Dihydropyridines as potent calcium channel blockers in neuronal cells

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Received 10 October 1982; revision received 14 December 1982

Nicardipine, one of the dihydropyridine derivatives, in a nanomolar concentration range suppressed the high K^+-induced neurotransmitter release from cultured neuronal cells (chick embryonic neural retina cells and clonal rat pheochromocytoma cells). The high K^+-induced Ca^{2+} uptake into pheochromocytoma cell was also blocked by nicardipine in the same concentration range. [^{3}H]Nitrendipine, another dihydropyridine derivative, bound specifically to pheochromocytoma cell homogenate in a saturable manner. We concluded that dihydropyridines block and bind to the high K^+-sensitive Ca^{2+} channels in neuronal cells.

Neuronal Ca^{2+} channel *Dihydropyridines* *Transmitter release* *Ca^{2+} uptake* *Radioligand binding* *Molecular probe*

1. INTRODUCTION

Calcium ions play important roles in regulating various neuronal functions, among which are membrane excitation [1], neurotransmitter release [2] and protein phosphorylation [3,4]. Voltage-sensitive Ca^{2+} channels are one of the key factors determining the cellular Ca^{2+} level. The Ca^{2+} antagonists that block the voltage-sensitive Ca^{2+} channels of neuronal cells in a nanomolar concentration range are so far not known [5]. Those drugs are longed for not only in physiological studies to suppress the Ca^{2+} channel-mediated functions with minimum interference with other ion channels but in biochemical and morphological experiments to identify the Ca^{2+} channels.

We report here that 1,4-dihydropyridine derivatives, known as Ca^{2+} channel blockers in heart and smooth muscle tissues [6], block the high K^+-induced Ca^{2+} uptake into and transmitter release from the cultured neuronal cells in the nanomolar range. These blockades suggest that dihydropyridine inhibits the function of the neuronal voltage-sensitive Ca^{2+} channel. A radioactive derivative of dihydropyridine bound to the neuronal cell and thus may serve as a molecular probe for the neuronal Ca^{2+} channel.

Abbreviations: BSS, balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; GABA, γ-aminobutyric acid; Hepes, *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid; NE, norepinephrine; Tris, tris (hydroxymethyl) aminomethane

2. MATERIALS AND METHODS

Neural retina cells were isolated from 8-day chick embryos. After dissociation with trypsin, 2×10^6 cells were plated on poly-D-lysine-coated 35 mm plastic dish (Falcon). The culture was maintained for 6 days in DMEM containing 5% newborn calf serum. Rat pheochromocytoma clone PC12 cells [7] (subclone h [8] was used) were plated on poly-D-lysine-coated 35 mm plastic dish (10^6 cells/dish) and maintained for 2 days in DMEM containing 5% newborn calf serum and 5% heat-inactivated horse serum. Assays for high K^+-induced transmitter release and Ca^{2+} uptake were made as described in [9]. See figure legends for brief descriptions. The high K^+-stimulation

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

was performed by an elevation of external K^+-concentration. Hypertonicity was not compensated, since preliminary experiments employing the hypertonic high K^+ solution and isotonic high K^+ (NaCl-subtracted) solution did not show the difference in the amount and time-course of transmitter release. For the radioligand binding assay, PC12 cells were disrupted by sonication in 50 mM Tris–HCl (pH 7.3) buffer and the homogenate (0.47 mg protein) was incubated in the 2 ml buffer containing [^{3}H]nitrendipine for 75 min at 25°C. Binding was terminated by a rapid vacuum filtration over a Whatman GF/B filter followed by several rinses with ice-cold buffer. The filter was counted for radioactivity. Specific binding was defined as that displaced by coincubation with 10^{-6} M nicardipine. Preliminary experiments revealed that the binding was linear in the range of 0–0.5 mg protein. The binding saturated over 45 min.

All experiments with dihydropyridine derivatives were done under darkness or dimmed light. Protein was determined after Lowry et al. [10] with bovine serum albumin as the standard.

The following materials were obtained from the companies indicated: $^{45}CaCl_2$ (spec. act. 32 Ci/g Ca) and [^{3}H]GABA (57 Ci/mmol), Amersham; [^{3}H]nitrendipine (88 Ci/mmol) and [^{3}H]norepinephrine (46.5 Ci/mmol), New England Nuclear; Hepes and poly-D-lysine, Sigma; DMEM and horse serum, Gibco; precolostrum newborn calf serum, Mitsubishi Chemical Ind. Ltd., Tokyo; nicardipine [11], gift from Yamanouchi Pharmaceutical Co., Tokyo.

3. RESULTS AND DISCUSSION

The cultured chick embryonic neural retina cells released the preloaded [^{3}H]GABA by an elevation of external K^+ concentration from 5.4 to 51.4 mM as reported in [12]. As shown in fig.1, nicardipine markedly suppressed the high K^+-induced [^{3}H]GABA release in a dose-dependent manner. The threshold of the inhibition was 10^{-9} M and 60% of the release was suppressed at 10^{-7} M. Nicardipine did not affect to the basal release seen under low K^+ conditions.

