British Journal of Pharmacology (1997) 121, 1136–1140

Voltage and pH dependent block of cloned N-type Ca^{2+} channels by amlodipine

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1 Two types of Ca^{2+} channel α_1-subunits were co-expressed in Xenopus oocytes with the Ca^{2+} channel α_2- and β_1-subunits. The Ba^{2+} current through the $\alpha_{1C}\alpha_2\beta$ and the $\alpha_{1B}\alpha_2\beta$ channels had electrophysiological and pharmacological properties of L- and N-type Ca^{2+} channels, respectively.

2 Amlodipine had a strong blocking action on both the L-type and N-type Ca^{2+} channels expressed in the oocyte. The potency of the amlodipine block on the N-type Ca^{2+} channel was comparable to that on the L-type Ca^{2+} channel. At -100 mV holding potential, the IC_{50} values for amlodipine block on the L-type and N-type Ca^{2+} channel were 2.4 and 5.8 μM, respectively.

3 The blocking action of amlodipine on the N-type Ca^{2+} channel was dependent on holding potential and extracellular pH, as has been observed with amlodipine block on the L-type Ca^{2+} channel. A depolarized holding potential and high pH enhanced the blocking action of amlodipine,

4 The time course of block development by amlodipine was similar for L-type and N-type Ca^{2+} channels. However, it was slower than the time course of block development by nifedipine for the L-type Ca^{2+} channel.

Keywords: N-type Ca^{2+} channel; amlodipine; dihydropyridine

Introduction

The dihydropyridines (DHPs) are a group of Ca^{2+} channel blocking agents that are used for the treatment of hypertension. The therapeutic role of Ca^{2+} channel blockers arises from their ability to lower blood pressure by relaxing the arteriolar smooth muscle and decreasing the peripheral vascular resistance.

A common side effect of DHPs is moderate reflex tachycardia mediated by the sympathetic system.

Amlodipine is a dihydropyridine compound characterized by a low incidence of tachycardia (Lopez et al., 1990), which is consistent with findings that the ability of amlodipine to stimulate the sympathetic system is very weak compared to other members of the DHP family (Kaplan, 1991; Leenen & Fourney, 1996). So far, the unique action of the drug has been explained by its extremely slow pharmacokinetic properties (Burges et al., 1989). However, some studies have suggested that amlodipine may have modulating effects on the neurohormonal system (Donati et al., 1992; Leenen & Fourney, 1996).

Although it is generally accepted that the DHPs are specific blockers of the L-type Ca^{2+} channels, we hypothesized that amlodipine may also act on N-type Ca^{2+} channels. The activity of the N-type Ca^{2+} channels in nerve and secretory cells is linked to both sympathetic nerve tone and catecholamine release. The effect of amlodipine on the sympathetic and neurohormonal system may be explained by interaction with the N-type Ca^{2+} channel. To test this hypothesis, we compared the blocking effect of amlodipine and nifedipine on cloned L-type and N-type Ca^{2+} channels expressed in Xenopus oocytes. Our results clearly show that amlodipine has a strong blocking action on both the N-type and L-type Ca^{2+} channels.

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Methods

The plasmid containing the α_{1B} calcium channel cDNA (pKCRB3) has been described previously (Fujita et al., 1993). The 7.3 kb HindIII/HindIII fragment from pKCRB3 was ligated with the HindIII-cleaved pSP64AS to yield pSPB3. The 6.8 kb HindIII/HindIII fragment containing the entire coding region of the α_{1C} Ca^{2+} channel (Mikami et al., 1989) was inserted into the HindIII site of the pSPA2 vector (Nakamura et al., 1991), to yield pSPCDR. The pSP65 or pSP72 recombinant plasmids carrying the α_2- or β_{1a}-Ca^{2+} channel subunit cDNAs have been described previously (Mikami et al., 1989; Mori et al., 1991). cRNAs specific for the α_{1B}, α_{1C}, α_2 and β_{1a} Ca^{2+} channel subunit were synthesized in vitro by use of the MEGAscript SP6 kit (Ambion).

