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Short Communication

Effect of Benidipine on Depolarizing Stimulation-Induced Increase of Intracellular Calcium Concentration in Cultured Mouse Hippocampal Neurons

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ABSTRACT—We examined the effect of benidipine, a 1,4-dihydropyridine calcium channel blocker, on depolarizing stimulation-induced increases of intracellular calcium concentration ($[Ca^{2+}]_i$) in cultured mouse hippocampal neurons in comparison with those of nicardipine and nilvadipine. Benidipine (0.1–10 μM) inhibited the $[Ca^{2+}]_i$ increase compared with the no drug control response. This effect was stronger than those of nicardipine and nilvadipine. The inhibitory effect of benidipine lasted even after washing out the drug for 125 min, while those of nicardipine and nilvadipine disappeared more rapidly. This is the first report that demonstrates that benidipine inhibits the $[Ca^{2+}]_i$ increase in the neuron itself.

Keywords: Benidipine, Intracellular calcium concentration, Neuron

The high voltage-activated L-type Ca^{2+} channel is known to be blocked by the dihydropyridine derivatives (1). Benidipine is a type of dihydropyridine Ca^{2+} channel blocker (2, 3). In the vascular system, benidipine causes vasodilation in vertebral and coronary arteries in anesthetized dogs (4). This vasodilative effect of benidipine is more long-lasting and potent than those of the other dihydropyridine Ca^{2+} channel blockers in vivo and in vitro (5, 6). In the nervous system, it is known that benidipine improves neurological symptoms induced by cerebral ischemia in rats (7). However, it is unclear whether benidipine is effective in the neuron itself. In this study, we investigated the alteration of intracellular calcium concentration ($[Ca^{2+}]_i$) induced by depolarizing stimulation in cultured mouse hippocampal neurons to determine if benidipine directly protects the stimulated neurons. Furthermore, we compared the effect of benidipine with those of other dihydropyridine derivatives, nicardipine and nilvadipine.

Primary culture of hippocampal neurons was performed as previously described (8). Briefly, hippocampal tissues were dissected from the brains of day 16–18 embryonic mice. The cells were dispersed with 0.25% trypsin and 0.01% DNase I digestion and plated at a density of $1.8–2.0 \times 10^5$ cells/cm^2 on a poly-L-lysine-coated glass coverslip with a silicon rubber wall. The culture of hippocampal cells was maintained in serum-free Dulbecco's modified Eagle's medium supplemented with 1 mg/ml bovine serum albumin, 10 μg/ml insulin, 0.1 mg/ml transferrin, 0.1 nM L-thyroxine, 30 nM sodium selenite, 1 μg/ml aprotinin (Funakoshi, Tokyo) and 0.1 mg/ml streptomycin-100 U/ml penicillin (Gibco BRL, Life Technologies Inc., Rockville, MD, USA) (9). The cells were cultured at 37℃ in a humidified atmosphere containing 5% CO_2.

Intracellular Ca^{2+} concentration was measured by a modified procedure as described previously (8). The hippocampal neurons cultured for 5 days were used. The culture medium was replaced with a basal salt solution (composition: 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 5.5 mM D-glucose, 20 mM HEPES-NaOH, pH 7.3) containing 10 μM fura-2/acetoxymethyl ester (Dojindo Laboratories, Kumamoto), and the cells were kept for 60 min at 37℃. The neurons were then superfused continuously with the basal salt solution at 33–34℃, and drugs were added to the superfusion medium 10 min before and during the depolarizing stimulation (60 mM of potassium chloride and 10 μM of (±)BAY K 8644 (Research Biochemicals International, Natick, MA, USA)). The time course of fura-2 fluorescence changes in the neurons was monitored at excitation wavelengths of both 340 and 380 nm. The data were analyzed with an Argus 200 system (Hamamatsu Photonics, Hamamatsu) to calculate the ratio of fura-2 fluorescence value at 340

