Control of Ion Conduction in L-type Ca$^{2+}$ Channels by the Concerted Action of S5–6 Regions

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ABSTRACT Voltage-gated L-type Ca$^{2+}$ channels from cardiac ($\alpha_{1C}$) and skeletal ($\alpha_{1S}$) muscle differ from one another in ion selectivity and permeation properties, including unitary conductance. In 110 mM Ba$^{2+}$, unitary conductance of $\alpha_{1S}$ is approximately half that of $\alpha_{1C}$. As a step toward understanding the mechanism of rapid ion flux through these highly selective ion channels, we used chimeras constructed between $\alpha_{1C}$ and $\alpha_{1S}$ to identify structural features responsible for the difference in conductance. Combined replacement of the four pore-lining P-loops in $\alpha_{1C}$ with P-loops from $\alpha_{1S}$ reduced unitary conductance to a value intermediate between those of the two parent channels. Combined replacement of four larger regions that include sequences flanking the P-loops (S5 and S6 segments along with the P-loop-containing linker between these segments (S5–6)) conferred $\alpha_{1S}$-like conductance on $\alpha_{1C}$. Likewise, substitution of the four S5–6 regions of $\alpha_{1C}$ into $\alpha_{1S}$ conferred $\alpha_{1C}$-like conductance on $\alpha_{1S}$. These results indicate that, comparing $\alpha_{1C}$ with $\alpha_{1S}$, the differences in structure that are responsible for the difference in ion conduction are housed within the S5–6 regions. Moreover, the pattern of unitary conductance values obtained for chimeras in which a single P-loop or single S5–6 region was replaced suggest a concerted action of pore-lining regions in the control of ion conduction.

INTRODUCTION

The voltage-gated L-type Ca$^{2+}$ channels from cardiac muscle ($\alpha_{1C}$) and skeletal muscle ($\alpha_{1S}$), though closely related in structure, differ from one another in a number of important functional ways. The high degree of sequence conservation between $\alpha_{1C}$ and $\alpha_{1S}$ has facilitated structure-function analysis for these channels. For example, structural elements regulating channel activation (Nakai et al., 1994) and mediating excitation-contraction coupling (Tanabe et al., 1990) have been identified using strategies that rely on this sequence similarity.

A substantial body of work has also been directed toward understanding the structural basis of ion selectivity in Ca$^{2+}$ channels. Earlier work had led to the conclusion that selectivity in ion transport was mediated by preferential binding of Ca$^{2+}$ over Na$^+$, the two principal competitors for transport through Ca$^{2+}$ channels under physiological conditions (Almers and McCleskey, 1984; Hess and Tsien, 1984). More recent work using site-directed mutagenesis has identified amino acid residues that form the selectivity filter that binds Ca$^{2+}$ in the pore (Tang et al., 1993; Yang et al., 1993; Ellinor et al., 1995; Cibulsky and Sather, 2000; Koch et al., 2000; Wu et al., 2000).

Despite the fact that tight binding of Ca$^{2+}$ is essential for selection against nonpreferred permeants such as Na$^+$, the observed rate of Ca$^{2+}$ conduction through the pore nonetheless requires fast Ca$^{2+}$ unbinding and transit. For highly selective ion channels generally, no simple relationship between selectivity and conduction exists. Thus, for example, all voltage-gated K$^+$ channels are highly selective for K$^+$, yet their unitary conductance values range over two orders of magnitude (Hille, 2001). Likewise, voltage-gated Ca$^{2+}$ channels are all highly selective for Ca$^{2+}$, and though less extreme than in the case of K$^+$ channels, different kinds of Ca$^{2+}$ channels differ among themselves in unitary conductance. In particular, the unitary conductance of $\alpha_{1C}$ Ca$^{2+}$ channels is roughly double that of $\alpha_{1S}$ channels.

Regions of Ca$^{2+}$ channels that may be involved in specifying ion conduction include the P-loops, four pore-lining structures in each channel molecule that together contribute to formation of the selectivity filter. The P-loops are thought to line the extracellular portion of the pore in members of the voltage-gated ion channel family, which includes Ca$^{2+}$ and K$^+$ channels (MacKinnon, 1995). Evidence provided by the crystal structure of a bacterial K$^+$ channel, an ancestor of both voltage-gated K$^+$ channels and Ca$^{2+}$ channels, has strengthened this view (Doyle et al., 1998). This bacterial K$^+$ channel structure also shows that transmembrane segments homologous to S6 contribute to the intracellular portion of the pore, the portion that opens into the cytosol; the S6 segment appears to help form the intracellular portion of the pore in voltage-gated K$^+$ channels of higher organisms as well (del Camino et al., 2000). Consonant with this basic structural model, P-loops have been implicated in the control of unitary conductance in many members of the family of voltage-gated ion channels. In some cases, the P-loop or the entire S5–S6 linker that encompasses the P-loop has been suggested as the sole determinant of unitary conductance (Hartmann et al., 1991; Goulding et al., 1993; Yatani et al., 1994; Repunette et al., 1999). In other cases, flanking S5 and S6 segments were
additionally shown to influence conduction (Aiyar et al., 1994; Sheih and Kirsch, 1994; Immke et al., 1998). Sequences even farther from the P-loop, including the cytosolically-disposed S4–S5 linker and C-terminal tail, have been implicated as determinants of unitary conductance (Isacoff et al., 1991; Slesinger et al., 1993; Choe et al., 2000). In the present work, we have used a systematic set of chimeras constructed between the α1C and α1S Ca^{2+} channel isofoms to identify domains that determine these channel’s characteristic ion transport rates. The aim of work such as this is to understand how ion channels that are very similar in ion selectivity can differ significantly in rate of ion transport.