Xenopus oocytes were injected either with 0.3 μg μl^{-1} α_{1B} or α_{1C} cRNA in combination with 0.2 μg μl^{-1} α_2 cRNA and 0.1 μg μl^{-1} β_{1a} cRNA. The injected oocytes were incubated for 2 to 5 days and then were subjected to electrophysiological measurements at 21 ± 2°C (Takao et al., 1994). Oocytes were bathed in a chamber perfused with an extracellular solution containing (mM): Ba^{2+} 10, TMA-OH 50, K^+2, HEPES 5 and niflumic acid 0.3 (pH 7.5 with methanesulphonic acid), and I_{Ba} flowing through the Ca^{2+} channels was measured by a two-microelectrode voltage-clamp method by a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA, U.S.A.). The experimental chamber was 0.5 ml in volume and was perfused continuously (2.0 to 3.0 ml min^{-1}) with the extracellular solution. Commercial software (pClamp version 6.0, Axon Instruments) was used for generating voltage pulses, acquiring data and analysing the currents. Typically, the ooctyes were clamped at -100 or -80 mV, and depolarized $+10$ mV every 15 s. Microelectrodes were filled with 3M KCl, and those showing a resistance of 0.5–1.2 ΩM were used.

Statistical data are presented as the mean $\pm$ s.e.mean. Fits of experimental data to mathematical equations were performed by use of a non-linear least squares fitting algorithm. Amlodipine was a generous gift from Sumitomo Pharmaceuticals Co., Ltd. (Tokyo, Japan). All other drugs were purchased from Sigma (U.S.A.).

Results

Figure 1a illustrates inward Ba^{2+} currents (I_{Ba}) recorded from Xenopus ooctyes that had been injected with α_{1C} (upper panel) or α_{1B} (lower panel) cRNA in combination with cRNAs of the α_2-subunit (Mikami et al., 1989) and the β_{1a}-subunit (Mori et al., 1991) of the Ca^{2+} channel. A step depolarization from a holding potential of -80 mV to 10 mV produced inward current in ooctyes injected with either α_{1B} or α_{1C} subunit cRNA. Mean peak inward current through the expressed L-type Ca^{2+} channels ($\alpha_{1C}\alpha_2\beta$) and N-type Ca^{2+} channels ($\alpha_{1B}\alpha_2\beta$) was 1.34 ± 0.5 μA (n = 23) and 1.62 ± 0.4 μA (n = 48), respectively. In the control study, we observed that the Ba^{2+} current (I_{Ba}) through both the L-type (n = 10) and N-type (n = 8) Ca^{2+} channels was stable over 30 min. Each type of current was completely blocked by its specific pharmacological antagonist as shown in Figure 1a (Mikami et al., 1989; Ellinor et al., 1995). Thus, the expressed channel subunits had typical pharmacological characteristics for L- and N-type Ca^{2+} channels. The Xenopus oocytes have an endogenous Ca^{2+} channel and this endogenous Ca^{2+} channel is insensitive to both dihydropyridines and ω-conotoxin (Bouron et al., 1995). I_{Ba} through the endogenous Ca^{2+} channels had a maximal inward current amplitude of -62.0 ± 11.3 nA (n = 10) at around +20 mV, under our experimental conditions in the oocytes injected only with cRNAs of the α_2- and β_{1a}-subunit. The fraction of I_{Ba} through the endogenous Ca^{2+} channels is considered to be less than 5% of the total current amplitude. Therefore, contamination of the endogenous Ca^{2+} channel was considered to be negligible when the effect of a drug on expressed Ca^{2+} channel was measured. The right lower panel of Figure 1a shows the effect of nifedipine on N-type Ca^{2+} channels ($\alpha_{1B}\alpha_2\beta$). Nifedipine had little effect on the channel at a concentration of 10 μM.

Figure 1b shows a representative trace illustrating the effect of amlodipine on I_{Ba} through an N-type Ca^{2+} channel. Amlodipine blocked the I_{Ba} in a dose-dependent manner. When the membrane potential was depolarized to +10 mV from a holding potential of -100 mV, maximal peak inward current was 1.42 μA in the control. When amlodipine was added to the extracellular solution, the I_{Ba} decreased gradually. A steady state inhibition was obtained within about 5 min after beginning of amlodipine superfusion. I_{Ba} was decreased to 0.98 μA by 1 μM amlodipine. The amlodipine concentration was then increased to 10 μM and the I_{Ba} was observed for another 5 min. The current was further decreased to 0.43 μA. As shown in Figure 1c, amlodipine did not affect the threshold of I_{Ba} or the membrane potential at which the maximal peak inward current was obtained.