nm to that at 380 nm ($R_{340/380}$). Under our conditions, the relationship between Ca^{2+} concentration ($[Ca^{2+}]$) to $R_{340/380}$ was linear from about 50 nM to 250 nM of $[Ca^{2+}]$. Therefore, the increase in $R_{340/380}$ was used as an index of relative $[Ca^{2+}]_i$ increase to evaluate a drug's effect. Data were obtained from the cells in a total of 3 cultures that showed an increase in $R_{340/380}$ larger than 0.4 in response to the depolarizing stimulation; data were expressed as the mean±S.E.M. of the percent of the control (no drug), which was calculated using "C" and "D" values as shown in Fig. 1. In the recovery study, after the stimulated cells were washed with the superfusion medium without drug during a given period, the cells were restimulated and the fluorescence was measured as above. In the case of benidipine, the first restimulation of the cells was started after 45-min washing because the recovery from the blockade of $[Ca^{2+}]_i$ increase by benidipine was hardly observed in a 10-min washing period in the preliminary experiments.

Benidipine hydrochloride, nicardipine hydrochloride and nilvadipine were synthesized at the Pharmaceutical Research Institute of Kyowa Hakko Kogyo Co., Ltd. Dulbecco's modified Eagle's medium was from Nissui Pharmaceutical Co., Ltd. (Tokyo). Trypsin, DNase I, poly-L-lysine and other culture reagents were from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

The $R_{340/380}$ was elevated by the depolarizing stimulation in primary cultures of fetal mouse hippocampal neuron, and the elevation was blocked by 1 μM benidipine (Fig. 1). This effect of benidipine was compared with those of nicardipine and nilvadipine. All these drugs suppressed the depolarizing stimulation-induced elevation of the $R_{340/380}$. These inhibitory effects were concentration-dependent in a range from 10^{-7} to 10^{-5} M (Table 1). The decreasing rank order of the inhibitory effect of drugs was benidipine > nicardipine > nilvadipine.

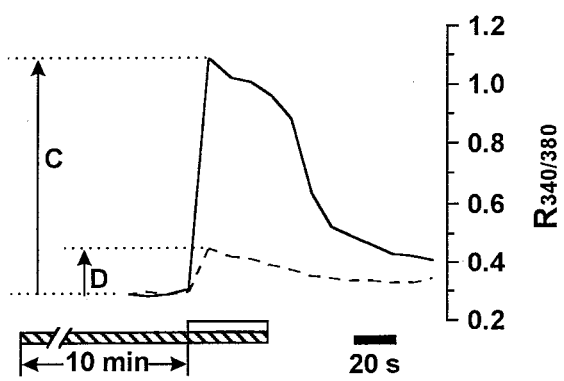

Fig. 1. Typical time course of $R_{340/380}$ change obtained from a single mouse hippocampal cultured neuron that was stimulated with 60 mM potassium chloride and 10 μM (±)BAY K 8644 (depolarizing stimulation, denoted as dep. stim.) in the absence (solid line) or presence (dashed line) of 1 μM benidipine. The drug was added 10 min before and during the dep. stim. The white and hatched bars indicate the duration of dep. stim. and administration of the drug (40 s), respectively. C (100%) and D denote the control response (without drug) and the response with the drug, respectively.

Table 1. Effects of Ca^{2+} channel blockers on the $R_{340/380}$ response in mouse hippocampal cultured neurons

	Benidipine	Nicardipine	Nilvadipine
Concentration (μM)			
0.1	56.3±1.9 (54)	90.1±1.6 (54)	92.8±1.6 (55)
1	16.3±0.8 (84)	59.8±1.1 (84)	67.9±1.0 (84)
10	3.5±0.1 (56)	9.1±0.4 (91)	39.9±1.5 (90)

Values are expressed as percent of the control±S.E.M. (number of neurons recorded).

