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A region of N-type Ca^{2+} channel critical for blockade by the dihydropyridine amlodipine

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ABSTRACT

Amlodipine, a dihydropyridine derivative, has been shown to block not only L-type but also N-type Ca^{2+} channels. Aiming to understand the mechanism underlying such a selective blockade by amlodipine, the interaction of amlodipine with N-type channels was investigated using the *Xenopus* oocyte expression system together with the two-microelectrode voltage-clamp technique and the binding assay for [^{3}H] amlodipine. When expressed as the $\alpha_{1B}\alpha_{2/}\delta_1\beta_{1a}$ combination, the N-type channel formed a high affinity binding site for [^{3}H]amlodipine (K_d, 3.08 nM) and was profoundly blocked by amlodipine (IC_{50}, 2.7 μM at −60 mV). By contrast, R-type ($\alpha_{1E}\alpha_{2/}\delta_1\beta_{1a}$) channels did not possess a high affinity binding site for [^{3}H] amlodipine and their channel activities were not influenced by amlodipine. In comparison of amino acid sequences in the transmembrane regions IIIS5, IIIS6 and IVS6 of the α_1 subunit, which are involved in dihydropyridine binding in L-type channels, the two amino acid residues Lys^{1287} (corresponding to Met^{1295} in α1B) and Phe^{1699} (corresponding to Leu^{1697} in α_{1B}) were unique in α_{1E}. An amino acid substitution of Lys1287Met in IIIS5 of α_{1E} conferred a high affinity binding site for amlodipine (K_d, 13.1 nM) and a sensitivity to amlodipine (IC_{50}, 11.3 μM). In N-type channel, reversely, an amino acid substitution of Met1295Lys in IIIS5 of α_{1B} deprived a high affinity binding site for amlodipine and reduced the channel blockade by amlodipine (IC_{50}, 29.6 μM). The results indicate that Met^{1295} in the region IIIS5 of α_{1B} is critical for amlodipine to efficiently bind and block the N-type Ca^{2+} channel.

1. Introduction

High voltage-activated Ca^{2+} channels in excitable cells such as myocytes, smooth muscle cells and neurons play important roles including contraction of myocytes, electrical excitement in neurons, and modulation of hormone and neurotransmitter release (Tsien et al., 1991). High voltage-activated Ca^{2+} channels are pharmacologically classified into at least five different subclasses (L-, N-, P-, Q- and R-type), of which characteristics are considered to be determined by the pore-forming α_1 subunit. The α_{1C} ($Ca_v1.2$), α_{1D} ($Ca_v1.3$) and α_{1S} ($Ca_v1.1$) subunits form L-type Ca^{2+} channels and dihydropyridines (DHPs), phenylalkylamines and benzothiazepines bind them with high affinity, whereas the α_{1B} ($Ca_v2.2$), α_{1A} ($Ca_v2.1$) and α_{1E} ($Ca_v2.3$) subunits form N-, P/Q- and R-type Ca^{2+} channels, respectively, which show low affinities for these drugs (Hering et al., 1998; Hockerman et al., 1997; Striessnig et al., 1998). Because nifedipine, the prototype of the DHPs, exclusively blocked muscular L-type Ca^{2+} channels (Fleckenstein, 1983), DHPs had been considered as selective blockers for L-type channels.

Recent studies have shown, however, that some DHPs such as amlodipine (Furukawa et al., 1997, 1999), cilnidipine (Fujii et al., 1997; Furukawa et al., 1999; Uneyama et al., 1997) and benidipine (Furukawa et al., 1999), blocked N- and P/Q-type Ca^{2+} channels as well. By contrast, these DHP derivatives fail to block R-type channels (Furukawa et al., 1999). These findings indicate that DHPs are no longer considered as the specific blockers of L-type Ca^{2+} channels. DHPs are widely used clinically in the treatment of hypertension, angina pectoris and cerebrovascular diseases. However, the molecular mechanism by which some DHPs can preferentially block N- and P/Q-type Ca^{2+} channels remains unclear.

Non-L-type Ca^{2+} channels are diversely distributed in peripheral and central nervous cells (Tsien et al., 1991). However, native neuronal cells and cell lines posses multiple subtypes of Ca^{2+} channels in a single cell, which hampers quantitative comparison of effects of a given drug on a single subtype of Ca^{2+} channel. To address these issues at the molecular level, a single α_{1B} or α_{1E} subclass of the Ca^{2+} channel and its mutant α_1 with an amino acid substitution were co-expressed with the same auxiliary α_2/δ and β subunits in *Xenopus* oocytes, and then amlodipine was examined on its membrane binding properties and current blocking effects. In the present study, Met^{1295} in the transmembrane region IIIS5 of the α_{1B} subunit of N-type

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channel had key roles in high affinity binding of amlodipine and channel blocking by amlodipine.

