NIH Public Access

Author Manuscript

Circ Res. Author manuscript; available in PMC 2009 June 8.

Published in final edited form as:
Circ Res. 2006 February 3; 98(2): e11–e18. doi:10.1161/01.RES.0000202692.23001.e2.

β-Adrenergic Stimulation of L-type Ca^{2+} Channels in Cardiac Myocytes Requires the Distal Carboxyl Terminus of α_{1C} but Not Serine 1928

Anand N. Ganesan, **Christoph Maack**, **David C. Johns**, **Agnieszka Sidor**, and **Brian O'Rourke**
Institute of Molecular Cardiobiology (A.N.G., C.M., A.S., B.O'R.), Department of Medicine; and the Department of Neurosurgery (D.C.J.), The Johns Hopkins University, Baltimore, Md.

Abstract

β-Adrenoceptor stimulation robustly increases cardiac L-type Ca^{2+} current (I_{CaL}); yet the molecular mechanism of this effect is still not well understood. Previous reports have shown in vitro phosphorylation of a consensus protein kinase A site at serine 1928 on the carboxyl terminus of the α_{1C} subunit; however, the functional role of this site has not been investigated in cardiac myocytes. Here, we examine the effects of truncating the distal carboxyl terminus of the α_{1C} subunit at amino acid residue 1905 or mutating the putative protein kinase A site at serine 1928 to alanine in adult guinea pig myocytes, using novel dihydropyridine-insensitive α_{1C} adenoviruses, coexpressed with β_2 subunits. Expression of α_{1C} truncated at 1905 dramatically attenuated the increase of peak I_{CaL} induced by isoproterenol. However, the point mutation S1928A did not significantly attenuate the β-adrenergic response. The findings indicate that the distal carboxyl-terminus of α_{1C} plays an important role in β-adrenergic upregulation of cardiac L-type Ca^{2+} channels, but that phosphorylation of serine 1928 is not required for this effect.

Keywords

protein kinase A; adenovirus; ion channel; calcium current; cAMP

Voltage-gated L-type ($Ca_v1.2$) Ca^{2+} channels play a central role in controlling cardiac function, providing the trigger for intracellular Ca^{2+} release during excitation-contraction coupling, contributing to the plateau phase of the cardiac action potential, and modulating pacemaker activity in the sinoatrial node.[1-3] The L-type Ca^{2+} channel is a multiprotein complex, comprised of the pore-forming α_1 subunit and the auxiliary β_2 and $\alpha_2\delta$ subunits.[4] In the heart, the molecular identity of the β subunit remains a subject of ongoing investigation.[5,6]

A number of receptor-mediated signal transduction pathways regulate Ca^{2+} influx via $Ca_v1.2$ channels, the most prominent of which is the β-adrenergic/cAMP signaling pathway. β-Adrenergic stimulation is the dominant mechanism of positive chronotropy, inotropy, and lusitropy in the heart.[7] Activation of β-adrenergic receptors strongly enhances cardiac L-type

© 2006 American Heart Association, Inc.

Correspondence to Brian O'Rourke, Johns Hopkins University, 1059 Ross Building, 720 Rutland Ave, Baltimore, MD 21205. E-mail E-mail: bor@jhmi.edu.

Under a licensing agreement between Excigen Inc and the Johns Hopkins University, D.C.J. is entitled to a share of royalty and milestone payments received by the University on sales of products described in this article. The terms of this agreement are being managed by the Johns Hopkins University in accordance with its conflict-of-interest policies.

This manuscript was sent to Harry A. Fozzard, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Ca^{2+} current (I_{CaL}) via cAMP generation and the activation of protein kinase A (PKA), presumably by phosphorylating specific sites on the channel protein.[8,9] Nevertheless, the structural determinants of PKA action on the Ca^{2+} channel in native adult cardiomyocytes are unknown.

Previous biochemical studies have implicated the carboxyl terminus as a target of PKA-mediated phosphorylation: for example, a truncated form of the channel was reportedly not phosphorylated by PKA in vitro.[10] The consensus phosphorylation site at serine 1928 was later identified as the main target of PKA.[11,12] On the other hand, phosphorylation sites have also been mapped to the β subunit of the channel and localized to serines 478 and 479 of β_{2a}.[13,14]

Mixed results have been obtained when attempts have been made to confirm the functional role of these sites in the regulation of Ca^{2+} channel activity, in part, because of the difficulty of reconstituting PKA-mediated channel activation in heterologous expression systems. Whereas some studies have observed PKA-mediated activation of the channel in heterologous systems,[10,14] others have observed no such stimulation.[15,16] Other groups have used PKA inhibitors to argue that $Ca_v1.2$ channels are phosphorylated at baseline when expressed in cultured cell lines.[17-19]

The failure to reproduce the strong upregulation of the L-type Ca^{2+} current in nonnative cells has been attributed to the lack of A-kinase anchoring proteins (AKAPs) in heterologous systems. By coexpressing AKAP 79, Gao et al[20] were able to reconstitute forskolin-mediated upregulation of Ca^{2+} channels expressed in HEK 293 cells, and the authors concluded that S1928 was necessary to produce PKA-mediated activation of the L-type current. In contrast, a subsequent study by the same group reported PKA-mediated activation of Ca^{2+} current through L-type channels with a mutant α_{1C} truncated at 1905 and provided evidence indicating that β-subunit phosphorylation was important.[14] Additionally, other investigators have been unable to reproduce PKA-mediated upregulation of Ca^{2+} channels in HEK 293 cells, even in the presence of AKAP 79,[21] and it has been suggested that the primary role of AKAP 79 is to facilitate α_1 subunit trafficking.[22]

The equivocal findings in heterologous expression systems suggest that the actual site responsible for β-adrenergic regulation of L-type Ca^{2+} channel in cardiac myocytes remains undefined. In the present study, we use novel adenoviral gene transfer vectors to express mutant α_{1C} subunits in native guinea pig cardiomyocytes to address this fundamental question of L-type Ca^{2+} channel physiology. The evidence supports a role for the distal carboxyl terminus of the α_1 subunit in the β-adrenergic enhancement of I_{CaL}. However, serine 1928 phosphorylation is not required.

