Two Domains in Dihydropyridine Receptor Activate the Skeletal Muscle Ca^{2+} Release Channel

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ABSTRACT The II-III cytoplasmic loop of the skeletal muscle dihydropyridine receptor (DHPR) α_1-subunit is essential for skeletal-type excitation-contraction coupling. Single channel and [^{3}H]ryanodine binding studies with a full-length recombinant peptide ($p^{666-791}$) confirmed that this region specifically activates skeletal muscle Ca^{2+} release channels (CRCs). However, attempts to identify shorter domains of the II-III loop specific for skeletal CRC activation have yielded contradictory results. We assessed the specificity of the interaction of five truncated II-III loop peptides by comparing their effects on skeletal and cardiac CRCs in lipid bilayer experiments; $p^{671-680}$ and $p^{720-765}$ specifically activated the submaximally Ca^{2+}-activated skeletal CRC in experiments using both mono and divalent ions as current carriers. A third peptide, $p^{671-690}$, showed a bimodal activation/inactivation behavior indicating a high-affinity activating and low-affinity inactivating binding site. Two other peptides ($p^{681-690}$ and $p^{681-685}$) that contained an RKRRK-motif and have previously been suggested in in vitro studies to be important for skeletal-type E-C coupling, failed to specifically stimulate skeletal CRCs. Noteworthy, $p^{671-690}$, $p^{681-690}$, and $p^{681-685}$ induced similar subconductances and long-lasting channel closings in skeletal and cardiac CRCs, indicating that these peptides interact in an isoform-independent manner with the CRCs.

INTRODUCTION

Excitation-contraction (E-C) coupling in striated muscle is a signal transduction event that leads to the activation of sarcoplasmic reticulum (SR) Ca^{2+} release channels (CRCs, also known as ryanodine receptors or RyRs). In mammalian skeletal muscle, this process is thought to be mediated by a direct physical interaction between dihydropyridine-sensitive (L-type) Ca^{2+} channels (dihydropyridine receptors or DHPRs), located in the surface membrane and tubular infoldings (T-tubule), and the CRCs (Rios and Pizarro, 1991). In contrast to its role as a voltage-sensor in skeletal muscle, the cardiac DHPR isoform mediates an influx of Ca^{2+} ions that open closely apposed CRCs in cardiac muscle. The skeletal CRC is a large channel composed of four large ~565-kDa subunits and four small 12-kDa FK506 binding proteins (Coronado et al., 1994; Meissner, 1994; Franzini-Armstrong and Protasi, 1997; Ondrias et al., 1998). The mammalian skeletal muscle DHPR is composed of five subunits, α_1, α_2, β, γ, and δ (Catterall, 1995). Co-immunoprecipitation (Marty et al., 1994) and cross-linking of the skeletal muscle DHPR and CRC (Murray and Ohlendieck, 1997) as well as morphological evidence (Block et al., 1988) suggest a well-defined interaction between the two receptors. Clusters of four particles called tetrads that represent four DHPRs are located opposite the four subunits of every other RyR (Block et al., 1988). The clustering of DHPRs into tetrads is dependent on the presence of the skeletal CRC (Protasi et al., 1998), whereas their targeting to junctional domains of T-tubules is not (Takekura and Franzini-Armstrong, 1999).

Microinjection of skeletal and cardiac cDNAs and their chimeric constructs into dysgenic myotubes that lack the skeletal muscle DHPR α_1-subunit first suggested that the cytosolic II-III loop region (residues 666–791) of the DHPR α_1 subunit is responsible for mediating skeletal-type E-C coupling (Tanabe et al., 1990). A recombinant peptide corresponding to the II-III loop increased [^{3}H]ryanodine binding to skeletal muscle SR vesicles and increased skeletal, but not cardiac, CRC activity in single channel experiments, thus implying that the II-III loop region may specifically and directly interact with the skeletal CRC (Lu et al., 1994). Synthetic peptides corresponding to different regions of the II-III loop have been used to narrow the amino acid residues required to activate the skeletal CRC. $Peptide^{671-690}$ and $p^{681-690}$ increased [^{3}H]ryanodine binding to and increased Ca^{2+} release from skeletal muscle SR vesicles (El-Hayek et al., 1995; El-Hayek and Ikemoto, 1998). Single channel measurements confirmed an interaction of $p^{671-690}$ with the skeletal CRC (Dulhunty et al., 1999; Gurrola et al., 1999). A second regulatory region in the DHPR α_1-subunit II-III loop was identified using a peptide corresponding to Glu^{724}-Pro^{760} that by itself had no effect, but which blocked the activating effects and conformational changes induced by $p^{671-690}$ or T-tubule depolarization (El-Hayek et al., 1995; Saiki et al., 1999). Microinjection of skeletal and cardiac muscle chimeric cDNAs into dysgenic myotubes indicated that skeletal residues 711–765, and to a lesser extent residues 725–742, were sufficient to evoke skeletal-type E-C coupling (Nakai et al., 1998). Noteworthy, the in vivo studies challenged the results obtained with a DHPR-derived peptide corresponding to the N-terminal part of the II-III loop, as this region did not appear to be critical for

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$E^{666}AESLT^{671}SAQKAKAEER^{681}KRRK^{685}M$
$SRGL^{690}PDKTEEEKSVMAKKLEQKP$
$KGEGIPTTAKL^{720}KVDEF^{725}ESNVNEV$
$KDPYPSADFPGD^{744}DEEDEPEIPVSPR$
$PRPLAELQ^{765}LKEKAVPIPEASSFFIFS$
$PTNKVRVL^{791}$

FIGURE 1 Amino acid sequence of cytoplasmic DHPR α_1 subunit II-III loop. Peptides used in this study are indicated by numbering the amino acids that correspond to the first and last residue of the peptides.

skeletal muscle E-C coupling (Nakai et al., 1998; Proenza et al., 2000). Additional regions of the DHPR α_1-subunit (Leong and MacLennan, 1998; Slavik et al., 1997) and the β-subunit of the DHPR (Beurg et al., 1999) have been reported to contribute to the functional coupling of the skeletal DHPR and CRC.

In the present study, we examined the effects of the full-length II-III loop peptide ($p^{666-791}$) and five shorter peptides ($p^{671-690}$, $p^{671-680}$, $p^{681-690}$, $p^{681-685}$, $p^{720-765}$) (Fig. 1) on single skeletal and cardiac muscle CRCs. Isoform-specificity and regulation of channel activity were determined in single channel measurements using submaximally and maximally Ca^{2+}-activated CRCs with 250 mM KCl as current carrier. Our results indicate that the full-length II-III loop peptide and two shorter regions ($p^{671-680}$ and $p^{720-765}$) specifically activate the submaximally Ca^{2+}-activated skeletal CRC in a concentration-dependent manner. Activation of skeletal CRCs by $p^{671-680}$ and $p^{720-765}$ was confirmed in experiments using Ca^{2+} as current carrier. We also present evidence that a region ($p^{681-690}$), previously proposed to be critical for skeletal-type E-C coupling, fails to significantly activate submaximally Ca^{2+}-activated skeletal CRCs. Rather, this peptide induced similar subconductance states in both the skeletal and cardiac CRC, which suggests an isoform-independent action.

