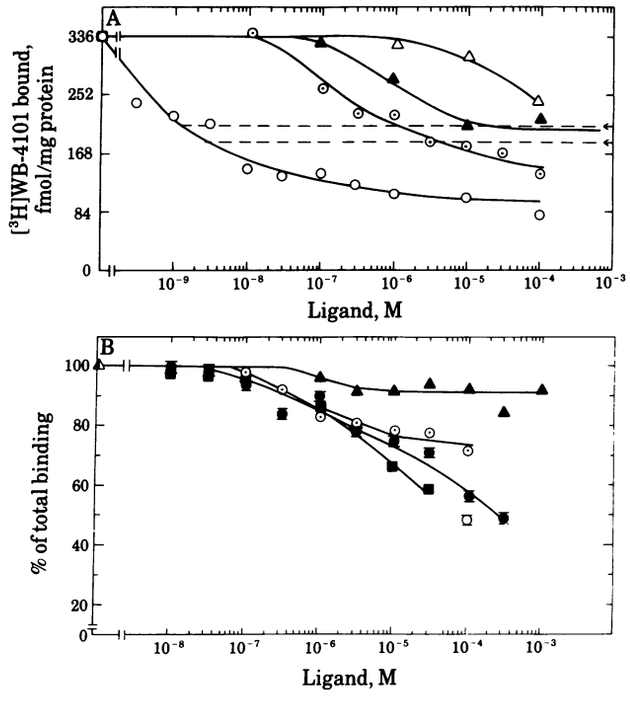

FIG. 3. Displacement of [^{3}H]WB-4101 from rat brain membrane preparations by various α-adrenergic ligands and Ca^{2+} antagonists. All binding assays were performed in 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$. Incubations were for 40 min at 25°C with 110 μg of rat membrane protein. (A) Displacement of [^{3}H]WB-4101 (4.3 nM) by WB-4101 (○), phentolamine (⊙), (−)NE (▲), and (+) NE (△). Total binding was 336 fmol/mg of protein. The transition from specific to nonspecific binding is depicted by the broken lines. These represent the fraction of [^{3}H]WB-4101 displaced by (−)NE (upper line) or phentolamine (lower line) at the indicated concentrations. (B) Displacement of [^{3}H]WB-4101 (24 nM) by (−)NE (▲), phentolamine (⊙), D600 (●), YC-93 (■), and WB-4101 (○). Total binding in this assay was 1800 fmol/mg of protein. (−)NE displaced only 8% of the total binding; 10 μM phentolamine displaced 21% of the total binding.

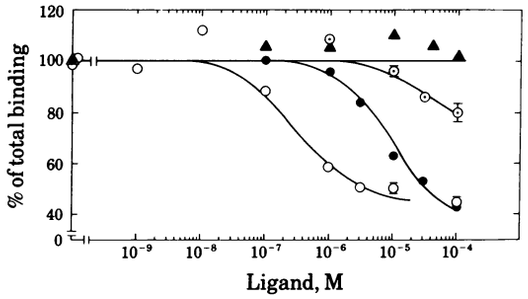

FIG. 4. Displacement of [^{3}H]WB-4101 by (−)NE (▲), phentolamine (⊙), D600 (●), and WB-4101 (○) in neuroblastoma–glioma hybrid cells (NG108-15). Binding mixtures contained, in the standard binding buffer, 105 μg of protein from NG108-15 washed membrane preparations, 24.2 nM [^{3}H]WB-4101, and the ligands mentioned above in the indicated concentrations. Incubations were for 40 min at 25°C. Total binding to the cell membranes was 1680 fmol/mg of protein. Subtracting nonspecific binding (determined in the presence of 100 μM WB-4101) yields 976 fmol/mg of protein.

The α-adrenergic effects of D600 and verapamil should be considered when interpreting data on Ca^{2+} blockage (7) and in clinical use of these agents (1, 17).