Materials and Methods

Vector Construction

A full-length, dihydropyridine (DHP)-insensitive rabbit $Ca_v1.2$ (X15539) α_1 subunit adenoviral shuttle vector pAdEcd-$\alpha_{1C(D-)}$ was prepared as previously described.[23] A serine to alanine substitution of $\alpha_{1C(D-)}$ at S1928 ($\alpha_{1C(D-)-S1928A}$) was constructed by site-directed mutagenesis with overlapping primers, using the QuikChange XL kit (Stratagene). A carboxyl-terminal truncation mutant, pAdEcd-$\alpha_{1C(D-)\Delta1905}$, in which the carboxyl terminus of the α_{1C} was truncated after amino acid 1905, was also created. Hence, this construct also lacked the putative phosphorylation site at serine 1928. Mutations at Thr1066 (to Tyr) and Glu1089 (to Met) were also incorporated in this construct to make the channel DHP insensitive, as for $\alpha_{1C(D-)}$ and $\alpha_{1C(D-)-S1928A}$, so that the current contributed by the mutated channels could be discriminated from that of native $Ca_v1.2$ channels.[23] Concentration-response experiments with

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

nitrendipine were performed on $\alpha_{1C(D-)}$, $\alpha_{1C(D-)S1928A}$, and $\alpha_{1C(D-)\Delta1905}$ in A549 cells as previously described[23] to verify that each construct produced DHP-insensitive current. A full-length wild-type α_{1C} fused to green fluorescent protein at its carboxyl terminus, $\alpha_{1C\text{-GFP}}$, was also constructed for the Western blot experiments shown in Figure 2.

The adenoviral shuttle plasmid pAd-β_{2a}, was created by subcloning the rat β_{2a} cDNA (NM053851) into the *Eco*R1 site of the adenoviral shuttle vector pAdLox. To avoid potential confounding effects of β-subunit phosphorylation on the response, the S478A/S479A double mutation of β_{2a} (pAd-$\beta_{2a\text{-S478/S479A}}$; $\beta_{2a\text{-AA}}$) was also created via site-directed mutagenesis with overlapping primers, using the QuikChange XL kit. pAd-VgRXR, a bicistronic vector containing the elements of the ecdysone receptor, was created as previously described.[23,24]

Adenoviral Construction

Adenoviruses were constructed by the Cre-*lox* technique, according to the methods of Hardy et al,[25] with variations as previously described.[23] The α_{1C} adenovirus shuttle vectors pAdEcd-$\alpha_{1C(D-)}$, pAdEcd-$\alpha_{1C(D-)S1928A}$, or pAdEcd-$\alpha_{1C(D-)\Delta1905}$ were cotransfected onto CRE-FB cells, along with ψFB6 adenoviral DNA as previously described.[23] Resulting viruses were expanded in 3 rounds and purified by plaque assay on 911-FB cells. The plaque purified virus was further expanded before purification on CsCl gradients, and dialyzed against 10 mmol/L Tris-HCl/1 mmol/L $MgCl_2$. Viral titers were estimated by particle counts and calculated by plaque assay performed on 911-FB cells.

Ad-$\beta_{2a\text{-AA}}$ and AdVgRXR were grown by cotransfection of their respective shuttle plasmids with ψ5 adenoviral DNA,[25] onto standard Cre-8 cells.[25] These viruses were expanded in 3 rounds and purified on CsCl gradients, as described previously.[23,26] Viral titers were estimated by particle counts and calculated by plaque assay performed on 911 cells.[27]

Adult Cardiac Myocyte Expression Experiments

Animal protocols used were in accordance with the Guide for Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Isolated guinea pig ventricular myocytes were obtained by enzymatic digestion as previously described.[28] Freshly isolated myocytes (20 to 40×10^3/mL) were plated on laminin-coated coverslips and bathed in DMEM supplemented with 5% FBS and 1% penicillin-streptomycin. Virus was added after allowing cells to adhere to coverslips for 3 to 4 hours. For experiments involving overexpression of $\beta_{2a\text{-AA}}$, cells were transduced with Ad-$\beta_{2a\text{-AA}}$ at $\approx1\times10^9$ plaque-forming units (PFU).

For the DHP-insensitive α_{1C} experiments, cells were transduced with Ad-VgRxR at 3×10^8 PFU/mL and Ad-$\beta_{2a\text{-AA}}$ at 1×10^9 PFU/mL, along with either Ad-$\alpha_{1C(D-)}$, Ad-$\alpha_{1C(D-)S1928A}$ or Ad-$\alpha_{1C(D-)\Delta1905}$ at $\approx5\times10^9$ PFU/mL. As we have described previously, coexpression of the α_{1C} constructs with Ad-$\beta_{2a\text{-AA}}$ was necessary to enhance I_{CaL} amplitude, which facilitates detection of the DHP-insensitive Ca^{2+} currents.[23]

After incubation with the viruses, the medium was replaced with fresh DMEM containing 10 μmol/L ponasterone A (H101-01, Invitrogen). Electrophysiological recordings were made at 36 to 48 hours following isolation.

Western Blot Experiments

The anti-$Ca_v1.2$ antibody used in these studies was an affinity purified rabbit polyclonal antibody, raised against a peptide made from amino acids 1 to 46 of the rabbit α_{1C} subunit N terminus (Alomone Labs, Jerusalem, Israel). The anti-green fluorescence protein (GFP) antibody was a mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif).

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

The lysate was prepared by scraping cultured cardiac myocytes into ice-cold RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate in PBS containing 5 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L β-glycerol phosphate, 1 mmol/L L-phenylalanine, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, and the following protease inhibitors: pepstatin A, 2 μg/mL; leupeptin, 10 μg/mL; aprotinin, 10 μg/mL; elastinal, 2 μg/mL; benzamidine, 0.5 mg/mL; calpain inhibitor peptide 10 μg/mL; and 1 mmol/L phenylmethane-sulfonyl fluoride, 0.4 mmol/L iodoacetic acid) and passed through a 25-gauge needle 15 times. This homogenate was lysed on ice for 1 hour, before centrifugation at 2000*g* for 20 minutes at 4°C.

The concentration of the supernatant protein was measured with the Bio-Rad DC protein assay, according to the instructions of the manufacturer, and samples were divided into aliquots and frozen at -20°C. Samples were mixed with 5:1 Laemmli sample buffer and incubated at room temperature for 60 minutes, before gel loading.

