Transfer of L-type Calcium Channel IVS6 Segment Increases Phenylalkylamine Sensitivity of α1A*

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Conditioned ("use-dependent") inhibition by phenylalkylamines (PAAs) is a characteristic property of L-type calcium (Ca\(^{2+}\)) channels. To determine the structural elements of the PAA binding domain we transferred sequence stretches of the pore-forming regions of repeat III and/or IV from the skeletal muscle α1 subunit (α1S) to class A α1 subunit (α1A) and expressed these chimeras together with β1A, and α2δ subunits in Xenopus oocytes. The corresponding barium currents (I\(_{Ba}\)) were tested for PAA sensitivity during trains of depolarizing test pulses (conditioned block). I\(_{Ba}\) of oocytes expressing the α1A subunit were only weakly inhibited by PAAs (less than 10% conditioned block of I\(_{Ba}\) during a 100-ms pulse train of 0.1 Hz). Transfer of the transmembrane segment IVS6 from α1S to α1A produced an enhancement of PAA sensitivity of the resulting α1A/α1S chimera comparable to L-type α1S subunits (about 35% conditioned block of I\(_{Ba}\) during a 100-ms pulse train of 0.1 Hz). Our results demonstrate that substitution of 11 amino acids within the segment IVS6 of α1A with the corresponding residues of α1S is sufficient to transfer L-type PAA sensitivity into the low sensitive class A Ca\(^{2+}\) channel.

Voltage-gated Ca\(^{2+}\) channels mediate the depolarization-induced influx of Ca\(^{2+}\) into excitable cells, thereby regulating cellular processes such as muscle contraction, propagation of action potentials, secretion, and gene expression. They are heterooligomeric complexes formed by at least an α, β, and α2δ subunit (1). The α subunit is the pore-forming membrane protein consisting of four homologous repeats (I–IV), each of them composed of six transmembrane segments (S1–S6) (2). Based on different pharmacological and biophysical properties, various types of voltage-dependent Ca\(^{2+}\) channels (T, L, N, P, Q, and R) can be distinguished (3–5). Their sensitivity to Ca\(^{2+}\) antagonists or toxins is determined by the α subunit (4, 6). At least six different α1 subunit genes have been isolated so far (for nomenclature, see Ref. 3). The α1 subunit classes C (α1C), D (α1D), and S (α1S) mediate the high affinity of L-type Ca\(^{2+}\) channels toward DHP antagonists, such as 1,4-dihydropyridines (DHPs), benzothiazepines, and phenylalkylamines (PAAs) (6, 7). In contrast, classes A, B, and E are considered to be DHP-insensitive.

To localize Ca\(^{2+}\) antagonist interaction domains within L-type α1 subunits we have recently shown (8) that sensitivity for DHP Ca\(^{2+}\) channel blockers and activators can be transferred to class A α1 subunits (α1A) by substituting regions close to the channel pore in repeats III and IV (segments III5S, III56, IVS6, and the respective SS5-S6 linkers) with the corresponding L-type α1S sequences (from α1S or α1C). The DHP sensitivity was lost after replacement of short sequence stretches within these regions by the α1A sequence. For example, when segment III5S was replaced by α1A sequence, DHP sensitivity disappeared. The same effect was observed after replacing the IVS5–IVS6 linker. These results suggest that the DHP molecules interact with multiple amino acid residues located within distant regions of the primary structure.

Hockerman et al. (9) recently identified three amino acid residues within segment IVS6 of a L-type Ca\(^{2+}\) channel α1C subunit (Tyr-1463, Ala-1467, and Ile-1470, numbering according to α1C) as critical determinants for high affinity block by PAAs. Mutation of these residues within α1C to non-L-type resulted in a decrease of PAA sensitivity.

In our present work we studied the importance of the IVS6 segment for the formation of PAA interaction domains by investigating whether this region also supports PAA sensitivity in a non-L-type sequence environment. We addressed this question by testing whether the characteristics of L-type channel block by PAAs can be transferred to α1A that forms a non-L-type Ca\(^{2+}\) channel. We demonstrated that currents through α1A expressed in Xenopus oocytes are less sensitive to PAAs than L-type currents. Transfer of the skeletal muscle IVS6 segment into α1A resulted in a chimeric α1A construct that displayed PAA sensitivity comparable to L-type currents. We therefore conclude that L-type IVS6 also supports PAA sensitivity in a non-L-type sequence environment.