MATERIALS AND METHODS

Materials

Phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL). The recombinant DHPR α_1-subunit full-length II-III loop peptide was expressed and purified as described (Lu et al., 1994). The truncated II-III loop peptides were synthesized and purified in the Molecular Biology and Biotechnology Micro-Protein Chemistry Facility at the University of North Carolina. All other chemicals were of analytical grade.

Preparation of heavy SR vesicles and purification and reconstitution of Ca^{2+} release channels

SR vesicle fractions enriched in [^{3}H]ryanodine binding and Ca^{2+}-release channel activities were prepared from rabbit skeletal and canine cardiac muscle in the presence of protease inhibitors (100 nM aprotinin, 1 μM leupeptin, 1 μM pepstatin, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride) (Meissner, 1984; Meissner and Henderson, 1987). The 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)-solubilized skeletal and cardiac muscle 30S CRC complexes were isolated by rate density gradient centrifugation and reconstituted into proteoliposomes by removal of CHAPS by dialysis (Lee et al., 1994). Proteoliposomes were sedimented by centrifugation, resuspended in 0.3 M sucrose, 5 mM KPipes, pH 7.4, quick-frozen in small aliquots, and stored at -80°C.

Single channel measurements

Unless otherwise indicated, single channel recordings were performed in symmetric KCl solutions (250 mM, 10 mM KHepes, pH 7.3) containing additions as indicated. Proteoliposomes containing the purified skeletal or cardiac muscle CRCs were added to the *cis* chamber of a bilayer apparatus and fused in the presence of an osmotic gradient with Mueller-Rudin type planar bilayers containing a 4:1 mixture of bovine brain phosphatidylethanolamine and phosphatidylcholine (30–40 mg of total phospholipid/ml n-decane). Unless otherwise indicated, the gradient was formed across the bilayer membrane with 250 mM *cis* KCl and 20 mM *trans* KCl solutions. After appearance of single channel activity, an increase in *trans* KCl concentration to 250 mM prevented further fusion of proteoliposomes. The number of channels incorporated in the bilayer was determined in the presence of maximally Ca^{2+}-activating conditions (see Fig. 8). For experiments using submaximally Ca^{2+}-activating CRCs, the free Ca^{2+} concentration was subsequently lowered to $\sim$1 μM. The *trans* side of the bilayer was defined as ground. Measurement of the sensitivity of the channels to μM cytosolic Ca^{2+} indicated that in a majority of recordings (>98%) the cytosolic side of the CRC faced the *cis* side and the lumenal side faced the *trans* side of the bilayer. Peptides were added to the *cis* (cytosolic) side of the bilayer from stock solutions made in 250 mM KCl, 10 mM KHepes, pH 7.3 solution. To test the possibility that added peptides bind to the bilayer chamber or associate with the membrane and thereby reduce the concentration of free peptide, we verified calculated concentrations spectrophotometrically for peptide concentrations >1 μM. Similarly, we ensured spectrophotometrically using antipyrazolo III that all peptides used in this study did not bind Ca^{2+}, and thereby lower the free Ca^{2+} concentration at higher doses. After a stirring period of 60 s, channel activities were recorded at +40 mV and −40 mV holding potential using a commercially available patch-clamp amplifier with a bilayer headstage (Axopatch 1D, Axon Instruments, Burlingame, CA). Unless otherwise indicated, recordings were filtered at 4 kHz through an eight-pole low-pass Bessel filter (Frequency Devices, Inc., Haverhill, MA) and digitized at 20 kHz. Data acquisition and analysis were performed with the software package pClamp 6.0.1. (Axon Instruments) using an IBM-compatible computer and a 12 bit A/D - D/A converter (Digidata 1200, Axon Instruments). Data files were directly acquired using the continuous Fetchex mode. Channel parameters were calculated from current recordings of 2 min duration (Tripathy and Meissner, 1996). Because all peptides used in this study, with the exception of $p^{681-685}$ (Fig. 6), lacked subconductances >50% in submaximally Ca^{2+} activating conditions and amplitude histogram analysis indicated that subconductances contributed to <10% of the overall open events, channel open probability (P_o) was obtained by setting the threshold level at 50% of the current amplitude between the closed and open states. P_o values in multichannel recordings were calculated using the formula $\Sigma\, iP_{o,i}/N$, where N is the total number of channels, and $P_{o,i}$ is channel open probability of the ith channel. Mean $P_{oControl}$ values ± SE for submaximally Ca^{2+}-activated skeletal CRCs were 0.0168 ± 0.002 (+40 mV; n = 127); 0.0163 ± 0.002 (−40 mV, n = 127); 0.0069 ± 0.002 (0 mV, n = 27). Submaximally Ca^{2+}-activated cardiac CRCs exhibited mean P_o values of 0.037 ± 0.012 (+40 mV, n = 42) and 0.036 ± 0.012 (−40 mV, n = 42) in the absence of peptide. Reversibility of channel activation induced by the activating peptides was verified by a decrease of activity following perfusion of the chamber solution. The positions of substates relative to the fully open amplitude were determined using current amplitude histograms

obtained from 2-min single channel recordings and visual inspection of the corresponding current traces. The positions of the major subconductances in each histogram were obtained by best-fitting Gaussian curves to the current amplitude histograms.

Determination of free Ca^{2+} concentrations

Different free Ca^{2+} concentrations were prepared by mixing $CaCl_2$ and EGTA as determined using the stability constants and computer program published by Schoenmakers et al. (1992). Free Ca^{2+} concentrations were verified using a Ca^{2+}-selective electrode (World Precision Instruments, Inc., Sarasota, FL).

Data analysis

Results are given as mean ± SE with the number of experiments in parentheses. Unless otherwise indicated, significance compared to the control group (channel activity in the absence of peptide) was determined using one-way analysis of variance (ANOVA) followed by a post hoc test (Dunnett's method) in cases where ANOVA demonstrated statistical significance. Differences were regarded to be statistically significant at $p < 0.05$.