MATERIALS AND METHODS

Ca^{2+} channel chimeras

Three kinds of chimeras were constructed between cDNAs encoding the α1C (Mikami et al., 1989; EMBL/GenBank accession number X15539) and α1S (Tanabe et al., 1987; Kim et al., 1990; accession number X05921) L-type Ca^{2+} channel subunits. In the first kind of chimeras, P-loop sequence was substituted from α1S into α1C. Based on the better-known structure of P-loops in voltage-gated K+ channels (Yellen et al., 1991), P-loops of Ca^{2+} channels were, in this work, considered to be 18-residue sequences within the linker between the S5 and S6 transmembrane segments. However, in motif IV, 20-residue P-loop sequences were substituted to include one additional difference in sequence between the two parent channels. Numbering the EEEE locus glutamates as position 0 in each motif, the substituted P-loop regions comprised residue positions −13 to +4, amino to carboxy, or in the case of motif IV, positions −13 to +6. In α1C, the P-loop segments for motifs I–IV were bounded by residues G279/D296, P601/P602, P723/S740, L1132/E1149, and P1433/M1452. In α1S, the P-loop sequences were bounded by residues I301/A380, L1001/Q1018, and P1310/L1329; for α1C, the P-loops were bounded by A380/D397, P723/S740, L1132/E1149, and P1433/M1452. In the other two kinds of chimeras, the entire sequence from the beginning of the S5 transmembrane segment through the end of the S6 segment (S5–6) was transferred from α1C to α1S, and vice versa. Hydropathy plot analysis has identified the S5 (20 residues) and S6 (25 residues) transmembrane segments in L-type Ca^{2+} channels (Tanabe et al., 1987), and the S5–6 regions of the four motifs range 100–136 residues in length. In α1S, the S5–6 segments for motifs I–IV were bounded by residues I599/S334, L651/V661, I931/I1065, and V1270/I1384; for α1C, the S5–6 segments were bounded by I301/S435, L684/V783, I1106/K1196, and V1393/M1506. The quadruple chimeras and the parent α1C and α1S subunits are diagrammed in Fig. 1. The single-motif chimeras for P-loop and S5–6 regions are not illustrated.

Chimeras were constructed using polymerase chain reaction (PCR) strategies. All PCR reactions were carried out using the Expand High Fidelity PCR kit (Boehringer-Mannheim, Indianapolis, IN). For construction of α1C-based chimeras bearing P-loop sequence from α1S, a four-primer strategy was used. Sense and antisense oligonucleotide fusion primers (primers 1 and 2; 51-mers) consisted of 32 bases of α1C P-loop sequence flanked on one side by −19 bases that were complementary to α1C sequence. Single fusion primers did not span the entire P-loop sequence for a given motif, but their lengths were such that the 5′ ends (α1S sequence) of sense and antisense fusion primers overlapped by 10 complementary bases. In two separate steps of PCR, either sense or antisense fusion primers were used in combination with a downstream or upstream flanking primer that was complementary to α1C sequence (primers 3 and 4; 18mers). These reactions yielded a 5′ and a 3′ fusion fragment, which were then combined and allowed to anneal to one another by virtue of the 10-base complementary sequence. In a final PCR step, the annealed fragments were extended for five thermocycles, then the two flanking primers from the first rounds of PCR (primers 3 and 4) were added to the reaction mix, and the product was amplified in 15 additional thermocycles. The final PCR product and the vector bearing α1C (pCARDHE) were subsequently digested with a pair of motif-specific restriction enzymes and gel-purified. Each P-loop chimera was completed by ligating the PCR cassette (396–659 bp, depending upon motif) into pCARDHE. α1C-based chimeras bearing single P-loops from α1S were referred to as C1S, C1S, C1S, and C1S; the subscripted Roman numeral indicates the motif within which the P-loop exchange was made. These individual P-loop chimeras were combined to produce an α1C-based chimera in which all four P-loops were replaced by their counterparts in α1S, and this construct is denoted CQuadS.

Chimeras in which S5–6 sequence from α1S was substituted into α1C, for each of the four single-motif chimeras, denoted as C1SS5–6S, C1SS5–6S, C1SS5–6S, and C1SS5–6S. A four-motif chimera produced by combining the four S5–6 single-motif chimeras is referred to as CQuadS. The S5–6 single-motif chimeras were constructed using a 5-primer strategy. In the first round of PCR, α1C S5–6 sequence fused at either end to a short stretch of α1C sequence was produced using an α1S template and a pair of fusion primers (typically 39-mers; primers 1 and 2) that included 5′ overhangs (24 bases in length) corresponding to α1C sequence located either immediately upstream of S5 or downstream of S6. In a second round of PCR, the gel-purified product of the first round, a primer complementary to upstream α1C sequence (primer 3; 30-mer), primer 1, and an α1C template were used to amplify the α1C sequence upstream of S5. To avoid amplifying nonchimeric, WT α1C in the final round of PCR, primer 3 included a 15-base, 5′-terminal, non-sense sequence that was complementary to neither α1C nor α1S. Primer 1 was added to this second-round reaction only after completing five thermocycles. In the third and final round of PCR, the gel-purified second-round product, a downstream primer complementary to α1C sequence (primer 4; 18-mer), an upstream primer complementary to the nonsense sequence of primer 3 (primer 5; 15-mer), and α1C template were used to amplify α1C sequence downstream of S6. Primer 5 was added to the reaction.
mix after completing five thermocycles. The final PCR product and the pCARDHE vector were digested with a pair of motif-specific restriction enzymes and gel purified. Each S5–6 chimera was completed by ligating the PCR cassette (600–1260 bp) into pCARDHE.

A chimera in which the S5–6 sequences of the four motifs of α1S were replaced by the corresponding sequences in α1C is referred to as SQuadS5–6C. To make this chimera, the SacII–BglII fragment of α1S, corresponding to most of the coding region, was first subcloned into pGEMHE (Liman et al., 1992) to make use of advantageous restriction sites in this construct. The strategy used to construct the SQuadS5–6C chimera was conceptually similar to that described for the CQuadS5–6C chimera.

