Characterization of a novel, hydrophilic dihydropyridine, NKY-722, as a Ca^{2+} antagonist in bovine cultured adrenal chromaffin cells

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1 To characterize NKY-722, a novel hydrophilic dihydropyridine derivative, as a Ca^{2+} antagonist, we examined its effects on $^{45}\text{Ca}^{2+}$ influx, intracellular free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$), and release of noradrenaline and adrenaline in bovine cultured adrenal chromaffin cells.

2 NKY-722 had little effect on basal $^{45}\text{Ca}^{2+}$ influx into the resting cells, but inhibited high K^+ (35.9 mM)-evoked $^{45}\text{Ca}^{2+}$ influx in a concentration-dependent manner with an IC_{50} value of 5.2 nM.

3 NKY-722 inhibited high K^+ -evoked increases in $[\text{Ca}^{2+}]_i$ in a concentration-dependent manner without effect on the resting $[\text{Ca}^{2+}]_i$.

4 NKY-722 had little effect on basal release of noradrenaline and adrenaline but inhibited high K^+ -evoked release of noradrenaline and adrenaline in a concentration-dependent manner with IC_{50} values of 5.0 nM and 4.8 nM, respectively.

5 Nicardipine, a prototype of NKY-722, also inhibited high K^+ -evoked $^{45}\text{Ca}^{2+}$ influx and release of noradrenaline and adrenaline in a concentration-dependent manner: the IC_{50} value for high K^+ -evoked $^{45}\text{Ca}^{2+}$ influx was 51 nM, and the values for high K^+ -evoked release of noradrenaline and adrenaline were 52 nM and 50 nM, respectively.

6 These results show that NKY-722 is a hydrophilic Ca^{2+} antagonist ten times more potent than nicardipine.

Keywords: Adrenal chromaffin cells; Ca^{2+} antagonist; Ca^{2+} influx; intracellular free Ca^{2+} concentration; Ca^{2+} channel; catecholamine release; dihydropyridine

Introduction

Voltage-dependent Ca^{2+} channels play an important role in the cellular regulation of Ca^{2+} movement. Biophysical and pharmacological criteria define at least three types of voltage-dependent Ca^{2+} channels, designated L (long-lasting), N (neuronal) and T (transient) in excitable cells (Hofmann *et al.*, 1987). The best characterized of these is the L channel, the target for a number of drugs. The 1,4-dihydropyridines (DHPs), phenylalkylamines and benzothiazepines, represented by nifedipine, verapamil and diltiazem, respectively, are the best known groups of compounds. These classic Ca^{2+} antagonists are widely employed therapeutically in a number of cardiovascular disorders and experimentally as tools for characterization of voltage-dependent, L-type Ca^{2+} channels and their cloning (Tanabe *et al.*, 1987; Mikami *et al.*, 1989). However, because of their hydrophobic character, their routes of administration and hence, their usage seem to be limited. To overcome the problem of hydrophobicity, a highly hydrophilic compound has recently been synthesized using nicardipine, a DHP, as a prototype. This compound, 3-(4-allyl-1-piperazinyl)-2,2-dimethyl propyl methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate dihydrochloride (NKY-722), has been shown to produce a long-lasting decrease in systemic arterial blood pressure in conscious spontaneously hypertensive rats (Osumi *et al.*, 1988), to inhibit Ca^{2+} -induced contraction of dog isolated mesenteric arteries (Osumi *et al.*, 1988) and to increase coronary blood flow (Imagawa *et al.*, 1989). From these indirect studies and because NKY-722 is a derivative of a classic dihydropyridine Ca^{2+} antagonist nicardipine, NKY-722 is considered to be a Ca^{2+} antagonist. As it may be important to have a hydrophilic Ca^{2+} antagonist from clinical and experimental viewpoints, we attempted to define NKY-722 as a Ca^{2+} antagonist by examining its effects on $^{45}\text{Ca}^{2+}$ influx, intracellular free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$), and release

of noradrenaline and adrenaline in bovine cultured adrenal chromaffin cells.