Following separation by 7.5% SDS-PAGE, proteins were transferred to polyvinylidene sulfonyl fluoride membranes (Bio-Rad) in Tris/glycine transfer buffer containing 5% methanol and 0.05% sodium dodecyl sulfate. Membranes were blocked with 5% nonfat milk in PBS supplemented with 0.05% Tween 20 (PBS-T), for 1 hour at room temperature, and then incubated with primary antibody (1:50 to 1:200) overnight at 4°C. The membrane was washed 6 times for 5 minutes with PBS-T and incubated with horseradish peroxidase-conjugated donkey anti-rabbit antibody diluted 1:50 000 in PBS-T. Following 6 further washes in PBS-T, blots were visualized with the Lumi-light Plus blotting substrate (Roche), according to the instructions of the manufacturer.

Immunofluorescence

Immunofluorescence labeling experiments were undertaken using myocytes cultured with $\alpha_{1C\text{-}GFP}$ using an anti-GFP antibody (Abcam-6556). Cultured myocytes were fixed for 20 minutes at room temperature in freshly prepared 3% paraformaldehyde in PBS. Fixed cells were permeabilized in 0.5% Triton X-100 in PBS/10 mmol/L glycine for 3 minutes. After 2 further washes in PBS/10 mmol/L glycine, myocytes were incubated for 1 hour in PBS/10% goat serum to block nonspecific binding. Following 2 washes in PBS/10 mmol/L glycine, the cells were incubated in primary antibody diluted 1:500 in PBS/10 mmol/L glycine supplemented with 4% goat serum, for 1 hour. The cells were then washed 2 times in PBS/10 mmol/L glycine, before a 1-hour incubation with secondary antibody, Alexa 568 goat anti-rabbit IgG (H+L) (Invitrogen), and diluted 1:500 in PBS/10 mmol/L glycine supplemented with 4% goat serum. After 2 final washes with PBS/10 mmol/L glycine, cells were mounted on coverslips. Experiments to determine nonspecific binding were performed with cells transduced with VgRXR alone, and control experiments were also carried out with secondary antibody alone.

Images were recorded using a 2-photon laser scanning microscope (Bio-Rad, MRC-1024MP) with excitation at 820 nm (Tsunami Ti:Sa laser, Spectra-Physics). The red emission of Alexa 568 was collected at 605±25 nm.

Electrophysiological Recordings

Electrophysiological recordings were performed using the whole-cell variation of the patch clamp technique. All experiments were performed at room temperature. Currents were recorded with an Axopatch 200B amplifier (Axon Instruments, Union City, Calif) and sampled at 5 kHz. The cells were superfused with an external solution comprised of (mmol/L): 140 NaCl, 5 CsCl, 1 $MgCl_2$, 10 HEPES, 2 $CaCl_2$, and 10 D-glucose. Patch pipettes (2.0 to 5.0 MΩ) were filled with an intracellular solution containing (mmol/L): 110 CsCl, 0.4 $MgCl_2$, 5

D-glucose, 10 HEPES, 20 tetraethylammonium, and 5 BAPTA (1,2-bis[2-aminophenoxy] ethane-*N,N,N'*,*N'*-tetraacetic acid). In experiments involving the use of DHP-insensitive α_1 subunits, 10 μmol/L nitrendipine (Sigma, St Louis, Mo) was washed in at the end of a subset of experiments to verify the expression of the exogenous α_{1C}. Voltage pulses were applied and data recorded with custom software. Leak currents were subtracted by a P/4 leak subtraction protocol. Total series resistance after establishing the whole-cell configuration was typically 10 MΩ and was electronically compensated by ≈70%. Thus we estimate the voltage error for a 1-nA current (the amplitude of isoproterenol [ISO]-stimulated peak inward I_{CaL}) to be <3 mV. I_{CaL} was elicited by 300-ms pulses to different test potentials (-80 to +50mV), following a 200-ms prepulse to -40 mV, from a holding potential of -80 mV. I_{CaL} measurements were normalized to cell capacitance, measured by integration of the area under an uncompensated -20 mV pulse from -80 mV. The current-density to voltage relationships (I-V) recorded were fitted to a modified Boltzmann equation:

$$I = G_{\max}(\mathrm{V} - \mathrm{V}_{\mathrm{rev}}) / (1 + \exp(-(\mathrm{V} - V_{1/2})/k)$$

where G_{max} is the maximal conductance (nS/pF), V_{rev} is the reversal potential (mV), $V_{1/2}$ is the midpoint voltage of activation (mV), and k is the slope factor.

Data analysis was performed offline, with custom software (Ionview, B.O'R.). Statistical analysis was performed with Origin 6.0 (Microcal). All data are presented as the mean±SEM. Statistical significance was calculated using an unpaired Student's *t* test, and differences were considered significant at $P<0.05$.

Results

β-Adrenergic Response in Adult Cardiomyocytes With $\beta_{2a\text{-}AA}$ Expression

The primary objective of this study was to investigate the role of the distal carboxyl terminus of α_{1C} in the β-adrenergic response. In a prior study, we determined that replacement of the native Ca^{2+} channels with virally expressed mutants was most effectively achieved when β subunits were coexpressed,[23] consistent with earlier reports that β-subunit expression is a limiting factor for Ca^{2+} channel membrane trafficking in myocytes.[5] However, our initial experiments indicated that the expression of wild-type β_{2a} subunits led to a marked hyperpolarizing shift (approximately -12.2 mV) in the midpoint of voltage-dependence for I_{CaL} activation ($V_{1/2}$) and a diminished ISO response, presumably attributable to the enhanced Ca^{2+} channel activity under basal conditions (data not shown). Mutation of the phosphorylation sites of β_{2a} at S478 and S479 to alanine lessened the hyperpolarizing shift in Ca^{2+} channel activation, but preserved the ability of the β subunit to facilitate Ca^{2+} channel trafficking. Expression of $\beta_{2a\text{-}AA}$ subunits resulted in a marked increase in baseline L-type Ca^{2+} current density (uninfected myocytes -3.2±0.7 pA/pF versus $\beta_{2a\text{-}AA}$ expressing -9.2±1.6 pA/pF; $P<0.05$) and a hyperpolarizing shift in $V_{1/2}$ of just -4.8 mV (Table). Importantly, a robust β-adrenergic response could be observed under these conditions. In Ad-$\beta_{2a\text{-}AA}$-transduced myocytes, ISO (100 nmol/L) caused a 95% increase in peak I_{CaL} (from -9.2±1.6 pA/pF to -17.9 ±1.4 pA/pF, $P<0.05$; Figure 1B and 1D) and a significant hyperpolarizing shift in $V_{1/2}$ (Table). This was comparable to the ISO-induced increase in peak inward L-type Ca^{2+} currents in uninfected cultured myocytes (Figure 1A and 1C; I_{CaL} baseline: -3.2±0.3 pA/pF versus 100 nmol/L ISO: -11.8±1.7 pA/pF). Thus, $\beta_{2a\text{-}AA}$ coexpression was used for the subsequent experiments with α_{1C} mutants.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Functional Expression of DHP-Insensitive α_{1C} Subunits in Cardiac Myocytes

