Calcium Current in Rat Cardiomyocytes Is Modulated by the Carboxyl-terminal Ahnak Domain* 

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Ahnak, a protein of 5643 amino acids, interacts with the regulatory β-subunit of cardiac calcium channels and with F-actin. Recently, we defined the binding sites among the protein partners in the carboxyl-terminal domain of ahnak. Here we further narrowed down the β2-interaction sites to the carboxyl-terminal 188 amino acids of ahnak by the recombinant ahnak protein fragments P3 (amino acids 5456–5556) and P4 (amino acids 5556–5643). The effects of these P3 and P4 fragments on the calcium current were investigated under whole-cell patch clamp conditions on rat ventricular cardiomyocytes. P4 but not P3 increased significantly the current amplitude by 22.7 ± 5% without affecting its voltage dependence. The slow component of calcium current inactivation was slowed down by both P3 and P4, whereas only P3 slowed significantly the fast one. The composite recombinant protein fragment P3−P4 induced similar modifications to the ones induced by each of the ahnak fragments. In the presence of carboxyl-terminal ahnak protein fragments, isoprenaline induced a similar relative increase in current amplitude and shift in current kinetics. The actin-stabilizing agents, phalloidin and jasplakinolide, did not modify the effects of these ahnak protein fragments on calcium current in control conditions nor in the presence of isoprenaline. Hence, our results suggest that the functional effects of P3, P4, and P3−P4 on calcium current are mediated by targeting the ahnak-β2-subunit interaction rather than by targeting the ahnak-F-actin interaction. More specifically they suggest that binding of the β2-subunit to the endogenous subsarcosome giant ahnak protein re-primes the α1C/β2-subunit interaction and that the ahnak-derived proteins relieve the β2-subunit from this inhibition.

Ca2+ influx through the L-type Ca2+ channel (ICaL) initiates and modulates cardiac contraction. L-type Ca2+ channels activate with membrane depolarization and inactivate over time. Inactivation is an important property in regulating action potentials duration and intracellular Ca2+ transients. The Ca2+ channels are multimeric proteins, built up of at least three channel subunits, α1, α2δ, and β. The α1-subunit serves as the channel pore and voltage sensor. The intracellular β-subunit modifies the properties of the channel complex both by chaperoning the translocation of the α1-subunit to the plasma membrane and by allosteric modulation of the α1C-subunit function. In cardiomyocytes, the subunit isoforms α1C (Ca1.2) and β2 constitute the channel complex (reviewed in Ref. 1).

The most potent mechanism to enhance myocardial contractility occurs via sympathoadrenergic stimulation (2). Activation of the β-adrenergic receptor results in an increase in peak inward current and a slowing of inactivation of (ICaL) via protein kinase A (PKA)-dependent phosphorylation of the channel subunits α1C (3−5) and β2 (6−8). In an attempt to define the molecular details of channel subunit phosphorylation, we have identified the 700-kDa ahnak protein as the prominent PKA target in mammalian cardiomyocytes that is recovered in anti-β2-subunit immunoprecipitates (9).

Ahnak, a protein of 5643 amino acids, has been implicated in different cell type-specific functions as diverse as cell differentiation (10, 11), signal transduction (9, 12−14), Ca2+ homeostasis (15), and regulated exocytosis (16). The ahnak protein can be divided into three regions: unique amino-terminal and carboxyl-terminal portions flank a large central region with multiple repeated units (10, 17). The carboxyl-terminal ahnak domain encompassing 1002 amino acid residues contains both nuclear localization signals and nuclear export signals that are believed to determine the subcellular distribution of ahnak (14, 18). We next characterized ahnak in normal human myocardium as a peripheral membrane protein associated with the cytoplasmic side of the sarcotubular structures (19). By using truncated ahnak fragments, we demonstrated that the high affinity interaction (Kd ~50 nM) between β2-subunit and ahnak is mediated by the most carboxyl-terminal 382 amino acids of ahnak, designated as the ahnak-C2 domain. This ahnak-C2 domain was also defined to be responsible for F-actin binding (19). Together, the localization and interaction partner suggest a role of cardiac ahnak in the regulation of Ca2+ channel activity either directly via β2-subunit interaction or indirectly via F-actin interaction. In fact, recent studies (20−22) revealed the cytoskeletal actin filament organization as an important regulator of the Ca2+ channel inward current.