Methods

Isolation and culture of bovine adrenal chromaffin cells

Bovine adrenal chromaffin cells were isolated, purified and cultured as described previously (Fenwick *et al.*, 1978; Waymire *et al.*, 1983; Lee *et al.*, 1990). In brief, chromaffin cells were isolated by the retrograde perfusion of bovine adrenal medullae with collagenase and deoxyribonuclease I (DNase I), and then purified from other types of cells by density gradient centrifugation on Percoll and by differential plating. To determine catecholamine release and $^{45}\text{Ca}^{2+}$ uptake, purified cells were plated as monolayers in poly-L-lysine-coated 24 well- and 6 well-cluster plates (Costar, Cambridge, MA, U.S.A.) at a density of 4×10^5 and 2×10^6 cells/well, respectively. For determination of $[\text{Ca}^{2+}]_i$, the cells were kept in suspension on 150 mm-diameter plastic dishes (Falcon 1058; Becton, Dickinson and Co., Cockeysville, MD, U.S.A.) at a density of $1.0\text{--}1.6 \times 10^7$ cells/dish. The culture medium used to maintain the purified cells was Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal calf serum, 10 mM HEPES, 20 mM NaHCO_3 , anti-mitotics (5-fluoro-2-deoxyuridine, 10^{-5} M; uridine, 10^{-5} M; and cytosine arabinoside, 10^{-5} M) to inhibit fibroblast growth, and antibiotics (penicillin G, 100 units ml^{-1} ; streptomycin, 100 ng ml^{-1} ; and nystatin, 25 units ml^{-1}). Cultures were maintained at 37°C in a humid atmosphere gassed with 5% CO_2 in air, and experiments were performed after 3 or 4 days of culture.

Measurement of $^{45}\text{Ca}^{2+}$ uptake

$^{45}\text{Ca}^{2+}$ uptake into cultured chromaffin cells was determined as described recently (Lee *et al.*, 1990). The cells were washed three times with Ca^{2+} -free Krebs-HEPES buffer (composition

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(mm): NaCl 140, KCl 4.7, MgCl₂ 1.2, KH₂PO₄ 1.2, glucose 11 and HEPES 15; adjusted to pH 7.4), and then preincubated in the same buffer at 37°C for 10 min. After preincubation, the cells were stimulated by incubating them in Ca²⁺-free high K⁺ (35.9 mM) Krebs-HEPES buffer (with an equimolar reduction in Na²⁺ concentration) containing 2 μ Ci of ⁴⁵CaCl₂ (specific activity, 10–40 mCi mg⁻¹ of calcium) at 37°C for various times (total ⁴⁵Ca²⁺ uptake). For determination of basal uptake of ⁴⁵Ca²⁺, the cells were incubated without stimulation in Ca²⁺-free Krebs-HEPES buffer containing 2 μ Ci of ⁴⁵CaCl₂. When the effects of Ca²⁺ antagonists were examined, the drugs were present during preincubation and incubation. At the end of each incubation period, the reaction was terminated by rapidly aspirating the ⁴⁵Ca²⁺-containing medium, and the cells were washed four times with 3 ml of ice-cold Ca²⁺-free Krebs-HEPES buffer. After washing, the cells were solubilized in 1 ml of 10% Triton X-100, and the ⁴⁵Ca²⁺ taken up into the cells was determined in a Packard Tri-Carb 4000 liquid scintillation counter. In the text, the data are shown for only high K⁺-evoked ⁴⁵Ca²⁺ uptake, which is the difference between total and basal uptake. ⁴⁵Ca²⁺ uptake was expressed in terms of the initial specific activity in the incubation medium.

Measurement of [Ca²⁺]_i

[Ca²⁺]_i was determined according to the method of Grynkiewicz *et al.* (1985) as modified by Lee *et al.* (1990) using a Ca²⁺ indicator fura-2. The cells, which had been kept in suspension, were washed twice with fura-2 loading buffer and collected by centrifugation. The composition of the fura-2 loading buffer was the same as that of Ca²⁺-free Krebs-HEPES buffer except that it contained 1 mM Ca²⁺. The cells were resuspended in the fura-2 loading buffer containing 3 μ M fura-2 acetoxymethylester at a density of 2 $\times$ 10⁶ cells ml⁻¹ and incubated at 37°C for 30 min for loading of fura-2. After the incubation, the cells were washed with bovine serum albumin/fura-2 loading buffer (fura-2 loading buffer containing 5 mg ml⁻¹ bovine serum albumin) to remove unloaded fura-2. The cells were resuspended in 2 ml of fresh fura-2 loading buffer and transferred to a quartz cuvette for measurement of fluorescence. Fluorescence measurements were made in a JASCO (Japan Spectroscopic Co., Ltd., Tokyo, Japan) Ca²⁺ analyzer (model CAF-100). Excitation wavelengths were set at 340 nm and 380 nm and an emission wavelength at 510 nm (Grynkiewicz *et al.*, 1985). [Ca²⁺]_i values were calculated from fura-2 fluorescence ratios (R) according to the following equation:

