Calcium channel activation: A different type of drug action

(calcium flux/neuroblastoma/dihydropyridines)

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ABSTRACT Depolarization of NG108-15 (neuroblastoma-glioma) cells causes an increase in $^{45}\text{Ca}^{2+}$ influx. This effect is blocked by low concentrations of dihydropyridines such as nitrendipine and by other blockers of voltage-sensitive calcium channels such as D-600, diltiazem, and Cd^{2+} . Two other dihydropyridines, BAY K8644 and CGP 28392, have the opposite effect. Low concentrations of these compounds enhance depolarization-induced $^{45}\text{Ca}^{2+}$ influxes. BAY K8644 is more effective than CGP 28392. Both agents have no effect on fluxes measured under nondepolarizing conditions. The effects of BAY K8644 and CGP 28392 can be inhibited by nitrendipine, D-600, diltiazem, or Cd^{2+} . Whereas the interaction between nitrendipine and BAY K8644 is shown to be competitive in nature, that between BAY K8644 and D-600 is shown to be noncompetitive. These results indicate that dihydropyridines show a variety of effects on calcium channels, ranging from agonistic through partially agonistic to antagonistic. Moreover, the results also indicate that dihydropyridines and D-600 exert their effects on calcium channels at different sites.

The last few years have seen the development of a group of drugs that appear to block the flow of Ca^{2+} through voltage-sensitive calcium channels (VSCC) (1-4). For example, drugs such as verapamil, diltiazem, and the dihydropyridine nifedipine are now widely used in the treatment of angina, cardiac arrhythmias, and hypertension. Elucidating the molecular basis of the action of such agents is clearly a subject of great interest at present. Calcium channel blocking drugs fall into a variety of chemical types (1, 4). It is quite possible that different drug classes block VSCC by different mechanisms (5-11). Recently, a dihydropyridine (BAY K8644) has been reported that causes smooth muscle to contract and stimulates cardiac contractility (12). It was suggested that this agent might act by opening VSCC rather than blocking them. We now demonstrate VSCC activation by this compound and show that this property is shared by another dihydropyridine, CGP 28392.

METHODS AND MATERIALS

Cells were grown as monolayers under 5% CO_2 in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and 2 mM glutamine.

Cells were subcultured onto 60-mm tissue culture plates and allowed to grow for 3 days. To induce cellular differentiation (13), growth media was supplemented with 10 μM prostaglandin E_1 and 50 μM 3-isobutyl-1-methylxanthine. In parallel experiments, other workers in our laboratory have shown that this treatment will increase intracellular cyclic AMP levels by 10- to 100-fold (unpublished observations). Cells were examined visually for morphological differentiation and were assayed for net $^{45}\text{Ca}^{2+}$ uptake at various time points after treatment as indicated in the figure legends. Sup-

plemented medium was replaced every 2 days or when required.

$^{45}\text{Ca}^{2+}$ Uptake Studies. Cells (passage 5-20) were subcultured onto 60-mm tissue culture plates and induced to differentiate as above. During the assay, tissue culture plates were supported in an open-air waterbath at 37°C. Cells were incubated for 5 min at 37°C before the assay. Details of $^{45}\text{Ca}^{2+}$ flux studies are given in the individual figure legends. The uptake of $^{45}\text{Ca}^{2+}$ was measured for increasing periods of time in Hepes (20 mM) buffered Eagle's minimal essential medium containing 135.7 mM NaCl, 5 mM KCl, 0.44 mM KH_2PO_4 , 0.34 mM Na_2HPO_4 , 2.62 mM NaHCO_3 , 1.3 mM CaCl_2 , 0.81 mM MgSO_4 , and 5.6 mM glucose. Buffer with 50 mM KCl was the same except that NaCl was 90.7 mM. $^{45}\text{Ca}^{2+}$ was added to give a final concentration of 1 $\mu\text{Ci}/\text{ml}$ (1 Ci = 37 GBq). At the indicated times, plates were aspirated, washed immediately, and inverted to allow to dry. Cells were solubilized with 5 ml of 0.2% sodium dodecyl sulfate and samples were removed for estimation of $^{45}\text{Ca}^{2+}$ uptake and protein content by fluorescence (14).

Materials. $^{45}\text{Ca}^{2+}$ (10-40 mCi/mg of Ca^{2+}) was obtained from Amersham. Nitrendipine and BAY K8644 were from Miles Laboratories (New Haven, CT), D-600 from Knoll (Ludwigshafen, F.R.G.), diltiazem from Marion Laboratories (Kansas City, MO), and CGP 28392 from CIBA-Geigy (Summit, NJ).