RESULTS

Effects of full-length II-III loop peptide on submaximally Ca^{2+}-activated skeletal and cardiac CRCs

Previous studies have shown that the cytoplasmic II-III loop of the DHPR α_1-subunit has a key role in mediating skeletal-type E-C coupling (Tanabe et al., 1990; Lu et al., 1994). In preliminary studies we confirmed that the full-length II-III loop peptide activates skeletal but not cardiac CRCs. Fig. 2 *A* shows the results of a representative channel recording using skeletal CRC. A single channel was recorded at +40 mV and −40 mV holding potentials in symmetric 250 mM KCl medium containing a submaximally activating concentration of ~1 μM cytosolic free Ca^{2+}. The lumenal Ca^{2+} concentration in all experiments investigating submaximally Ca^{2+}-activated CRCs was ~1 μM to minimize lumenal to cytosolic Ca^{2+} fluxes through the CRC (Tripathy and Meissner, 1996). The two top traces show a low channel open probability (P_o) for skeletal CRC in the absence of the II-III loop peptide and are characterized by a low number of brief, often not fully resolved open events, shown as upward and downward deflections from the closed state (marked "C"). Addition of 100 nM II-III loop peptide to the cytosolic (*cis*) side of the bilayer increased P_o 1.3- and 3-fold (second two traces). A further increase in P_o was observed at both holding potentials as the cytosolic II-III loop peptide concentration was raised from 100 nM to 750 nM and 3 μM (third and last recordings). Clearly defined subconductances were not detected at either holding potential. The peptide-induced increase in P_o was due to an increase in the number of events, as there was no change in the mean open time of the channel openings in the presence of peptide. The mean relative increase in P_o of the skeletal CRC as a function of cytosolic peptide concentration from four to nine experiments is shown in Fig. 2 *B* (*circles*). The normalized P_o values reached a maximum level (~7-fold) at both holding potentials at a peptide concentration of ~3 μM, and slightly declined as the peptide concentration was raised to 7.5 μM.

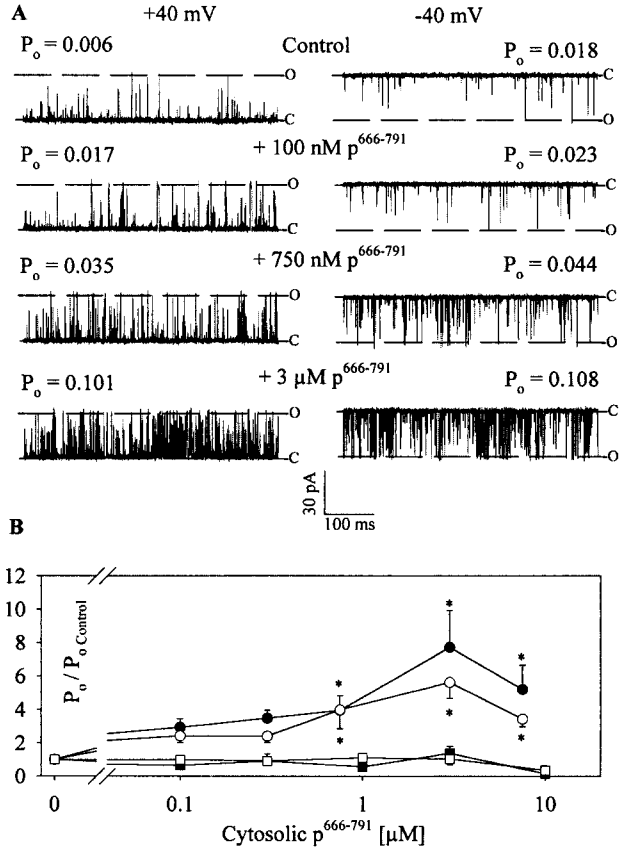

FIGURE 2 DHPR full-length II-III loop peptide specifically activates the submaximally Ca^{2+}-activated skeletal muscle CRC. (*A*) Shown are eight recordings from one experiment with a single skeletal CRC. Single channel currents, shown as upward or downward deflections from closed levels (marked "C"), were recorded in symmetric 250 mM KCl, 10 mM KHepes, pH 7.3. The *cis* (cytosolic) and *trans* (lumenal) free Ca^{2+} was ~1 μM. Current recordings were measured at +40 mV (*left panel*) and −40 mV (*right panel*) holding potential at 0 (Control), 100 nM, 750 nM and 3 μM $p^{666-791}$. (*B*) Relative mean P_o values (±SE) from four to nine experiments using skeletal (*circles*) and from three to six experiments using cardiac (*squares*) CRCs at different peptide concentrations and recorded at holding potentials of +40 mV (*open symbols*) and −40 mV (*closed symbols*). *Significantly different ($p < 0.05$) from control as determined by ANOVA and Dunnett's method.

The effects of the II-III loop peptide in single channel measurements were initially studied in our laboratory (Lu et al., 1994) using CHAPS-solubilized skeletal CRCs. The previous data indicated a much greater level of activation of the skeletal CRC due to an increase in both frequency of open events and mean open time. However, because subsequent studies have indicated that residual CHAPS in the lipid bilayer can alter channel response and evoke non-

physiological effects (Xiong et al., 1998), all current experiments were performed using reconstituted liposomes. Fig. 2 *B* also summarizes data (not shown) using cardiac CRCs at a submaximally activating free Ca^{2+} concentration of ~1 μM. In contrast to the skeletal CRC, the full-length II-III loop peptide did not activate the cardiac CRC (Fig. 2 *B*, *squares*).

Effects of truncated II-III loop peptides on submaximally Ca^{2+}-activated skeletal and cardiac CRCs

The effects of shorter peptides corresponding to different regions of the II-III loop were investigated to assess the specificity of their CRC interactions and to determine the minimum sequences that mediate skeletal-type E-C coupling. Minimally Ca^{2+}-activated skeletal and cardiac CRCs (~1 μM free Ca^{2+}) were used to test for activating effects of the peptides with K^+ as current carrier. Neither the full-length nor the truncated II-III loop peptides had any effect on the unmodified bilayer. Concentration-dependent stimulation by two truncated peptides was confirmed with Ca^{2+} as the current carrier and using physiological KCl concentrations of 150 mM. Because some of the peptides caused inhibition of channel activity and the appearance of subconductance states, we also explored their effects on maximally Ca^{2+}-activated channels.

Peptide$^{671-690}$

Peptide$^{671-690}$ corresponds to the N-terminal region of the II-III loop (Fig. 1) and was previously found to contain a critical sequence for mediating skeletal-type E-C coupling (El-Hayek et al., 1995; El-Hayek and Ikemoto, 1998; Dulhunty et al., 1999). We found that $p^{671-690}$ displayed activating and inhibiting effects on the skeletal CRC caused by an increase and subsequent decrease in the frequency of open events. In Fig. 3 *A*, addition of 30 nM $p^{671-690}$ activated a single skeletal CRCs at +40 mV and −40 mV holding potentials. Increasing $p^{671-690}$ concentration to 3 μM preserved channel activity, whereas further increase in peptide concentration to 30 μM decreased P_o at both holding potentials. Low-conductance substates (*arrows*) were identified at +40 mV at peptide concentrations in excess of 0.5 μM (Fig. 3 *A*, *third and bottom traces, left panel*). The ability of $p^{671-690}$ to induce subconductances in skeletal and cardiac CRCs is analyzed in greater detail in Fig. 8, *A* and *B*, respectively, using maximally Ca^{2+}-activated channels.

