Research Paper

Auxiliary β Subunits Differentially Determine PKA Utilization of Distinct Regulatory Sites on Caᵥ1.3 L Type Ca²⁺ Channels

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Original manuscript submitted: 02/01/07
Revised manuscript submitted: 04/06/07
Manuscript accepted: 04/12/07

Previously published online as a Channels E-Publication: http://www.landesbioscience.com/journals/channels/article/4284

KEY WORDS
Caᵥ1.3, cAMP, kinase, PKA, phosphorylation, L type calcium channel, auxiliary β subunit

ACKNOWLEDGEMENTS
We are especially grateful to Dr. Diane Lipscombe for providing us with the plasmids used in this study. We thank Alex Dopico and Angela Cantrell for helpful comments. This work was supported by a Beginning Grant-In-Aid from the American Heart Association-Southeast Affiliate (0365289B), NIH (NS46661), and start-up funds provided by the University of Tennessee Health Science Center and the UTHSC Neurobiology of Brain Disease Center of Excellence.

NOTE
Supplementary material can be found at: www.landesbioscience.com/supplement/LiangCHAN1-2-sup.pdf

ABSTRACT
L-type calcium channels (Caᵥ1.1–Caᵥ1.4) link Ca²⁺ influx to membrane depolarization and serve a critical role in regulating membrane excitability, muscle contraction, hormone secretion, and gene transcription. In many tissues, L-type calcium channel activity (Caᵥ1.1 and Caᵥ1.2) is enhanced by transmitters and hormones that activate the cAMP-dependent protein kinase (PKA), which is largely thought to be mediated via phosphorylation of the pore-forming α subunit. However, the ability of PKA to regulate Caᵥ1.3 and the sites contributing to effective modulation of channel activity remains to be established. Using HEK 293 cells, we demonstrate that currents carried by the long C-terminal splice variant of Caᵥ1.3 (Caᵥ1.3L) are selectively enhanced compared to the short C-terminal splice variant (Caᵥ1.3S) when the catalytic subunit of PKA is introduced into the cell via the whole-cell recording electrode. However, the persistence of this regulation is dependent on the identity of the auxiliary β subunit, such that PKA produces only a transient increase in the presence of β₂ while a persistent increase is observed in the presence of the β₂a subunit. Site-directed mutagenesis of consensus PKA phosphorylation sites revealed that Ser1964 and Ser1743 in Caᵥ1.3L were the predominant sites controlling PKA modulation in the presence of the β₂ and β₂a auxiliary subunits, respectively. Therefore, β subunits determine the contribution of distinct sites within Caᵥ1.3 towards PKA-mediated enhancement of channel activity. These data suggest that auxiliary β subunits govern the access of signaling enzymes to L-type calcium channels.

ABBREVIATIONS
AKAP, A kinase anchoring protein; cAMP, 3’,5’ cyclic adenosine monophosphate; DMEM, Dulbecco’s Modified Eagle’s Medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HEK 293, human embryonic kidney 293; I–V, current voltage; PBS, phosphate buffered saline; PKA, cAMP dependent protein kinase; PKI, protein kinase inhibitor (5-24) amide; RT PCR, reverse transcriptase polymerase chain reaction; SAN, sino atrial node

INTRODUCTION
By linking Ca²⁺ influx to membrane depolarization, the family of voltage-gated L-type Ca²⁺ channels (Caᵥ1) contribute to muscle contraction, transmitter and hormone secretion, membrane excitability, synaptic plasticity, activation of signaling cascades, and gene transcription. L-type channels are derived from four genes, encoding Caᵥ1.1–1.4. Each member of the Caᵥ1 family of channels displays unique and sometimes overlapping tissue distributions. Although the molecular, biochemical, pharmacological, and biophysical properties of Caᵥ1.1 and Caᵥ1.2 have been extensively examined, it was commonly thought that other Caᵥ1 family members would display similar properties given the homology exhibited at the molecular level. Recent studies, however, indicate that Caᵥ1.3 exhibits an activation threshold that is ~15 mV hyperpolarized compared to Caᵥ1.2, as well as a reduced sensitivity to dihydropyridine antagonists. Likewise, Caᵥ1.4 not only exhibits an activation threshold negative to that of Caᵥ1.2 and reduced sensitivity to dihydropyridine antagonists, but also lacks Ca²⁺-dependent inactivation. Collectively, these studies suggest that each member of the L-type Ca²⁺ channel family may vary in hallmark properties associated with the prototypical Caᵥ1.2 family member. Thus, the question arises as to whether other modes of regulation associated with Caᵥ1.1 and Caᵥ1.2 can be extended to the Caᵥ1.3 channels.
Numerous studies have implicated Ca_1.1 and Ca_1.2 L-type channel activity as a critical target of regulation by hormone and neurotransmitter activated G-protein coupled receptors (GPCRs). Ligand binding to these receptors leads to G_{αs} mediated stimulation of adenyl cyclase, activation of the cAMP-dependent protein kinase (PKA), and phosphorylation of Ca_1.1 and Ca_1.2 at specific sites that cause upregulation of L-type channel activity.^{1,11} This pathway provides an important mechanism for sympathetic control of skeletal muscle and cardiac contraction, as well as for hormone secretion. The question of whether this pathway applies to Ca_1.3 remains unclear. However, primary sequence analysis of Ca_1.3 indicates that several potential PKA phosphorylation sites are present.^{12,13} Further, recombinant Ca_1.3 can be phosphorylated in vitro by incubation with the PKA catalytic subunit, with several potential PKA phosphorylation sites in the C-terminal tail of Ca_1.3 having been identified.^{14} However, the Ca_1.3 gene undergoes extensive alternative splicing yielding multiple mRNA transcripts and protein products.^{3,4,13,15} The most notable processing occurs in the C-terminals of Ca_1.3, giving rise to proteins that differ in length by over 500 amino acids (Fig. 1).^{13} The longer form of the C-terminal tail has been demonstrated to be a target for several regulatory protein-protein interactions as well as possessing several regulatory motifs common with other Ca_1 family members.^{16-20} (Fig. 1). Importantly, it is within this long form of the channel where the majority of the in vitro identified PKA sites exist (Fig. 1), leading us to hypothesize that PKA may differentially regulate Ca_1.3 C-terminal splice variants.