To eliminate the difficulties in interpretation of experimental results due to the presence of non-neuronal cells and the heterogeneity among neurones in the cultured neural tissues, we used for further analyses a rat pheochromocytoma clone PC12, which shares many cellular properties with sympathetic neurones [7,8,13–15]. Figure 2 shows the inhibition of high K^+-induced [^{3}H]NE release from PC12 cells by nicardipine. The inhibition was dose-dependent and complete at 10^{-7} M. The dose of half inhibition was about 7×10^{-9} M.

In the presence of 1 mM $CaCl_2$, the high K^+-induced [^{3}H]NE release from PC12 cells was inhibited by 69% with 10^{-8} M nicardipine (% releases over 1 min were 3.6 and 5.2 with and without nicardipine, respectively). When $CaCl_2$

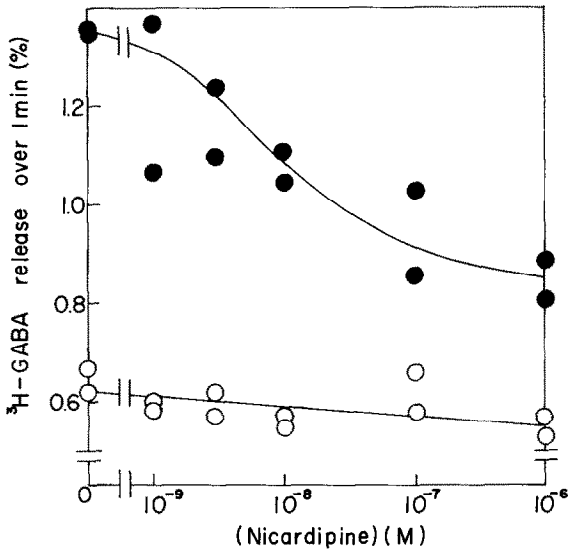

Fig.1. Dose-dependent inhibition of high K^+-induced [^{3}H]GABA release from cultured neural retina cells by nicardipine. The assay was made as follows: The cell was exposed for 1 h to the balanced salt solution (BSS, composed of 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose, 50 mM Hepes-NaOH pH 7.3) supplemented with [^{3}H]GABA (0.5 μCi/dish). After another incubation with BSS containing no [^{3}H]GABA for 1 h, BSS was replaced 5 times at 1 min intervals. At the 5th replacement, BSS was strengthened with KCl (51.4 mM in final conc.). Nicardipine was included at the 3rd replacement and thereafter. The release was expressed by a percentage of the radioactivity of the 5th replacement to that of the cells solubilized after the experiment (●). For the release under low KCl, the ratio of the 4th replacement to the cell lysate was plotted (○). The value plotted represents each one experimental point. The total [^{3}H]GABA uptaken in the cell prior to the release was 81021 ± 3646 cpm/dish.

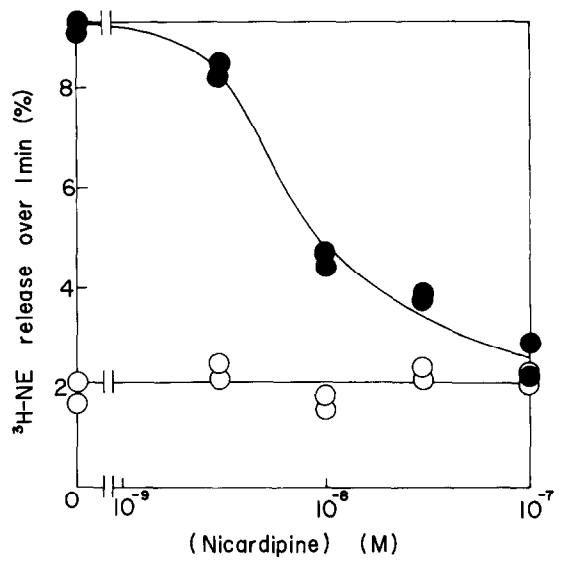

Fig.2. Dose-dependent inhibition of high K^+-induced [^{3}H]NE release from PC12 cells by nicardipine. The assay method was the same as in fig.1 but for [^{3}H]NE (0.25 μCi/dish) instead of [^{3}H]GABA. Ascorbic acid (1.2 mM) was added to BSS. Releases under 5.4 and 51.4 mM KCl were indicated by ○ and ●, respectively. The value plotted represents each one experimental point. The total [^{3}H]NE uptaken in the cell prior to release was 21430 ± 717 cpm/dish.