Following a strategy similar to our previous study,[23] α_{1C} mutants were engineered to be dihydropyridine insensitive, to enable pharmacological discrimination of L-type Ca^{2+} current contributed by exogenously expressed channels from that of native Ca^{2+} channels. Consistent with our earlier results, a concentration of 10 μmol/L nitrendipine blocked more than 85% of I_{CaL} in myocytes transduced with Ad-$\beta_{2a\text{-}AA}$ alone, whereas only ≈50% of the total I_{CaL} was blocked in $\alpha_{1C(D\text{-})}$+$\beta_{2a\text{-}AA}$-transduced cells (Figure 2). Similar fractions of DHP-insensitive current were observed for myocytes expressing $\beta_{2a\text{-}AA}$ with either $\alpha_{1C(D\text{-})S1928A}$ or $\alpha_{1C(D\text{-})\Delta 1905}$ (Figure 2C).

Significant expression of mutant α_{1C} subunits was also confirmed in biochemical studies. A full-length α_{1C} fused to green fluorescent protein at the carboxyl terminus was used to discriminate exogenous and native α_{1C} subunits by molecular weight on Western blots and to provide a specific tag for the heterologous channels. Two intense high molecular mass bands appeared (>220 kDa) in samples from myocytes infected with Ad-$\alpha_{1C\text{-}GFP}$ (Figure 2) using an anti-$Ca_v1.2$ antibody, in addition to the lowermost band representing native α_{1C}. Densitometry confirmed that the signal from the uppermost bands in $\alpha_{1C\text{-}GFP}$ cells represented 63±6% of total anti-$Ca_v1.2$ signal intensity (Figure 2). When reprobed with an anti-GFP antibody, only the uppermost band was evident, verifying the presence of the fusion protein and suggesting that the middle band was a carboxyl-terminal cleavage product of the Ca^{2+} channel.

Ad-$\alpha_{1C\text{-}GFP}$ was next used to examine the spatial localization of the expressed mutant channels. Immunofluorescence staining of fixed cardiac myocytes with an anti-GFP antibody conjugated to the fluorescent dye Alexa 568 revealed a membrane distribution of expressed α_{1C} subunits including strong transverse-bands consistent with t-tubular localization, in accord with the known distribution of α_{1C} subunits in myocytes. Hence, expression of mutant α_{1C} subunits was confirmed both functionally and biochemically.

Distal Carboxyl Terminus of α_{1C} Is Necessary for β-Adrenergic Regulation of Ca^{2+} Channels

To ensure that the observed responses could be exclusively attributed to the expressed α_{1C} mutants, we first examined the effects of β-adrenergic stimulation on I_{CaL} in the presence of 10 μmol/L nitrendipine. Under these conditions, 100 nmol/L ISO induced a 50±11% increase of peak I_{CaL} in cells expressing $\alpha_{1C(D\text{-})}/\beta_{2a\text{-}AA}$ (Figure 3A and 3D). Remarkably, the ISO response was almost completely eliminated (I_{CaL} increased by only 3±7%) when the α_{1C} carboxyl terminus was truncated at amino acid residue 1905 ($\alpha_{1C(D\text{-})\Delta 1905}/\beta_{2a\text{-}AA}$; Figure 3B and 3D). To determine whether the loss of responsiveness involved impairment of the pathway between the β-adrenergic receptor and PKA, we also repeated the experiments using the membrane permeable cAMP analogue 8-bromo-adenosine 3′5′-cyclic monophosphate (8-Br-cAMP) to directly activate the kinase. Similar results were obtained, with a substantial reduction in 8-Br-cAMP induced Ca^{2+} current augmentation with $\alpha_{1C(D\text{-})\Delta 1905}$ compared with $\alpha_{1C(D\text{-})}$ (Figure 3E).

The ISO response was also examined in the absence of nitrendipine, to eliminate any possibility that the results were somehow dependent on the presence of concomitant Ca^{2+} channel block. ISO increased peak I_{CaL} by 104±23% in myocytes expressing $\alpha_{1C(D\text{-})}$ and by only 25±16% in myocytes expressing $\alpha_{1C(D\text{-})\Delta 1905}$. The small increase in the latter case is consistent with the presence of a small fraction of native L-type channels that would be available to be activated by ISO. In addition to the significant effect of the 1905 truncation to blunt the ISO effect on peak I_{CaL}, it is notable that the hyperpolarizing shift in V½ was also markedly reduced in myocytes expressing $\alpha_{1C(D\text{-})\Delta 1905}$ (Table).

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

S1928 Is Not Required for β-Adrenergic Regulation of Ca^{2+} Channels

In contrast to the effect of truncating the carboxyl terminus, the S1928A point mutation of the α_{1C} did not significantly diminish the ISO response, either in the presence or in the absence of nitrendipine (Figures 3 and 4). In myocytes expressing $\alpha_{1C(D-)S1928A}$, ISO increased I_{CaL} by 82±16% in the absence of nitrendipine (Figure 4) and a prominent leftward shift in $V_{1/2}$ was still observed (Table).

Discussion

The 2 main findings of the study were (1) that the distal carboxyl terminus of α_{1C} is required for the β-adrenergic stimulation of L-type Ca^{2+} channels in cardiac cells and (2) that serine 1928 phosphorylation is not required. This was most evident in the failure of Ca^{2+} currents to be enhanced by ISO in $\alpha_{1C(D-)\Delta1905}/\beta_{2a-AA}$-transduced cells in the presence of nitrendipine. The β-adrenergic response was also significantly blunted in $\alpha_{1C(D-)\Delta1905}/\beta_{2a-AA}$-transduced cells in the absence of nitrendipine, but the presence of ISO-responsive native L-type Ca^{2+} channels made the distinction less prominent. The ISO response was not significantly diminished in myocytes expressing the S1928A α_{1C} point mutant.