The goal of the present study was to elucidate whether ahnak affects Ca2+ channel gating properties. Small ahnak

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1 The abbreviations used are: PKA, protein kinase A; ISO, isoprenaline; aa, amino acids; GST, glutathione S-transferase.
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fragments were expressed encompassing the 188 carboxyl-terminal amino acid residues of human ahnak (P3, aa 5456–5556, and P4, aa 5556–5643); their effects on Ca2+ current, I_{CaL}, were investigated under whole-cell patch clamp conditions on ventricular rat cardiomyocytes. The rationale for this approach was that we narrowed down the interaction sites between ahnak and β2-subunit to the composite ahnak protein fragment encompassing the carboxyl-terminal amino acids 5026–5463 as an intrinsic regulatory protein of the L-type Ca2+ channel complex.

EXPERIMENTAL PROCEDURES

Materials—Plasmid DNA of human ahnak was kindly provided by Dr. Emma Shvivelman (University of San Francisco). The ahnak-C2 fragment encompassing the carboxyl-terminal amino acids 5262–5463 was constructed from the plasmid z7 (10) and was expressed as GST fusion protein as described (19). The construct for the Ca2+ channel β2-subunit was prepared from the expression plasmid pCDNAβ2 encoding the rabbit β2 (546) kindly provided by Dr. Franz Hofmann (Technical University, Munich, Germany). The β2-subunit was expressed as GST fusion protein as described previously (19). The antibody against the β2-subunit was characterized previously (9, 24).

Recombinant Ahnak Fragments—The carboxyl-terminal ahnak fragments were generated by PCR using z7 as template and the following primers: P1–P2, forward, 5’-agctgaatctgtgatgtgaacctgccag-3’; reverse, 5’-gatctcgagcttcaaactccagcgt-3’. P3–P4, forward, 5’-agctgaatctgtgatgtgaacctgccag-3’; reverse, 5’-gatctcgagaccttcaaactccagcgt-3’. P1–P3, forward, 5’-agctgaatctctgaagtcaaactg-3’; reverse, 5’-gatctcgagcagtttgacttcagactc-3’. P1, forward, 5’-gatctcgagcagtttgacttcagactc-3’; reverse, 5’-gatctcgagcagtttgacttcagactc-3’. P2 (aa 5255–5463). These recombinant proteins showed the expected molecular masses (Fig. 1B). The β2-subunit binding of the carboxyl-terminal ahnak fusion proteins was studied in an enzyme-linked immunosorbent assay-based competition approach. For this purpose recombinant rabbit cardiac β2-subunit was bound to ahnak-C2 capturing microtiter plates in the absence and presence of the carboxyl-terminal ahnak fusion proteins. The binding was monitored by immunoreaction with an anti-β2-subunit specific antibody (Fig. 1C). As expected, the binding of β2-subunit to captured ahnak-C2 was efficiently prevented by the inclusion of a 10-fold molar excess of ahnak-C2 in the binding assay. In contrast, inclusion of the P1–P2 truncation mutant as competitor reduced the β2-subunit binding only marginally, indicating that this portion does not confer ahnak-C2-β2-subunit interaction. Consistent with this result, the carboxyl-terminal truncate P3–P4 efficiently inhibited the interaction (Fig. 1C). The P3 and P4 ahnak carboxyl-terminal domains resulted in partial competition of the β2-subunit interaction. Taken together, these results define binding sites to the Ca2+ channel β2-subunit in both carboxyl-terminal ahnak sub-fragments P3 and P4.