Voltage-gated calcium channels are heteromultimeric complexes consisting of the pore forming α subunit, as well as auxiliary β, γ, and α_δ subunits.\(^1\) These auxiliary subunits have been associated with alterations of surface expression, kinetics, and voltage-dependent properties.\(^{21}\) Although most attention regarding PKA mediated modulation of L-type calcium channels has focused on phosphorylation dependent modulation of the pore forming α subunit, several studies have also suggested that the β_2α_2 subunit may also constitute a target for PKA mediated upregulation of Ca_1.2 channel activity.\(^{22,23}\) Therefore, we sought to determine whether PKA regulates Ca_1.3 splice forms and whether such regulation is influenced by the identity of coexpressed β subunits. Our data suggest that auxiliary β subunits play an important role in determining which PKA regulatory sites in the long isoform of Ca_1.3 are used to modify channel activity.

**MATERIALS AND METHODS**

Reverse transcriptase-polymerase chain reaction (RT-PCR). Adult rats were sacrificed by overdose with pentobarbital in accordance with institutional protocols. The heart was dissected out and placed into an oxygenated calcium-free phosphate buffered saline (PBS). Atria and ventricular tissue were microdissected. Total RNA was harvested and treated with DNase I using the RNaseasy kit (Qiagen; Valencia, CA) following the manufacturer’s protocol. One-step RT-PCR (Qiagen) reaction was performed using ~1 µg of total RNA. The following RT-PCR primers were used: sense (5’-AGCTACTTGGACAGTTGTC-3’) and antisense #1 (5’-GCTGAAGGACTCTCTCCTCTA-3’) as previously described to detect the presence of long and short forms of Ca_1.3 (designated here as Ca_1.3L and Ca_1.3Sb).\(^{15}\) Sense (as above) and antisense #2 (5’-ACAGATGAGCCA CACACGC-3’), were used to assess the presence of the short form of the channel previously cloned from brain and superior cervical ganglion (which we designate as Ca_1.3S). Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from (IDT; Coralville, IA). All RT-PCR reactions were carried out on a thermocycler (Techne; Cambridge, UK) using the following protocol: 94°C for 2 min for initial denaturation, 47°C for 30 min for reverse transcription, 94°C for 15 min to inactivate the reverse transcriptase. The subsequent PCR was carried out using 30 cycles. Each cycle consisted of 1 min denaturing at 95°C, 1 min annealing at 47°C, and 1 min extension at 72°C. The resulting PCR products were resolved on 2.5% agarose gels. To adjust for differences in expression, 6 µl of Ca_1.3 RT-PCR product from right atria and left ventricle, while 2 µl of Ca_1.3 RT-PCR product from brain was loaded onto gels in a 10 µl total sample volume. Control GAPDH RT-PCR product (1 µl) from each tissue was loaded onto gel in a 10 µl total sample volume. Gels were stained with ethidium bromide and visualized using a Chemiimager 4400 (Alpha Innotech; San Leandro, CA).
Expression constructs. cDNA plasmids encoding for rat Ca\(_{\text{1.3}}\) (exons +11/-32/+42a), Ca\(_{\text{1.3L}}\) (exons +11/-32/+42), \(\beta_3\), and \(\alpha_\delta\delta\)-1 subunits were as previously described\(^3\) and generously provided by Dr. Diane Lipscombe (Brown University). The cDNA plasmid encoding for the rat brain \(\beta_3\) has been described.\(^{24}\) Site-directed mutagenesis was performed using the Quik-Change XLII mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Incorporation of the appropriate and lack of unwanted mutations were verified by automated sequencing performed by the University of Tennessee Health Science Center’s Molecular Resource Center.

Cell cultures and transfections. HEK 293 cells (CRL-1573) were obtained from ATCC (Manassas, VA) at passage 36 and were used at passages 38–44. HEK 293 cells were passaged by trypsinization every 4–5 days and plated at low density (~50,000 cells) on 15-mm round glass coverslips and placed in 12-well tissue culture plates. HEK 293 cell cultures were maintained in DMEM (Invitrogen; Carlsbad, CA) with 10% FBS (Hyclone; Logan, UT) supplemented with penicillin-streptomycin (Invitrogen). Cells were transiently transfected by the calcium phosphate precipitation method 6 or 30 hours following plating using 0.5 \(\mu\)g of each construct and 0.2 \(\mu\)g enhanced green fluorescent protein (GFP) as a transfection marker. Following transfection, cells were washed with PBS for 5–10 min, which was then replaced with fresh media supplemented with 10 mM MgCl\(_2\). As HEK 293 cells have a depolarized resting membrane potential (~ -30 to -20 mV; S.J. Tavalin, unpublished observations), the addition of MgCl\(_2\) was included to help guard against excessive calcium entry in cells expressing the channel which could lead to cell death and thus selection against these cells.