Fig. 3 *B* shows the mean relative changes in P_o values calculated from 13 to 16 experiments with skeletal CRCs plotted as a function of peptide concentration. A broad plateau of maximal channel activation was observed in the presence of 30 nM to 3 μM peptide, indicating a high affinity activation site. Higher peptide concentrations (10 and 30 μM at +40 mV and 30 μM at −40 mV) resulted in a significant decrease in channel activity compared to levels observed in the presence of 0.1 μM to 3 μM and 0.3 μM peptide concentration, respectively. Significance was determined as described in the Materials and Methods section using one-way ANOVA analysis followed by the LSD method as post hoc test.

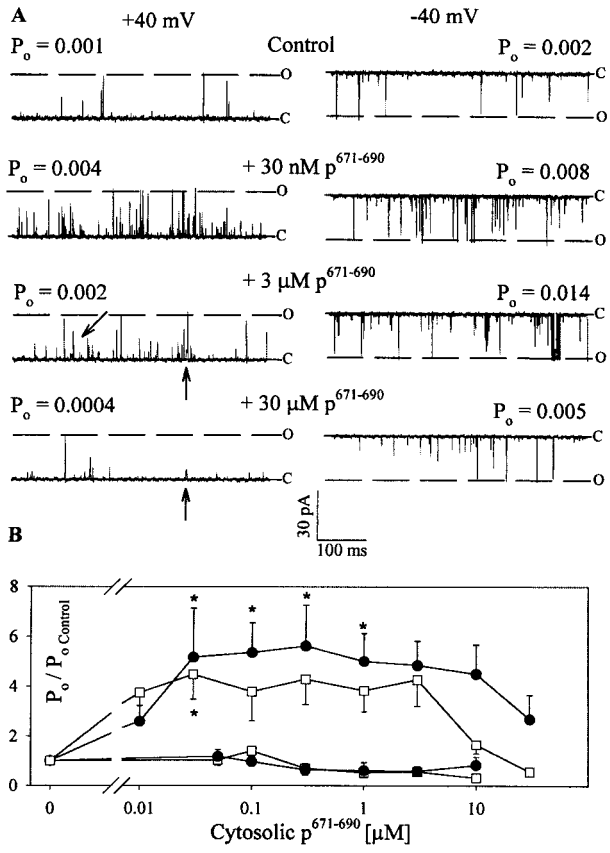

FIGURE 3 Effects of $p^{671-690}$ on submaximally Ca^{2+}-activated skeletal and cardiac CRCs. (*A*) Shown are eight recordings from one experiment with 1 skeletal CRCs at 0 (Control) and the indicated peptide concentrations. Single channel currents, shown as upward or downward deflections from closed levels (marked "C"), were recorded as in Fig. 2. (*B*) Relative mean P_o values (±SE) from 13 to 16 experiments using skeletal (*circles*) and from four to five experiments using cardiac (*squares*) CRCs at different peptide concentrations and recorded at holding potentials of +40 mV (*open symbols*) and −40 mV (*closed symbols*). *Significantly different ($p < 0.05$) from control as determined by ANOVA and Dunnett's method.

Like the full-length $p^{666-791}$, $p^{671-690}$ failed to activate the submaximally Ca^{2+}-activated cardiac CRC and caused a slight decrease in channel activity at concentrations >1 μM (Fig. 3 *B*, *squares*) due to a decreased number of open events. Subconductance states similar to those observed in submaximally Ca^{2+}-activated skeletal channels (Fig. 3 *A*, *left panel*) were observed at +40 mV and at peptide concentrations of ≥3 μM (not shown).

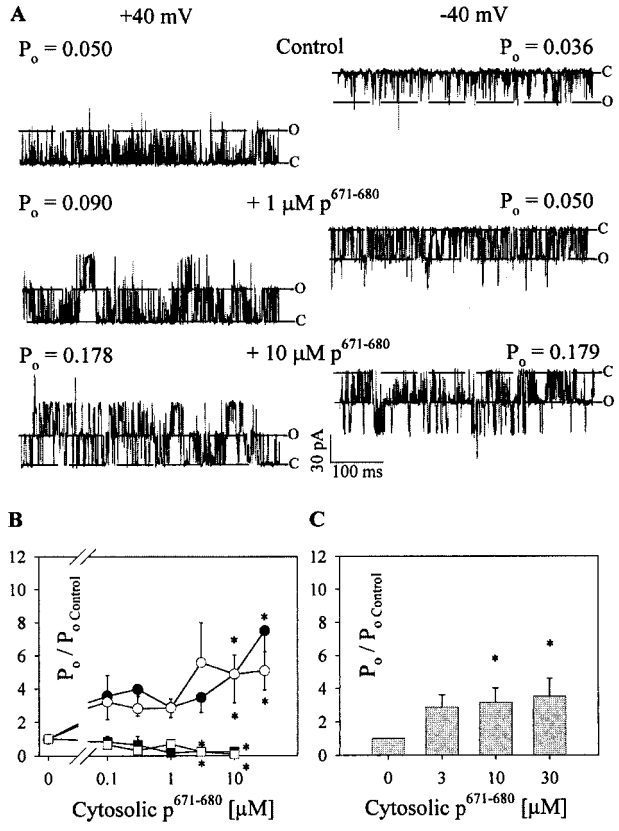

FIGURE 4 Effects of $p^{671-680}$ on submaximally Ca^{2+}-activated skeletal muscle and cardiac muscle CRC activities. (*A*) Shown are six recordings from one experiment with three skeletal muscle CRCs. Single channel currents, shown as downward or upward deflections from closed levels (marked "C"), were recorded as in Fig. 2. (*B*) Relative mean P_o values (±SE) from 6 to 15 experiments with skeletal (*circles*) and from three to four experiments with cardiac (*squares*) CRCs at holding potentials of +40 mV (*open symbols*) and −40 mV (*closed symbols*). (*C*) Relative mean P_o values from 8 to 11 experiments with skeletal CRCs under more physiological conditions using symmetrical 150 mM KCl and 10 mM lumenal Ca^{2+} as current carrier at 0 mV holding potential. The mean open probability used in the calculation of the $P_o/P_{oControl}$ ratios was 0.0024 ± 0.0004 (n = 15). To decrease noise, channel recordings were filtered at 300 Hz before analysis. *Significantly different ($p < 0.05$) from control as determined by ANOVA and Dunnett's method.

Peptide$^{671-680}$

Fig. 4, *A* and *B* show the effects of $p^{671-680}$ (N-terminal half of $p^{671-690}$) on submaximally Ca^{2+}-activated skeletal and cardiac CRCs with K^+ as current carrier. The channel traces of three skeletal CRCs are shown in Fig. 4 *A*, and the mean relative changes in P_o values obtained from 6 to 15 experiments as a function of peptide concentration are shown in Fig. 4 *B*. Peptide$^{671-680}$ significantly activated the skeletal CRC at micromolar concentrations increasing channel activities ~6-fold at −40 and +40 mV holding potentials (Fig. 4 *B*, *circles*). Peptide$^{671-680}$ did not activate the cardiac CRC, rather the channels were significantly inhibited in the presence of micromolar peptide concentrations (Fig. 4 *B*, *squares*). For both isoforms modification of channel activity was due to an increase (skeletal) or decrease (cardiac CRCs) in the number of open events rather a change in mean open time. Unlike $p^{671-690}$ or $p^{681-690}$, elevated concentrations of $p^{671-680}$ did not induce subconductance states in the submaximally Ca^{2+}-activated skeletal CRC (*bottom traces* in Fig. 4 *A*) or cardiac CRC (not shown). The lack of subconductances was confirmed using maximally Ca^{2+}-activated skeletal and cardiac CRCs (data not shown).