DNA sequences for all chimeras were confirmed by restriction digests and dyeoxy chain termination sequencing of both strands of all PCR-amplified regions.

**Ca²⁺ channel expression in Xenopus oocytes**

cRNAs encoding α1 subunits were synthesized using vectors for α1C- and α1S-based constructs that yielded high functional expression in Xenopus oocytes. Before construction of α1C-based chimeras, the α1C insert was subcloned into a modified version of pGEMHE, a vector that incorporates the 5′ and 3′ untranslated regions of the Xenopus β-globin gene (Liman et al., 1992). In the subcloning process, several in-frame start- and stop-codons in the 5′ untranslated region of the original α1C clone were deleted, and a Kozak consensus sequence for initiation of translation was inserted immediately upstream of the true α1C start codon. The resulting high-expression construct, termed pCARDHE, was used in the fabrication of all α1C-based chimeras.

To enhance expression of α1S in Xenopus oocytes, the 3′ coding region was truncated (Ren and Hall, 1997; Morrill and Cannon, 2000). One α1S construct was truncated after the codon specifying amino acid 1662 (Beam et al., 1991; Ren and Hall, 1997). When subcloned into pGEMHE, neither the full-length α1S cDNA nor the two 3′-truncated forms of α1S yielded highly-expressed cRNAs (~100–500 nA whole-oocyte Ba²⁺ currents when coexpressed with α2δ1β and β1b). When subcloned into pAGA2 (Ren and Hall, 1997), the version of α1S truncated after codon 1698 produced significantly larger currents. Therefore, after the SQuadS5–6C chimera was constructed in pGEMHE, the SacII–BglII fragment of the chimera was subcloned into the pAGA2 vector to enhance chimera expression.

To further enhance functional expression of Ca²⁺ channels, cDNAs for the ancillary subunits α2δ1 (rabbit; Mikami et al., 1989; the 3′ noncoding region was truncated), β2d (rabbit; Hullin et al., 1992; EMBL/GenBank accession number X64298), and β1b (rat; Pragnell et al., 1991; accession number X61394) were subcloned into the modified version of pGEMHE that was used for α1C. Ca²⁺ channel subunit cRNAs were transcribed in vitro using the mMESSAGE mACHINE T7 RNA synthesis kit (Ambion, Austin, TX). Equimolar concentrations of α1S, α2S- and β-subunit cRNAs were injected into Xenopus laevis oocytes. α1C- and α1S-based chimeras were coexpressed with α2δ1β and β1b, whereas α1S- and α1C-based chimeras were coexpressed with α2δ1α and β1b, except where noted. According to the most recently proposed systematic nomenclature, the subunit makeup of these channels is written Ca1.1αβδ2α2δ1β1b for α1C-based channels, and Ca1.1αβδ2α2δ1α for α1S-based channels (Ertel et al., 2000). Oocytes were dissociated from ovarian tissue by shaking in a Ca²⁺-free OR-2 solution (in mM: 82.5 NaCl, 2 KCl, 1 MgCl₂, 5 n-(2-hydroxyethyl)piperazine-n'-2-ethanesulfonic acid) (HEPES), pH 7.5 with NaOH containing 2 mg/ml collagenase B (Boehringer-Mannheim) for 60–90 min. Injected oocytes were incubated in ND-96 solution (in mM: 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.6 with NaOH supplemented with 2.5 mM sodium pyruvate (Sigma, St. Louis, MO), 100 U/ml penicillin (Sigma) and 0.1 mg/ml streptomycin (Sigma). Injected oocytes were maintained at 18°C, and were studied 3–14 days postinjection.

**Two-electrode voltage clamp recording**

Whole-oocyte currents were recorded as described previously (Sather et al., 1993). The bath was continuously perfused with a Cl⁻-free, nominally 40 mM Ba²⁺ solution (mM: 40 Ba(OH)₂, 52 TEA-OH, 5 HEPES, pH 7.4 with methanesulfonic acid). Owing to precipitation, Ba²⁺ concentration was substantially lower than the nominal value, and was measured as ~10 mM (Williamson and Sather, 1999). To test the Mg²⁺ permeability of α1S channels, 40 nM or 100 mM Mg(OH)₂ solutions were used (in mM: 40 Mg(OH)₂, 52 TEA-OH, 5 HEPES, pH 7.4 with methanesulfonic acid, or 100 mM Mg(OH)₂, 5 HEPES, pH 7.4 with methanesulfonic acid). Currents were measured with a model OC-725C amplifier (Warner Instruments), filtered at 500 Hz (4-pole Bessel filter, Warner Instruments) and sampled at 1 kHz. Data were acquired and analyzed using software custom-written in AxoBasic (Axon Instruments, Foster City, CA). For voltage pulses of size P, peak currents were subtracted using the average of 10 pulses to −P/4. For the Cd²⁺ block experiments, a 1 mM CdCl₂ stock solution was diluted to a final concentration of 1 μM in the Ba²⁺ solution.

**Single-channel recording**

The vitelline membrane was manually stripped from an oocyte after soaking in a hyperosmotic solution (Sather et al., 1993). Single-channel currents were recorded in cell-attached patches while the stripped oocyte was bathed in a high K⁺ solution that zeroed the membrane potential (in mM: 100 KCl, 10 HEPES, 10 ethylene glycol-bis(beta-aminoethy1 ether)-n,n,n',n'-tetraacetic acid) (EGTA), pH 7.4 with KOH). The L-type Ca²⁺ channel agonist PPL 64176 (RBI, Natick, MA) was included in the bath solution at a concentration of 2 μM to prolong channel openings. Pipettes were pulled from borosilicate glass (Warner Instruments, Hamden, CT), coated with Sylgard (Dow Corning, Midland, MI) and heat-polished. Pipettes typically had resistances of 25–40 MΩ when filled with the recording solution of (in mM) 110 BaCl₂, 10 HEPES (pH 7.4 with TEA-OH). Single-channel records were obtained using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). The amplifier’s internal filter was set to 10 kHz and an external filter (8-pole Bessel filter, Frequency Devices, Haverhill, MA) was set to 2 kHz, yielding a ~3 dB frequency for the cascaded filters of 1.96 kHz. The data were sampled at 10 kHz using a Digidata 1200A (Axon Instruments) A/D converter and Pulse software (HEKA, distributed by Instutech Corp., Great Neck, NY). Single-channel current amplitudes were determined by cursor analysis of long-duration openings (Pulse, HEKA).