Electrophysiology and data analysis. Twenty-four hours after transfection, cells were visually selected for recording by GFP epifluorescence. Whole-cell recordings were performed with an Axopatch 200B or Multiclamp 700 amplifier (Axon Instruments; Union City, CA). Patch pipettes (2–4 M\(\Omega\)) contained: 140 mM Cs methanesulfonate, 10 mM HEPES, 5 mM adenosine triphosphate (Na salt), 5 mM MgCl\(_2\), 2 mM CaCl\(_2\), and 10 mM BAPTA (pH 7.4). PKA catalytic subunit (Sigma; St. Louis, MO) and PKI(6-22amide; Biomol; Plymouth Meeting, PA) were added to the pipette solution from frozen concentrated stocks that were stored in small aliquots. PKA catalytic subunit (Sigma; St. Louis, MO) and PKI(6-22amide; Biomol; Plymouth Meeting, PA) were added to the pipette solution from frozen concentrated stocks that were stored in small aliquots.

The extracellular recording solution contained: 150 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 10 mM HEPES, 10 mM glucose (pH 7.4) and was perfused at 1–2 ml.

All experiments were initiated within 30 seconds of establishing the whole-cell configuration and were performed at a holding potential of ~80 mV at 20°C. Whole-cell capacitance and series resistance were compensated (90–95%). Currents were digitized at 5–10 kHz and filtered at 1–2 kHz with a Digidata 1322A board and Clampex 9 software (Axon Instruments). Only cells with stable series resistance <6 m\(\Omega\) were included for analysis. All recordings commenced and terminated with a 300 ms voltage ramp protocol from -80 mV to +50 mV, which allowed us to generate the current-voltage (I-V) relationships and determine input resistance from the linear portion of the I-V curve (~80 mV to -65 mV). All cells included for analysis had stable input resistances ≥1 G\(\Omega\). I-V relationships generated from the voltage ramp protocol quantitatively and qualitatively reproduced those generated from a conventional series of test pulses (not shown). To reduce quantitative errors in the I-V relationships, the leak current was estimated by linear regression analysis of the data points between -80 and -65 mV, extrapolating the fit throughout the entire voltage range and digitally subtracting this line from the raw data. Data points (every 10 mV from -80 mV to +50) were extracted from the leak subtracted I-V for display and quantitative purposes. For each cell, I-V data were normalized to the current evoked at 0 mV in response to the initial I-V protocol. A liquid junction potential of -12 mV existed and was not corrected for, thus all voltages reported were -12 mV depolarized from their true values. After the initial I-V ramp protocol, membrane currents were elicited by standard 100 ms square test pulses using a p/4 protocol from -80 to 0 mV applied at 0.1 Hz for 5 min. Cells in which the initial test pulse current appeared to inactivate by >10% were discarded, as this was accompanied by a time dependent reduction of a slowly activating net outward current (presumably delayed rectifier) at positive potentials reflecting poor initial whole-cell access and intracellular solution exchange. Current measurements during the test pulse were taken as the average current within a 1 ms window about the peak. For all cells, current measurements were normalized to the peak current evoked by the initial test pulse. All cells included in this study (except those in Fig. 3) had current amplitudes >100 pA. Data were analyzed using Clampfit (Axon Instruments) and Origin Pro 7.0 (Microcal; Northampton, MA). Statistical analysis comprised of ANOVA followed by Student’s t-test. All data are displayed as means ± the s.e.m.

RESULTS

Expression profile of Ca\(_{\text{1.3}}\) C-terminal splice variants. In cardiac tissue, Ca\(_{\text{1.3}}\) contributes to the diastolic depolarization thus controlling heart rate.\(^{25,26}\) Several studies have suggested that Ca\(_{\text{1.3}}\) is selectively expressed in nodal (sino-atrial node (SAN) and atrioventricular node (AVN)) and atrial tissue but absent in ventricular tissue.\(^{25,27}\) Although selective processing of the channel appears to favor the presence or absence of specific exons in some tissues including heart and brain,\(^{3,15}\) the expression pattern for C-terminal splice forms in the heart remains to be assessed. Alternative splicing of the C-terminal region causes a 58 base pair (bp) deletion in Ca\(_{\text{1.3}}\) mRNA.\(^{13}\) This deletion results in a frameshift that produces a premature termination codon giving rise to Ca\(_{\text{1.3}}\) \(\alpha\) subunits that differ in length by 535 amino acids. We employed the same
primers that were used to initially detect the 58 bp difference in Ca\textsubscript{v}1.3 isoforms found in a rat beta cell line and originally referred to as rCACN4A and rCACN4B (for Ca\textsubscript{v}1.3L and Ca\textsubscript{v}1.3Sb).\textsuperscript{13} As shown in (Fig. 2), we detected a 344 bp RT-PCR product in right atria and brain consistent with the presence of the long form of the channel. In addition, a variably present, but faint, 344 bp product was also observed in left ventricle. This is consistent with recent reports indicating that Ca\textsubscript{v}1.3 is present in this region of the heart, albeit at low copy number.\textsuperscript{25,28,29} In contrast, the anticipated 288 bp product corresponding to the short form was absent. An additional short form of the channel with a divergent C-terminus has been cloned from rat brain and superior cervical ganglion (originally referred to as rbII and designated here as Ca\textsubscript{v}1.3S).\textsuperscript{3,12} Therefore, RT-PCR reactions employing the same forward primer but with a reverse primer specific to this short isoform were performed on mRNA harvested from these tissues. This primer set yielded a 406 bp product from brain but did not yield detectable product from atrial or ventricular tissue (Fig. 2). Control RT-PCR reactions with primers directed against GAPDH revealed a 432 bp product from all three tissues (Fig. 2), verifying that similar amounts of mRNA were used in all reactions. Collectively, these data suggest that the long form is predominantly expressed in cardiac tissue while the long and a short form of the channel are expressed in brain.