EXPERIMENTAL PROCEDURES

Materials—The phenylalkylamines (–D888) (–devapamil, –desmethoxyverapamil), (–)D600 (–gallopamil, –methoxyverapamil), and (–)emopamil were kindly provided by Dr. Traut (Knoll AG, Ludwigshafen, Germany).

Construction of Chimeric α1 cDNAs—α1 chimeras (AL21, AL22, and AL23; for nomenclature, see Fig. 1A) consisting of α1A from rabbit brain (B1-2) (11) and α1S from carp skeletal muscle (12) were constructed and inserted into the polyadenylating transcription plasmid pNK52 (provided by O. Pongs). Polymerase chain reaction (PCR) was used to create common restriction sites by introducing silent cDNA mutations. Mutations were then introduced into forward and reverse primer primers or by the *gene SOEing* technique (13). Amplification of cDNA by PCR (Thermo- mycorder 60, Biomed) was performed with 35 cycles at low stringency (1 min at 94 °C, 30 s at 42 °C, 1.5 min at 72 °C) using proofreading Pfu-polymerase (Stratagene). Chimeras AL21, AL22, and AL23 were constructed as follows (PCR-generated restriction sites are indicated by asterisks): AL21 (amino acid numbers in parentheses): A(1–1723), S(1311–1437), A(1856–2424). The *SfiI-ClaI* fragment (nucleotide
Numbers in parenthesis refer to A(4296–4925) was ligated into the SfiI (4296A) and CiaI* (4925A) sites of the chimeric construct AL9:pNKS52 (8). AL22: A(1–1723), S(1311–1402), A(1821–2424). A BamHI* site at position 4303 (5) was created by "gene SOEing" in the KpnI*-BglII fragment of chimeric construct AL12s (8). This PCR product was ligated into the KpnI* (5467A) and BglII (6185A) sites of AL1 (4296A), A(1–1791), S(1374–1402), A(1821–2424), KpnI*-BamHI* fragment of A(5467–5667) was ligated into the KpnI* (5467A) and BamHI* (4303S) sites of AL22. The construction of chimeras Ls, L, and L4 was described previously (8, 14). The correct nucleotide composition of the chimeras was verified by extensive restriction endonuclease mapping and by cDNA sequencing with the dideoxy chain termination method (15).

Expression of α1L chimeras in Xenopus laevis oocytes—Preparation of stage V–VI oocytes from X. laevis and injection of cRNA were described in detail elsewhere (14). The capped run-off poly(A)* cRNA transcripts from XbaI linearized cDNA templates were synthesized according to the procedures of Kirig and Melton (16). α1L cRNAs (15 mg/50 ml) were co-injected with approximately equimolar ratios of β4a (17) and α1L (18) subunit cRNAs.

Voltage Clamp Measurements—Ba2+ inward currents (Ib) through voltage-gated Ca2+ channels were measured between 2 and 7 days after injection of the oocytes (19) using the two-microelectrode voltage-clamp technique (Turbo Tec: 01C, NPI-Electronic, Germany). Endogenous Ca2+ channel currents were studied after injection of β4 and α1L subunit cRNAs. Endogenous currents were present only in a minority of the tested oocyte batches and reached levels of expression between 5 and 80 nA. Only oocytes displaying Ib were used for further analysis. Voltage recording and current-injection microelectrodes were filled with 2.8 M CsCl, 0.2 M CsOH, 10 mM EGTA, 10 mM HEPES (pH 7.4) and had resistances of 0.3–2 megohm. Oocytes were injected 20–40 min before the voltage-clamp experiments with 50 nl of a 0.1 M 1,2-bis(2-aminophenylethanesulfonic acid) (N,N,N',N'-tetraacetic acid solution to block endogenous Ca2+–activated Cl− conductance (see Ref. 20). All experiments were carried out at room temperature in bath solution with the following composition (in mM): 40 Ba(OH)2, 40 2-methyl-D-glucamine, 10 glucose (pH adjusted to 7.4 with methanesulfonylic acid). The recording chamber (150–μl total volume) was continuously perfused at a flow rate of 1 ml/min with control- or drug-containing solutions. Leakage current correction was performed by using average values of scaled leakage currents elicited by a 10-mV hyperpolarizing voltage step. The pClamp software package (version 5.51, Axon Instruments, Inc.) was used for data acquisition and analysis. Data were filtered at 1 kHz, digitized at 1 kHz, and stored on a computer hard disk.