**RESULTS**

In two-electrode voltage-clamp recordings, channels containing α1 subunits of predominantly α1S-based or α1C-based origin carried currents of roughly similar size, with peak inward currents of typically ~1–3 μA in the 40 mM Ba²⁺ solution. The resulting similarity of voltage clamp quality and of single-channel event frequency facilitated comparisons among channel constructs.

The chimeric constructs were designed to study ion permeation. However, as an indicator of the specificity in effect of the structural manipulations, we examined whether channel gating might have been altered in the chimeras. We found that wild-type and chimeric channels containing α1C-based subunits exhibited the fast activation kinetics expected for α1C channels, whereas channels containing α1S-based subunits exhibited the slow activation kinetics characteristic of the skeletal muscle Ca²⁺ channel (Fig. 2A) (Tanabe et al.,...
During a test pulse to +20 mV, \( \tau_{\text{act}} \) for WT \( \alpha_{1C} \) channels was 3.2 \( \pm \) 0.1 ms (mean \( \pm \) SE; \( n = 6 \)), whereas \( \tau_{\text{act}} \) for \( \alpha_{1S} \) channels was 21.8 \( \pm \) 1.0 ms (\( n = 6 \)). The \( \alpha_{1C} \)-based chimeras \( \text{CQuadPS} \) (\( \tau_{\text{act}} = 2.4 \pm 0.3 \) ms, \( n = 6 \)) and \( \text{CQuadSS6S5} \) (\( \tau_{\text{act}} = 1.3 \pm 0.1 \) ms, \( n = 6 \)) activated with time courses like that of wild-type \( \alpha_{1C} \), and the \( \alpha_{1S} \)-based chimera \( \text{SQuadSS5} \) activated with a time course like that of \( \alpha_{1S} \) (21.6 \( \pm \) 3.7 ms, \( n = 6 \)). Thus as judged by the general similarity of chimeras to their parents in regard to activation gating, these manipulations of pore structure appear to have had restricted effects on the behavior of the channels.

### Selective permeability properties of wild-type and chimeric channels

In contrast to the lack of effect of altered pore structure on activation gating, indices of ion permeability were significantly affected by the structural alterations. Reversal potentials for whole-oocyte currents in 40 mM Ba\(^{2+} \) (Fig. 2 B) were modestly different between wild-type \( \alpha_{1C} \) (\( E_{\text{rev}} = 73.2 \pm 0.9 \) mV, \( n = 12 \)) and \( \alpha_{1S} \) (\( E_{\text{rev}} = 67.7 \pm 1.0 \) mV, \( n = 15 \)). Each of the three quadruple chimeras exhibited reversal potentials that were less positive than for either of the wild-type channels, with \( \text{SQuadSS5} \) being the least selective for Ba\(^{2+} \) (\( E_{\text{rev}} = 46.3 \pm 1.7 \) mV, \( n = 9 \), for \( \text{SQuadSS6S5} \); 61.1 \( \pm \) 2.1 mV, \( n = 6 \), for \( \text{CQuadSS6S5} \); 63.9 \( \pm \) 1.5 mV, \( n = 8 \), for \( \text{CQuadSS6S5} \)). The fact that preference for Ba\(^{2+} \) over K\(^+ \) was reduced in all three chimeras relative to either parent channel suggests that interactions between the transferred sequences and the bulk of the channel protein were different from the corresponding interactions within the parent channels, with the implication that these specific interactions are important in the normal high selectivity of calcium channels. In addition, the observation that \( E_{\text{rev}} \) was reduced to a greater extent in the \( \alpha_{1S} \)-based chimera than in the \( \alpha_{1C} \)-based chimeras suggests that structural features specifying this measure of ion selectivity are different between \( \alpha_{1C} \) and \( \alpha_{1S} \).

Percent block of Ba\(^{2+} \) current by 1 \( \mu \)M Cd\(^{2+} \) (Fig. 2 C) was also different between wild-type \( \alpha_{1C} \) (56.9 \( \pm \) 3.2\%, \( n = 4 \)) and \( \alpha_{1S} \) (68.9 \( \pm \) 6.7\%, \( n = 3 \)). Block of \( \text{CQuadSS5} \) (62.4 \( \pm \) 2.2\%, \( n = 10 \)) was intermediate between that of the two wild-type channels and block of \( \text{SQuadSS6S5} \) (78.4 \( \pm \) 0.7\%, \( n = 6 \)) was somewhat greater than that of \( \alpha_{1S} \). \( \text{SQuadSS6S5} \), however, was significantly more sensitive to Cd\(^{2+} \) block than was either parent (96.0 \( \pm \) 2.5\%, \( n = 6 \)). Based on the 1:1 binding that describes Cd\(^{2+} \) block of Cd\(^{2+} \) channels, these percent block values correspond to calculated half-block (\( IC_{50} \)) values of 757 nM and 451 nM for \( \alpha_{1C} \) and \( \alpha_{1S} \); to 603 nM and 276 nM for the \( \text{CQuadSS5} \) and \( \text{SQuadSS6S5} \) chimeras; and to 42 nM for the \( \text{SQuadSS6S5} \) chimera. Thus in all three cases, chimeric substitution increased the channel’s affinity for Cd\(^{2+} \) relative to the parents. This systematic enhancement of Cd\(^{2+} \) affinity in chimeras relative to the parent channels suggests that, as for the reversal potential measurements, interactions between the transferred amino acids are important in determining the degree of selectivity for Ca\(^{2+} \).
acid sequence and the bulk of the channel protein are likely to be important in determining the structure and selectivity behavior of the pore.