The classification of α-adrenoreceptors into two distinct groups, α_1 and α_2, is now well established (18–21). [^{3}H]WB-4101 labels specifically α_1-receptors, whereas [^{3}H]clonidine labels specifically α_2-receptors. Because the Ca^{2+} antagonists were found to bind strongly to α_1-receptors, it was of interest to examine their affinity for α_2-receptors. The IC_{50} for displacement of [^{3}H]clonidine by D600 was found to be higher than 300 μM (data not shown).

Binding of [^{3}H]WB-4101 to NG108-15 Cells. The neuroblastoma–glioma hybrid cells were recently shown to possess α_2-receptors by indirect binding studies using [^{3}H]clonidine (8). However these cells appear to lack α_1-receptors. This conclusion is based on the finding that [^{3}H]WB-4101 binding to NG108-15 cells is not displaceable by (−)NE or low concentrations (≤10 μM) of phentolamine. From Fig. 4, it is apparent that (−)NE up to 100 μM does not displace [^{3}H]WB-4101. Phentolamine at 10 μM displaced 4% of total binding to the cell membranes and 20% at 100 μM. D600 displaced almost 40% of total binding to the membrane at 10 μM and 55% at 100 μM. Unlabeled WB-4101 at 1.0 μM and 10 μM displaced 50% and 55% of total binding, respectively. Total binding to the NG108-15 membrane was 1680 fmol/mg of protein. The α_2-receptor population in this preparation was found to be 62 fmol/mg of protein, as measured by [^{3}H]clonidine binding (data not shown).

Characteristics of Action Potentials in NG108-15 Cells. The control cells that were used for electrophysiological studies had resting membrane potentials ranging between −40 and −65 mV (mean ± SEM = −51.6 ± 1.4 mV, n = 43). When the membrane potential was preset to −90 mV, the action potential in normal medium had an amplitude of 65.3 ± 1.84 mV and a rate of rise of 86.3 ± 7.4 V/sec (n = 23) (Fig. 5A). Upon addition of 3 μM TTX or replacement of external Na^+ by Tris, (Fig. 5C), a smaller and much slower spike was observed. The amplitude, overshoot, and rate of rise of this spike varied with external Ca^{2+} (Fig. 5E). These findings suggest that the slow spike is carried by Ca^{2+}, whereas the spike in normal medium is carried by both Na^+ and Ca^{2+}. Regenerative responses in the neuroblastoma clone N1E-115 (22) and the neuroblastoma–glioma hybrid clone 108CC25 (23) have also been shown to involve increases in permeability to both Na^+ and Ca^{2+}.

Effect of D600 and WB-4101 on Electrical Excitability. The Ca^{2+} inhibitor D600 and the α-adrenergic antagonist WB-4101 were tested for their ability to alter voltage-dependent Ca^{2+}