**Auxiliary \(\beta\) subunits enhance Ca\textsubscript{v}1.3 current density.** In light of our expression data for Ca\textsubscript{v}1.3S and Ca\textsubscript{v}1.3L from native tissues, we sought to determine the ability of PKA to modulate currents formed by these channels. Previous studies suggested that Ca\textsubscript{v}1.3 required auxiliary \(\beta\) subunits in order to properly form functional channels when either stably transfected in CHO cells or upon cRNA injection in *Xenopus* oocytes.\textsuperscript{7,13} In contrast to these studies, we found, in ~20 to 25% of GFP-positive cells, that both Ca\textsubscript{v}1.3S and Ca\textsubscript{v}1.3L form functional currents when transiently transfected in HEK 293 cells which activate near -50 mV and peak at -10 mV consistent with the voltage-dependence of Ca\textsubscript{v}1.3 in the presence of additional auxiliary subunits (Fig. 3A and B).\textsuperscript{3-7,13} These data further indicate that the unique voltage-dependence of these L-type Ca\textsuperscript{2+} channels is intrinsic to the pore-forming subunit. Barium currents carried by Ca\textsubscript{v}1.3S were significantly larger than those carried by Ca\textsubscript{v}1.3L (Ca\textsubscript{v}1.3S: 13.8 ± 4.7 pA/pF, \(n = 3\) vs. Ca\textsubscript{v}1.3L: 4.9 ± 0.7 pA/pF, \(n = 5\), \(p < 0.05\); (Fig. 3C). Likewise, coexpression of the \(\alpha_{2}\delta-1\) subunit...
produced currents with similar voltage-dependence and current density (Fig. 3). Currents in all the above conditions were typically much smaller than 100 pA and cells harboring these currents tended to be unstable thus precluding further analysis. It is important to note that we observed, in ~5% of tested cells, that expression of either Ca_{1.3S} or Ca_{1.3L} alone could also produce transient currents reminiscent to those reported for other Ca\textsuperscript{2+} channel α subunits expressed in the absence of auxiliary subunits\textsuperscript{30} (data not shown). Coexpression of the β_{3} subunit with either Ca_{1.3S} or Ca_{1.3L} (with the α_{2}δ-1 subunit) dramatically enhanced current density by ~10-20 fold (Ca_{1.3S} + β_{3} + α_{2}δ-1: 118.7 ± 40.2 pA/pF; n = 7; Ca_{1.3L} + β_{3} + α_{2}δ-1: 107.4 ± 30.1 pA/pF; n = 8; p<0.05; (Fig. 3C). Likewise, coexpression of the β_{2a} subunit with either Ca_{1.3S} or Ca_{1.3L} (and α_{2}δ-1 subunit) increased the current density, albeit to a lesser extent (Ca_{1.3S} + β_{2a} + α_{2}δ-1: 49.3 ± 10.9 pA/pF; n = 7; Ca_{1.3L} + β_{2a} + α_{2}δ-1: 27.7 ± 4.8 pA/pF; n = 6; (Fig. 3C). Coexpression of either β_{3} or β_{2a} subunits also increased the likelihood of detecting currents to ~50–60% of GFP-positive cells. Collectively, these data are in agreement with the well-documented role of auxiliary β subunits in promoting Ca\textsuperscript{2+} channel surface expression.\textsuperscript{21}