Although $\text{Cd}^{2+}$ block of $\text{Ba}^{2+}$ current clearly differed between $\alpha_{1C}$ and $\alpha_{1S}$, the differences were not so large that chimeras could be readily used to identify pore features responsible for differences in this property of the parent channels. And because $\text{Cd}^{2+}$ sensitivity of the chimeras did not fall between that of the parents, $\text{Cd}^{2+}$ block of $\text{Ba}^{2+}$ current was not used for comparative structure-function analysis of $\alpha_{1C}$ and $\alpha_{1S}$ channels.

Previous work on native $\text{Ca}^{2+}$ channels in skeletal muscle indicated that monovalent cation current carried by $\alpha_{1S}$ would be orders-of-magnitude less sensitive to block by $\text{Cd}^{2+}$ than monovalent current carried by $\alpha_{1C}$ (compare Almers et al., 1984 with Yang et al., 1993), but we found no large difference between $\alpha_{1S}$ and $\alpha_{1C}$ in potency of $\text{Cd}^{2+}$ block of monovalent current: $\text{Cd}^{2+}$ blocked current carried by 100 mM $\text{Li}^{+}$ through these two channels with roughly similar potency when the channels were expressed in oocytes (data not shown). It has also been reported that native skeletal muscle L-type $\text{Ca}^{2+}$ channels can carry $\text{Mg}^{2+}$ current (Almers and Palade, 1981; McCleskey and Almers, 1985), in contrast to the case for cardiac L-type channels (Hess et al., 1986; Lansman et al., 1986). For wild-type $\alpha_{1S}$ channels expressed in oocytes, however, we were unable to detect inward $\text{Mg}^{2+}$ (40 mM or 100 mM) current. Thus because $\alpha_{1C}$ and $\alpha_{1S}$ differed only modestly or not at all in reversal potential, $\text{Cd}^{2+}$ block, and $\text{Mg}^{2+}$ permeability, we have focused our investigation of structural determinants of $\text{Ca}^{2+}$ channel permeation upon the robust difference in unitary conductance between $\alpha_{1C}$ and $\alpha_{1S}$ channels, as described below.

**Unitary conductance: P-loop transfer from $\alpha_{1S}$ to $\alpha_{1C}$**

Unitary current-voltage relationships in 110 mM $\text{Ba}^{2+}$ for $\alpha_{1C}$ and $\alpha_{1S}$ are plotted in Fig. 3. The relationships for both wild-type channels as well as all of the chimeras are slightly curvilinear. They were, however, reasonably well fit with linear regressions. We used such fits to estimate unitary conductance (slope of the fit to data over the range $-20$ to $+20$ mV), which allows comparisons to be made with work by others. $\alpha_{1C}$ had a unitary conductance of 28.9 pS, which is in close agreement with the value of 29.1 pS measured from ventricular myocytes by Yue and Marban (1990). Conductance for $\alpha_{1S}$ was 16.3 pS, which is also similar to that measured from native channels, in this case, in skeletal myotubes (14.3 pS; Dirksen et al., 1997). The small difference between the two values for $\alpha_{1S}$ may be due to the difference in voltage range over which unitary current amplitude was measured: Dirksen et al. (1997) used $-20$ to $+20$ mV, whereas we used $-100$ to $+20$ mV, and curvature in the current-voltage relationship results in steepening of the

![FIGURE 3 Unitary current-voltage relationships for $\alpha_{1C}$, $\alpha_{1S}$, and mutants in which P-loop sequence from $\alpha_{1S}$ was substituted into $\alpha_{1C}$ and chimeras were coexpressed in oocytes with $\beta_{1\alpha}$ and $\beta_{1\beta}$-subunits, whereas $\alpha_{1S}$ was coexpressed with $\alpha_{1C}$ and $\beta_{1\alpha}$-subunits. Currents in cell-attached patches were measured with 110 mM $\text{Ba}^{2+}$ in the pipette. From the holding potential of $-80$ mV, a 25 or 50 ms prepulse of $+20$ to $+80$ mV was usually applied immediately before the 300 ms test pulse, with no interval between the prepulse and test pulse. The prepulse facilitated channel activation, and $\alpha_{1S}$ generally required stronger facilitation ($+80$ mV for 50 ms). Mean unitary current amplitude $\pm$ SE ($n = 3$–7 patches at each potential) is plotted versus test pulse voltage for $\alpha_{1S}$ (O), $\alpha_{1C}$ (■), CIPS (□), CIIPS (△), CIIIPS (□), CIVPS (□), and CIQuadPS (□). Solid lines represent linear regression fits to the data. Representative single-channel currents recorded during a test pulse to $-40$ mV are displayed in the lower part of the figure.](image-url)
slope at more negative voltages. The transfers of P-loop sequences from $\alpha_{1S}$ to $\alpha_{1C}$, one motif at a time, each had a small effect on unitary conductance (Fig. 3). P-loop replacement in motif II reduced $\alpha_{1C}$ conductance to 27.2 pS ($C_{\text{II P}}S$), in motif III to 27.4 pS ($C_{\text{III P}}S$), in motif IV to 27.8 pS ($C_{\text{IV P}}S$), and in motif I to 28.6 pS ($C_{\text{I P}}S$). When all four P-loops were transferred together, conductance was reduced to a level intermediate between those for $\alpha_{1C}$ and $\alpha_{1S}$ ($C_{\text{Quad P}}S$; 22.9 pS). This observation, that replacement of all four $\alpha_{1C}$ P-loops with the corresponding $\alpha_{1S}$ P-loops did not fully transfer an $\alpha_{1S}$-like conductance to $\alpha_{1C}$, suggests that additional parts of the channel influence unitary conductance.