RESULTS AND DISCUSSION

We have shown that several clonal cell lines express VSCC that are similar to those found in smooth muscle in that they are blocked by low concentrations of dihydropyridines (13, 15). This is the case, for example, with the neuroblastoma-glioma cell line NG108-15. As can be seen in Fig. 1A, when these cells are subjected to depolarization by 50 mM K^+ they show a rapid increase in the net uptake of $^{45}\text{Ca}^{2+}$. Other studies have established that this influx is through VSCC (13, 15). For example, it is not blocked by tetrodotoxin and is independent of the presence of external Na^+ . Moreover, the depolarization-induced influx is potently inhibited by dihydropyridines such as nitrendipine at nanomolar concentrations (nitrendipine IC_{50} = 6.5 nM). Other organic calcium channel blockers are also effective, as are inorganic channel blockers such as Cd^{2+} and Co^{2+} .

In Fig. 1A, it can be seen that addition of BAY K8644 (structure in Fig. 2) to NG108-15 cells under nondepolarizing conditions (5 mM K^+) produces little effect on $^{45}\text{Ca}^{2+}$ uptake. A small increase in uptake is observed. A quite different effect is seen in the presence of 50 mM K^+ . Fig. 1B shows that, in the presence of 50 mM K^+ , $^{45}\text{Ca}^{2+}$ uptake is stimulated as discussed above. The simultaneous addition of BAY K8644 dramatically enhances the effect of 50 mM K^+ .

Abbreviation: VSCC, voltage-sensitive calcium channel.

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FIG. 1. Time courses of net $^{45}\text{Ca}^{2+}$ uptake into NG108-15 cells. Cells were grown as monolayers under 10% CO_2 in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and 2 mM glutamine. NG108-15 cells (500,000 cells per plate) were subcultured onto 60-mm tissue culture plates and allowed to grow for 3 days. To induce cellular differentiation, growth medium was supplemented with 10 μM prostaglandin E_1 and 50 μM 3-isobutyl-1-methylxanthine. Supplemented medium was replaced every 2 days or when required. During the assay of $^{45}\text{Ca}^{2+}$ uptake tissue culture plates were supported in an open air waterbath at 37°C. Cells were incubated for 5 min at 37°C before the assay. The uptake of $^{45}\text{Ca}^{2+}$ was measured for increasing periods of time in Hepes (20 mM) buffered Eagle's minimal essential medium. Buffer with 50 mM KCl was prepared by adjusting Na^+ reciprocally. $^{45}\text{Ca}^{2+}$ was added at a final concentration of 1 $\mu\text{Ci}/\text{ml}$. At the indicated times, plates were aspirated, washed immediately four times in 175 mM choline chloride/2 mM EGTA, and inverted to dry. Cells were solubilized with 5 ml of 0.2% sodium dodecyl sulfate and samples were removed for estimation of $^{45}\text{Ca}^{2+}$ uptake and protein content by fluorescence (14). Each point represents single experimental observations performed in duplicate. Each curve is a typical experimental result that has been obtained on at least three separate occasions. (A) Effect of 1 μM BAY K8644 under nondepolarizing conditions. Δ , 5 mM K^+ ; $\circ$, 5 mM K^+ + 1 μM BAY K8644; $\bullet$, 50 mM K^+ . (B) Effect of 1 μM BAY K8644 under depolarizing conditions. Δ , 5 mM K^+ ; $\bullet$, 50 mM K^+ ; $\blacksquare$, 50 mM K^+ and 1 μM BAY K8644.

FIG. 2. Structures of dihydropyridines.

FIG. 3. (A) Dose-response curves for the enhancing effects of BAY K8644 ($\circ$) and CGP 28392 ($\bullet$) upon high K^+ (50 mM KCl) stimulated $^{45}\text{Ca}^{2+}$ uptake into NG108-15 cells. Uptake was measured for 10 min (see Fig. 1). Results are expressed as percent maximal response for each drug. Results are mean $\pm$ SEM of triplicate determinations performed on at least three separate occasions. (B) Comparative effects of 1 μM BAY K8644 and CGP 28392 on net $^{45}\text{Ca}^{2+}$ uptake into NG108-15 cells. Results are the mean $\pm$ SEM of quadruplicate determinations. Conditions are as follows, from left to right: 5 mM KCl; 50 mM KCl; 50 mM KCl and 1 μM CGP 28392; 50 mM KCl and 1 μM BAY K8644.

In the presence of a maximally effective concentration of BAY K8644, the effect of 50 mM K^+ is enhanced approximately 4-fold. Another dihydropyridine, CGP 28392 [4-(2-difluoromethoxy)phenyl-1,4,7,7-tetrahydro-2-methyl-5-oxofuro-pyridine-3-carboxylic acid ethyl ester (Fig. 2)], has effects that are qualitatively the same as those of BAY K8644. It can be seen in Fig. 3A that both agents are very potent. Effects of BAY K8644 are apparent at concentrations as low as 3 nM. CGP 28392 is approximately an order of magnitude less potent than BAY K8644. Thus, these two dihydropyridines exert their effects over concentration ranges similar to those over which other dihydropyridines such as nifedipine block VSCC. Another interesting feature is illustrated in Fig. 3B. It can be seen that in addition to being more potent, BAY K8644 also produces larger effects than CGP 28392. Thus, a maximal concentration of CGP 28392 (1 μ M) is only about 60% as effective as a maximal concentration of BAY K8644. Similar effects of both agents are also seen in another cell line, NCB-20 (neuroblastoma-brain cell hybrid). We have established that this cell line also possesses VSCC that have properties similar to those in NG108-15 cells (13, 15). However, BAY K8644 had little ef-

fect on $^{45}Ca^{2+}$ uptake into 3T3 fibroblasts under nondepolarizing (5 mM K^+) or depolarizing (50 mM K^+) conditions.