To evaluate the blocking potency of amlodipine, we compared the dose-dependent effect of amlodipine and nifedipine on an N-type Ca^{2+} channel. The effect of each drug was evaluated at 5 min after beginning the superfusion. Similar experiments were repeated to evaluate the actions of both drugs on the L-type Ca^{2+} channel. Dose-response curves for the effect of amlodipine and nifedipine on the L-type and N-type Ca^{2+} channel are shown in Figure 2a. Both drugs blocked the L-type Ca^{2+} channel. However, under our experimental conditions, the drug concentration for 50% blockage of the current amplitude (IC_{50}) was about 10 times higher for amlodipine. Amlodipine blocked the N-type Ca^{2+} channel in a dose-dependent manner. The IC_{50} for amlodipine block on the N-type Ca^{2+} channel was 5.8 μM. The Hill coefficient for drug binding was 0.92, which suggests 1 : 1 binding of the drug to the channel (n = 12). As previously shown (Tsien et al., 1991), nifedipine had virtually no effect on the N-type Ca^{2+} channel.

The time course of block development for each drug at 10 μM was compared at a holding potential of -100 mV. When the change in current amplitude was described by a single exponential process, the time constant of block development by amlodipine was 75.1 ± 10.2 s for L-type Ca^{2+} channels (n = 8) and 93.8 ± 7.4 s for N-type Ca^{2+} channels (n = 6), respectively. The time constant of block development

