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SHORT COMMUNICATION

Inhibition of Sodium Channels by D600

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SUMMARY

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D600, an inhibitor of the slow inward Ca^{++}/Na^{+} current during the plateau phase of the cardiac action potential, also inhibits veratridine-activated fast sodium channels in both cultured heart cells and cultured neuroblastoma cells. The inhibition is competitive with respect to veratridine and is antagonized by Ca^{++}. The K_I for D600 in the presence of 1.8 mM Ca^{++} is approximately 3 μM.

INTRODUCTION

Verapamil and its methoxy derivative D600 reduce myocardial contractility by inhibiting the slow inward current carried by Ca^{++} and Na^{+} ions during the plateau phase of the cardiac action potential (1, 2). At concentrations of D600 lower than 1 μM, the drug has little effect on the rapidly activated inward current carried by Na^{+} ions during the rising phase of the cardiac action potential. At higher concentrations, however, reduction of the rate of rise of the cardiac action potential is observed (3). These two effects of D600 may be due to different activities of the two enantiomers present in the racemic mixtures used in most experiments (4). Since the inward current during the initial phase of the cardiac action potential is due to rapid activation of tetrodotoxin-sensitive Na^{+} channels, the reduction in rate of rise of the cardiac action potential by D600 may reflect an effect of D600 on the tetrodotoxin sensitive Na^{+} channels. These ion channels in heart are similar pharmacologically and electrophysiologically to Na^{+} channels responsible for action potential generation in nerve.

Veratridine and related drugs and toxins cause persistent activation of Na^{+} channels in electrically excitable cells (5-7). Considerable evidence indicates that the ion channels activated by these drugs and toxins are identical to those activated by membrane depolarization in nerve. Both are inhibited by low concentrations of tetrodotoxin (5-7). Variant neuroblastoma cells that lack Na^{+} channels are unresponsive to veratridine and related drugs (6). In these experiments, we have analyzed the inhibition of veratridine-activated Na^{+} channels by Ca^{++} and D600 in heart cells and neuroblastoma cells in cell culture.

Chemicals were obtained from the following sources: $^{22}NaCl$ and [4,5 ^{3}H]leucine from New England Nuclear; ouabain from Sigma Chemical Company; veratridine from Aldrich; and tetrodotoxin and N-2 hydroxyethyl piperazine, N'-2 ethanesulfonic acid (HEPES) from Calbiochem; fetal calf serum and horse serum from Colorado Serum Co.; 1X crystalline trypsin from Worthington Biochemical; and medium M-199 from Microbiological Associates. D600

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was a gift of Knoll Pharmaceutical, Ludwigshafen an Rhein, Federal Republic of Germany. Embryonated leghorn chicken eggs were obtained from Truslow Farms, Chestertown, MD.

The growth medium for all cell cultures consisted of medium M-199 supplemented with 4% fetal calf serum and 2% heat-inactivated horse serum. Horse serum was heat inactivated by incubation for 30 min at 57°.

Heart cell cultures were prepared by a modification of the method of DeHaan (8). Hearts were removed, placed in Ca^{++} and Mg^{++} free Puck's Saline G, dissected free of major blood vessels, cut open, and rinsed with Ca^{++} and Mg^{++} free Saline G to remove blood. The hearts were then minced with fine scissors and incubated with 0.025% (w/v) trypsin in Ca^{++} and Mg^{++} free Saline G in a rotary shaking water bath at 37° for 10 min. The supernatant, obtained after allowing the pieces of tissue to settle, was removed, diluted into M-199 containing 10% heat inactivated horse serum, and saved at room temperature. Four successive trypsin treatments were carried out. The first supernatant contained mainly fibroblasts and was discarded. The subsequent three supernatants were combined and the cells were sedimented at 1000 rpm in a desk top centrifuge for 8 min. The cells were resuspended in growth medium and incubated for 45 min at 37° in a humidified atmosphere of 5% CO_2/95% air. During this incubation, nearly 95% of the fibroblasts in the suspension adhere to the dish. The heart cells are then transferred to culture vessels.

Cells were seeded at a density of 1×10^5 cells/cm^2 in collagen-coated multiwell plates (1.6 cm diameter, Linbro Chemical Co.). On the third day of incubation, the medium was changed and on day 5 or 6 the medium was replaced with growth medium containing 0.2 μCi/ml [^{3}H]leucine. The cultures were used on day 6 or day 7.

Cultures of neuroblastoma clone N18 were prepared as previously described (6).

Measurements of $^{22}Na^+$ uptake were carried out as described by Catterall and Nirenberg (1973). Cultures in multiwell plates were placed in a 37° water bath. $^{22}Na^+$ uptake was initiated by addition of uptake medium consisting of 50 mM HEPES (adjusted to pH 7.4 with NaOH), 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose, 1.0 mM NaH_2PO_4 (adjusted to pH 7.4 with NaOH), 10 μCi/ml ^{22}NaCl, and the effectors noted in the figure legends. Uptake was terminated by removing the radioactive uptake medium and washing 3 times in 20 sec at room temperature with wash medium consisting of 164 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.0 mM NaH_2PO_4 (adjusted to pH 7.4 with NaOH). Cells were suspended in 0.4 N NaOH and the radioactivity was determined by liquid scintillation counting. Under these conditions, $^{22}Na^+$ uptake remains linear for 5 min in the presence and absence of veratridine. All values reported are initial rates derived from measurements after 2 min of incubation.