Ca_{1.3S} is resistant to alterations in PKA activity. The majority of studies examining recombinant Ca_{1.3} channels have employed α_{2}δ subunits in conjunction with either the β_{3} or β_{2a} auxiliary subunits.\textsuperscript{3,7,13,22} As the β_{2a} is also a PKA substrate that may contribute to augmentation of the related Ca_{1.2},\textsuperscript{22,23} we initially performed whole-cell recordings from HEK 293 cells transiently transfected with Ca_{1.3S}, β_{3}, and α_{2}δ-1 to avoid any potential confounding contribution of the β_{3} subunit. Barium currents mediated through this combination exhibited a voltage-dependence similar to previous reports.\textsuperscript{3,4,6,31} Following a 5-min series of test pulses to 0 mV at 0.1 Hz, currents declined in a linear fashion by 16.7% ± 3.5% (Fig. 4A). The I-V relationship shows that the current declined to a similar extent without alteration of its voltage-dependence (Fig. 4B1). Increasing PKA activity within the cell by inclusion of the catalytic subunit of PKA (100U/ml) in the whole-cell pipette solution did not alter the activation threshold, peak current, or rundown of the channel (Fig. 4A and B2). The lack of effect of PKA on the current could arise if the channel was already phosphorylated. Therefore, the specific PKA inhibitory peptide, PKI (1 μM) was included in the whole-cell recording solution in order to favor the activity of countervailing phosphatases. However, PKI was also without effect on the current (Fig. 2). The lack of effect of PKI is not likely to be due to an insufficient concentration of the inhibitor as this concentration has been demonstrated to be effective against other PKA regulated targets.\textsuperscript{32,33} Collectively, these data suggest that Ca_{1.3S} is resistant to PKA modulation.

Transient enhancement of Ca_{1.3L} by PKA. Parallel recording conditions were used to determine whether Ca_{1.3L}, cotransfected with β_{3} and α_{2}δ-1 subunits, was subject to regulation by PKA activity. Currents were relatively stable under control conditions, declining by 8.1 ± 5.4% (Fig. 5A) over the 5-min recording period, which was not significantly different from the short form of the channel. PKA infusion into the cell led to a significant increase in current that peaked at 1–1.5 min (PKA (1.5 min): 19.2 ± 5.3%,
PKA Regulation of Ca\(_{1.3}\).

Figure 5. Transient enhancement of Ca\(_{1.3L}\) by PKA. HEK 293 cells were transiently transfected with Ca\(_{1.3L}\), \(\beta_2\), and \(\alpha_{2,\delta-1}\) subunits. Control whole-cell pipette solution was used (squares), supplemented with 100 U/ml PKA catalytic subunit (circles), or supplemented with the PKA inhibitory peptide PKI (1 \(\mu\)M) (triangles). (A) Average time course of peak current normalized to the initial test pulse is plotted versus time. Number of observations for each condition is indicated in parentheses. (B) (Left) Representative traces to the initial test pulse (black trace) and at two minutes (gray trace) for each condition are shown. (Right) Averaged I-V relationships are shown in response to ramp protocol (-80 mV to 50 mV; 300 ms) for initial I-V (open symbols) and at termination of recording at 5 min (closed symbols). I-Vs were normalized to the current at 0 mV for the initial I-V. Conditions (and mean initial amplitudes ± s.e.m.) are as follows: [1] control (770.5 ± 143.4 pA); [2] PKA (602.1 ± 220.4 pA); [3] PKI (841.4 ± 196.1 pA).

n = 9 vs. control (1.5 min): 2.1 ± 2.5%, n = 8; p < 0.05; Fig. 5A, B1 and B2) after initiating recording, but declined to control levels by 5 min (0.5 ± 5.4%; n = 9). In contrast, PKI did not modify the time course compared to controls, further suggesting that both forms of Ca\(_{1.3}\) are unlikely to be basally regulated by PKA under our recording conditions. In conjunction with our data on Ca\(_{1.3S}\), these data support the idea that the long form of the channel is selectively modulated by PKA.

The \(\beta_2,\alpha\) auxiliary subunit is not sufficient to confer PKA sensitivity to Ca\(_{1.3S}\). Previous studies indicated that the \(\beta_2,\alpha\) auxiliary subunit is a substrate for PKA and sufficient to confer PKA regulation to a truncated form of Ca\(_{1.3L}\).22,23 Therefore, we tested whether expression of the \(\beta_2,\alpha\) subunit could endow Ca\(_{1.3S}\) with sensitivity towards PKA. In the presence of the \(\beta_2,\alpha\) and \(\alpha_{2,\delta}\)-subunits, currents decayed by 12.8 ± 5.3% over the 5-min recording period (Fig. 6A and B1), similar to that observed when \(\beta_3\) was present. Elevation or reduction of cellular PKA activity by inclusion of PKA or PKI, respectively, had no effect on time course of the current or shape of the I-V relationship (Fig. 6A, B2 and B3). These results further strengthen the idea that the short form of Ca\(_{1.3}\) is resistant to PKA modulation. Moreover, these results indicate that although the \(\beta_2,\alpha\) subunit is known to be a PKA substrate,22,23 it is insufficient to confer kinase sensitivity to the short form of the channel.

Sustained upregulation of Ca\(_{1.3L}\) by PKA in the presence of the \(\beta_3\) subunit. Although the \(\beta_2,\alpha\) subunit may not be sufficient to confer PKA sensitivity to Ca\(_{1.3S}\), it may be able to modify the PKA responsiveness of Ca\(_{1.3L}\). In order to test this hypothesis, Ca\(_{1.3L}\) was coexpressed with \(\beta_3\) and \(\alpha_{2,\delta-1}\). Under control conditions, currents carried by this subunit combination declined by 4.9 ± 3.7% during the 5-min recording period (Fig. 7A and B1). However, PKA infusion led to a rapid 29.0 ± 12.9% (n = 8) increase in the current over the first minute of the recording which remained elevated throughout the 5-min recording period (25.3 ± 10.5%; Fig. 7A and B2). Both were significantly (p < 0.05) greater than that observed in control recordings at the corresponding time points. In contrast, infusion of PKI did not alter the current compared to control cells as the current declined by 12.2 ± 7.5% (n = 6) over the same period (Fig. 7A and B3). Furthermore, none of the conditions altered the shape of the I-V relationship. Together with our previous findings, these data suggest that \(\beta_3\) subunits influence the stability of Ca\(_{1.3L}\) modulation by PKA.