In the recovery study, the depolarizing stimulation-induced elevations of the $R_{340/380}$, which had been suppressed by nicardipine and nilvadipine, recovered to approximately 80% of the control responses after a 10-min washing period (Fig. 2). In contrast, the blockade by benidipine of the depolarizing stimulation-induced elevation persisted even after 45-min and 125-min washing: the $R_{340/380}$ did not recover to more than 40% of the control response (Fig. 2). In repeated depolarizing stimulation

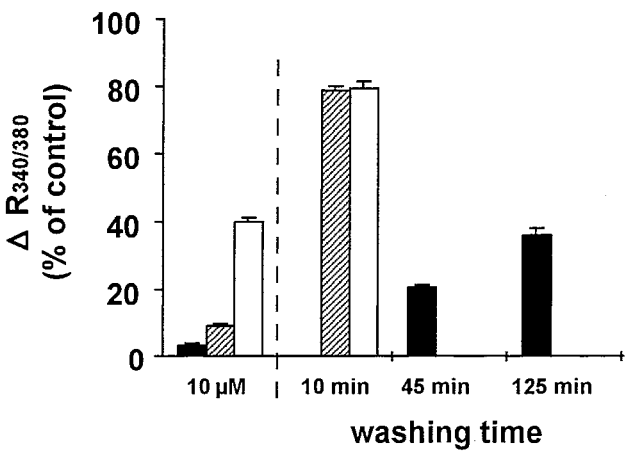

Fig. 2. Recovery from the blockade by Ca^{2+} channel blockers. After the measurement of the cellular response in the presence of Ca^{2+} channel blockers, the neurons were washed by continuous superfusion (without drugs) during the time stated in the figure: 10 min for nicardipine and nilvadipine, and 45 to 125 min for benidipine. After the wash, responses evoked by depolarizing stimulation were measured. Data of 45 and 125 min washing for benidipine were obtained from the same neurons. "10 μM" represented the same data as 10 μM in Table 1. Black, hatched and white columns indicate benidipine, nicardipine and nilvadipine, respectively. Each column and bar shows the mean and S.E.M. of recorded neurons, respectively. Cell numbers recorded were 56, 91 and 90 for benidipine, nicardipine and nilvadipine, respectively.

(total of 4 times), the 4th $[Ca^{2+}]_i$ increase by the stimulation was maintained to 97% of the first response (data not shown). Therefore, in this study, it is thought that the long-lasting effect of benidipine is scarcely influenced by repeated depolarizing stimulation-dependent attenuation of the $[Ca^{2+}]_i$ increase.

In this study, we observed that the dihydropyridine-type Ca^{2+} channel blockers, benidipine, nicardipine and nilvadipine, suppressed the $[Ca^{2+}]_i$ increase induced by the depolarizing stimulation in concentration-dependent manners in cultured mouse hippocampal neurons. Benidipine exhibited about a tenfold stronger inhibitory effect than the other two drugs since the potency of benidipine at a concentration of 0.1 μM was almost equal to or stronger than those of nicardipine and nilvadipine at 1 μM. This higher potency of benidipine compared with the other dihydropyridine Ca^{2+} channel blockers was also observed in the vascular system (2, 5). Thus it seems that in hippocampal neurons, there exists an L-type Ca^{2+} channel of the same type as that in the vascular system. Indeed, it is reported that the L-type Ca^{2+} channel is distributed in central neurons (10). It remains to be clarified how much the blockade of $[Ca^{2+}]_i$ increase by benidipine in neurons contributes to the suppression of ischemic damages.

We elucidated the difference of benidipine from the other two drugs by a recovery study. The effect of benidipine did last after washing much longer than those of the other two. Such pharmacological properties of benidipine may be in part derived from the slow dissociation rate of this drug. The dissociation rate of (+/−)[^{3}H]benidipine from the dihydropyridine receptor sites in rat heart membranes is 50 times slower than that of (+/−)[^{3}H]nitrendipine (11). Alternatively or additionally, the long-lasting effect of benidipine may be due to an additional interaction with Ca^{2+} channels or lipid membrane (12).

In conclusion, we found that the dihydropyridine-type Ca^{2+} channel blockers benidipine, nicardipine and nilvadipine blocked the increase of $[Ca^{2+}]_i$ induced by the depolarizing stimulation in cultured mouse hippocampal neurons. Among them, benidipine had the highest potency and showed the longest duration of action in blocking the $[Ca^{2+}]_i$ increase. This is the first report that demonstrates that benidipine inhibits the $[Ca^{2+}]_i$ increase in the neuron itself.

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