**Unitary conductance: S5–6 transfer from $\alpha_{1S}$ to $\alpha_{1C}$**

For voltage-gated K$^+$ channels, evolutionary relatives of voltage-gated Ca$^{2+}$ channels, structure-function studies have suggested that the cytoplasmic portion of the S6, and perhaps S5, transmembrane segments may line part of the inner pore (Choi et al., 1993; Aiyar et al., 1994; Lopez et al., 1994; Shieh and Kirsch, 1994; Taglialatela et al., 1994; Liu et al., 1997; del Camino et al., 2000). Structure-function studies have also implicated the intracellular loop between S4 and S5 in formation of the innermost part of the K$^+$ channel pore (Isacoff et al., 1991; Slesinger et al., 1993). In voltage-gated Ca$^{2+}$ channels, evidence that S6 amino acids are critical for binding of pore-blocking phenylalkylamines indicates that S6 may form part of the inner pore in these channels as well (Streissnig et al., 1990; Hockerman et al., 1997). We therefore examined the role in ion conduction of the S5–6 region, which is composed of S5 and S6 segments and the entire sequence connecting S5 and S6, including the P-loop.

The size of the effect of transfer of S5–6 from $\alpha_{1S}$ to $\alpha_{1C}$ was motif-specific (Fig. 4). Replacement of S5–6 in motif I or in motif II had larger effects, lowering unitary conductance from the wild-type $\alpha_{1C}$ value of 28.9 pS to 24.4 pS in the CIS5–6S chimera or to 24.9 pS in the CIIS5–6S chimera. Transfer of S5–6 in either motif III or IV had almost negligible effect on unitary conductance (28.3 pS in $C_{\text{III S5–6 S}}$ and 30.0 pS in $C_{\text{IV S5–6 S}}$). The effect of single motif S5–6 transfers was in no case as large as the combined transfer of all four P-loops ($C_{\text{Quad P}}S$). However, replacement of all four S5–6 regions in $\alpha_{1C}$ produced a channel with an $\alpha_{1S}$-like conductance: in fact, the conductance of $C_{\text{Quad S5–6 S}}$ (14.1 pS) was slightly smaller than that of wild-type $\alpha_{1S}$ (16.3 pS). The similarity in conductance between wild-type $\alpha_{1S}$ and the $C_{\text{Quad S5–6 S}}$ chimera suggests that, for wild-type $\alpha_{1C}$ versus wild-type $\alpha_{1S}$, the differences in pore structure that are responsible for differences in unitary conductance are contained within the S5–6 regions.

Based on the results of previous work (Dirksen et al., 1997), our finding that the $C_{\text{I S5–6 S}}$ chimera did not exhibit an $\alpha_{1S}$-like conductance was unexpected. Dirksen and colleagues (1997) had found that the makeup of the region linking S5 with S6 in motif I, a sequence that formed part of the swapped region in our $C_{\text{I S5–6 S}}$ chimera, was largely responsible for the difference in unitary conductance be-
tween $\alpha_{1C}$ and $\alpha_{1S}$. Among potential explanations for the contrasting findings, evidence that channel $\alpha_{2\delta}$- and $\beta$-subunits may influence unitary conductance (Meir and Dolphin, 1998) raised the possibility that our result with CIS5–6S might be attributable to its ancillary subunits. In the work reported here, we used a skeletal muscle $\alpha_{2\delta}$ isoform, similar to the experimental situation in the work by Dirksen and colleagues (1997). However, we used a $\beta_{2b}$-subunit in the work described above, in contrast to Dirksen and colleagues’ reliance on the skeletal muscle $\beta_{1a}$- and $\beta_{1b}$-subunits (Ren and Hall, 1997). We therefore re-examined the unitary conductance of $\alpha_{1C}$ and $\alpha_{1C}$-based chimeras, but with a skeletal muscle $\beta$-subunit coexpressed in place of $\beta_{2b}$. When coexpressed with $\beta_{1b}$, CIS5–6S had a unitary conductance (24.9 pS) that was little changed from its conductance when coexpressed with $\beta_{2b}$ (24.4 pS). Nor was unitary current at $/C255$ 0 mV different when CIS5–6S was expressed with $\beta_{1b}$ ($/C255$ 3.16 ± 0.03 pA, $n = 4$) versus $\beta_{2b}$ ($/C255$ 3.04 ± 0.04 pA, $n = 5$). Unitary current amplitudes for CQuadS5–6S and wild-type $\alpha_{1C}$ were also unchanged by coexpression with $\beta_{1b}$. Thus under these conditions, $\beta$-subunit isoform does not appear to modulate the effects of transferred S5–6 sequences on unitary conductance.

**Reciprocity of chimeric effects on unitary conductance: S5–6 transfer from $\alpha_{1C}$ to $\alpha_{1S}$**

The diminishment of unitary conductance produced by chimeric manipulation of the $\alpha_{1C}$ pore can be interpreted in competing ways. It might reflect the straightforward transfer of $\alpha_{1S}$-like ion transport behavior along with $\alpha_{1S}$ pore sequence, or it might arise from incompatibility of the transferred $\alpha_{1S}$ sequence with the host $\alpha_{1C}$ sequence, resulting in misfolding in the pore region and retarded ion flux. To discriminate between these alternatives, we examined the unitary conductance of an $\alpha_{1S}$-based chimera in which the four S5–6 regions were replaced with the corresponding sequences from $\alpha_{1C}$. For this SQuadS5–6C chimera, complementary to CQuadS5–6S, we specifically tested whether transfer of $\alpha_{1C}$ sequence into the $\alpha_{1S}$ host would yield a chimera with $\alpha_{1C}$-like unitary conductance. Indeed, as illustrated in Fig. 5, the unitary conductance of the SQuadS5–6C (30.0 pS) chimera closely approximated that of the wild-type $\alpha_{1C}$ channel.