Estimation of Conditioned I b Block by PAAs—Conditioned ("use-dependent") block of Ib by the PAAs (−)D888 and (−)D600 was measured during trains of either 100- or 800-ms test pulses. Test pulses were applied after a 3-min equilibration period in drug-containing solution at a standard frequency of 0.1 Hz. The conditioned channel block by PAAs was estimated as the inhibition of peak Ib after 12 depolarizing test pulses (Figs. 1B and 2). The holding potential was −80 mV and test potentials were applied to the peak potential of the current voltage relationship of the Ca2+–channel constructs. Because of incomplete current recovery from inactivation of Ib some chimeras displayed a decay in peak Ib during the pulse trains in the absence of drug. To estimate the peak Ib decay under control conditions we applied similar test pulses in the absence of drug, which were preceded by a 3-min rest period (see Figs. 1B and 2). The peak Ib inhibition during the first pulse after a 3-min equilibration in the drug-containing solution was defined as "resting-state dependent block." Data are given as ranges or mean ± S.E. Statistical significance of Ib block by PAA compared to current decay under control conditions was calculated according to unpaired Student’s t test.

RESULTS

PAA Effects on L-type Ca2+ Channel Currents—To investigate if Xenopus oocytes are an appropriate expression system for studying PAA effects on Ca2+ channels, we compared the PAA sensitivity of wild type α1A with the previously described L-type α1L chimeras L1 and L4 (8, 14) after expression in oocytes. The injection of α1L subunit cRNAs, together with β4 and α2β6 subunit cRNAs, resulted in expression of calcium channels with barium current (Ib) amplitudes exceeding those of endogenous currents at least 10-fold (see "Experimental Procedures").

Both L-type chimeras are sensitive to DHP Ca2+ channel agonists and antagonists and have been characterized previously (8, 14, 21). Chimeras L1 (Fig. 1A) corresponds to α1A (22) but with its NH2 terminus replaced by the respective sequence from the carp skeletal muscle α1S (black transmembrane segments and bold lines) and of the cardiac α1C (white segments and thin lines). To transfer PAA sensitivity to the α1A (sequence is indicated as gray segments and thin lines) sequence stretches from L-type channel α1L were inserted into α1S thus generating chimeras AL1 and AL21-23. Alternatively, PAA sensitivity of chimeras L1 was reduced by replacing repeat IV and the carboxyl terminus by α1S sequence (chimera AL4). B, comparison of the conditioned Ib block of the α1A subunits as depicted in A by different phenylalkylamines. The block of Ib was measured as cumulative current inhibition (in percent) during 12 depolarizing pulses (100 or 800 ms) after a 3-min incubation of the Xenopus oocytes in either 50 μM (−)D888, 100 μM (−)D600, or 100 μM (−)emepamil as indicated. Oocytes were depolarized to the peak potential of the current voltage relationship. Duration of test pulses during a train was either 100 ms (black bars) or 800 ms (gray bars). Drop in peak Ib during the pulse protocol under control conditions indicates an incomplete recovery of Ib from inactivation during the train. Bars represent the mean ± S.E. of 3–19 experiments; *, p < 0.05; **, p < 0.01.
of chimera Lₙ after 3 min of incubation with 50 μM (-)-D888 (9 ± 2%, n = 9, see Fig. 2A) was small and indistinguishable from current run-down during the corresponding period in drug-free solution (5.5 ± 1.6%, n = 10). As PAA action on L-type Ca²⁺ channels is crucially dependent on channel activation (see Ref. 23), we estimated the sensitivity of the expressed α₅ chimera for PAA as cumulative Iₚα inhibition during a pulse train (see "Experimental Procedures"). Figs. 1B and 2A illustrate the conditioned block of chimera Lₙ and Lₜ induced by 50 μM (-)-D888 or 100 μM (-)-D600. Prolongation of the test pulse duration from 100 to 800 ms substantially increased the extent of block by (-)-D888 (from 30 to 47% for Lₙ, and 26 to 45% for Lₜ; Figs. 1B and 2A). PAA-induced block of Lₙ and Lₜ was accompanied by an acceleration in current decay (Fig. 2A) which was most prominent for the slowly inactivating chimera Lₙ in the presence of drug Iₚα recovered by 61 ± 7% (mean for Lₙ, n = 6) from conditioned block during a 3-min rest at −80 mV.