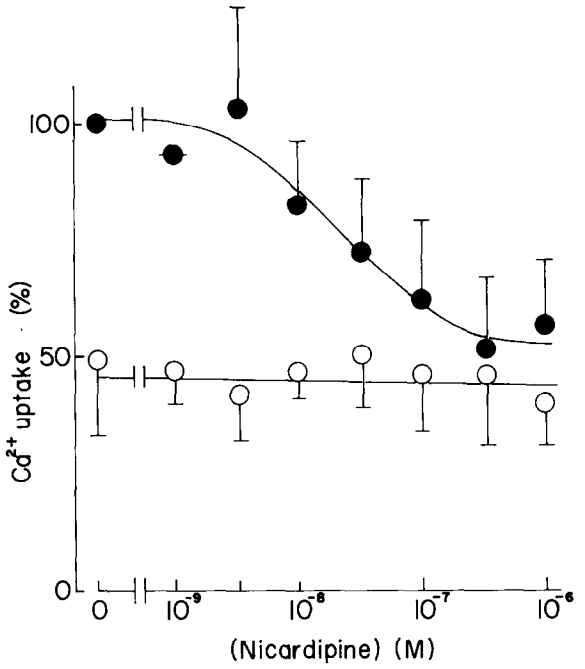

Fig.3. Dose-dependent inhibition of high K^+-induced Ca^{2+} uptake into PC12 cells by nicardipine. After replacement of the culture medium with BSS, the cells were exposed to BSS containing nicardipine of indicated concentration for 2 min. Then, the solution was replaced with BSS containing nicardipine. $^{45}CaCl_2$ (1 μCi/dish) and KCl (●, 51.4 mM; ○, 5.4 mM, in final conc.). After 30 s exposure to the ^{45}Ca-containing BSS, the cells were rinsed thoroughly four times with ice-cold BSS. Finally the cells were alkali-solubilized and radioactivity was counted. Preliminary experiments showed that the uptake increased linearly with time up to 40 s. Means ± SD from 4 successive experiments were shown after standardization. The mean value of Ca^{2+} uptake in 51.4 mM KCl without nicardipine (2.55 ± 0.68 nmol/mg protein/30 s) was taken as 100%.

was raised to 3 mM, the release was inhibited by 37% with 10^{-8} M nicardipine (% releases were 2.3 and 3.6 with and without nicardipine, respectively). Such antagonistic effect of external Ca^{2+} is common to the known Ca^{2+} channel blockers [6].

Exposure to high K^+ environment causes Ca^{2+} uptake into PC12 cells through direct activation of the voltage-sensitive Ca^{2+} channels [16]. Nicardipine inhibited the high K^+-induced Ca^{2+} uptake into PC12 cells in a dose-dependent manner (fig.3). The dose of half inhibition was about 2×10^{-8} M.

These results suggest that nicardipine blocked the function of the high K^+-sensitive (probably identical to the so-called voltage-sensitive) Ca^{2+} channel of neural cells. This was also indicated by an observation that [^{3}H]NE release induced by maitotoxin, a dinoflagellate toxin considered to directly activate the Ca^{2+} channels [9], was blocked by nicardipine (not shown here).

Recent reports showed that [^{3}H]nitrendipine, another dihydropyridine derivative, specifically bound to the membrane fractions from rat brain [17–20]. It is not clear whether those [^{3}H]nitrendipine bindings were of neuronal origin, since brain contains non-neuronal cells as glia and blood vessels. We found that [^{3}H]nitrendipine specifically bound to PC12 cell homogenate in a saturable manner (fig.4). Scatchard plot revealed the dissociation constant (K_D) of 1.5×10^{-10} M and the maximal binding capacity (B_{max}) of 6.5 fmol/mg protein. In comparison with brain homogenates (K_D, $0.9–1.6 \times 10^{-10}$ M and B_{max}, 4–15 fmol/mg wet tissue weight [17–20], PC12 cells had similar K_D and smaller B_{max}.

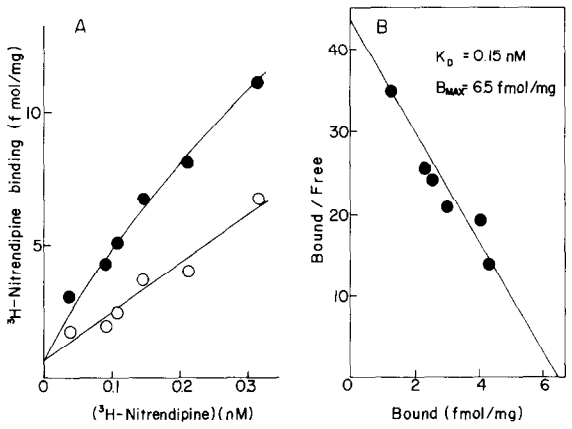

Fig.4. (A) Binding of [^{3}H]nitrendipine to PC12 cell homogenate as a function of concentration of added nitrendipine. Points were determined in triplicate and non-specific binding was determined in parallel samples which contained 10^{-6} M nicardipine (●, total binding; ○, non-specific binding). (B) Scatchard plot of specific [^{3}H]nitrendipine binding to PC12 cell homogenate based on data in A.

From these results, dihydropyridine derivatives can be useful chemical tools for the study of Ca^{2+} channel of neurones.

ACKNOWLEDGEMENTS

We are grateful to Dr H. Hatanaka of this institute for providing PC12h cells, to Dr M. Mitsuka for the culture of retina cells and to Dr T. Amano for encouragement.

NOTE ADDED IN PROOF

After submission of this manuscript, a report with conclusions qualitatively the same as ours was published [L. Toll. (1982) J. Biol. Chem. 257, 13189–13192].

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