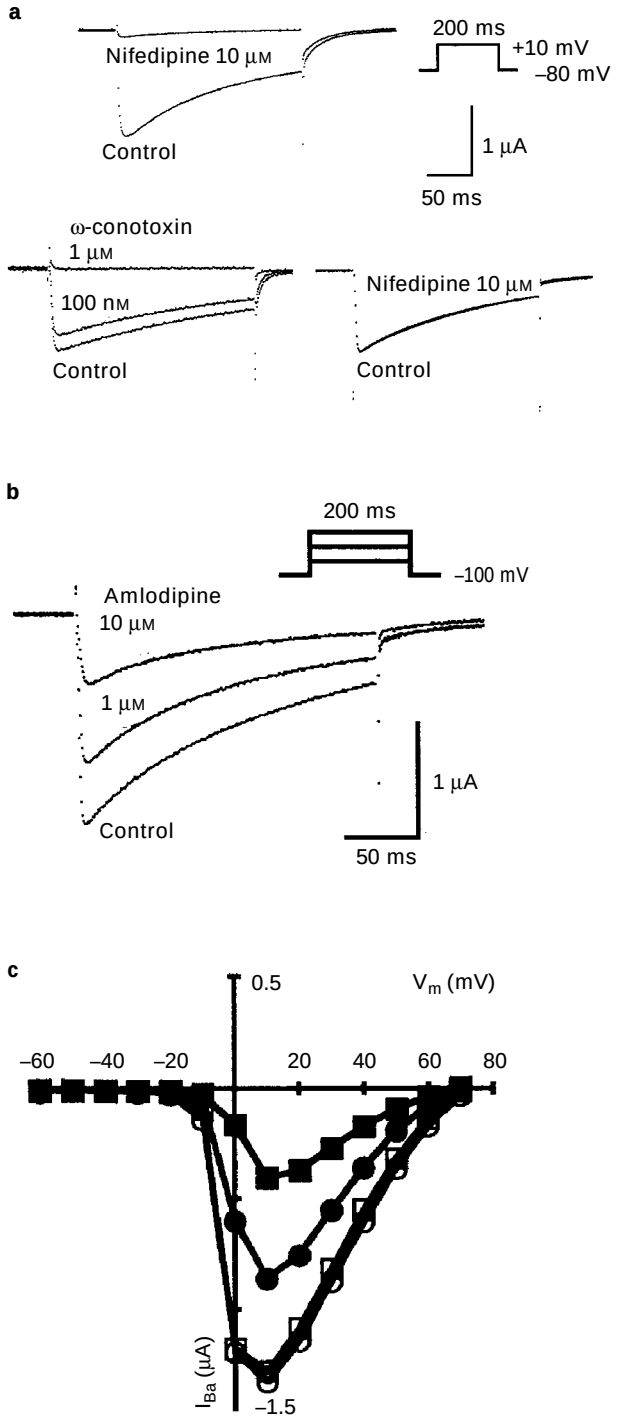

Figure 1 Effect of nifedipine and amlodipine on L- and N-type Ca^{2+} channels expressed in Xenopus oocytes. (a) Superimposed traces of the Ba^{2+} current (I_{Ba}) are shown in the control and after 5 min of superfusion with the Ca^{2+} channel blockers. I_{Ba} was elicited by a step depolarization to +10 mV from a holding potential of -80 mV. The peak I_{Ba} amplitude in the oocyte expressing $\alpha_{1C}\alpha_2\beta$ (L-type) channel was 1.68 μA in the control, and was reduced to 0.11 μA by nifedipine 10 μM (upper panel). I_{Ba} in an oocyte expressing $\alpha_{1B}\alpha_2\beta$ (N-type) channel (lower panel left) was 1.27 μA. The current was almost completely blocked by 1 μM ω-conotoxin. On the other hand, I_{Ba} through the N-type Ca^{2+} channel was insensitive to nifedipine 10 μM (lower right panel). Nifedipine at 10 μM had virtually no effect on the current. (b) Amlodipine blocked the N-type Ca^{2+} channel in a dose-dependent manner. (c) The current-voltage relationships in the same experiment as shown in (b). (○) Control responses; effect of amlodipine (□) 0.1 μM, (●) 1 μM and (■) 10 μM. Amlodipine did not change the threshold of, or membrane potential at which peak inward currents were obtained.

by nifedipine for L-type Ca^{2+} channels was 12.5 ± 5.2 s (n = 8), which was significantly smaller than the time constant for amlodipine block of the L-type Ca^{2+} channels ($P < 0.01$). After washout of amlodipine the I_{Ba} through the N-type Ca^{2+} channels partially recovered. In 5 experiments, the I_{Ba} recovered to $82.5 \pm 8.2\%$ of control values with 5 min washouts.

The effect of amlodipine on the L-type Ca^{2+} channel is known to be dependent on holding potential and extracellular pH. Therefore, we evaluated whether the effect of amlodipine on the N-type Ca^{2+} channel was dependent on these parameters. In Figure 2b, original current traces of an N-type Ca^{2+} channel at two different holding potentials (−100 mV and −80 mV), and two different extracellular pHs (7.5 and 10.5) are shown. The block of I_{Ba} by 1 μM amlodipine was more prominent at a depolarized holding potential and high pH, similar to the effect of amlodipine of L-type Ca^{2+} channel (Kass & Arena, 1989). To confirm the effect of the holding potential and pH, we measured the dose-dependent effect of amlodipine. As clearly shown in Figure 2c, the blocking effect of amlodipine was enhanced by a depolarized holding poten-