Effects of Ahnak Fragments on Ca2+ Current—Cardiac myocytes were investigated under whole-cell patch clamp conditions using a pipette solution that was added with 10 μM of one of the short carboxyl-terminal sequences of ahnak P3, P4, or P3–P4, or with GST as a control, such a concentration was far above the apparent K_D of 50 nM for binding to ahnak-C2 domain. The intracellular perfusion of a cell with a pipette solution that contained the combined P3–P4 ahnak protein fragment induced a slowly developing increase in peak I_{CaL}, elicited at 0 mV and reached a maximum within about 3–4 min, a time needed for diffusion of the internal pipette solution. I_{CaL}, inactivation kinetics were simultaneously modified showing an increase in both τ_{r} and τ_{i}, the fast and slow time constants that described the two components of I_{CaL}, inactivation (Fig. 2A). The results of similar experiments with adding 10 μM GST, P3, P4, or P3–P4 ahnak protein fragments are summarized in Fig. 2B (see also Table I). The addition of GST had no effect on I_{CaL} amplitude and kinetics. Thus in some cases, data are pooled results of about the same number of cells investigated in con-
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**Fig. 1.** Molecular structure of ahnak and dissection of carboxyl-terminal binding sites to the Ca\(^{2+}\) channel \(\beta_{2}\)-subunit. A. scheme of ahnak indicating the molecular structure and the location of the carboxyl-terminal fragments P1–P2, P3–P4, P3, and P4. B. C-terminal ahnak proteins fused to GST. C. competition assay for Ca\(^{2+}\) channel \(\beta_{2}\)-subunit binding. The competitors are truncation mutants of ahnak-C2 shown in A. Optical densities measured for bound \(\beta_{2}\)-subunit in the absence of competitors were 0.513 ± 0.011 (X ± S.E., n = 3). Results of a representative experiment are shown out of three similar experiments.

Control conditions or with GST-added pipette solution. Added at 10 \(\mu\)M, P4 had a significant positive effect on \(I_{Ca,L}\) amplitude that was increased by 22.7 ± 5.0% from a control value of 13.2 ± 1.1 pA/pF \((n = 23, 16; p < 0.01)\). The combined ahnak fragment P3–P4 also significantly increased peak \(I_{Ca,L}\) by 34.8 ± 7.1%, whereas fragment P3 had no significant effect. All fragments significantly slowed down both the fast and slow components of \(I_{Ca,L}\) inactivation except P4 that did not significantly alter the fast component of inactivation (Table I). Altogether, in the presence of one of the ahnak protein fragments, the increase in current amplitude or slowing of its inactivation led to a 50% increase in the amount of charges carried by Ca\(^{2+}\) (see also Fig. 3).

The carboxyl-terminal ahnak fragments-induced increase in current amplitude occurred at all voltages, whereas there was no clear shift in the whole I/V curves. However, a detailed study of the activation and inactivation parameters indicates that in the presence of P4 and P3–P4, half-activation but not half-

inactivation was shifted leftward by about 4 mV, although this effect did not reach significance (Table I). The \(\beta\)-adrenergic stimulatory effects of isoprenaline (ISO, 1 \(\mu\)M) on \(I_{Ca,L}\), which include increases in \(I_{Ca,L}\) amplitude and \(\tau_{f}\) and a leftward shift in voltage dependence, were not significantly affected by the presence of the ahnak fragments except for a significant prolongation of the fast inactivation phase by P4 and P3–P4, such that the quantity of entering Ca\(^{2+}\) charges could then be increased up to nearly 3-fold in all three experimental conditions (Fig. 3; Table I).

The actin-stabilizing agents, a 5-h incubation with 100-\(\mu\)M phallaloid as well as the acute application of 10 \(\mu\)M jasplakolide did not modify the effects of the two P3 and P4 fragments on \(I_{Ca,L}\) amplitude or inactivation kinetics, upon a depolarization at 0 mV in control conditions as well as in the presence of isoprenaline (not shown).

**Effects of Ahnak Protein Fragments on Voltage-dependent Ca\(^{2+}\) Current Facilitation**—High voltage pre-depolarizing steps are inducing a partial recovery from inactivation or voltage-dependent facilitation of \(I_{Ca,L}\) that is accompanied by a slowing of \(I_{Ca,L}\) inactivation kinetics. \(I_{Ca,L}\), voltage-dependent facilitation had similar relative amplitude in each experimental condition with pipette solution containing 10 \(\mu\)M GST, P3, P4, or P3–P4 fragment. Voltage-dependent facilitation was not affected by ISO (not shown). The changes in current inactivation kinetics were further analyzed following a depolarizing prepulse to +60 mV (Fig. 4). The depolarizing prepulse induced an increase in both \(\tau_{f}\) and \(\tau_{s}\) characterizing current inactivation during the test pulse to 0 mV. Nevertheless, \(\tau_{s}\) was further increased in the presence of each ahnak fragment, and the effect was slightly more marked in the presence of isoprenaline.