$$[\text{Ca}^{2+}]_i = K(R - R_{\min})/(R_{\max} - R)$$

where $R_{\min}$ and $R_{\max}$ are the ratios, e.g., 340 nm/380 nm, obtained at zero and saturating Ca²⁺ concentrations and K is the product $K_D(\text{Fo}/\text{Fs})$, where K_D is the effective dissociation constant (224 nm), Fo is the 380 nm excitation signal in the absence of Ca²⁺, and Fs is the 380 nm excitation signal at the saturating Ca²⁺ concentration (Grynkiewicz *et al.*, 1985). For determination of the saturating Ca²⁺ concentration, digitonin, a detergent, was added to the incubation medium at a final concentration of 20 μ M. For determination of the zero Ca²⁺ concentration, EGTA, a Ca²⁺ chelator together with 100 μ l of 1 M Tris-HCl (pH 8.6), was added to the incubation medium at a final concentration of 5 mM after treatment with digitonin.

Determination of catecholamine release

When catecholamine release from cultured chromaffin cells was measured, the culture medium was discarded, and the cells were washed three times with fresh Krebs-HEPES buffer (composition mm: NaCl 140, KCl 4.7, MgCl₂ 1.2, KH₂PO₄ 1.2, CaCl₂ 2.2, glucose 11 and HEPES 15; adjusted to pH 7.4). After the cells were preincubated in Krebs-HEPES buffer at 37°C for 10 min, the cells were stimulated by incubating them in 1 ml of high K⁺ Krebs-HEPES buffer at 37°C for 6 min, the

minimum time required for catecholamine release to reach a plateau (Lee *et al.*, 1990). The reaction was terminated by rapidly removing the incubation medium which was transferred to another set of test tubes containing 1 ml of 0.2 M perchloric acid. For extraction of catecholamine remaining in the cells, the cells were lysed by addition of 1 ml 0.4 M perchloric acid to each well, and scraped off. When the effects of Ca²⁺ antagonists were examined, the drugs were present during preincubation and incubation. Catecholamines, which were released into the medium and remained in the cells, were quantified by high performance liquid chromatography (h.p.l.c.) (Hitachi LC 655; Hitachi Co., Ltd., Tokyo, Japan) with electrochemical detection as described in detail elsewhere (Miwa *et al.*, 1986; Lee *et al.*, 1987). Results were expressed as percentage release of the total cellular catecholamine content.

Drugs

Chemicals were obtained from the following sources: ⁴⁵CaCl₂, from Amersham, Arlington Heights, IL, U.S.A.; DNase I, 5-fluoro-2-deoxy-uridine and poly-L-lysine bromide, from Sigma, St. Louis, MO, U.S.A.; penicillin G and streptomycin, from Meiji Seika, Tokyo, Japan; cytosine arabinoside, from Kohjin, Tokyo, Japan; Percoll, from Pharmacia, Uppsala, Sweden; foetal calf serum, nystatin suspension and Dulbecco's modified Eagle's medium, from GIBCO, Grand Island, NY, U.S.A.; fura-2 acetoxymethyl ester, HEPES and EGTA, from Dohjin Chemical Institute, Kumamoto, Japan; bovine serum albumin, collagenase, uridine, digitonin and Triton X-100, from Wako Pure Chemical Industries, Osaka, Japan. NKY-722 was a generous gift from Kyoto Pharmaceutical Industries, Kyoto, Japan.

Statistical analysis

All results are expressed as means $\pm$ s.e.mean. The data were subjected to a two-way analysis of variance, and when significant F values were encountered, Newman-Keuls' multiple-range test was used to test for significant differences between treatment means (Steel & Torrie, 1960). A probability level of $P < 0.05$ was considered to be statistically significant.

To estimate the IC₅₀ values for inhibition of high K⁺-evoked catecholamine release and ⁴⁵Ca²⁺ uptake, sigmoid concentration-response curves were converted into straight lines by plotting values on the ordinate scale as log (Y/100 - Y): where Y is 100 $\times$ the ratio (the response in the presence of a given concentration of Ca²⁺ antagonist/the response in the absence of Ca²⁺ antagonist). The intercept with the abscissa scale (Y = 0) gives the IC₅₀ values.

Results and Discussion

Figure 1 shows the time course of high K⁺-evoked ⁴⁵Ca²⁺ uptake into cultured adrenal chromaffin cells in the absence or presence of NKY-722. NKY-722 had little effect on basal Ca²⁺ uptake into the resting cells (data not shown). When the cells were stimulated by high K⁺ in the absence of NKY-722, high K⁺-evoked ⁴⁵Ca²⁺ uptake into the cells increased rapidly and reached a plateau within 4 min. In the presence of 10⁻⁸ M NKY-722, the rate of the ⁴⁵Ca²⁺ uptake was markedly reduced but 20 min after addition of ⁴⁵Ca²⁺ to the medium, the amount of ⁴⁵Ca²⁺ taken up into the cells reached the same level as in the absence of NKY-722. In the presence of a higher concentration of NKY-722 (10⁻⁶ M), the ⁴⁵Ca²⁺ uptake was completely inhibited for up to 8 min.