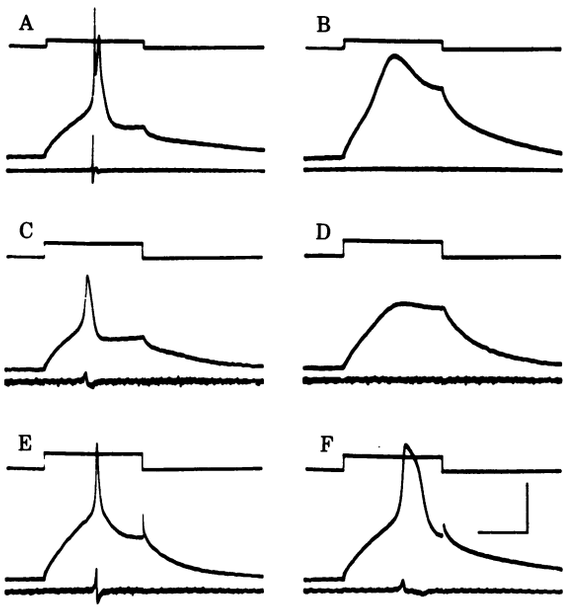

FIG. 5. Effect of WB-4101 on regenerative responses from NG108-15 cells. Three cells are shown prior to (left) and during (right) addition of 150 μM WB-4101 in Dulbecco's modified Eagle's medium (*A* and *B*), in a Na^+-free solution containing 1.8 mM Ca^{2+} (*C* and *D*), and in a Na^+-free solution containing 80 mM Ca^{2+} (*E* and *F*). Membrane potentials were adjusted to −90 mV prior to stimulation and records were taken 34 min (*B*), 29 min (*D*), and 22 min (*F*) after addition of WB-4101. The upper, middle, and lower traces in each panel show, respectively, the current pulse, the action potential, and its first derivative. The base line for the current trace indicates the 0-mV level for the action potential. Calibrations: the vertical bar denotes 4 nA, 40 mV, and 200 V/sec (*A* and *B*), 20 V/sec (*C* and *D*), or 40 V/sec (*E* and *F*). The horizontal bar represents 100 msec.

conductance, using the Ca^{2+} spike amplitude and rate of rise as criteria for action on Ca^{2+} channels.

In control cells the Ca^{2+} spike had an amplitude of 40.2 ± 0.85 mV and a rate of rise of 6.1 ± 0.41 V/sec (mean ± SEM, $n = 21$). Approximately 80% of the cells sampled displayed all-or-none behavior. After addition of D600 at concentrations of 35 μM or greater, the spike showed a dependence on stimulus intensity and underwent a depression in amplitude (Fig. 6), accompanied by a marked slowing in its rates of rise and fall. The IC_{50} values for inhibition of the Ca^{2+} spike amplitude and rate of rise were 80 and 24 μM, respectively. The concentrations of D600 required to block Ca^{2+} spikes in NG108-15 cells are considerably higher than those found effective in inhibiting voltage-sensitive Ca^{2+} responses in smooth and cardiac muscle (1, 2). They are consistent, however, with values reported for blockade of Ca^{2+} in other neuronal systems (24).

The actions of WB-4101 on the Ca^{2+} spike resembled closely those of D600 (Fig. 6). All spikes were graded at WB-4101 concentrations of 35 μM or greater. The IC_{50} for inhibition of the Ca^{2+} spike amplitude was estimated from Fig. 6 as 48 μM, making WB-4101 slightly more potent than D600 in this characteristic. Likewise, WB-4101 was somewhat more potent than D600 in reducing the maximal rate of rise of the Ca^{2+} spike (IC_{50} = 16 μM).

In addition to Ca^{2+} channels, D600 and WB-4101 also blocked voltage-sensitive Na^+ and K^+ channels. The IC_{50} for reduction of the Na^+ spike amplitude was estimated to be 230 and 105 μM for D600 and WB-4101, respectively (Fig. 7). Comparison of these IC_{50} values with those obtained for inhibition of the Ca^{2+} spike indicates that both agents are more effective in reducing Ca^{2+} conductance than Na^+ conductance. The selectivity is rather small, however, being approximately 3-fold

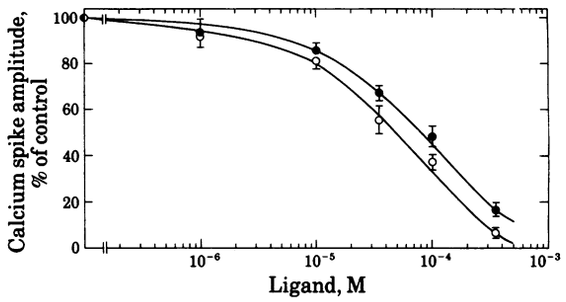

FIG. 6. Effect of various concentrations of D600 (●) and WB-4101 (○) on Ca^{2+} spike amplitudes of NG108-15 cells recorded in Na^+-free medium containing 1.8 mM Ca^{2+}. Ca^{2+} spikes were elicited with 100- to 300-msec depolarizing pulses. The membrane potential was preset to −90 mV prior to stimulation. The symbols show the mean ± SEM of data obtained from 7–11 cells.

for D600 and 2-fold for WB-4101.