Stimulation of submaximally Ca^{2+}-activated skeletal CRCs was confirmed at 0 mV holding potential under more physiological conditions using symmetrical 150 mM KCl solution and 10 mM lumenal Ca^{2+} as current carrier. Fig. 4 *C* summarizes data from 8 to 11 experiments with 3, 10, and 30 μM cytosolic $p^{671-680}$. The results suggest a concentration-dependent agonist function of $p^{671-680}$ leading to an ~3-fold increase in activity of the skeletal CRC at micromolar peptide concentrations.

Peptide$^{681-690}$

El-Hayek and Ikemoto (1998) localized a critical sequence for mediating skeletal-type E-C coupling to residues 681–690. We found that $p^{681-690}$ did not significantly activate the skeletal muscle CRC at submaximally activating Ca^{2+} (Fig. 5, *A* and *B*, *circles*). Peptide concentrations of 1 μM and 10 μM and greater caused inhibition of skeletal (*circles*) and cardiac (*squares*) channel activity, respectively, at +40 mV holding potential but were without a substantial effect at −40 mV. For both isoforms channel inhibition was the result of a decreased frequency of open events. Elevated peptide concentrations induced low-conductance substates at +40 mV holding potential, as indicated by arrows for the skeletal CRC (Fig. 5 *A*, *left panel*).

Peptide$^{681-685}$

A cluster of five positively charged amino acids (residues 681–685) in the II-III loop has been postulated to be part of the domain that interacts with the skeletal CRC (Gurrola et al., 1999). Fig. 6 shows the effects of $p^{681-685}$ on submaximally Ca^{2+}-activated skeletal CRCs. Peptide$^{681-685}$ induced long-lasting channel closings at +40 mV holding potential. At −40 mV, the peptide increased the number of channel events. However, current amplitude histogram analysis revealed that the additional events represented a subconductance of 60% of the control full conductance in the presence of 10 or 30 μM $p^{681-685}$ (*second and third traces in right column*). No increase in the frequency of the fully open state, as determined for full-length II-III loop peptide, $p^{671-680}$, and $p^{720-765}$ was recorded in the presence of $p^{681-685}$. An essentially identical behavior was observed for cardiac muscle CRCs (data not shown).

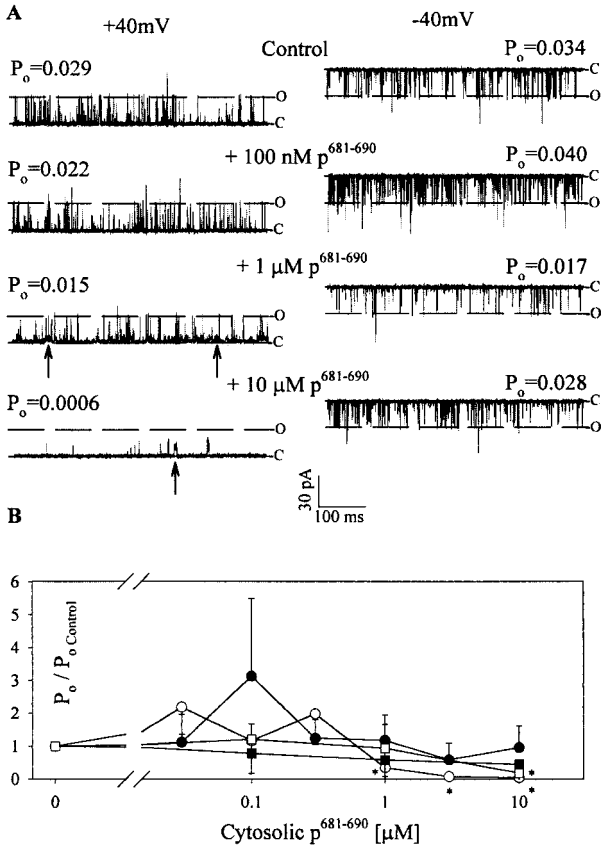

FIGURE 5 Effects of $p^{681-690}$ on submaximally Ca^{2+}-activated skeletal muscle CRC. (*A*) Shown are eight recordings from one experiment with two skeletal CRCs at 0 (Control) and the indicated peptide concentrations. Single channel currents, shown as upward or downward deflections from closed levels (marked "C"), were recorded as in Fig. 2. (*B*) Relative mean P_o values (±SE) from four to eight experiments using skeletal (*circles*) and four to five experiments using cardiac CRCs (*squares*) at holding potentials of +40 mV (*open symbols*) and −40 mV (*closed symbols*). *Significantly different ($p < 0.05$) from control as determined by ANOVA and Dunnett's method.

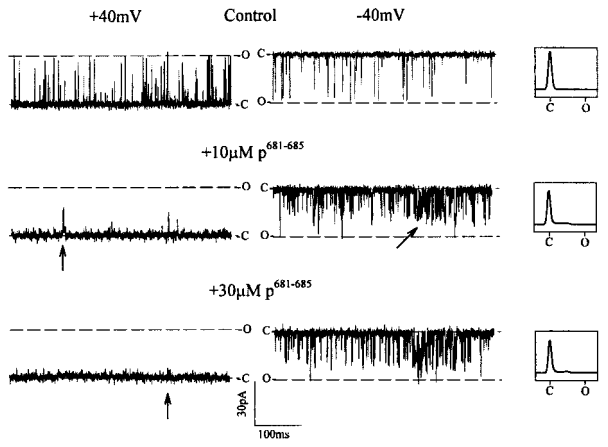

FIGURE 6 Effects of $p^{681-685}$ on submaximally Ca^{2+}-activated skeletal muscle CRC. Shown are six recordings from one representative ($n = 4$) experiment with one skeletal CRC at 0 (Control) and the indicated peptide concentrations. Single channel currents, shown as upward or downward deflections from closed levels (marked "C"), were recorded as in Fig. 2. Current amplitude histograms on the right indicate that the peptide induces at +40 mV a substate corresponding to 60% of the control full conductance.