The present study is the first to investigate the structural determinants of the α_{1C} subunit involved in the β-adrenergic regulation of L-type Ca^{2+} channels in native adult myocytes. This was enabled by the efficient expression of $Ca_V1.2$ mutants using novel adenoviral constructs. Discrimination between the heterologous channels and native channels was accomplished by engineering the former to be DHP-insensitive. Both functional and biochemical data indicated that the majority of the L-type Ca^{2+} channels expressed were derived from the viral construct, so that significant differences in function could be readily observed, even in the absence of the dihydropyridine.

An important technical issue was how to optimize Ca^{2+} channel expression using β-subunit coexpression without introducing confounding effects caused by alterations in baseline Ca^{2+} channel activity by the β subunit. We found that mutation of the consensus phosphorylation sites of β_{2a} prevented the pronounced hyperpolarization of the voltage dependence of L-type Ca^{2+} channel activation under basal conditions, permitting us to observe a robust ISO response. Beyond the technical advantage of this approach, the results with β_{2a-AA} indicate that β subunit phosphorylation is not obligatory for the β-adrenergic response, a conclusion that differs from previous studies of Ca^{2+} channel regulation for channels heterologously expressed in cultured cell lines.[14]

The present results also provide evidence against the current dogma that PKA-mediated Ser1928 phosphorylation is required for the β-adrenergic stimulation of I_{CaL}. Both the ISO-induced increase in peak inward I_{CaL} and the left shift in the $V_{1/2}$ of activation were preserved in cardiomyocytes expressing $\alpha_{1C(D-)S1928A}$. In this regard, the functional data supporting S1928 as the site of modulation have been equivocal. Gao et al[20] reported that Ba^{2+} currents through expressed Ca^{2+} channels were upregulated by forskolin or 8-Br-cAMP in an AKAP 79 stably transfected HEK 293 cell line and that this response was absent for the S1928A α_{1C} point mutation. However, only half of the control cells tested were clearly responsive to the effects of the PKA activators, suggesting a high degree of variability in this model system. In addition, forskolin increased Ba^{2+} current by only ≈50%, and 8-Br-cAMP by ≈15%, much less than that typically observed for PKA-mediated I_{CaL} enhancement observed in cardiac myocytes. Moreover, a later study from the same laboratory[14] reported that I_{CaL} mediated by the 1905 α_{1C} truncation mutant, lacking serine 1928, could be increased by PKA activation.

Other reports have also raised cautions about the conclusions reached in the earlier studies. Many investigators have failed to reproduce PKA modulation of L-type Ca^{2+} currents in

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

heterologous expression systems. Zong et al[16] tested 25 μmol/L PKA along with okadaic acid in CHO and HEK 293 cells expressing either $\alpha_{1C\text{-a}}$ or $\alpha_{1C\text{-b}}$ and were not able to observe PKA-mediated potentiation of Ba^{2+} currents. Mikala et al[15] systematically substituted 5 potential PKA phosphorylation sites in human α_{1C}, including the S1928 homolog, and found that modulation of baseline L-type Ca^{2+} current behavior could not account for the failure to observe PKA modulation. Altier et al[22] also failed to observe PKA-mediated activation of Ba^{2+} currents in HEK 293 cells, even in the presence of AKAP 79 overexpression.

Although the functional effects of S1928 may be questionable, there is agreement that S1928 can be readily phosphorylated by PKA in vitro.[11,12] However, according to a recent report, this site appears to be promiscuously phosphorylated by at least 3 isoforms of PKC (cPKCα, nPKCε, and aPKCζ) in addition to PKA.[29] This raises an important question about the physiological relevance of this in vitro biochemical evidence, considering that the considerably divergent physiological effects of PKA and PKC activation on channel activity.[8]

The present data suggest that although S1928 is not critical for the β-adrenergic effect on Ca^{2+} channel function, the distal α_{1C} carboxyl terminus beyond residue 1905 is required. One plausible explanation for this effect could be that the carboxyl terminus plays a central role in the assembly of a PKA signaling complex. For example, Hulme et al[30] recently demonstrated an interaction between AKAP 15 and a leucine zipper motif in the distal carboxyl terminus of α_{1C} using immunoprecipitation methods. Disruption of the interaction with competing AKAP 15 leucine zipper peptides substantially reduced ISO-induced upregulation of L-type Ca^{2+} currents in cardiac myocytes, suggesting that the AKAP 15-α_{1C} interaction was necessary for efficient β-adrenergic regulation of Ca^{2+} currents in cardiac myocytes.[30] Further investigation into the role of the leucine zipper motif in the response using the present strategy will be important.

The existing literature provides a number of potential alternative sites of PKA-mediated phosphorylation of α_{1C}. For example, Leach et al,[31] using phosphopeptide antibodies, reported that serines 1627 and 1700 were the primary sites of PKA-mediated phosphorylation of the cardiac Ca^{2+} channel α_{1C} subunit, whereas no phosphopeptide products could be detected for sites S1575, S1848, or S1928 on the carboxyl terminus. Other sites at S1829[32]and S1142[33] also have been proposed as possible sites of regulation. The present work reopens the issue and provides a new way to test potential sites.

There is also the possibility that phosphorylation of other Ca^{2+} channel-associated proteins could modify channel function. However, in contrast to previous reports,[14] the present findings do not point toward the β subunit as the functionally important site of regulation in adult cardiac cells.

The results have demonstrated the potential for structure/function studies of the α_{1C} subunit in native cells to complement investigations in heterologous systems. However, the potential limitations of this strategy need to be acknowledged. In this case, overexpressed β_2 subunits may form complexes with native α_{1C} subunits, or, conversely, overexpressed α_{1C} may complex with native β subunits. However, although the possibilities for such channel subpopulations pose challenges for the interpretation of studies of molecular regulation, the experimental evidence in our study suggests that the overexpressed subunits dominated the phenotype of L-type Ca^{2+} currents. In the case of the β_2 subunit, this was apparent with the changes in current density and the voltage dependence of activation. With α_{1C}-subunit overexpression, this was evident with changes in DHP-sensitivity and the β-adrenergic response.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Conclusions

Our findings demonstrate the distal carboxyl terminus beyond amino acid 1905 of the α_{1C} subunit is required for β-adrenergic enhancement of L-type Ca^{2+} currents in adult cardiomyocytes. However, our results do not support a functional role for phosphorylation of S1928. The present study also demonstrates that structure/function studies of the α_{1C} subunit of the L-type Ca^{2+} channel are possible using adenoviral gene transfer methods.

Acknowledgments

This study was supported by NIH grant R01HL61711 (to B.O'R.), an American Heart Association Predoctoral Fellowship (0315387U, to A.N.G.), Deutsche Forschungsgemeinschaft MA 2528/1.1 (to C.M.), and NIH grant RO1-NS044541 (to D.C.J.). We thank Dr Henry Colecraft for useful discussions.