The PAA (-)-emopamil exhibits 1–2 orders of magnitude lower affinity for the PAA binding domain of L-type Ca²⁺ channels than (-)-D888 and (-)-D600 (24). Unlike these PAA, (-)-emopamil (100 μM) did not induce conditioned block of Iₚα (shown for chimera Lₙ in Fig. 1B). This suggests that the observed PAA effects are mediated by specific interaction with the PAA binding domain.

PAA Effects on Ca²⁺ Channel Currents through α₅₉ Subunits—In contrast to the L-type Ca²⁺ channel α₅ chimera, PAA-induced block of Iₚα for α₅₉ was much less pronounced. Fig. 2B illustrates Iₚα recordings from an oocyte expressing α₅₉ during trains of test pulses in the absence and presence of 50 μM (-)-D888. In the absence of drug Iₚα decreased by 4 ± 1% (n = 7) during a 100-ms pulse train. This decrease in peak current amplitude was more pronounced (10 ± 4%, n = 5) after increasing the pulse length to 800 ms (Fig. 1B) and presumably resulted from incomplete recovery of Iₚα from inactivation. 50 μM (-)-D888 (Figs. 1B and 2B) or 100 μM (-)-D600 (Fig. 1B) caused a small but significant additional conditioned block of α₅₉ current during 100-ms pulse trains (corresponding to about 10% of the peak current value). Iₚα block by 50 μM (-)-D888 during a 800-ms pulse train was enhanced to 21 ± 4% (n = 5) (Fig. 1B). Taken together, these data demonstrate that Ca²⁺ channels formed by α₅₉ subunits are only weakly sensitive to PAA compared to the L-type chimeras Lₙ and Lₜ.

PAA Effects on Ca²⁺ Channel Currents through α₅₉/α₅₉ Subunits—To determine if the PAA sensitivity of L-type Ca²⁺ channels can be transferred from an L-type Ca²⁺ channel to α₅₉, we constructed a series of chimeras between α₅₉ and L-type sequence (Fig. 1A). When repeat IV and the adjacent carboxyl terminus of the PAA-sensitive chimera Lₚ were replaced by α₅₉ sequence PAA sensitivity of the resulting chimera AL₄ (Fig. 1A) was reduced to the level of the α₅₉ subunit (Fig. 1B).

AL₄ represents the first of four chimeras in which α₅₉ sequence was introduced into α₅₉ within repeats III and IV. It contains L-type sequences in the S5-S6 linkers and adjacent segments S6 in repeats III and IV (B) (Fig. 1A). A substantial fraction of Iₚα from chimera AL₁ did not recover from inactivation during the 10-s interpulse interval of the train in the absence of PAA. As shown for α₅₉ (Fig. 2B) this resulted in a decrease in Iₚα amplitude during frequent depolarizations and was more pronounced if prolonged test pulses were applied (data not shown). This prevented the analysis of conditioned block during trains of pulses longer than 100 ms. During 100 ms pulse trains the PAA sensitivity of AL₁ was comparable to constructs Lₙ and Lₜ: 50 μM (-)-D888 induced a conditioned block of 24% (n = 4) beyond the peak current decay of Iₚα observed in the absence of drug (Fig. 1B). In chimera AL₂ repeat III completely consisted of α₅₉ sequence. The observed PAA sensitivity still resembled that of AL₁ (Fig. 1B). Furthermore, neither the removal of L-type sequence on the cytoplasmic side of IVS₆ (generating chimera AL₂) nor of the IVS₅-IVS₆ linker (leading to chimera AL₂, see Figs. 1 and 2B) decreased the PAA sensitivity. Iₚα through chimera AL₂ exhibited less than 2% (n = 12) run-down and displayed the characteristic features of PAA sensitivity. Iₚα block of chimera AL₂ was less than 5% (n = 12), the fraction of Ca²⁺ channels blocked by PAA was dependent on the application of depolarizing test pulses (Iₚα was inhibited during a train of 100-ms pulses by 57 ± 6% (n = 7) in the presence of 50 μM (-)-D888 compared to 21 ± 4% (n = 12) under control conditions; Fig. 1B), and (iii) Iₚα recovered from conditioned block in the presence of 50 μM (-)-D888 by 95 ± 3% (n = 12) during a 3-min rest at −80 mV. As was the case with Lₙ and Lₜ, no frequency-dependent effect of (-)-emopamil was observed during trains of 100-ms test pulses in chimera AL₂ (Fig. 1B).