**DISCUSSION**

Our results provide evidence that the S5–6 regions, composed of transmembrane segment S5, the entire S5–S6 linker and transmembrane segment S6, contain the structural features that are responsible for the difference in unitary conductance between $\alpha_{1C}$ and $\alpha_{1S}$ L-type Ca$^{2+}$ channels. The combination of the four P-loops, which represents a subset of the S5–6 regions, does not fully determine ion transport rate. Rather, the S5–6 regions from at least two motifs, and possibly all four, are required to specify the rate of ion transport through these channels. The reciprocal nature of the effects on ion conduction of the quadruple S5–6 swaps in $\alpha_{1C}$ and $\alpha_{1S}$ strengthens the conclusion that no other regions account for the characteristic ion transport rates of these L-channels.
S5–6 regions control ion flux through α1C and α1S Ca\(^{2+}\) channels

Unitary conductance and unitary current results for all the chimeras studied are compared in Fig. 6. The results are scaled relative to the normalized difference between α1C and α1S in either conductance (Fig. 6A) or current (Fig. 6B). Dotted lines mark values for α1C (upper level in both panels) and α1S (lower level in both panels). In general, the pattern of results is similar for unitary conductance and unitary current. Thus whether comparing conductance or current results for the quadruple chimeras (black or white bars), the quadruple P-loop substitution shifted the ion transport rate only about halfway toward the donor rate whereas quadruple S5–6 substitution more or less completely transferred the ion transport rate of the donor. Roles for non-P-loop regions in controlling ion conduction have previously been suggested for voltage-gated K\(^+\) channels (Lopez et al., 1994; Aiyar et al., 1994; Shieh and Kirsch, 1994; Tagliafera et al., 1994; Immke et al., 1998) and for inward rectifier K\(^+\) channels (Choe et al., 2000), and the full S5–6 region has been specifically implicated in cyclic nucleotide-gated channels (Siefert et al., 1999).

In comparing the results for individual motifs, three points emerge. First, different motifs are differentially important in determining ion transport rate. Although P-loop transfers produce roughly similar, small changes in ion transport rate, S5–6 transfers clearly are distinct from one another in the size of their effects. Thus among the S5–6 chimeras, transfers in motifs I and II produced the largest changes whereas transfer in motifs III and IV had lesser effects. Regarding the magnitude of effects produced by S5–6 substitution, the ordering of motifs is different for ion transport rate than it is for ion selectivity: for ion conduction, motifs I and II are most influential, whereas for ion selectivity, selectivity filter glutamate residues in motifs III and then II are most consequential (Yang et al., 1993; Ellinor et al., 1995). This contrast reiterates the point that ion conduction and selectivity are divergent phenomena in Ca\(^{2+}\) channels.

Second, the effects on conductance produced by single-motif sequence transfers are not in every instance additive: the magnitude of the change in ion transport rate produced by a quadruple transfer is not necessarily predicted by summing the magnitudes of the four corresponding single-motif transfers. In the most striking case, substituting all four α1S S5–6 regions into α1C (CQuadSS5–6S) reduced unitary conductance by about twice that of the summed reductions produced by the four individual S5–6 transfers. When considering instead unitary currents or the results for P-loops, the evidence for non-additivity was much weaker. Nonetheless, the absence of additivity of conductance for the S5–6 transfers raises the possibility of cooperative or synergistic interaction among the four motifs.

Third, interactions between P-loop sequence and other parts of the S5–6 region seem to be complex. The data summarized in Fig. 6 show that although individual S5–6 substitutions caused distinctive decrements in ion conduction, individual P-loop substitutions produced approximately similar, small decrements in ion conduction. Regarding motif IV, for example, P-loop substitution produced bigger changes in ion conduction than did S5–6 substitution, as though the effects of P-loop transfer could be reversed by transfer of structural features contained in the non-P-loop components of the S5–6 region. Alternatively, this finding might indicate that “improper” interactions of transplanted P-loop residues with host channel residues led to local protein misfolding and diminished ion conduction. This view may also account for our finding that Na\(^{+}\) channel P-loops not only fail to confer Na\(^{+}\) selectivity on Ca\(^{2+}\) channels.
channels, but the resulting Na\(^+\) channel/Ca\(^{2+}\) channel chimera also failed to carry Ca\(^{2+}\) or Ba\(^{2+}\) current (unpublished data).

Our findings generally agree with previous work by Dirksen et al. (1997) in that the motif I P-loop and S5–6 region house the key determinants of ion conduction, but our results using systematic sets of P-loop and S5–6 region chimeric constructs reveal significant participation of other motifs as well. In the study by Dirksen and colleagues (1997), substitution of \(\alpha_{1S}\) sequence into the motif I S5–S6 linker of \(\alpha_{1C}\), which left the flanking S5 and S6 segments of \(\alpha_{1C}\) in place, displaced unitary conductance \(\sim 75\%\) of the way toward the \(\alpha_{1S}\) value. In contrast, we have found that substitution of a larger region in motif I, encompassing the S5–S6 linker but also including the flanking S5 and S6 regions in Ca\(^{2+}\) segments, displaced unitary conductance only \(\sim 35\%\) (\(C_{ISS-S}\)), Fig. 6 A) of the way toward the \(\alpha_{1S}\) value. In our work, we found that full transfer of \(\alpha_{1S}\)-like conductance required substitution of all four S5–6 regions. Various explanations for the apparent discrepancy between the two studies can be proposed, but a likely one stems from the fact that different chimeras were studied. As discussed above, interactions between the S5–S6 linker and surrounding parts of the S5–6 region may be important in determining conductance, and in the absence of appropriate interactions between these parts of the conductance-determining S5–6 regions, unitary conductance might consequently be reduced. The fact that swapping the four S5–6 regions reciprocally transferred unitary conductance between \(\alpha_{1C}\) and \(\alpha_{1S}\) confirms the idea that the S5–6 regions contain the structural features responsible for the difference in ion conduct between \(\alpha_{1C}\) and \(\alpha_{1S}\). Comparison of results with our chimeras and those of Dirksen and colleagues (1997) also supports the idea, discussed above, that structural features contained within the S5–6 regions but outside the S5–S6 linker specify unitary conductance in these two L-type Ca\(^{2+}\) channels.