Previous experiments have shown that veratridine activates Na^+ channels in cultured heart cells and that the increased Na^+ permeability is inhibited by low concentrations of tetrodotoxin (9). In nerve and neuroblastoma cells, the activation of Na^+ channels is also inhibited competitively by Ca^{++} and other divalent cations (10). In order to study the effect of Ca^{++} on activation of Na^+ channels in heart cells by veratridine, titration experiments were carried out at increasing veratridine concentrations in the presence of 1, 3 or 5 mM Ca^{++}. The results, presented as a double reciprocal plot in Figure 1A, show that Ca^{++} is a competitive inhibitor of activation by veratridine. A secondary plot of the apparent activation constants ($K_{0.5}$) for veratridine at different Ca^{++} concentrations (Fig. 1B) is linear, suggesting that binding of Ca^{++} excludes binding of veratridine in this concentration range. The K_I for Ca^{++} is 0.19 mM.

D600 inhibits beating of cultured heart cells at concentrations ranging from 30 nM to 1 μM depending upon the embryonic age of the hearts from which the cultures were prepared (9, 11). This inhibition is due to block of slow Ca^{++}/Na^+ channels required for beating (9). Higher concentrations of D600 inhibit veratridine-activated Na^+ channels (Fig. 2). Half-maximal inhibition is observed at 30 μM. Double reciprocal analysis of veratridine titration curves carried out at different fixed D600 concentra-

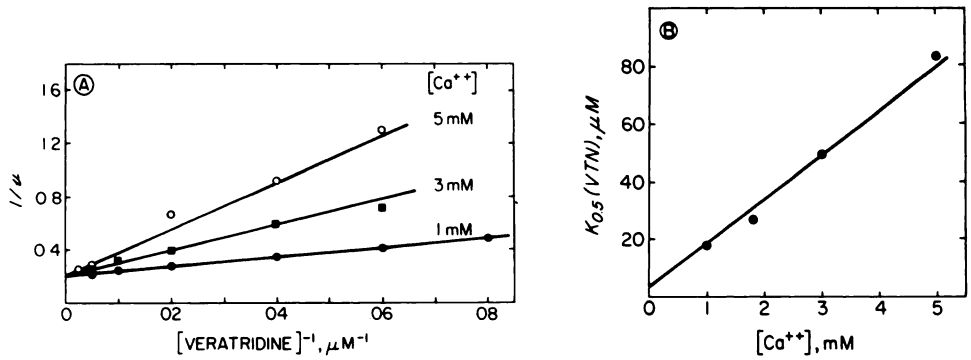

FIG. 1. *Competitive inhibition of veratridine-activated sodium channels by Ca^{++}*

A. $^{22}Na^+$ uptake was measured as described in the presence of the indicated concentrations of veratridine and 1, 3, or 5 mM Ca^{++}. Velocity (v) is presented in nmol Na^+/min per cell culture. B. Values for $K_{0.5}$ for veratridine activation determined in experiments like the one illustrated in panel A are plotted vs Ca^{++} concentration.

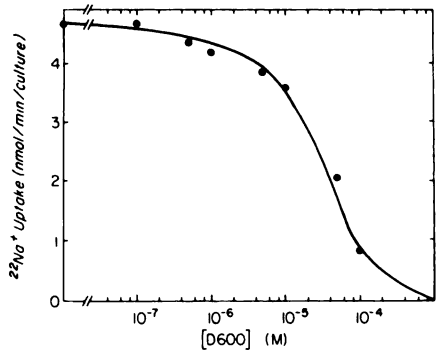

FIG. 2. *Inhibition of veratridine-activated sodium channels by D600*

$^{22}Na^+$ uptake was measured in the presence of 500 μM veratridine and the concentrations of D600 indicated on the abscissa. The rate of uptake by untreated control cultures has been subtracted.

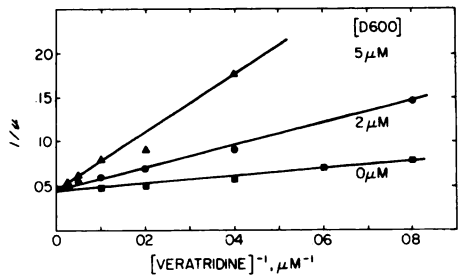

FIG. 3. *Competitive inhibition of veratridine-activated sodium channels by D600*

$^{22}Na^+$ uptake was measured in the presence of the indicated concentrations of veratridine and 0, 2, or 5 μM D600. Velocity (v) is presented in nmol Na^+/min per cell culture.