PKA enhancement of Ca\(_{1.3L}\) in the presence of the \(\beta_3\) subunit predominantly relies on Ser1964. Sequence analysis suggested the presence of four consensus PKA phosphorylation sites within the long C-terminal tail of Ca\(_{1.3}\): Ser1743, Ser1816, Ser1842, and Ser1964. Of these, only Ser1743 has been ascertained to be phosphorylated by PKA using an in vitro assay. However, Ser1743 appears responsible for only ~25% of the phosphorylation induced by incubation with PKA, suggesting that one or more of the remaining sites may be responsible for the remaining phosphorylation signal. Of these remaining sites, Ser1964 appeared to be the most likely candidate, as this site appears to be analogous to the Ser1928 site in Ca\(_{1.2}\). Therefore, site-directed mutagenesis was employed to mutate Ser1964 to a non-phosphorylatable alanine.
residue (S1964A). Upon PKA infusion into the cells the current declined by 9.3 ± 7.7% (n = 7) (Fig. 8A and B). This decline was similar to that observed for the wild-type channel in the absence of PKA suggesting that Ser1964 is necessary for PKA mediated enhancement of the current. In contrast, when Ser1743 was mutated to alanine (Ca1.3(S1743A)), PKA infusion maximally increased the current by 17.4 ± 5.5% (n = 6) nearly 4 min after initiation of the recording (Fig. 8A and B). The magnitude of the PKA induced increase was similar to that observed for the wild-type form of the channel. However, the time to peak enhancement was delayed, and the subsequent decline to baseline appeared to be blunted compared to the wild-type channel. These data suggest that while Ser1743 is not necessary for modulation of channel activity of this subunit combination, it appears to influence PKA access to Ser1964.

PKA enhancement of Ca1.3L in the presence of the β2a subunit predominantly relies on Ser1743. Parallel experiments were performed to assess whether modulation of Ca1.3 in the presence of the β2a also showed the same dependence on putative PKA phosphorylation sites. In contrast to the lack of modulation observed in the presence of β1, when Ca1.3(S1964A) was coexpressed with β2a and α2δ-1, PKA enhanced the current by 30.2 ± 11.0% (n = 6). While the magnitude of the enhancement was similar to that observed for the wild-type channel in the presence of the β2a subunit, the time course for enhancement was dramatically slowed (Fig. 9). These results suggest that while Ser1964 is not absolutely required for PKA modulation of Ca1.3L in the presence β2a subunit, it determines the speed by which modulation of Ca1.3L activity occurs within the context of the β2a subunit. The fact that PKA enhanced CaV1.3L(S1964A), in the presence of the β2a subunit, suggested that at least one other site also contributes to regulation of the current. Therefore, we examined whether the aforementioned Ser1743 could be required to support modulation of Ca1.3L by PKA when the β2a and α2δ subunits are present. Using this subunit combination, currents carried by Ca1.3(S1743A) declined by 5.8 ± 6.8% (n = 9; p < 0.05 compared to wild-type) when PKA was infused into the cells (Fig. 9). This decline was similar to that observed for the wild-type channel in the absence of PKA (Fig. 7). Thus Ser1743 appears to be required for PKA modulation of Ca1.3L in the presence of the β2a subunit. Collectively, our data demonstrate that auxiliary β subunits determine the degree to which distinct sites in Ca1.3L contribute to current modulation by PKA.

**DISCUSSION**

Our data suggests that Ca1.3L may serve as a potential downstream target for hormones and transmitters that lead to PKA activation. Given the negative activation threshold of Ca1.3, which is near resting potentials for many excitable cells,2 PKA modulation of Ca1.3L may be particularly poised to mediate the effects of hormone and transmitters in controlling of electrophysiological rhythms and repetitive firing. For example, in the pacemaker cells of the SAN, Ca1.3 contributes to the diastolic depolarization and thereby helps to set heart rate.25,26 Sympathetic discharge via β-adrenergic receptors and subsequent activation of PKA is known...
PKA Regulation of Ca\(_{1.3}\).

Figure 7. Persistent enhancement of Ca\(_{1.3}\) by PKA in the presence of the \(\beta_2\) subunit. HEK 293 cells were transiently transfected with Ca\(_{1.3}\), \(\beta_2\), and \(\alpha_\delta\) subunits. Control whole-cell pipette solution was used (squares), supplemented with \(100 \text{ U/ml}\) PKA catalytic subunit (circles), or supplemented with the PKA inhibitory peptide PKI (1 \(\mu\text{M}\)) (triangles). (A) Average time course of peak current normalized to the initial test pulse is plotted versus time. Number of observations for each condition is indicated in parentheses. (B) (Left) Representative traces normalized to the initial test pulse (black trace) and at two minutes (gray trace) for each condition are shown. (Right) Averaged I-V relationships are shown in response to ramp protocol (−80 mV to 50 mV; 300 ms) for initial I-V of observations for each condition is indicated in parentheses. (B) (Left) Representative traces to the initial test pulse (black trace) and at two minutes (gray trace). (B) (Right) Averaged I-V relationships are shown in response to ramp protocol (−80 mV to 50 mV; 300 ms) for initial I-V of observations for each condition is indicated in parentheses. (B) (Right) Averaged I-V relationships are shown in response to ramp protocol (−80 mV to 50 mV; 300 ms) for initial I-V of observations for each condition is indicated in parentheses. (B) (Right) Averaged I-V relationships are shown in response to ramp protocol (−80 mV to 50 mV; 300 ms) for initial I-V of observations for each condition is indicated in parentheses.