References

1. Reuter H. Properties of two inward membrane currents in the heart. Annu Rev Physiol 1979;41:413–424. [PubMed: 373598]
2. Reuter H. The dependence of slow inward current in Purkinje fibres on the extracellular calcium-concentration. J Physiol 1967;192:479–492. [PubMed: 6050160]
3. Bers DM. Cardiac excitation-contraction coupling. Nature 2002;415:198–205. [PubMed: 11805843]
4. Catterall WA. Structure and regulation of voltage-gated Ca2+ channels. Annu Rev Cell Dev Biol 2000;16:521–555. [PubMed: 11031246]
5. Colecraft HM, Alseikhan B, Takahashi SX, Chaudhuri D, Mittman S, Yegnasubramanian V, Alvania RS, Johns DC, Marban E, Yue DT. Novel functional properties of Ca(2+) channel beta subunits revealed by their expression in adult rat heart cells. J Physiol 2002;541:435–452. [PubMed: 12042350]
6. Foell JD, Balijepalli RC, Delisle BP, Yunker AM, Robia SL, Walker JW, McEnery MW, January CT, Kamp TJ. Molecular heterogeneity of calcium channel beta-subunits in canine and human heart: evidence for differential subcellular localization. Physiol Genomics 2004;17:183–200. [PubMed: 14762176]
7. Xiao RP. Beta-adrenergic signaling in the heart: dual coupling of the beta2- adrenergic receptor to G (s) and G(i) proteins. Sci STKE 2001;2001:RE15. [PubMed: 11604549]
8. Kamp TJ, Hell JW. Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C. Circ Res 2000;87:1095–1102. [PubMed: 11110765]
9. Keef KD, Hume JR, Zhong J. Regulation of cardiac and smooth muscle Ca(2+) channels (Ca(V)1.2a,b) by protein kinases. Am J Physiol Cell Physiol 2001;281:C1743–C1756. [PubMed: 11698232]
10. Yoshida A, Takahashi M, Nishimura S, Takeshima H, Kokubun S. Cyclic AMP-dependent phosphorylation and regulation of the cardiac dihydropyridine sensitive Ca channel. FEBS Lett 1992;309:343–349. [PubMed: 1325377]
11. Mitterdorfer J, Froschmayr M, Grabner M, Moebius FF, Glossmann H, Striessnig J. Identification of PK-A phosphorylation sites in the carboxyl terminus of L-type calcium channel alpha 1 subunits. Biochemistry 1996;35:9400–9406. [PubMed: 8755718]
12. De Jongh KS, Murphy BJ, Colvin AA, Hell JW, Takahashi M, Catterall WA. Specific phosphorylation of a site in the full-length form of the alpha 1 subunit of the cardiac L-type calcium channel by adenosine 3′,5′-cyclic monophosphate-dependent protein kinase. Biochemistry 1996;35:10392–10402. [PubMed: 8756695]
13. Haase H, Bartel S, Karczewski P, Morano I, Krause EG. In-vivo phosphorylation of the cardiac L-type calcium channel beta-subunit in response to catecholamines. Mol Cell Biochem 1996;163-164:99–106. [PubMed: 8974044]
14. Bunemann M, Gerhardstein BL, Gao T, Hosey MM. Functional regulation of L-type calcium channels via protein kinase A-mediated phosphorylation of the beta(2) subunit. J Biol Chem 1999;274:33851–33854. [PubMed: 10567342]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

15. Mikala G, Klockner U, Varadi M, Eisfeld J, Schwartz A, Varadi G. cAMP-dependent phosphorylation sites and macroscopic activity of recombinant cardiac L-type calcium channels. Mol Cell Biochem 1998;185:95–109. [PubMed: 9746216]
16. Zong X, Schreieck J, Mehrke G, Welling A, Schuster A, Bosse E, Flockerzi V, Hofmann F. On the regulation of the expressed L-type calcium channel by cAMP-dependent phosphorylation. Pflugers Arch 1995;430:340–347. [PubMed: 7491257]
17. Perets T, Blumenstein Y, Shistik E, Lotan I, Dascal N. A potential site of functional modulation by protein kinase A in the cardiac Ca2+ channel alpha 1C subunit. FEBS Lett 1996;384:189–192. [PubMed: 8612821]
18. Singer-Lahat D, Lotan I, Biel M, Flockerzi V, Hofmann F, Dascal N. Cardiac calcium channels expressed in Xenopus oocytes are modulated by dephosphorylation but not by cAMP-dependent phosphorylation. Receptors Channels 1994;2:215–226. [PubMed: 7874448]
19. Perez-Reyes E, Yuan W, Wei X, Bers DM. Regulation of the cloned L-type cardiac calcium channel by cyclic-AMP-dependent protein kinase. FEBS Lett 1994;342:119–123. [PubMed: 8143862]
20. Gao T, Yatani A, Dell'Acqua ML, Sako H, Green SA, Dascal N, Scott JD, Hosey MM. cAMP-dependent regulation of cardiac L-type Ca2+ channels requires membrane targeting of PKA and phosphorylation of channel subunits. Neuron 1997;19:185–196. [PubMed: 9247274]
21. Dai S, Klugbauer N, Zong X, Seisenberger C, Hofmann F. The role of subunit composition on prepulse facilitation of the cardiac L-type calcium channel. FEBS Lett 1999;442:70–74. [PubMed: 9923607]
22. Altier C, Dubel SJ, Barrere C, Jarvis SE, Stotz SC, Spaetgens RL, Scott JD, Cornet V, De Waard M, Zamponi GW, Nargeot J, Bourinet E. Trafficking of L-type calcium channels mediated by the postsynaptic scaffolding protein AKAP79. J Biol Chem 2002;277:33598–33603. [PubMed: 12114507]
23. Ganesan AN, O'Rourke B, Maack C, Colecraft H, Sidor A, Johns DC. Reverse engineering the L-type Ca(2+) channel alpha(1c) subunit in adult cardiac myocytes using novel adenoviral vectors. Biochem Biophys Res Commun 2005;329:749–754. [PubMed: 15737650]
24. Hoppe UC, Marban E, Johns DC. Adenovirus-mediated inducible gene expression in vivo by a hybrid ecdysone receptor. Mol Ther 2000;1:159–164. [PubMed: 10933926]
25. Hardy S, Kitamura M, Harris-Stansil T, Dai Y, Phipps ML. Construction of adenovirus vectors through Cre-lox recombination. J Virol 1997;71:1842–1849. [PubMed: 9032314]
26. Maack C, Ganesan A, Sidor A, O'Rourke B. Cardiac sodium-calcium exchanger is regulated by allosteric calcium and exchanger inhibitory peptide at distinct sites. Circ Res 2005;96:91–99. [PubMed: 15550690]
27. Fallaux FJ, Kranenburg O, Cramer SJ, Houweling A, Van Ormondt H, Hoeben RC, Van Der Eb AJ. Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. Hum Gene Ther 1996;7:215–222. [PubMed: 8788172]
28. O'Rourke B, Ramza BM, Marban E. Oscillations of membrane current and excitability driven by metabolic oscillations in heart cells. Science 1994;265:962–966. [PubMed: 8052856]
29. Yang L, Liu G, Zakharov SI, Morrow JP, Rybin VO, Steinberg SF, Marx SO. Ser1928 is a common site for Cav1.2 phosphorylation by protein kinase C isoforms. J Biol Chem 2005;280:207–214. [PubMed: 15509562]
30. Hulme JT, Lin TW, Westenbroek RE, Scheuer T, Catterall WA. Beta-adrenergic regulation requires direct anchoring of PKA to cardiac CaV1.2 channels via a leucine zipper interaction with A kinase-anchoring protein 15. Proc Natl Acad Sci U S A 2003;100:13093–13098. [PubMed: 14569017]
31. Leach RN, Brickley K, Norman RI. Cyclic AMP-dependent protein kinase phosphorylates residues in the C-terminal domain of the cardiac L-type calcium channel alpha1 subunit. Biochim Biophys Acta 1996;1281:205–212. [PubMed: 8664319]
32. Takahashi E, Fukuda K, Miyoshi S, Murata M, Kato T, Ita M, Tanabe T, Ogawa S. Leukemia inhibitory factor activates cardiac L-Type Ca2+ channels via phosphorylation of serine 1829 in the rabbit Cav1.2 subunit. Circ Res 2004;94:1242–1248. [PubMed: 15044319]
33. Erxleben C, Gomez-Alegria C, Darden T, Mori Y, Birnbaumer L, Armstrong DL. Modulation of cardiac Ca(V)1.2 channels by dihydropyridine and phosphatase inhibitor requires Ser-1142 in the domain III pore loop. Proc Natl Acad Sci U S A 2003;100:2929–2934. [PubMed: 12601159]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