Peptide$^{720-765}$

Expression of chimeric cDNAs in dysgenic myotubes and studies with DHPR-derived peptides have revealed a second region (residues 711–765) in the skeletal II-III loop of DHPR α_1-subunit that interacts with the skeletal CRC (El-Hayek et al., 1995; Nakai et al., 1998; Saiki et al., 1999), with conflicting roles ascribed to this region. Expression studies suggest an agonist function (Nakai et al., 1998), whereas peptide studies suggest a blocking function for DHPR residues 724–760 in skeletal muscle E-C coupling (Saiki et al., 1999). Fig. 7 *A* shows that $p^{720-765}$ activated submaximally Ca^{2+}-activated skeletal CRCs in single channel measurements. The activity of the channels progressively increased at both holding potentials as the peptide concentration was increased from 0.3 μM to 30 μM (Fig. 7 *A*). The increase in channel activity was due to an increase in the number of open events rather than an increase in mean open times. In separate experiments we determined that the activating effect of this peptide on skeletal CRCs was reversible (data not shown). Thirty μM $p^{720-765}$ increased skeletal CRCs activity ~8-fold compared to control levels (Fig. 7 *B*, *circles*) while no significant activation of cardiac CRC activity was observed in the tested peptide range from 100 nM to 30 μM (Fig. 7 *B*, *squares*). To confirm skeletal CRC stimulation by $p^{720-765}$ under more physiological conditions, single channel measurements were recorded at 0 mV holding potential in symmetrical 150 mM KCl with 10 mM lumenal Ca^{2+} as current carrier. Fig. 7 *C* summarizes results from five to six experiments with 3, 10, and 30 μM cytosolic $p^{720-765}$. We observed a concentration-dependent activation of submaximally Ca^{2+}-activated skeletal CRCs with an ~20-fold activation in the presence of 30 μM $p^{720-765}$.

Effects of truncated DHPR II-III loop peptides on maximally Ca^{2+}-activated skeletal and cardiac CRCs

The potential of the peptides to induce subconductances in skeletal and cardiac CRCs was further examined under maximally Ca^{2+}-activating conditions at a free cytosolic and lumenal Ca^{2+} concentration of 20 μM. At this Ca^{2+} concentration both channel isoforms are maximally activated by Ca^{2+} under the recording conditions used in this study (Xu et al., 1998). Addition of the full-length II-III loop peptide, $p^{671-680}$ or $p^{720-765}$, to maximally Ca^{2+}-activated skeletal or cardiac CRCs did not result in any detect-

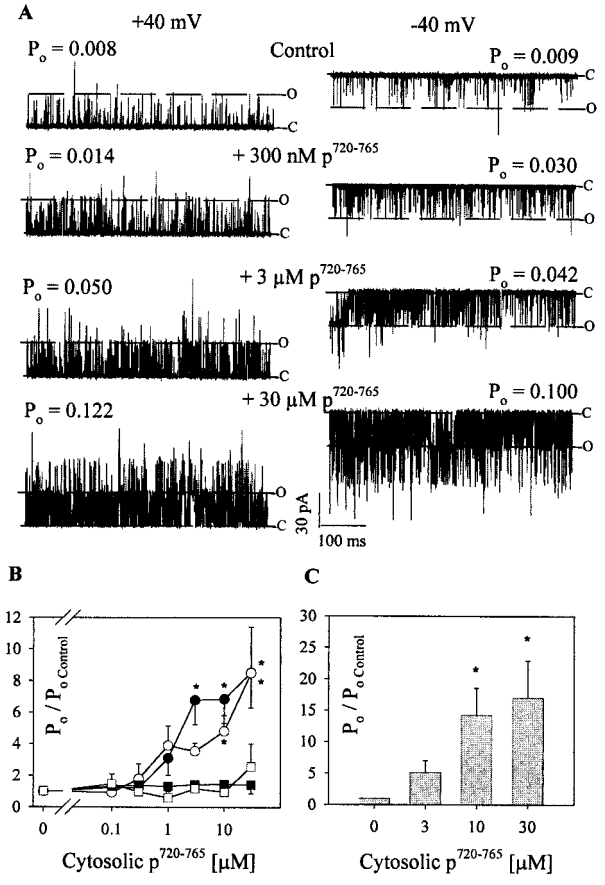

FIGURE 7 Effects of $p^{720-765}$ on submaximally Ca^{2+}-activated skeletal muscle and cardiac muscle CRC activities. (*A*) Shown are eight recordings from one experiment with three skeletal CRCs. Single channel currents, shown as downward or upward deflections from closed levels (marked "C"), were recorded as in Fig. 2 at +40 mV (*left panel*) and −40 mV (*right panel*) holding potentials at 0 (Control) and the indicated peptide concentrations. (*B*) Relative mean P_o values (±SE) from 4 to 11 experiments with skeletal CRCs (*circles*) and 9–17 experiments with cardiac CRCs (*squares*) at different peptide concentrations at +40 mV (*open symbols*) and −40 mV (*closed symbols*) holding potentials. (*C*) Relative mean P_o values from five to six experiments with skeletal CRCs under more physiological conditions using symmetrical 150 mM KCl and 10 mM lumenal Ca^{2+} as current carrier at 0 mV holding potential. The mean open probability used in the calculation of the $P_o/P_{oControl}$ ratios was 0.0037 ± 0.0025 ($n = 8$). To decrease noise, channel recordings were filtered at 300 Hz before analysis. *Significantly different ($p < 0.05$) from control as determined by ANOVA and Dunnett's method.

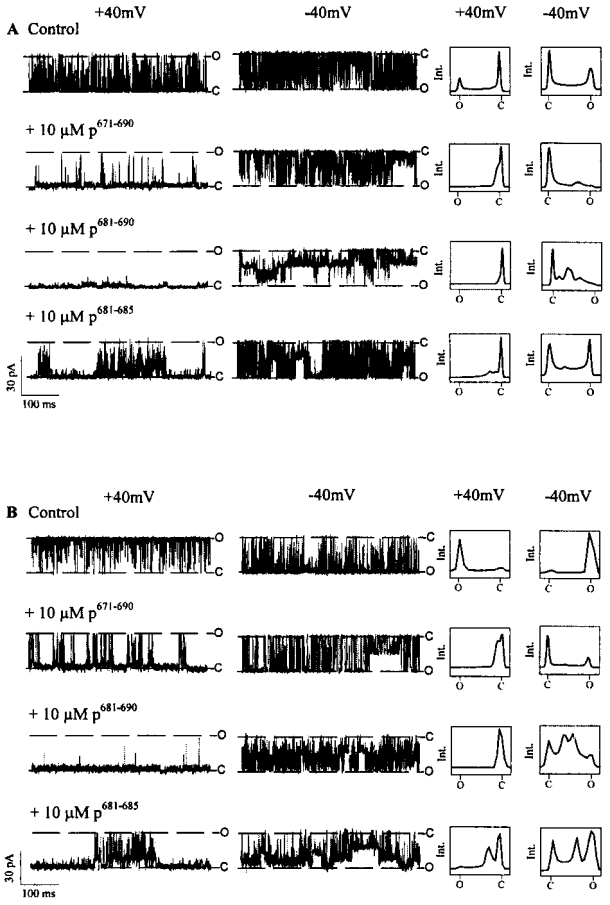

FIGURE 8 Truncated DHPR α_1-subunit II-III loop peptides containing the sequence RKRRK induce similar subconductances in maximally Ca^{2+}-activated skeletal (*A*) and cardiac (*B*) CRCs at +40 mV and −40 mV holding potentials. The cytosolic and lumenal free Ca^{2+} concentrations were 20 μM. Three truncated peptides, $p^{671-690}$, $p^{681-690}$, and $p^{681-685}$, induced subconductances and long channel closings. The major subconductance states induced by the peptides are shown in the accompanying current amplitude histograms, and are summarized in Table 1. The control traces and current amplitude histograms are from an experiment in which the effects of 10 μM $p^{671-690}$ were tested. The control traces and current amplitude histograms for experiments in the presence of $p^{681-690}$ and $p^{681-685}$ (not shown) were very similar to the one shown.

able subconductances (data not shown). However, $p^{671-690}$, $p^{681-690}$, and $p^{681-685}$, all of which contain a cluster of five positively charged amino acids, induced subconductance states in both skeletal and cardiac CRCs reminiscent of those observed in submaximally Ca^{2+}-activated channels.