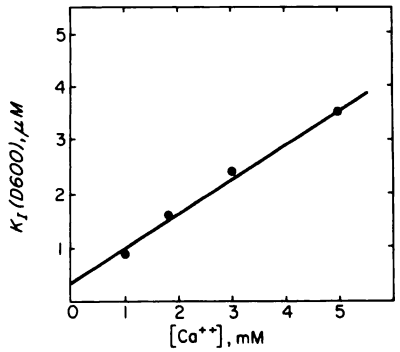

FIG. 4. *Competitive interaction between Ca^{++} and D600*

The K_I for D600 inhibition was measured in experiments like the one illustrated in Figure 3 at Ca^{++} concentrations of 1, 1.8, 3, and 5 mM Ca^{++}.

tions give competitive inhibition patterns (Fig. 3). The experiment of Figure 3 was carried out at 1.8 mM Ca^{++}. Similar experiments were performed at 1, 3, and 5 mMCa^{++} and the K_I for D600 was determined at each Ca^{++} concentration. These K_I values are plotted versus Ca^{++} concentration in Figure 4. These results show that the K_I for D600 depends linearly on Ca^{++} concentration consistent with the conclusion that Ca^{++}, D600, and veratridine act at a common receptor site associated with Na^+ channels in cultured heart cells. This conclusion is considered in more detail below.

In order to determine whether the effect of D600 on Na^+ channels is unique to heart cells, we also studied neuroblastoma cells under identical conditions. As observed with heart cells, we find that high concentrations of D600 completely inhibit veratridine-activated Na^+ channels. Half maxi-

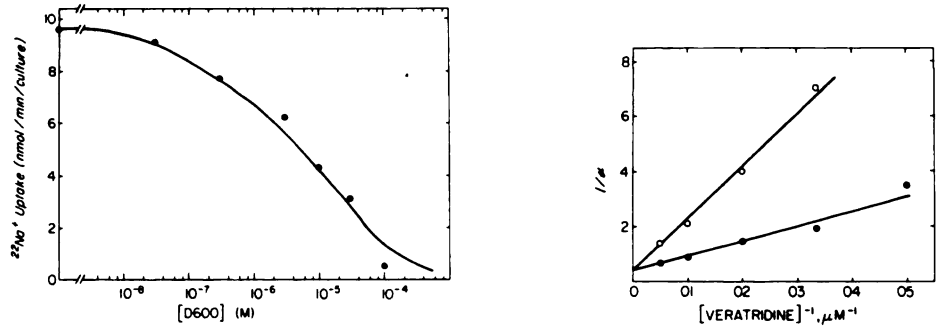

FIG. 5. *Inhibition of veratridine-activated sodium channels in neuroblastoma cells by D600*
A. $^{22}Na^+$ uptake was measured in the presence of 200 μM veratridine and the concentrations of D600 indicated on the abscissa. The rate of uptake by untreated control cultures has been subtracted. B. $^{22}Na^+$ uptake was measured in the presence of the indicated concentrations of veratridine and 0 μM D600 (●) or 5 μM D600 (○). Velocity (v) is presented in nmol Na^+/min per culture.

mal inhibition was obtained at 8 μM D600 (Fig. 5A). Double reciprocal analysis of veratridine titration curves showed that the inhibition was competitive with a K_I of 3 μM (Fig. 5B).

The data presented here are consistent with a linear competitive binding interaction among veratridine, Ca^{++}, and D600. This result suggests that binding of Ca^{++} or D600 excludes binding of veratridine over the concentration ranges studied. Our results indicate that the site(s) of action of these agents is associated with the rapidly activated, tetrodotoxin-sensitive Na^+ channels present in heart cells. Therefore, we conclude that D600, at higher concentrations than required to inhibit the slow inward current during the plateau of the cardiac action potential, will bind to sites associated with rapidly activated Na^+ channels and inhibit their activation by veratridine. The reduction in rate of rise of the cardiac action potential by D600 (3) may be caused by this interaction with Na^+ channels.

Although our results demonstrate competitive binding of veratridine Ca^{++}, and D600 over the concentration range studied, they do not distinguish whether this interaction represents simple competition for a common binding site or competitive allosteric interactions among multiple binding sites. Veratridine interacts with a specific receptor site associated with sodium channels and allosterically activates them by binding with high affinity to the active state of the ion channel (12). The competitive inhibition by Ca^{++} and D600 could be caused by direct interaction of Ca^{++} and D600 with the veratridine receptor site, by interaction of Ca^{++} and D600 with a separate site which reduces the affinity for veratridine, or by interaction of Ca^{++} and D600 with a separate site which increases the energy required to activate the sodium channel (12).

Both the inhibition of rapidly activated sodium channels by D600 and the inhibition of slow Ca^{++}/Na^+ channels by D600 are antagonized by Ca^{++}. It seems likely that each of these two different ion channels has a site sensitive to Ca^{++} and D600. These sites may reflect common structural features between the two different types of ion channels. In any case, it seems clear that D600 can inhibit both slow and fast sodium channels at concentrations greater than 1 μM and therefore that experiments using these relatively high concentrations of D600 should be interpreted carefully.

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