**Effects of Ahnak Protein Fragment on Use-dependent Ca\(^{2+}\) Current Facilitation**—In rat cardiomyocytes, use-dependent facilitation that leads to an increased Ca\(^{2+}\) influx is mostly attributable to a slowing of the fast inactivation phase that is maximal after the 4th or 5th pulse. This effect is dependent on reduced sarcoplasmic reticulum-Ca\(^{2+}\) release with increasing stimulation frequency and is suppressed by \(\beta\)-adrenergic stimulation (25, 27). The effects of the three ahnak fragments were investigated on use-dependent facilitation by varying the basal stimulation frequency of the 0-mV depolarizing test from 0.25 to 2 Hz after a 15-s rest period and analyzing changes in the two inactivation phases of \(I_{Ca,L}\). The use-dependent increase in \(\tau_{s}\) seen during facilitation was not observed in the presence of P3 and of P3–P4 that had already increased \(\tau_{s}\). There was no effect of P4 on \(\tau_{s}\). There was also no significant effect of these ahnak fragments on \(\tau_{f}\).

**DISCUSSION**

This study demonstrates that carboxyl-terminal ahnak protein fragments modulate Ca\(^{2+}\) channel properties. Reversible binding between the protein fragments and the cardiac \(\beta_{2}\)-subunit suggests this interaction to be responsible for Ca\(^{2+}\) channel modulation. Carboxyl-terminal fragment addition to the pipette solution induces an increase in \(I_{Ca,L}\) amplitude and a slowing of \(I_{Ca,L}\) inactivation. Furthermore, voltage-dependent but not use-dependent \(I_{Ca,L}\) facilitation is affected. None of the isoprenaline effects on Ca\(^{2+}\) current are affected. Taking into account that the presence of the \(\beta_{2}\)-subunit increases \(I_{Ca,L}\), these results suggest a stronger interaction of endogenous \(\alpha_{1}\) and \(\beta_{2}\)-Ca\(^{2+}\) channel subunits occurs in the presence of carboxyl-terminal ahnak protein fragments by preventing the ahnak-\(\beta_{2}\)-subunit binding. The results are thus consistent with the hypothesis that endogenous ahnak exerts a sustained inhibitory effect on the cardiac Ca\(^{2+}\) channel by binding to the \(\beta_{2}\)-subunit.

\(\beta\)-Subunits induce generic as well as specific modifications in
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The carboxyl-terminal ahnak fragments increased Ca²⁺ current and slowed its kinetics. A time constant of peak I_{Ca,L} and τ_f and τ_s, the time constant of the fast and slow components of inactivation shown here following a 1-min delay after breaking the patch with a pipette that contained the P3–P4 ahnak protein fragment. Stimulation frequency was 0.125 Hz. Inset, typical recordings of the Ca²⁺ current elicited at 0 mV at the indicated time. B, peak I_{Ca,L} densities elicited at 0 mV depolarization in control conditions and with a pipette solution added with 10 μM GST, P3, P4, or P3–P4 protein fragment. *, p < 0.05 compared with control.

**Table I**

<table>
<thead>
<tr>
<th>Ca²⁺ current</th>
<th>Activation</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>I_{Ca,L} (pA/pF)</td>
<td>A_f</td>
</tr>
<tr>
<td>Control + ISO</td>
<td>23</td>
<td>13.2 ± 1.1</td>
</tr>
<tr>
<td>GST + ISO</td>
<td>11</td>
<td>22.0 ± 1.5</td>
</tr>
<tr>
<td>P3 + ISO</td>
<td>15</td>
<td>22.0 ± 1.3</td>
</tr>
<tr>
<td>P4 + ISO</td>
<td>16</td>
<td>23.6 ± 1.9</td>
</tr>
<tr>
<td>P3–P4 + ISO</td>
<td>4</td>
<td>16.2 ± 0.9</td>
</tr>
</tbody>
</table>