**Reversal potential, Cd\(^{2+}\) block and unitary conductance**

Whereas the unitary conductance results are interpretable in a straightforward manner, the effects of chimeric substitution on two other measures of ion permeability, reversal potential, and Cd\(^{2+}\) block, are not as readily rationalized. The parent \(\alpha_{1C}\) and \(\alpha_{1S}\) channels differed little from one another in \(E_{\text{rev}}\) and in estimated \(I_{50}\) for Cd\(^{2+}\) block, but as a general trend, the three quadruple chimeras (C\(_{\text{QuadPS}}\), C\(_{\text{QuadSS-}S}\), S\(_{\text{QuadSS-}C}\)) differed from their parents: in the quadruple chimeras, \(E_{\text{rev}}\) was as much as 20 mV less positive (S\(_{\text{QuadSS-}C}\)) and Cd\(^{2+}\) \(I_{50}\) was as much as 10-fold lower (S\(_{\text{QuadSS-}C}\)) relative to the parent channels. Thus quadruple chimera-genesis seemingly reduced ion selectivity if judged from bi-ionic reversal potential, but increased ion selectivity if judged from Cd\(^{2+}\) binding affinity. The S\(_{\text{QuadSS-}C}\) chimera represents the most striking case, with the lowest preference for Ba\(^{2+}\) over K\(^+\) (\(E_{\text{rev}}\)) but the highest preference for Cd\(^{2+}\) over Ba\(^{2+}\) (\(I_{50}\)). Part of the explanation for this situation may be that these two measures of ion selectivity differ in the ions compared and in the direction of ion flow, so that inward Ba\(^{2+}\) competes with outward K\(^+\) in one case but inward Cd\(^{2+}\) competes with inward Ba\(^{2+}\) in the other.

It is noteworthy that \(E_{\text{rev}}\) and \(I_{50}\) were altered in the quadruple chimeras despite the fact that all four selectivity filter glutamates (EEEE locus) were present in these chimeras. One explanation is that the EEEE locus is very sensitive to structural context, so that incompatibility between \(\alpha_{1C}\) and \(\alpha_{1S}\) sequence in the chimeras results in altered EEEE locus configuration and altered selectivity. Alternatively, non-EEE locus mutations have previously been found to affect Ca\(^{2+}\) channel selectivity, suggesting the possibility that altered pore structure elsewhere in the transplanted region might account for the changes in selectivity (Williamson and Sather, 1999; Feng et al., 2001).

**Differences between \(\alpha_{1C}\) and \(\alpha_{1S}\) in S5–6 sequence and ion conduction**

Sequence comparison suggests ways that the S5–6 regions might potentially control ion conduction in Ca\(^{2+}\) channels. In motif III, previous work comparing \(\alpha_{1C}\) with \(\alpha_{1A}\) (P/Q-type Ca\(^{2+}\) channel) sequence led to the finding that the side-chain volume of a residue neighboring the EEEE locus influenced unitary conductance (Williamson and Sather, 1999). The residue at this neighbor position is conserved between \(\alpha_{1C}\) and \(\alpha_{1S}\), however, and in general, there are few remarkable differences in P-loop sequence between \(\alpha_{1C}\) and \(\alpha_{1S}\), which may account for the inability of quadruple P-loop substitution to fully transfer conduction behavior. In the regions flanking the P-loops, the S5–S6 linkers differ between \(\alpha_{1C}\) and \(\alpha_{1S}\) at several positions. Examining these differences in motif I, \(\alpha_{1C}\) has a net charge of −5 relative to \(\alpha_{1S}\), which has previously been suggested to attract permeant cations into the extracellular pore entrance and thereby impart higher conductance on \(\alpha_{1C}\) channels (Dirksen et al., 1997). Considering this idea in light of the evidence that surrounding parts of S5–6 are important in specifying conduction rate, electrostatic enhancement of permeant ion entry rate may not be a dominant factor in conduction. Indeed, evidence against electrostatic focusing of permeant divalent metal cations at the mouth of L-type Ca\(^{2+}\) channels under the experimental conditions used here has been obtained (Kuo and Hess, 1992).

Regarding our evidence that the S5–6 region is crucial in controlling flux through Ca\(^{2+}\) channels, in the homologous K\(^+\) channels the cytosolically-disposed part of S6 and possibly S5 is thought to contribute to the pore lining (Aiyar et al., 1994; Lopez et al., 1994; Shi et al., 1994; Liu et al., 1997; Doyle et al., 1998; del Camino et al., 2000). The cytosolic halves of S5 and S6 in \(\alpha_{1C}\) and \(\alpha_{1S}\) are highly hydrophobic, which is consistent with the hydrophobicity of...
homologous sequences lining the central pool and inner pore of the KcsA K⁺ channel (Doyle et al., 1998). In Ca²⁺-channels, differences in these hydrophobic sequences may therefore help to determine conduction rate through the cytoplasmic part of the pore, as has been proposed for the KcsA channel. Additionally, the more extracellularly-disposed parts of the S5 and S6 segments may be involved, based on the fact that the number of differences in sequence between α₁C and α₁S is greater in the extracellular halves of S5 and S6 than in the intracellular halves. Whether amino acid residues in S5 or S6 contribute directly to pore formation in Ca²⁺-channels, for example at the extracellular entrance, is unknown. However, S5 and S6 may act by exerting indirect effects that influence the conformation of the more external portion of the pore, particularly the P-loop.

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