It is generally accepted that the slope of the early increasing phase of ⁴⁵Ca²⁺ uptake curve is considered to represent the rate of Ca²⁺ influx through Ca²⁺ channels (Borle 1981; Artalejo *et al.*, 1987). In contrast, the late plateau phase is considered to represent the size of the intracellular exchangeable Ca²⁺ pool (Borle 1981; Artalejo *et al.*, 1987). On the other hand, in the adrenal chromaffin cells, depolarization of the

Figure 1 Time course of high K^+ -evoked $^{45}\text{Ca}^{2+}$ uptake into cultured adrenal chromaffin cells in the absence or presence of NKY-722. After preincubation in Ca^{2+} -free Krebs-HEPES medium at 37°C for 10 min, the cells were stimulated by incubating them in high K^+ medium containing $2\mu\text{Ci}$ of $^{45}\text{CaCl}_2$ at 37°C for various times and the $^{45}\text{Ca}^{2+}$ taken up into the cells was determined (total uptake). Basal $^{45}\text{Ca}^{2+}$ uptake (data not shown) was determined by incubating cells in normal K^+ medium. When the effects of NKY-722 on $^{45}\text{Ca}^{2+}$ uptake were examined, NKY-722 was added to both preincubation and incubation medium. The data shown are high K^+ -evoked $^{45}\text{Ca}^{2+}$ uptake, which was determined by subtracting basal uptake from total uptake: (○) high K^+ -evoked $^{45}\text{Ca}^{2+}$ uptake in the absence of NKY-722; (●) and (▲) high K^+ -evoked $^{45}\text{Ca}^{2+}$ uptake in the presence of 10^{-8}M and 10^{-6}M NKY-722, respectively. Data are means of three separate experiments, each done in triplicate; vertical bars show s.e.mean. ** $P < 0.01$; significantly different from the control value obtained in the absence of NKY-722.

plasma membrane by high K^+ stimulation is known to activate voltage-dependent, L-type Ca^{2+} channels in the membrane (Cena *et al.*, 1983; Vaghy *et al.*, 1987). Thus the present results suggest that NKY-722 inhibits Ca^{2+} influx through

Figure 2 Effects of various concentrations of NKY-722 or nicardipine on high K^+ -evoked $^{45}\text{Ca}^{2+}$ uptake into cultured adrenal chromaffin cells. After preincubation in Ca^{2+} -free Krebs-HEPES medium for 10 min at 37°C , the cells were stimulated by incubating them in high K^+ medium containing $2\mu\text{Ci}$ of $^{45}\text{CaCl}_2$ at 37°C for 1 min and the $^{45}\text{Ca}^{2+}$ taken up into the cells was determined (total uptake). Basal $^{45}\text{Ca}^{2+}$ uptake (data not shown) was determined by incubating cells in normal K^+ medium. When the effects of Ca^{2+} antagonists on $^{45}\text{Ca}^{2+}$ uptake were examined, Ca^{2+} antagonists were added to both preincubation and incubation medium. The data shown are high K^+ -evoked $^{45}\text{Ca}^{2+}$ uptake, which was determined by subtracting basal uptake from total uptake: (●) NKY-722; (○) nicardipine; (◐) represents both NKY-722 and nicardipine. Data are means of three separate experiments, each done in triplicate; vertical bars show s.e.mean.

Figure 3 Typical tracings illustrating the effects of NKY-722 on high K^+ -evoked increases in $[\text{Ca}^{2+}]_i$ in suspended adrenal chromaffin cells. Suspended cells were loaded with fura-2 and transferred to a quartz cuvette for measurement of the fluorescence. Ten minutes after addition of $50\mu\text{l}$ of vehicle or NKY-722 solution, the cells were stimulated by addition of $40\mu\text{l}$ 1.8M KCl (final concentration, 35.9mM) to the cuvette as indicated by arrowheads in the absence (a) or presence of NKY-722 (b, 10^{-8}M ; c, 10^{-6}M).

voltage-dependent, L-type Ca^{2+} channels but it does not affect the size of the intracellular exchangeable Ca^{2+} pool.