In contrast to the presence of a predominant single isoform in the heart, both short and long forms of the channel are present in pancreatic \(\beta\)-cells and brain. The detection of a single splice variant of the C-terminus in the heart is consistent with previous observations for preferential expression of other Ca\(_{1.3}\) exons in the heart.\(^{34}\) Exon 32, which is selectively expressed in the heart,\(^{35}\) was not present in the isoforms tested here. However, this exon encodes for a region which would not be expected to contribute to PKA modulation of Ca\(_{1.3}\), since it resides in the extracellular region between segments 3 and 4 of repeat IV of the channel. Consistent with other reports, we detected Ca\(_{1.3}\) in atrial tissue and in ventricular tissue.\(^{15,25,27-29}\) Although we did not detect Ca\(_{1.3}Sb\), this additional short form of the channel contains a unique consensus PKA site\(^{36}\) that is not present in either form tested here and may correspond to a recombinant 180 kDa isoform of Ca\(_{1.3}\) which is also enhanced by PKA.\(^{38}\) Given our data for a lack of short Ca\(_{1.3}\) splice variants in the heart, we suggest that a recently detected 180 kDa form of the channel in the heart may reflect proteolytic processing of the expected −240 kDa long form of the channel. This would be in line with the presence of a conserved proteolytic cleavage site which exists in Ca\(_{1.1}\)–Ca\(_{1.3}\) L-type Ca\(^{2+}\) channels.\(^{18}\) Posttranslational processing of the longer form of the channel, in cardiac tissue, at this site would be expected to yield the observed 180 kDa fragment and also retain Ser1743 as a core PKA regulatory site. This would also strengthen the proposed link between proteolytic processing of L-type Ca\(^{2+}\) channels in the heart and regulation by PKA.\(^{16}\) Because proteolytic cleavage is not thought to occur for the L-type Ca\(^{2+}\) channels within recombinant systems,\(^{16,18}\) future studies will need to examine whether such processing impacts regulation of Ca\(_{1.3}\) by PKA.

The long form of Ca\(_{1.3}\) was selectively enhanced by PKA, regardless of auxiliary \(\beta\) subunit isoform tested, indicating that the long C-terminal provides the principle target for PKA modification of the channel’s phosphorylation state and function. This agrees with an in vitro study which found that phosphate incorporation, upon incubation with PKA, was localized to at least two sites within this region of the channel.\(^{14}\) Consistent with this in vitro study, we have now identified two sites (Ser1964 and Ser1743) that are required for PKA enhancement of Ca\(_{1.3}\) currents, albeit each of these sites plays distinct and complementary roles depending on the identity of the auxiliary \(\beta\) subunit. Although we did not directly examine whether the two remaining consensus PKA sites contribute to regulation of
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Figure 8. PKA enhancement of Ca_{1.3L} in the presence of the β_{3} subunit predominantly relies on Ser1964. HEK 293 cells were transiently transfected with Ca_{1.3(1964A)} (circles) or Ca_{1.3(1743A)} (squares) plus β_{3} and α_{2δ1} subunits. The whole-cell pipette solution supplemented with [100 U/ml] PKA catalytic subunit. (A) Average time course of peak current normalized to the initial test pulse is plotted versus time. Number of observations for each condition is indicated in parentheses. (B) (Left) Representative traces to the initial test pulse (black trace) and at five minutes (gray trace) for each condition are shown. (Right) Averaged I-V relationships are shown in response to ramp protocol (-80 mV to 50 mV; 300 ms) for initial I-V (open symbols) and at termination of recording at five minutes (closed symbols). I-Vs were normalized to the current at 0 mV for the initial I-V. Conditions (and mean initial amplitudes ± s.e.m.) are as follows: (1) Ca_{1.3(1964A)} + β_{3} + α_{2δ1} (685.3 ± 236.7 pA); (2) Ca_{1.3(1743A)} + β_{3} + α_{2δ1} (681.8 ± 290.7 pA).

the channel, it would appear that neither of these sites is sufficient to support regulation of channel activity, as the enhancing action of PKA can be ablated (Figs. 8 and 9) despite the continued presence of these sites. Presently, the architecture of the C-terminus of Ca_{1.3} is largely unknown. However, our finding that Ser1743 and Ser1964 reciprocally influence the rate by which each contributes toward enhancement of Ca_{1.3L} suggests that phosphorylation of these residues may affect a common structural element, though with differing efficacies. Future studies will be necessary to delineate how auxiliary β subunits impact the local architecture of this C-terminal region of the favoring the regulation described here.