Biophysical Properties of Chimera AL₂—In 40 mM Ba²⁺ solution Iₚα of chimera AL₂ activated at a threshold of approximately −10 mV and reached peak current values between 10 and 20 mV (16 ± 1.2 mV, n = 26). The Iₚα of AL₂ had a similar threshold as AL₂ (−10 mV) and reached a peak current at 13 ± 1.4 mV (n
During a 100-ms test pulse to 10 mV, whereas only 14 elements of segment IVS6 in inactivation of membrane domain IVS6 of a skeletal muscle high-affinity PAA binding domain, we investigated if the transfer of L-type sequence into a non-L-type channel (9) did not require the introduction of other than the IVS6 sequence.

When compared to PAA block of L-type channels in various mammalian cells (23, 28, 31). As previously observed, e.g. for DHPs (8) and Ca\(^{2+}\) antagonist Ro 40-5697 (32), the effective drug concentrations for channel block after expression in Xenopus oocytes were higher than required in electrophysiological studies using mammalian cells (see Refs. 9 and 28).

The low PAA sensitivity of \(\alpha_{1A}\) suggests that additional interaction sites are provided by \(\alpha_{1A}\). Low PAA sensitivity was also observed for N-type Ca\(^{2+}\) channels (\(\alpha_{1B}\)) and for L-type channels (\(\alpha_{1C}\)) lacking the high affinity determinants for PAA sensitivity in segment IVS6 (9). Therefore, additional regions of PAA interaction may be localized in sequence stretches conserved among these \(\alpha_1\) subunits. Future studies will concentrate on the possible involvement of these regions in the interaction of Ca\(^{2+}\) channels with PAA.

As previously shown in mammalian cells, PAA block of I\(_{Ba}\) in Xenopus oocytes is also dependent on the application of depolarizing test pulses (Fig. 2). PAAAs are believed to interact selectively with the open Ca\(^{2+}\) channel conformation (28, 29) which complicates an estimation of drug association and dissociation rate constants (30). Inhibition of open Ca\(^{2+}\) channels by PAAAs is also supported by the observed acceleration of I\(_{Ba}\) decay in chimeras L\(_1\) and L\(_2\) in the presence of PAAAs (Fig. 2A).

The mechanism of Ca\(^{2+}\) channel block in Xenopus oocytes appeared to be similar to PAA block of L-type channels in various mammalian cells (23, 28, 31). As previously observed, e.g. for DHPs (8) and Ca\(^{2+}\) antagonist Ro 40-5697 (32), the effective drug concentrations for channel block after expression in Xenopus oocytes were higher than required in electrophysiological studies using mammalian cells (see Refs. 9 and 28).

An additional finding of our study was that introducing L-type sequence into \(\alpha_{1A}\) did not only enhance PAA sensitivity but also changed the inactivation kinetics of I\(_{Ba}\). Interestingly, the implantation of the transmembrane segment IVS6 from the slowly inactivating L-type chimera L\(_5\) into \(\alpha_{1A}\) did not result in a transfer of the slower L-type inactivation kinetics into the faster inactivating \(\alpha_{1A}\). Unexpectedly, this sequence substitution accelerated the inactivation kinetics compared to that of \(\alpha_{1A}\). This finding gives an example where kinetic properties of Ca\(^{2+}\) channels are not simply transposed by swapping corresponding sequences between different \(\alpha_1\) subunits as was previously shown for structural elements of repeat I as well as III and IV (14, 33, 34). Our observation, that inactivation of \(\alpha_{1A}\) Ca\(^{2+}\) channels is accelerated by changes in the amino acid sequence of segment IVS6 indicates a possible involvement of this region in inactivation gating in addition to its role in forming the PAA interaction domain.

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