At cytosolic concentrations in excess of 0.5 μM, $p^{671-690}$, $p^{681-690}$, and $p^{681-685}$ induced subconductance states in maximally Ca^{2+}-activated skeletal and cardiac CRCs. Representative current traces and the corresponding current amplitude histograms are shown for skeletal (Fig. 8 *A*) and cardiac CRCs (Fig. 8 *B*). Channels were recorded at +40 mV and −40 mV holding potentials in the absence (control) or presence of 10 μM $p^{671-690}$ (*first and second two traces* in Fig. 8, *A* and *B*), $p^{681-690}$ (*third two traces*), and $p^{681-685}$ (*fourth two traces*). The peptides induced a large number of different subconductances as identified by visual inspection of current traces. Subsets of consistently observed major substates were calculated as means ± SE from four to five amplitude histograms and are listed in Table 1. The two predominant substates induced by $p^{671-690}$ at +40 mV had conductances of 6.8 ± 1.1% and 13.1 ± 1.7% of the control full conductance. Additionally, long-lasting channel closings were observed at this holding potential (not shown).

TABLE 1 Subconductances in maximally Ca^{2+}-activated skeletal and cardiac CRCs induced by DHPR α_1-subunit II-III loop peptides containing a cluster of five positively charged amino acids

	Skeletal CRC		Cardiac CRC	
Peptide	+40 mV	−40 mV	+40 mV	−40 mV
$p^{671-690}$	6.8 ± 1.1	31.2 ± 2.0		
	13.1 ± 1.7	45.2 ± 4.7	11.6 ± 2.9	42.4 ± 2.3
		63.3 ± 2.1		64.8 ± 1.0
		86.6 ± 0.8		
$p^{681-690}$	4.8 ± 0.9	14.7 ± 4.4	5.6 ± 0.7	16.2 ± 1.8
		36.1 ± 4.1		
		48.7 ± 3.1		43.4 ± 2.8
		62.5 ± 2.2		61.6 ± 5.0
		86.5 ± 2.1		
$p^{681-685}$	11.9 ± 1.8	45.0 ± 3.2	14.0 ± 5.0	53.9 ± 2.9
	27.6 ± 1.3	61.5 ± 1.1	25.0 ± 2.8	84.6 ± 6.2

Subconductances are given as percent of control (no peptide) conductances. Data are the mean ±SE of four to five experiments.

$Peptide^{671-690}$ induced four predominant substates at −40 mV holding potential with conductances of 31.2 ± 2.0%, 45.2 ± 4.7%, 63.3 ± 2.1%, and 86.6 ± 0.8% of control full conductance. $Peptide^{681-690}$ and $p^{681-685}$ showed similar substate behavior, as a majority of the subconductances induced by these two peptides were close to those induced by $p^{671-690}$ (Table 1). Moreover, $p^{671-690}$, $p^{681-690}$, and $p^{681-685}$ induced subconductance states in the cardiac CRC that were similar to those induced in the skeletal CRC at positive and negative holding potentials (Fig. 8 and Table 1).

DISCUSSION

In the present study we have investigated various domains of the DHPR α_1-subunit II-III loop thought to be important for conferring skeletal-type E-C coupling. Comparison of the effects of the full-length II-III loop peptide and five shorter peptides on single skeletal and cardiac Ca^{2+} release channels allowed us to identify two regions ($p^{671-680}$ and $p^{720-765}$) that specifically activate the skeletal muscle CRC under physiologically relevant conditions. The finding that $p^{720-765}$ specifically activates the submaximally Ca^{2+}-activated skeletal CRC identifies a region of the DHPR II-III loop that has also been suggested by previous in vivo studies using dysgenic mice as being important for skeletal-type E-C coupling. Assessment of isoform-specificity at both submaximally and maximally Ca^{2+} activating conditions for each peptide further suggested that a region ($p^{681-690}$), previously implied to confer skeletal-specific E-C coupling, lacks isoform-specificity and induces similar subconductances in both skeletal and cardiac CRCs.

Full-length II-III loop peptide activates skeletal CRC in a concentration-dependent manner

In agreement with a previous study (Lu et al., 1994), the DHPR α_1-subunit full-length II-III loop peptide activated the purified skeletal CRC, but not the cardiac CRC, in single channel recordings. The full-length peptide activated the skeletal CRC by increasing the number of events, which suggests an increase in the close → open transition rate. The peptide at concentrations of up to 10 μM did not induce subconductance states in either submaximally or maximally Ca^{2+}-activated skeletal or cardiac CRCs. The results confirm that the II-III loop of DHPR α_1-subunit specifically interacts with the skeletal CRC.

Activation and inactivation of skeletal CRC by II-III loop peptides $p^{671-690}$, $p^{671-680}$, and $p^{681-690}$

Previous in vitro studies have suggested that residues 671–690 are critical for skeletal-type E-C coupling (El-Hayek et al., 1995; Gurrola et al., 1999; Dulhunty et al., 1999). At concentrations <10 μM, $p^{671-690}$ activated the skeletal CRC, whereas concentrations of >10 μM were inhibitory. El-Hayek and Ikemoto (1998) further localized the critical sequence to residues 681–690. A cluster of five positively charged residues RKRRK (residues 681–685) present in $p^{671-690}$ and $p^{681-690}$ was suggested to be essential for activation of the skeletal CRC by $p^{671-690}$ and $p^{681-690}$ (El-Hayek and Ikemoto, 1998; Zhu et al., 1999). Structural analysis using NMR indicated that $p^{671-690}$ consists of a helical segment extending from the N-terminus to Lys^{685}, followed by an unstructured region extending to the C-terminus (Casarotto et al., 2000). Thus, the cluster RKRRK is located in the region of the peptide that is structured, whereas in a shorter peptide (residues 681–687) RKRRK showed little if any structure. This may explain why this cluster by itself was only minimally effective in increasing channel activity (El-Hayek and Ikemoto, 1998; Casarotto et al., 2000). However, a role for this cluster was indicated by the observation that its interruption by a single negatively charged residue (R684E) abolished the increase in [^{3}H]ryanodine binding otherwise observed with the unmodified $p^{666-690}$ (Gurrola et al., 1999). Conversely, formation of a cluster of five positively charged residues (substitution of a glutamate with a lysine) in an inactive cardiac peptide corresponding to skeletal $p^{681-690}$ yielded an active peptide that induced subconductances in skeletal CRC (Zhu et al., 1999).