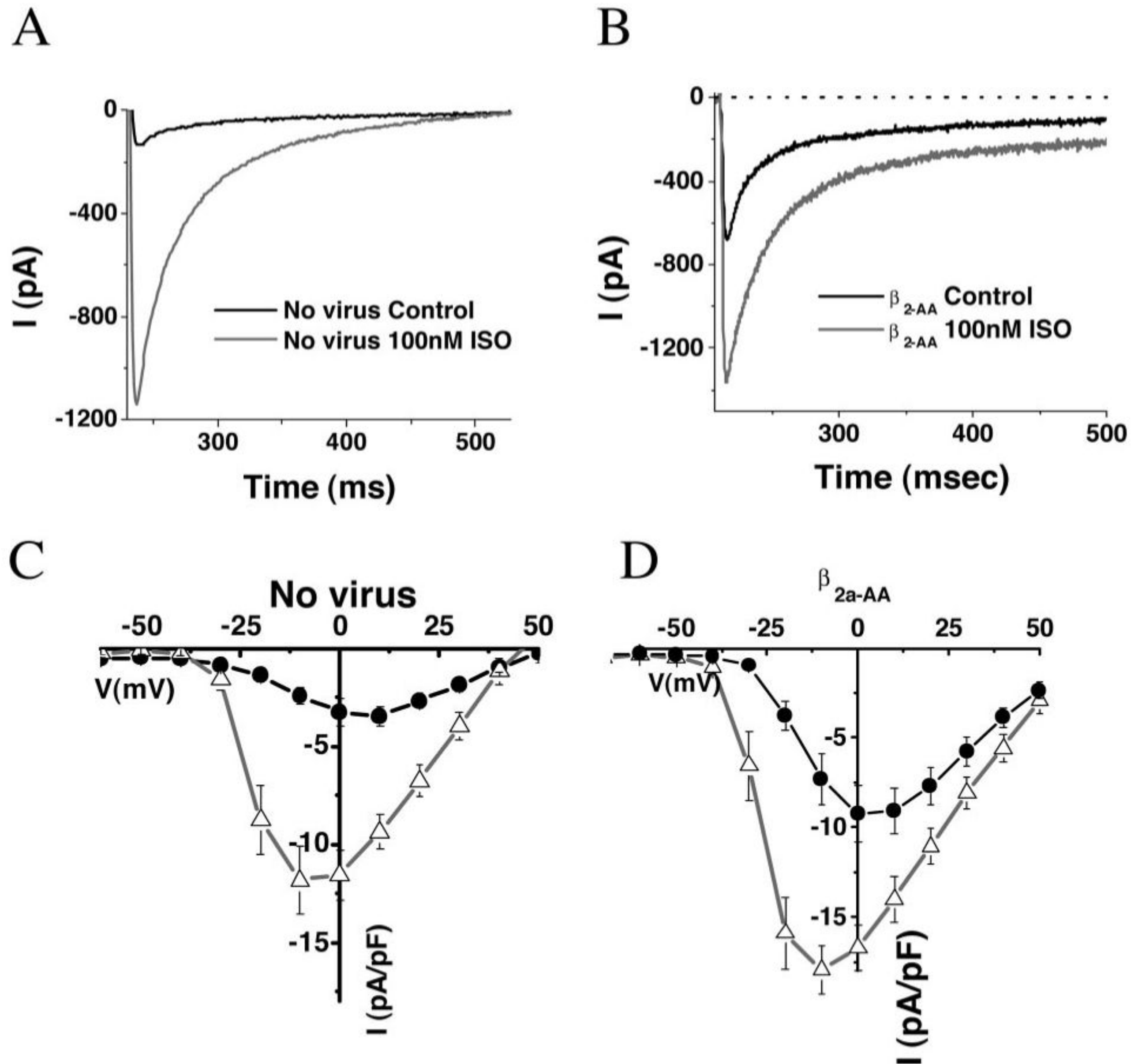

Figure 1.