Figure 2 shows the effect of various concentrations of NKY-722 or nicardipine on high K^+ -evoked $^{45}\text{Ca}^{2+}$ uptake into cultured adrenal chromaffin cells. Basal $^{45}\text{Ca}^{2+}$ uptake was virtually unaffected by NKY-722 or nicardipine (data not shown). High K^+ -evoked $^{45}\text{Ca}^{2+}$ uptake was inhibited by NKY-722 or nicardipine in a concentration-dependent manner. The IC_{50} values for inhibition of high K^+ -evoked $^{45}\text{Ca}^{2+}$ uptake were $5.2 \pm 0.2\text{ nM}$ ($n = 9$) for NKY-722 and $51 \pm 2\text{ nM}$ ($n = 9$) for nicardipine. Thus NKY-722 seems to be ten times more potent than its prototype nicardipine as a Ca^{2+} antagonist.

Figure 4 Effects of various concentrations of NKY-722 or nicardipine on high K^+ -evoked release of noradrenaline (a) and adrenaline (b) from cultured adrenal chromaffin cells. After preincubation in Krebs-HEPES medium at 37°C for 10 min, the cells were stimulated by incubating them in high K^+ medium at 37°C for 6 min, and noradrenaline and adrenaline released during that time were determined (total release). Basal release of noradrenaline and adrenaline (data not shown) was determined by incubating cells in normal K^+ medium. When the effects of Ca^{2+} antagonists on catecholamine release were examined, Ca^{2+} antagonists were added to both preincubation and incubation medium. The data shown are high K^+ -evoked catecholamine release, which was determined by subtracting basal release from total release: (●) NKY-722; (○) nicardipine; (◐) represents both NKY-722 and nicardipine. Data are means of three separate experiments, each done in triplicate; vertical bars show s.e.mean.

Figure 3 shows typical tracings illustrating the effects of NKY-722 on high K^+ -evoked increases in $[Ca^{2+}]_i$ in suspended adrenal chromaffin cells. Basal $[Ca^{2+}]_i$ in the resting cells was unchanged upon addition of NKY-722 at a final concentration of 10^{-8} M or 10^{-6} M. In the absence of NKY-722, $[Ca^{2+}]_i$ increased from $0.15 \mu M$ to $0.40 \mu M$ (Figure 3a), when the cells were stimulated by high K^+ . In the presence of 10^{-8} M NKY-722 (Figure 3b), the high K^+ -evoked increase in $[Ca^{2+}]_i$ was reduced to about 50% of the value in its absence and completely inhibited at 10^{-6} M NKY-722 (Figure 3c). By comparing these data with those in Figure 2, the concentration-inhibition relationship for high K^+ -evoked increases in $[Ca^{2+}]_i$ is consistent with that for high K^+ -evoked $^{45}Ca^{2+}$ uptake. These data suggest that NKY-722 is acting mainly on Ca^{2+} channels to reduce the depolarization-induced increases in $[Ca^{2+}]_i$.

Figure 4 shows the effects of NKY-722 or nicardipine on high K^+ -evoked release of noradrenaline and adrenaline from cultured adrenal chromaffin cells. Neither NKY-722 nor nicardipine had a significant effect on basal release of noradrenaline and adrenaline (data not shown). In contrast, high K^+ -evoked release of noradrenaline was inhibited by NKY-722 or nicardipine in a concentration-dependent manner (Figure 4a). The IC_{50} values for high K^+ -evoked noradrenaline release were 5.0 ± 0.3 nM ($n = 9$) for NKY-722 and 52 ± 2 nM ($n = 9$) for nicardipine. Essentially similar results

were obtained for adrenaline release (Figure 4b): the IC_{50} values were 4.8 ± 0.3 nM ($n = 9$) for NKY-722 and 50 ± 3 nM ($n = 9$) for nicardipine. Again, the concentration-inhibition relationship for high K^+ -evoked catecholamine release is in good agreement with that for high K^+ -evoked $^{45}Ca^{2+}$ uptake and that for high K^+ -evoked increases in $[Ca^{2+}]_i$. These results suggest that NKY-722, like nicardipine, reduces catecholamine release by inhibiting Ca^{2+} influx and a subsequent increase in $[Ca^{2+}]_i$.

To examine reversibility of the action of NKY-722, the cells were first preincubated in buffer containing various concentrations of NKY-722, and then stimulated by high K^+ in the drug-free buffer. Under these conditions, inhibition of high K^+ -evoked release of noradrenaline and adrenaline was no longer observed (data not shown), suggesting that the effect of NKY-722 is reversible.

In summary, the present investigation established a novel, highly hydrophilic DHP compound, NKY-722, as a potent, reversible Ca^{2+} antagonist.

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