2. Methods

2.1. PCR product preparation

All PCR reactions were performed in 50 μl reactions containing 1 U KOD DNA polymerase (Toyobo, Tokyo, Japan), 5 ng of plasmid and 1 pmol of each PCR primers for 30 cycles (94 °C 30 s, 55 °C 1 min, 72 °C 2 min). The chloramphenicol resistance gene from pGPS2.1 (New England BioLabs, Beverly, MA) was amplified using the following four sets of primers: R3S5-1, 5′-**C GTG GTG ACC TCC TTG AAG AAC GTC TTC AAC ATC CTC ATC GTA TAC** AGT TTA AAC GAT ATC GGA TCC AGG CGT T-3′ and R3S5-2, 5′-**G CTG CAC TGC GAT GAC AGC GAA GAT GAA CAT GAA GAG *CAT* GTA TAC** ATA CCT GTG ACG GAA GAT CAC TTC GCA G-3′, where the homology arms corresponding to the amino acid residues 1271–1286 and 1285–1300 of α_{1E} are in bold, a Lys1287Met substitution is in italic and a restriction site for *Bst*Z17I underlined; R4S6-1, 5′-**GT GAG CGC TGC GGC ACT GAC CTC GCC TAC GTC TAC TTT GTA AGC TT** AGT TTA AAC GAT ATC GGA TCC AGG CGT T-3′ and R4S6-2, 5′-**TAC GAA CAG GTT GAG CAT CAG GAA GGA GCA *GAG* GAA GAT GAA GCT T** ATA CCT GTG ACG GAA GAT CAC TTC GCA G-3′, where the homology arms corresponding to the amino acid residues 1681–1696 and 1694–1709 of α_{1E} are in bold, a Phe1699Leu substitution is in italic and a restriction site for *Hin*dIII underlined; N3S5-1, 5′-**T GTG GTG AAC TCA CTG AAA AAC GTC CTC AAC ATC CTG ATC GTA TAC** AGT TTA AAC GAT ATC GGA TCC AGG CGT T-3′ and N3S5-2, 5′-**G CTG CAC GGC AAT GAC GGC AAA GAT GAA CAT GAA GAG *CTT* GTA TAC** ATA CCT GTG ACG GAA GAT CAC TTC GCA G-3′, where the homology arms corresponding to the amino acid residues 1279–1294 and 1293–1308 of α_{1B} are in bold, a Met1295Lys substitution is in italic and a restriction site for *Bst*Z17I underlined; N4S6-1, 5′-**CC AGC GAG TGC GGC AGC GAC TTT GCC TAC TTT TAC TTC GTA AGC TT** AGT TTA AAC GAT ATC GGA TCC AGG CGT T-3′ and N4S6-2, 5′-**CAC AAA GAG GTT CAA CAT CAG AAA GGA GCA *GAA* GAA GAT GAA GCT T** ATA CCT GTG ACG GAA GAT CAC TTC GCA G-3′, where the homology arms corresponding to the amino acid residues 1679–1694 and 1692–1707 of α_{1B} are in bold, a Leu1697Phe substitution is in italic and a restriction site for *Hin*dIII underlined. The PCR products PCRP-R1, PCRP-R2, PCRP-N1 and PCRP-N2 amplified with the primer sets R3S5-1/2, R4S6-1/2, N3S5-1/2 and N4S6-1/2 were purified and resuspended in water (1 μg/μl), respectively.

2.2. Mutagenesis of α_{1E} and α_{1B} by using ET-recombination

The plasmid pSPB3S (Furukawa et al., 1998) carrying the entire protein-coding sequence of α_{1B} (Fujita et al., 1993) was partially digested with *Hin*dIII, blunted with T_4 DNA polymerase, and circularized with T_4 DNA ligase, to yield pSPB3H1. The plasmids pSPBII carrying the entire protein-coding sequence of α_{1E} (Niidome et al., 1992) and pSPB3H1 were digested with *Hin*dIII, blunted, and circularized to produce pSPB2H1 and pSPB3H2, respectively. In the plasmids pSPB2H1 and pSPB3H2, *Hin*dIII sites on vector were deleted.

Using *Escherichia coli* strain JC8679 (Gillen et al., 1981), electroporation-competent bacterial cells were prepared according to the procedures described previously (Angrand et al., 1999) and, then, transformed with the plasmid pSPB2H1 or pSPB3H2 by electroporation using a Gene Pulser (Bio-Rad, Hercules, CA) set at 2.5 kV, 200 Ω and 25 μF. Again, electroporation-competent bacterial cells were prepared from the transformed cells with each plasmid. JC8679 (Deposit No. HT017) was obtained from Health Science Research Resources Bank (Osaka, Japan).