Blockade of K^+ conductance was observed in normal medium as well as in Na^+-free solutions. Concentrations of D600 and WB-4101 between 1 and 10 μM produced a marked slowing in the falling phase of both Na^+ and Ca^{2+} spikes, whereas higher drug concentrations (≥200 μM for WB-4101 or ≥350 μM for D600) caused an almost complete block of delayed rectification. Inhibition of Na^+ and K^+ conductance by high concentrations of D600 have been reported previously (2, 25).

Antagonism of the WB-4101 Inhibition by Increases in External Ca^{2+}. The level of inhibition produced by WB-4101 on the Ca^{2+} spike was found to vary with Ca^{2+} concentration. This is illustrated in Fig. 5. When 150 μM WB-4101 was added to cells bathed in 1.8 mM Ca^{2+}, the Ca^{2+} spike amplitude and its rates of rise and fall were markedly depressed (Fig. 5*D*). However, when the Ca^{2+} concentration was raised to 80 mM, 150 μM WB-4101 had little effect on the spike amplitude and reduced its rate of rise by only 47% (Fig. 5*F*). The most prominent effect of WB-4101 in high Ca^{2+} was a depression in the rate of fall and prolongation of the decay phase of the Ca^{2+} spike, indicating a continued block of K^+ conductance. The ability of high Ca^{2+} concentrations to antagonize the blockade of Ca^{2+} spikes by WB-4101 is of interest because it implies a direct action of WB-4101 on the Ca^{2+} channel. The failure of 80 mM Ca^{2+} to antagonize the inhibition of K^+ conductance to the same degree suggests that the channels are blocked independently; hence the low selectivity encountered with WB-4101 is not necessarily a consequence of a general toxic effect on the mem-

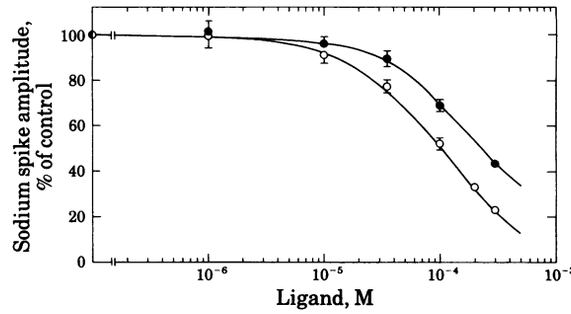

FIG. 7. Effect of various concentrations of D600 (●) and WB-4101 (○) on Na^+ spike amplitudes of NG108-15 cells recorded in Dulbecco's modified Eagle's medium. Although both Na^+ and Ca^{2+} carry the inward current in normal medium, the peak amplitude and maximal rate of rise of the action potential reflect principally the contribution of Na^+. Cells were stimulated by 100- to 300-msec depolarizing pulses from a background level of −90 mV. The symbols represent the mean ± SEM of data obtained from 7–10 cells.

brane. Other explanations for the interaction between elevated Ca^{2+} concentrations and WB-4101 are considered in *Discussion*.