Despite the identification of Ser1928 as a Ca_{1.2} PKA site that is reliably phosphorylated in both recombinant and native systems by biochemical measures, the extent to which this site contributes to modulation of the current has also been controversial. One hallmark feature of PKA-mediated modulation of native Ca_{1.2} is a negative shift in voltage-dependent activation by ~10 mV. At present, the contribution of phosphorylation of Ser1928 towards this effect is unclear as some reports suggest that in recombinant systems that PKA can induce a hyperpolarizing shift in activation while recent results suggest that this shift persists when Ca_{1.2} constructs lacking Ser1928 are expressed in cardiac myocytes. In the present study, sustained augmentation of Ca_{1.3L} activity occurs to a similar extent regardless of voltage (Supplementary Fig. 1) suggesting that PKA phosphorylation of Ca_{1.3L} (at Ser1964 or Ser1743) does not alter the voltage-dependent activation of the channel. Recent studies further suggest that the magnitude of current enhancement attributable to phosphorylation of Ca_{1.2} at Ser 1928 may be limited to ~20–30%, although this range may be extended depending on the degree of intracellular calcium buffering. Because Ser1964 of Ca_{1.3} appears conserved with Ser1928, and participates in PKA modulation of recombinant Ca_{1.3} channels to a similar extent, these findings suggest that both Ca_{1.1} family members may use overlapping strategies to achieve PKA-mediated enhancement of the current.

Efficient PKA phosphorylation and modulation of Ca_{1.1} and Ca_{1.2} is achieved via A-kinase anchoring proteins (AKAPs). In particular, AKAP15/18, AKAP79, and MAP2 have each been found to be complexed with these L-type Ca^{2+} channels thereby helping to recruit PKA to these channels and/or facilitating to regulation of these channels. Although Ca_{1.3L} possesses a conserved AKAP binding domain that also exists within Ca_{1.1} and Ca_{1.2}, it is not clear whether AKAPs associate with Ca_{1.3} and whether they contribute to efficient cAMP and PKA dependent regulation of the channel. While AKAPs may be important for targeting PKA to L-type calcium channels, it is unknown whether this localized PKA can discriminate among multiple proximal phosphorylation sites. Given the presence of multiple putative PKA phosphorylation sites within the C-terminus of Ca_{1.3L}, auxiliary β subunits may provide an additional mechanism to either control PKA access to such regulatory sites or the functional consequences linked to these sites. In fact, a previous study demonstrated that subunit interactions can be important for protein kinase C-mediated phosphorylation of Ca_{1.1}. If this is a more general property, whereby auxiliary β subunits dictate signaling enzyme accessibility to channels, then β subunits...
may also determine the ability of opposing phosphatases to regulate channel activity. Such a scenario may help to explain the observed β subunit-dependent differences in the stability of PKA regulation of Ca1.3, which are presumably mediated via phosphatases.

At present, the identity of β subunits endogenously associated with Ca1.3 is not known. However, the spectrum of auxiliary β subunits associated with Ca1.3 may differ in a tissue and/or cell-dependent manner in order to custom tailor channel properties.48 Although we have only detected augmentation of Ca1.3L by PKA, it must be noted that the PKA pathway has also been linked to suppression of L-type Ca2+ channel activity. Such a scenario may help to explain the observed PKA enhancement of Ca1.3L in the presence of the β2δ subunit predominantly relies on Ser1743. HEK 293 cells were transiently transfected with Ca1.3(1964A) [circles] or Ca1.3(1743A) [squares] plus β2 and α2δ-1 subunits. The whole-cell pipette solution was supplemented with 100 U/ml PKA catalytic subunit. (A) Average time course of peak current normalized to the initial test pulse is plotted versus time. Number of observations for each condition is indicated in parentheses. (B) (Left) Representative traces to the initial test pulse (black trace) and at 5 min (gray trace) for each condition are shown. (Right) Averaged IV relationships are shown in response to ramp protocol (−80 mV to 50 mV; 300 ms) for initial IV (open symbols) and at termination of recording at 5 min (closed symbols). I-Vs were normalized to the current at 0 mV for the initial I-V. Conditions (and mean initial amplitudes ± s.e.m.) are as follows: (1) Ca1.3(1964A) + β2δ-1 2.1 (217.2 ± 30.3 pA); (2) Ca1.3(1743A) + β2δ-1 (434.7 ± 45.3 pA).

Figure 9. PKA enhancement of Ca1.3L in the presence of the β2δ subunit predominantly relies on Ser1743. HEK 293 cells were transiently transfected with Ca1.3(1964A) [circles] or Ca1.3(1743A) [squares] plus β2 and α2δ-1 subunits. The whole-cell pipette solution was supplemented with 100 U/ml PKA catalytic subunit. (A) Average time course of peak current normalized to the initial test pulse is plotted versus time. Number of observations for each condition is indicated in parentheses. (B) (Left) Representative traces to the initial test pulse (black trace) and at 5 min (gray trace) for each condition are shown. (Right) Averaged IV relationships are shown in response to ramp protocol (−80 mV to 50 mV; 300 ms) for initial IV (open symbols) and at termination of recording at 5 min (closed symbols). I-Vs were normalized to the current at 0 mV for the initial I-V. Conditions (and mean initial amplitudes ± s.e.m.) are as follows: (1) Ca1.3(1964A) + β2δ-1 2.1 (217.2 ± 30.3 pA); (2) Ca1.3(1743A) + β2δ-1 (434.7 ± 45.3 pA).

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