α₁-subunit behavior. Increased channel expression, activity, and membrane targeting of the α₁-subunit are common modifications induced by all β-subunits, whereas changes in the voltage dependence and kinetics of activation and inactivation are more specific of a given pair of α₁/β-subunits. These effects appear to be mediated by high affinity interactions between the AID (α₂-interaction domain; located on the loop connecting domains I and II of each α₁-subunit) (28) and the BID (β-interaction domain; located on the beginning of the second conserved region of the β-subunit) (29). Additional interaction sites of lower affinity (on the amino- and carboxyl-terminal domains of the β-subunits and α₁-subunits) may also contribute to these regulations (30–33). Previously, we proposed potential regulation of Ca²⁺ channel activity by the interaction of
the β₂-subunit with ahnak, a 700-kDa PKA substrate (9) originally identified as neuroblast differentiation-associated protein (10). The most carboxyl-terminal 382 amino acids of ahnak, designated as the ahnak-C2 domain, were identified to mediate high affinity (Kᵣ ~ 50 nM) β₂-subunit binding that do not interfere with α₇-B₂-subunit interaction mediated by the AID (19). The first part of this work more precisely determines the interaction of carboxyl-terminal ahnak with the β₂-subunit by using the amino- and carboxyl-terminal truncation mutants of ahnak-C2, P1–P2, and P3–P4, respectively. In vitro binding experiments revealed that P3–P4 alone showed partial β₂-subunit binding observed with ahnak-C2. Because P3 and P4 alone showed partial β₂-subunit binding, we propose the existence of two binding sites within the 188 carboxyl-terminal amino acid residues of ahnak.

The increase in Ca²⁺ current amplitude induced by applying the carboxyl-terminal fragments indicates changes in channel gating. This observation could be accounted for by suggesting that β₂-subunits are made more available because co-expression of the β₂-subunit with the α₇-subunit enhances charge movement (34–36). β-Subunits are also known to modulate Ca²⁺ channel kinetics. Ca²⁺ channels are capable of undergoing three different types of inactivation processes: Ca²⁺-dependent inactivation, fast voltage-dependent inactivation, and slow inactivation (37). Increasing the depolarization voltage progressively replaced Ca²⁺-dependent inactivation in the fast phase of the decay of the Ca²⁺ current with rapid voltage-dependent inactivation. Ba²⁺ current, through the T-type Ca²⁺ channel, inactivates essentially by voltage-dependent mechanisms with fast and slow kinetics. Furthermore, the fractional inhibition of slow inactivation in mutants causes an acceleration of fast inactivation suggesting that fast and slow inactivation mechanisms are linked. Two major mechanisms have been implicated in voltage-dependent inactivation. The ball-and-chain mechanism or hinged-lid of an ion pore implies the occlusion by a positively charged segment that could be the amino terminus in K⁺ channel or, in Ca²⁺ channel, the I–II linker region that docks to a site comprising at least the domain II and III of S6 segments (38). The second C-type mechanism of slower K⁺ channel inactivation was found to involve a constriction of the pore by S6 segments (39). Recent results suggest that the slow inactivation of α₇-channel is mediated by an annular determinant composed of amino acid residues situated in the cytoplasmic ends of transmembrane segments S6 in repeats I–IV (40). This slow phase was not investigated in this study that uses depolarizing pulses in the hundreds millisecond range. Voltage- and Ca²⁺-dependent inactivation are intrinsic properties of the α₇-subunit. It was proposed that voltage and Ca²⁺ inactivate by using a “ball-and-chain” mechanism with blocking particles and binding sites encoded by homologous sequences therefore sensitive to the same molecular interactions with the β₂-subunit, with binding ensuring channel inactivation. The I–II loop represents an attractive candidate (38, 41, 42). The three ahnak protein fragments that are shown in this work to bind with the β₂-subunit induce an increase in Iₐᵤₐ₅ amplitude as well as a slowing of both components of Iₐᵤₐ₅ inactivation. Note, however, that these effects are not directly correlated because a lesser increase in peak Iₐᵤₐ₅ is observed when adding fragment P3 that also induces a more marked slowing of the fast inactivation component than P4.

Analysis of voltage-dependent activation shows a hyperpo-
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