DISCUSSION

In the present study we examined the properties of two different populations of sites labeled by [^{3}H]WB-4101. As previously reported (13–16), WB-4101 binds with high affinity to α_1-adrenergic receptors. Our results on rat brain membranes indicate that in addition to the α_1-adrenergic receptors (360 fmol/mg of protein) that are labeled with [^{3}H]WB-4101, a much larger population (2700 fmol/mg of protein) is also labeled by this ligand with about 1/100th the affinity. The Ca^{2+} antagonists D600 and verapamil displaced [^{3}H]WB-4101 effectively from both α-adrenergic receptor sites and the lower affinity sites. YC-93, an analog of nifedipine that differs structurally from D600, also competes at both sites labeled with [^{3}H]WB-4101. These results suggest that (*i*) WB-4101 binds to Ca^{2+} channels in addition to α_1-adrenergic receptors and (*ii*) the Ca^{2+} antagonists D600, verapamil, and YC-93 bind to α_1-adrenergic receptors in addition to Ca^{2+} channels. Blockade of Ca^{2+} conductance by D600 and WB-4101 was clearly demonstrated by the depression in the amplitude and rate of rise of the Ca^{2+} spike in the clonal NG108-15 cells. However, at concentrations affecting Ca^{2+} entry, both agents also had marked effects on Na^+ and K^+ conductance. This raises the possibility that the depression of Ca^{2+} influx may not represent a specific blockade of Ca^{2+} channels but reflects instead general perturbations of membrane structure. Such a proposal was recently made by Fairhurst *et al.* (7) to explain the multiple actions of the organic Ca^{2+} inhibitors applied at high concentrations.

While a nonspecific mechanism for channel blockade by D600 and WB-4101 is distinctly possible, the data can also be interpreted in terms of a more specific ligand action. First, D600 and WB-4101 displayed at least some selectivity in blocking the three principal ion channels, in the sequence $K^+ > Ca^{2+} > Na^+$. Moreover, the selectivity between Ca^{2+} and Na^+ may be considerably greater than actually observed. This is based on findings that slow Ca^{2+} spikes, but not fast Na^+ spikes, are enhanced by K^+ blockade (26). Consequently, the inhibition of K^+ conductance by D600 and WB-4101 would be expected to impair their ability to block the Ca^{2+} spike and lead to a lower apparent affinity of the ligands for the Ca^{2+} channel.

Second, the inhibition of the Ca^{2+} spike by WB-4101 was antagonized by an increase in the external Ca^{2+} concentration, which may indicate that WB-4101 and Ca^{2+} act competitively at the same or adjacent sites. These results do not, of course, rule out a nonspecific mechanism for WB-4101, because elevated Ca^{2+} concentrations are known to antagonize the effect of local anesthetics (27). If the WB-4101 action on channels is nonspecific, increases in the external Ca^{2+} concentration should also antagonize the drug-induced blockade of K^+ conductance. Although some alleviation in the blockade of K^+ conductance was detected (see Fig. 5), the interpretation of this finding is complicated by the existence of a Ca^{2+}-dependent K^+ conductance in neuroblastoma cells (22). As a result, it is not clear whether the alleviation of K^+ blockade represents an effect on K^+ channels or whether such changes are mediated secondarily by increases in Ca^{2+} concentration.

Third, the binding capacities of the low-affinity WB-4101 sites are in the same range as the Na^+ channel densities determined on rat brain synaptosomal preparations by using photoaffinity labels based on scorpion toxin (28) or on [^{3}H]TTX (29). Thus the WB-4101 site density is in a range appropriate for ion channels.

One of the major difficulties in characterizing the low-affinity WB-4101 binding sites is the low potency and poor selectivity of the available Ca^{2+} inhibitors on the systems employed. A lack of specificity of these agents on synaptosome preparations was recently reported by Nachshen and Blaustein (24). The relative ineffectiveness of the organic Ca^{2+} inhibitors on neuronal systems indicates a fundamental difference between these Ca^{2+} channels and those found in smooth and cardiac muscle. Preliminary electrophysiological data on the NBr10A cell line (which resembles the NG108-15 cell line in its electrical properties) indicate that the Ca^{2+} inhibitor YC-93 is more selective and approximately one order of magnitude more potent than D600, whereas nifedipine is similar to D600 in its potency and selectivity. Perhaps structural modifications of WB-4101 or YC-93 may lead to the development of more selective neuronal Ca^{2+} antagonists.

We thank Drs. Alex Levitzki and Marshall Nirenberg for helpful discussions and Dr. John W. Daly for support and encouragement. D.A. was a Fogarty International Research Fellow.