An amino acid substitution in α_{1E} (Lys1287Met or Phe1699Leu) and α_{1B} (Met1295Lys or Leu1697Phe) was performed using RecE and RecT mediated recombination (ET recombination) (Angrand et al., 1999). Briefly, the electroporation-competent cells transformed with pSPB2H1 were thawed on ice, added by the PCR product PCRP-R1 or PCRP-R2 (1 μg), and subjected to the electroporation. Similarly, the competent cells transformed with pSPB3H2 were subjected to the electroporation with the PCR product PCRP-N1 or PCRP-N2. These transformed cells after the electroporation were suspended in 600 μl L-broth and incubated for 1.5 h at 37 °C before plating on L-agar containing chloramphenicol (100 μg/ml), in order to identify bacteria cells containing homologously recombined plasmids (pSPB2-3SCm1, pSPB2-4SCm1, pSPB3-3SCm1 and pSPB3-4SCm1) with the PCR products PCRP-R1, PCR-R2, PCR-N1 and PCR-N2, respectively. To remove the chloramphenicol resistance gene, the recombined plasmids pSPB2-3SCm1/pSPB3-3SCm1 and pSPB2-4SCm1/pSPB3-4SCm1 were digested with *Bst*Z17I and *Hin*dIII, respectively, and circularized to yield pSPB2-3SH1, pSPB3-3SH1, pSPB2-4SH1 and pSPB3-4SH1. The 0.6 kb *Bsm*I/*Nsi*I and 1.5 kb *Nsi*I/*Bgl*II fragments excised from pSPB2-3SH1 and pSPB2-4SH1 were replaced by those from pSPB2H1 to yield pSPB2-3S5-1 and pSPB2-4S6-1, respectively. The 1.9 kb *Sfi*I/*Bsp*EI and 0.8 kb *Bsp*EI/*Srf*I fragments from pSPB3-3SH1 and pSPB3-4SH1 were replaced by those from pSPB3H2 to yield pSPB3-3S5-1 and pSPB3-4S6-1, respectively.

Subcloning and mutagenesis procedures were verified by restriction enzyme analysis and DNA sequencing. In pSPB2-3S5-1 and pSPB2-4S6-1, the amino acid residues Lys^{1287} and Phe^{1699} of α_{1E} were replaced with Met (a R-type mutant α_{1E}KM) and Leu (another R-type mutant α_{1E}FL), respectively. In pSPB3-3S5-1 and pSPB3-4S6-1, the amino acid residues Met^{1295} and Leu^{1697} of α_{1B} were replaced with Lys (an N-type mutant α_{1B}MK) and Phe (another N-type mutant α_{1B}LF), respectively.

2.3. In vitro transcription

cRNAs specific for the mutants α_{1E}KM, α_{1E}FL, α_{1B}MK and α_{1B}LF were synthesized *in vitro* using a MEGAscript SP6 kit (Ambion, Austin, TX). Methods for *in vitro* transcription of cRNAs specific for wild-type α_1 (α_{1E}, α_{1B} and α_{1A}) and the Ca^{2+} channel α_2/δ_1 and β_{1a} subunits and procedures for functional expression of Ca^{2+} channels in *Xenopus* oocytes were described previously (Furukawa et al., 1998, 1999).

The present study has been approved by the Animal Ethical Committee of the Tokyo Institute of Psychiatry. Female *Xenopus laevis* were anaesthetized with 3-aminobenzoic acid ethyl ester, and then, a mini-laparotomy was performed to obtain ovarian sacs. After removal of the follicular cell layer, *Xenopus* oocytes were injected with 0.3 μg/μl the mutant α_1, α_{1E} (Niidome et al., 1992), α_{1B} (Fujita et al., 1993) or α_{1A} (Mori et al., 1991) cRNA in combination with 0.2 μg/μl α_2/δ_1 (Mikami et al., 1989) cRNA and 0.1 μg/μl β_{1a} (Mori et al., 1991) cRNA.

2.4. Binding assay

After injection of channel subunit cRNAs, the oocytes were cultured for 2 to 4 days. Oocyte membranes were, then, prepared as described previously (Yamamoto et al., 1999). Briefly, after mechanical removal of vitelline envelope (Stuhmer, 1992), stage V–VI oocytes were frozen in liquid nitrogen and thawed three times. Then, oocytes were homogenized in 10 vol of a 1 mM $NaHCO_3$, 3 mM EDTA solution, which contained protease inhibitors such as pepstatin A (1 μg/ml), leupeptin (10 μg/ml), aprotinin (20 μg/ml), phenylmethanesulfonyl fluoride (200 μM), benzamidine (0.1 mg/ml) and calpain inhibitor I and II (8 μg/ml each). The homogenate was centrifuged at 1000*g* for 15 min, and then, the supernatant was centrifuged at 10 000*g* for 20 min. For binding assay, the 10 000*g* pellet was resuspended in the same solution. All procedures were carried out at 4 °C or on ice.

Radioligand binding assays with tritium-labelled amlodipine {2-[(2-aminoethoxy)-methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl 5-methyl ester, 82 Ci/mmol} were performed using 0.2–0.4 mg/ml of oocyte membranes (Nayler