As discussed above, both inorganic and organic VSCC blockers inhibit depolarization-induced $^{45}Ca^{2+}$ uptake into NG108-15 cells. This is also the case for the stimulation produced by both BAY K8644 and CGP 28392. This can be seen in Fig. 4. The effects of both drugs can be inhibited by nitrendipine, an "antagonist" dihydropyridine. Thus, nitrendipine blocks both the normal 50 mM K^+ -induced $^{45}Ca^{2+}$ uptake and the extra increment resulting from the presence of either BAY K8644 or CGP 28392. Fig. 5 shows that the inhibitory effect of nitrendipine on BAY K8644-induced stimulation is competitive in nature. Thus, as might be expected, both types of dihydropyridine appear to interact with the channel at the same site. The effects of BAY K8644 and CGP 28392 are also blocked by the organic VSCC blockers D-600 (methoxyverapamil) and diltiazem and also by the inorganic channel blocker Cd^{2+} (Fig. 4). Fig. 5 shows, however, that the blocking effects of D-600 are not of a simple competitive nature. This is in agreement with the suggestion of Schramm *et al.* (12). Clearly, therefore, dihydropyridines and D-600 interact with VSCC at different sites, a fact that has been predicted from biochemical labeling studies of VSCC utilizing [3H]nitrendipine (5-11).

It was observed (Fig. 3) that a maximal concentration of CGP 28392 produced less of an effect than BAY K8644. This

FIG. 4. Inhibition by calcium channel antagonists of $^{45}Ca^{2+}$ uptake into NG108-15 cells stimulated by 1 μ M BAY K8644 (A) and 1 μ M CGP 28392 (B). At the start of the incubation, 1 μ M BAY K8644 or 1 μ M CGP 28392 was added to high K^+ (50 mM KCl) buffer. The organic calcium channel antagonists, nitrendipine ($\bullet$), D-600 ($\circ$), and diltiazem ($\blacktriangle$), were added 5 min prior to the start of the incubation. Cadmium ($\blacksquare$) was added at the start of the incubation. Results are expressed as the percent inhibition of the maximal response produced by either 1 μ M BAY K8644 or 1 μ M CGP 28392. This dihydropyridine-stimulated uptake component was measured as the difference in $^{45}Ca^{2+}$ uptake over 10 min in HEPES-buffered Eagle's minimal essential medium containing either 5 or 50 mM KCl. The protocol was similar to that described in the legend to Fig. 3. Each point is the mean of triplicate determinations performed on at least three separate occasions. Bars indicate SEM.

FIG. 5. Mechanism of inhibition by nitrendipine (A) and D-600 (B) of the BAY K8644-stimulated $^{45}Ca^{2+}$ uptake into NG108-15 cells. BAY K8644 was added to high K^+ (50 mM KCl) uptake stimulating solutions at the concentrations indicated. $\bullet$, Controls with no antagonist present. (A) Nitrendipine, 3 nM ($\circ$) and 10 nM ($\blacktriangle$), and (B) D-600, 200 nM ($\circ$) and 500 nM ($\blacktriangle$), were added 5 min prior to the addition of BAY K8644. Results are expressed as the percent inhibition of the maximal response produced by 1 μ M BAY K8644. This 1 μ M BAY K8644 component was measured over a 10-min incubation as described in the legend to Fig. 1. Each point is the mean $\pm$ SEM of triplicate determinations.

suggests that CGP 28392 might act as a partial agonist. Indeed, CGP 28392 can inhibit the effects of BAY K8644. When 300 nM BAY K8644 and 1 μ M CGP 28392 are added together, the resulting enhancement of 50 mM K^+ -stimulated $^{45}Ca^{2+}$ uptake is 79% of that obtained with 300 nM BAY K8644 only.

Our results show that dihydropyridines can produce a range of effects on VSCC. Thus, compounds such as nitrendipine act as pure "antagonists," whereas those such as BAY K8644 act as "agonists." Other substances such as CGP 28392 have an intermediate action and may be considered partial agonists. In this respect, it is interesting to note at low concentrations nifedipine has been reported to have agonist effects (16).

It should be noted that in biochemical labeling studies, the total number of VSCC observed (B_{max}) appears to be dependent on the particular radioactive dihydropyridine utilized (17). It is possible that this is a result of differences in the "purity" of antagonist activity associated with different dihydropyridine structures.

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