1. Fleckenstein, A. (1977) *Annu. Rev. Pharmacol. Toxicol.* **17**, 149–166.
2. Rosenberger, L. & Triggle, D. J. (1978) *Calcium in Drug Action*, ed. Weiss, G. B. (Plenum, New York), pp. 3–31.
3. Ritchie, J. M., Rogart, R. B. & Strichartz, G. R. (1976) *J. Physiol. (London)* **261**, 477–494.
4. Ray, R., Morrow, C. S. & Catterall, W. A. (1978) *J. Biol. Chem.* **253**, 7307–7313.
5. Kass, R. S. & Tsien, R. W. (1975) *J. Gen. Physiol.* **66**, 169–192.
6. McGee, R., Jr. & Schneider, J. E. (1979) *Mol. Pharmacol.* **16**, 877–885.
7. Fairhurst, A. S., Whittaker, M. L. & Ehlert, F. J. (1980) *Biochem. Pharmacol.* **29**, 155–162.
8. Atlas, D. & Sabol, S. L., *Eur. J. Biochem.*, in press.
9. Sabol, S. L. & Nirenberg, M. (1979) *J. Biol. Chem.* **254**, 1913–1920.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
11. Peroutka, S. J., Greenberg, D. A., U'Prichard, D. C. & Snyder, S. H. (1978) *Mol. Pharmacol.* **14**, 403–412.
12. Rodbard, D., Munson, P. J. & DeLean, A. (1977) in *International Symposium on Radioimmunoassay and Related Procedures in Medicine*, (International Atomic Energy Commission, Vienna, Austria), pp. 469–504.
13. Kapur, H. & Mottram, D. R. (1978) *Biochem. Pharmacol.* **27**, 1879–1880.
14. Guicheney, P., Garay, R. P., Levy-Marchal, C. & Meyer, P. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 6285–6289.
15. Rehavi, M., Yavetz, B., Ramot, O. & Sokolovsky, M. (1980) *Life Sci.*, **26**, 615–621.
16. Kapur, H., Rouot, B. & Snyder, S. H. (1979) *Eur. J. Pharmacol.* **57**, 317–328.
17. Fleckenstein, A. (1975) *New Therapy of Ischemic Heart Disease*, ed. Lochner, W. (Springer, Berlin), pp. 56–65.
18. Langer, S. Z. (1974) *Biochem. Pharmacol.* **23**, 1793–1800.
19. Berthelsen, S. & Pettinger, W. A. (1977) *Life Sci.* **21**, 595–606.
20. Starke, K. & Langer, S. Z. (1979) in *Presynaptic Receptors*, eds. Langer, S. Z., Starke, K. & Dubocovitch, M. I. (Pergamon, Oxford), pp. 1–33.
21. Hoffman, B. B. & Lefkowitz, R. J. (1980) *Biochem. Pharmacol.* **29**, 452–454.
22. Moolenaar, W. H. & Spector, I. (1979) *J. Physiol.* **292**, 297–306.
23. Reiser, G., Heumann, R., Kemper, W., Lautenschlager, E. & Hamprecht, B. (1977) *Brain Res.* **130**, 495–504.
24. Nachshen, D. A. & Blaustein, M. P. (1979) *Mol. Pharmacol.* **16**, 579–586.
25. Galper, J. B. & Catterall, W. A. (1979) *Mol. Pharmacol.* **15**, 174–178.
26. Hagiwara, S. (1973) *Adv. Biophys.* **4**, 71–102.
27. Blaustein, M. P. & Goldman, D. E. (1966) *J. Gen. Physiol.* **49**, 1043–1063.
28. Beneski, D. A. & Catterall, W. A. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 639–643.
29. Agnew, W. A., Moore, A. C., Levinson, S. R. & Raftery, M. A. (1980) *Biochem. Biophys. Res. Commun.* **92**, 860–866.