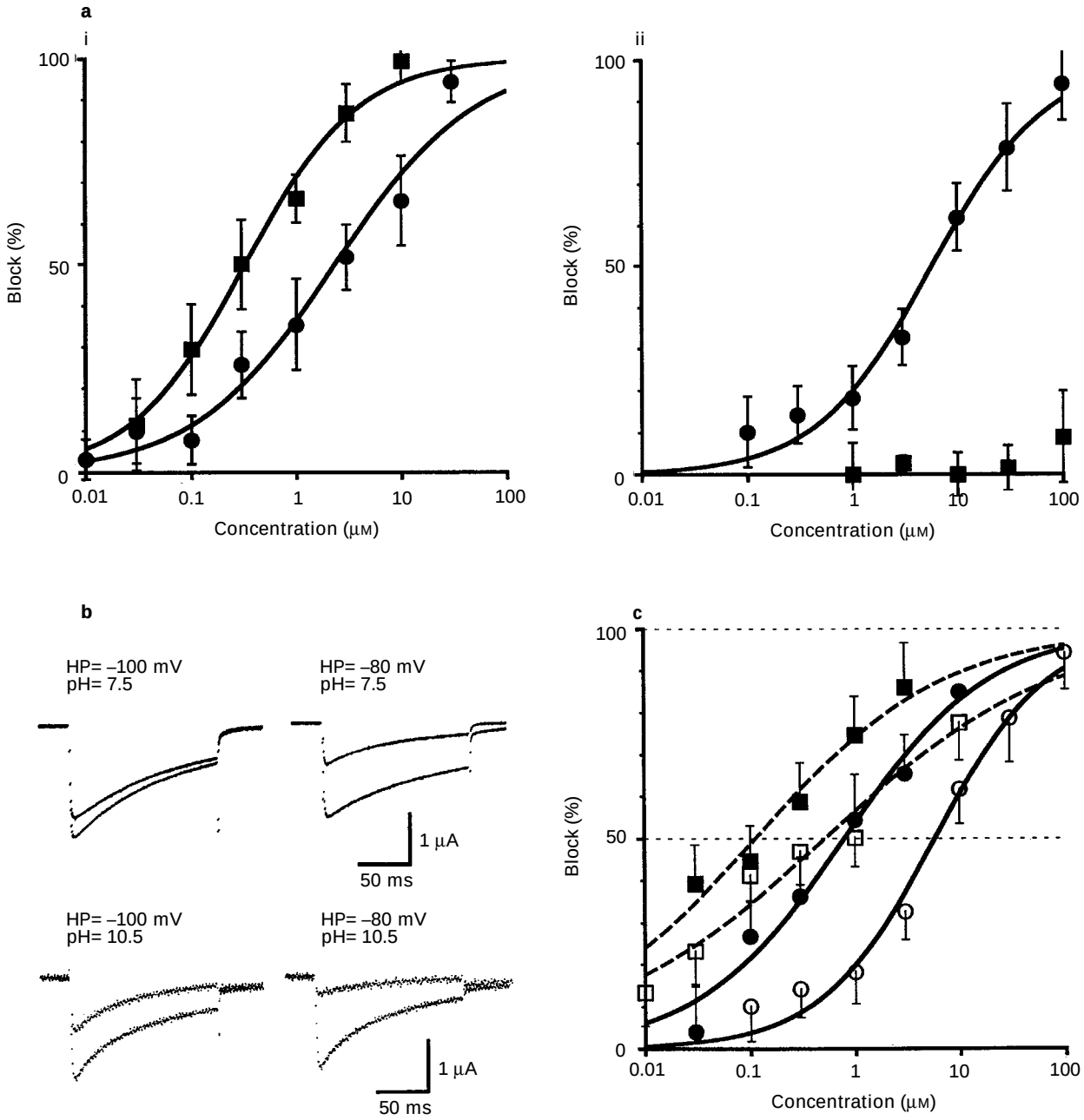

Figure 2 (a) Dose-response curve for effects of (■) nifedipine and (●) amlodipine on (i) L- and (ii) N-type Ca^{2+} channels. Block of I_{Ba} was plotted as a function of the drug concentration. All data were obtained after 5 min of superfusion of the drugs. Smooth lines shown in the figure are the results of least square fitting according to: $\text{Block} = 100/(1 + (IC_{50}/[D])^{n_H})$ where [D] denotes the drug concentration, and n_H denoted Hills coefficient. Values for the drug effect on L-type Ca^{2+} channel (i): $IC_{50} = 2.4$, $n_H = 0.7$ for amlodipine (n = 12) and $IC_{50} = 0.32$, $n_H = 0.9$ for nifedipine (n = 9). Values for amlodipine effect on N-type Ca^{2+} channel (ii): $IC_{50} = 5.8$ and $n_H = 0.92$ (n = 14). In (i) holding potential (HP) = −80 mV; in (ii) HP = −100 mV. (b) Original current traces showing the effect of holding potential and extracellular pH on amlodipine block of N-type Ca^{2+} channel. Note that the depolarized holding potential and high pH enhanced the block by amlodipine 1 μM. (c) Dose-response curves for amlodipine block on the N-type Ca^{2+} channel at two different holding potentials and extracellular pHs: (○) pH 7.5, HP −100 mV; (●) pH 7.4, HP −80 mV; (□) pH 10.5, HP −100 mV; (■) pH 10.5, HP −80 mV. IC_{50} and n_H were as following; 5.8 and 0.92 at HP = −100 mV and pH = 7.5; 0.52 and 0.89 at HP = −100 mV and pH = 10.5; 0.51 and 0.71 at HP = −80 mV and pH = 7.5; 0.08 and 0.62 at HP = −80 mV and pH = 10.5. Data shown are means of n = 7; vertical lines indicate s.e.mean.

tial and high extracellular pH. The effect of these two parameters on the dose-response curve was additive. The IC_{50} decreased to 0.08 μM when the holding potential was -80 mV and the extracellular pH was 10.5. The voltage and pH dependent block by amlodipine of the N-type Ca^{2+} channels is similar to the block of L-type Ca^{2+} channels by this drug (Kass & Arena, 1989).