Our results confirm that $p^{671-690}$ can activate and inhibit the skeletal CRC depending on peptide concentration. However, we find that the region in $p^{671-690}$ that specifically interacts with the skeletal CRC resides in residues 671–680 as $p^{671-680}$ activated the submaximally Ca^{2+}-activated skeletal, but not cardiac, CRC. To our knowledge, the effects of $p^{671-680}$ on the skeletal and cardiac CRCs have not been

documented previously. In contrast, $p^{681-690}$ interacted isoform-independently with the channels inducing substates in the skeletal and cardiac CRCs that were not observed for the full-length II-III loop peptide. A long mean open time of the subconductances could provide an explanation for the observation that the peptide increased [^{3}H]ryanodine binding to and SR Ca^{2+} release from skeletal SR vesicles (El-Hayek and Ikemoto, 1998; Gurrola et al., 1999; Zhu et al., 1999). In addition to lacking isoform-specificity, the proposed role of aa 681–690 was questioned by a recent report showing that microinjection of a construct with a scrambled sequence in residues 681–690 resulted in skeletal E-C coupling (Proenza et al., 2000). Thus, this study, which was published after the original submission of our work, also suggests that aa 681–690 are not crucial for conferring skeletal-specific E-C coupling.

The relevance of our finding concerning the role of aa 671–680 is at variance with expression studies in dysgenic myotubes. Microinjection of skeletal muscle and cardiac DHPR chimeric constructs into dysgenic myotubes indicated that residues 666–709 of the II-III cytoplasmic loop of the skeletal muscle DHPR α_1-subunit are not crucial skeletal muscle E-C coupling (Nakai et al., 1998). However, it is conceivable that due to 70% sequence identity in the N-terminal part of the DHPR II-III loop, studies with skeletal/cardiac chimeras may have failed to detect a role of aa 671–680 in mediating skeletal-type E-C coupling. Ultimately, the physiological relevance of our findings regarding $p^{671-680}$ remains to be determined using a cellular system.

Peptide$^{666-791}$ contains a second critical sequence for activating the skeletal CRC

Expression of skeletal muscle and cardiac DHPR chimeric constructs in dysgenic myotubes indicated that a 17-amino acid region (residues 725–742) of the putative II-III cytoplasmic loop of the DHPR α_1-subunit is a weak determinant of skeletal muscle E-C coupling (Nakai et al., 1998). Skeletal-type coupling was stronger in a chimera containing skeletal residues 711–765. These results are difficult to reconcile with observations that $p^{724-760}$ by itself had no effect, but antagonized the activating effects and conformational changes induced by both $p^{671-690}$ and T-tubule depolarization (El-Hayek et al., 1995; Saiki et al., 1999). Based on this evidence, Saiki et al. (1999) proposed that depolarization-induced activation of E-C coupling is mediated by the binding of an activator located in the II-III loop corresponding to $p^{671-690}$ to the skeletal CRC. Binding of a blocker/reprimer located in residues 724–760 to the same or another region of skeletal CRC removes the activator from its site(s) during T-tubule polarization. In contrast, Nakai et al. (1998) speculated that the midregion of the II-III loop (residues 725–742) might be an agonist that assumes the right configuration to bind to and activate the CRC only when the sarcolemma is depolarized. Our single channel studies support the model by Nakai et al. (1998) by showing that $p^{720-765}$ specifically activated the submaximally Ca^{2+}-activated skeletal CRC at micromolar concentrations. We conclude that the skeletal II-III loop contains two segments, residues 671–680 and 720–765, that specifically activate the skeletal CRC.

Induction of subconductance states in skeletal and cardiac CRCs

Our results suggest that the three peptides, $p^{671-690}$, $p^{681-690}$, and $p^{681-685}$, that all contain a cluster of five positively charged residues, interact with both the skeletal and the cardiac CRCs. Elevated levels of $p^{671-690}$ and $p^{681-690}$ inhibited the submaximally Ca^{2+}-activated skeletal and cardiac CRCs and formed detectable subconductance states at +40 mV at concentrations in excess of 0.5 μM. All three peptides induced subconductance states in both channel isoforms at +40 mV and −40 mV under maximally Ca^{2+}-activating conditions. Peptide$^{671-680}$, which does not contain a cluster of positive charges, failed to induce subconductances in skeletal and cardiac CRCs.

One explanation for the subconductances is that the short positively charged peptides enter the wide cytosolic vestibule of the CRCs (Radermacher et al., 1994; Serysheva et al., 1995) and bind to site(s) inaccessible for larger peptides containing the RKRRK motif (i.e., the full-length II-III loop peptide) and cause a partial block of the ion conductance pathway. Similar mechanisms have been proposed for other small basic peptides (Mead et al., 1998; Tripathy et al., 1998), large tetraalkyl ammonium ions (Tinker et al., 1992) and charged local anesthetics (Tinker and Williams, 1993; Xu et al., 1993) to explain subconductances in the skeletal and cardiac CRCs. In a recent study, the $p^{671-690}$-induced block of the skeletal CRC could be reversed by an increased permeant cation concentration in the *trans* (SR lumenal) bilayer chamber, also suggesting that the peptides can enter the cytoplasmic vestibule to induce subconductances (Dulhunty et al., 1999).

It is noteworthy that the motif RKRRK is also found in Imperatoxin A ($IpTx_a$), a 33-amino acid peptide isolated from the scorpion Pandinus imperator, which induces voltage-dependent subconductance states in both skeletal and cardiac CRCs (Tripathy et al., 1998). It has been suggested that $IpTx_a$ mimics the effects of II-III loop peptides as it displays structural and functional similarities with residues 666–690 of the skeletal DHPR α_1-subunit II-III loop (Gurrola et al., 1999). The toxin binds with nanomolar affinity to skeletal CRC (Gurrola et al., 1999) at a cytoplasmic site 11 nm from the transmembrane pore (Samso et al., 1999). The presence of a common motif of basic residues in $IpTx_a$ and the three truncated II-III loop peptides ($p^{671-690}$, $p^{681-690}$, and $p^{681-685}$) raises the possibility that the peptides do not act as channel blockers, but rather bind to regions outside

the channel pore, and induce subconductance states by a conformational change.

In conclusion, the present study shows that the DHPR α_1-subunit II-III loop has two regions, residues 671–680 and 720–765, that specifically activate the skeletal CRC. A II-III loop region containing a cluster of five positively charged residues (aa 681–685) induces subconductances in both skeletal and cardiac CRCs. The finding of an isoform-independent interaction among $p^{671-690}$, $p^{681-690}$, $p^{681-685}$, and CRCs suggests that the action of these peptides, while interesting from a biophysical point of view, could be unrelated to cellular function.

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