Discussion

In the Xenopus oocytes expression system, we discovered that amlodipine blocks N- and L-type Ca^{2+} channels with a comparable potency. This finding was quite unexpected, as DHP compounds are generally recognized as specific L-type Ca^{2+} channel blockers. Our results demonstrate the need to evaluate the Ca^{2+} channel subtype selectivity of each individual DHP compound.

Recent studies indentified the specific binding site of the DHP compounds within the L-type Ca^{2+} channel by making chimeric channels constructed from DHP sensitive and non-sensitive channels (Grabner et al., 1996), and by site directed mutagenesis (Peterson et al., 1996). Determining the binding site of amlodipine within the N-type Ca^{2+} channel may provide insight into the subtype differences seen between the interaction of amlodipine with the two types of Ca^{2+} channels.

Our results showed that block of the N-type Ca^{2+} channels by amlodipine was dependent on both membrane potential and extracellular pH. Previous studies have extensively investigated the voltage and pH dependent block of amlodipine on the L-type Ca^{2+} channel. The voltage-dependence was explained by state-dependent changes in the drug affinity due to alterations in the channel conformation (Kass & Arena, 1989). The pH dependence was attributed to the transition between the molecular and ionized forms of the drug (Nayler & Gu, 1991). The membrane potential and extracellular pH dependent block by amlodipine of the N-type Ca^{2+} channels may be controlled by similar mechanisms as those suggested for the L-type Ca^{2+} channels. To determine this detailed channel kinetic studies need to be performed.

The Xenopus oocyte expression system allowed us to compare the effects of amlodipine on N-type and L-type Ca^{2+} channels under identical experimental conditions. We found that, at a -100 mV holding potential with pH 7.5, 10 μM amlodipine blocked both the L-type and N-type Ca^{2+} channel with a time constant of block development around 1 min. Therefore, we demonstrated that the blocking potency of amlodipine on the expressed L- and N-type Ca^{2+} channels were comparable. However, the steady state block of amlodipine on the Ca^{2+} channels might not be evaluated well by this system. The development of block by amlodipine is known to be very slow at physiological pH, at which more than 90% of this compound is ionized (Mason et al., 1989). The development of block is known to be even slower at lower concentrations of the drug (Burges et al., 1989; Kass & Arena, 1989). In a binding assay on rat cardiac membrane, Nayler & Gu (1991) showed that amlodipine required several hours to reach steady state block. Such a long time span was not examined in the two microelectrode voltage clamp method with Xenopus oocytes, and we did not study the very slow component of block development. The steady state block of amlodipine on N-type Ca^{2+} channel needs to be revealed by further studies.

Several findings have suggested that amlodipine may affect the neurohormonal system by modulating the pressor response (Donati et al., 1992) and plasma catecholamine levels (Abernethy et al., 1988; Leenen & Fourney, 1996; Lopez et al., 1990). Some of these studies have shown the sympatholytic effect of amlodipine, which is exceptional for a DHP compound. We assumed that the sympatholytic effect might be related to the ability of amlodipine to block the N-type Ca^{2+} channel, which is mainly distributed in the sympathetic nerve terminals to trigger catecholamine release (Hirning et al., 1988). Our experimental conditions were far from the physiological state, and clinical implications of our results are limited. The effect of amlodipine in vivo has to be evaluated in other experimental systems. However, the minimal concentration at which we observed significant block of the channel was quite comparable to the clinical concentration of amlodipine (10^{-10} to 10^{-7} M) (Burges et al., 1989; Elliot & Meredith, 1991), and we believe that our discovery provides novel clues for understanding the effect of amlodipine on the cardiovascular system.

We are grateful to Drs Atsushi Mikami and Tsutomu Tanabe for providing us with α_{1C} and α_2 cDNA. We express special gratitude to Dr Nobuyuki Hara and Hiroshi Tamura for technical assistance.

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(Received November 1, 1996,
Revised March 11, 1997,
Accepted April 3, 1997)