Isoproterenol response in adult myocytes transduced with adenoviruses overexpressing $\beta_{2a\text{-}AA}$ subunits. Whole-cell currents were recorded using voltage clamp pulses from a holding potential of -80 mV to test potentials, following a 200 ms prepulse to -40 mV, before and after the application of 100 nmol/L ISO. A and B, Example current tracings following pulses to 0 mV, before and after 100 nmol/L ISO. C and D, Current-voltage relationships: before (black) and after (gray) the application of 100 nmol/L ISO in uninfected myocytes (C) and myocytes transduced with $\beta_{2a\text{-}AA}$ (D). $\beta_{2a\text{-}AA}$ transduction caused a baseline increase in I_{CaL} and a small hyperpolarizing shift in the voltage dependence of activation; however, the β-adrenergic response was preserved. Values are mean±SEM.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

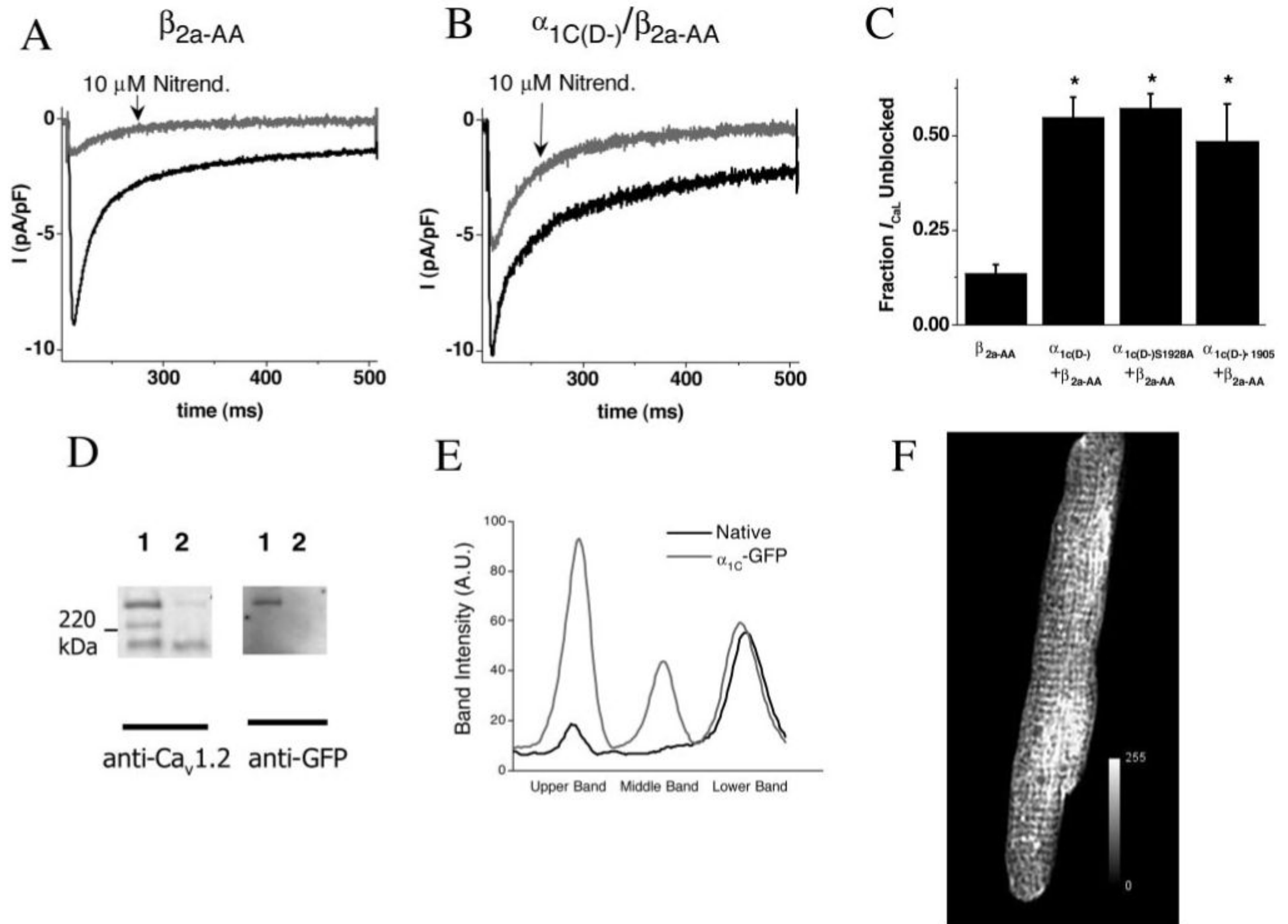

Figure 2.

Overexpression of DHP-insensitive α_{1C} enables pharmacological discrimination of native and exogenously expressed channels. Whole-cell current recordings were undertaken in adult myocytes transduced with α_{1C} adenoviruses and $\beta_{2a\text{-}AA}$ adenoviruses. A repeated-pulsing protocol at 0.5 Hz to 0 mV was used to obtain a steady state, following which 10 μmol/L nitrendipine was added. Example current tracings are shown in myocytes transduced with $\beta_{2a\text{-}AA}$ (A) or $\alpha_{1C(D\text{-})/}\beta_{2a\text{-}AA}$ (B) adenoviruses, before (black) and after (gray) the application of 10 μmol/L nitrendipine. In C, the fraction of I_{CaL} left unblocked after the application of nitrendipine is shown following transduction with each of the DHP-insensitive α_{1C} adenoviruses. D, Representative Western blot. Left, Lane 1 was transduced with $\alpha_{1C\text{-}GFP}$ and AdVgRXR; lane 2, with AdVgRXR alone. Right, The same membrane was reprobed with anti-GFP antibody, confirming that the strong upper band was attributable to $\alpha_{1C\text{-}GFP}$ expression. The absence of the middle band in lane 1 is indicative of carboxyl-terminal processing of $\alpha_{1C\text{-}GFP}$. In E, the anti-Ca$_v$1.2 signal intensity plot profile is shown for $\alpha_{1C\text{-}GFP}$ and control cells, demonstrating the presence of novel peaks in upper and middle bands caused by $\alpha_{1C\text{-}GFP}$ expression. F, Representative image of an $\alpha_{1C\text{-}GFP}$/AdVgRXR-transduced myocyte, stained with anti-GFP primary antibody, and an Alexa-Fluor 568 secondary antibody. *P<0.05 vs $\beta_{2a\text{-}AA}$.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

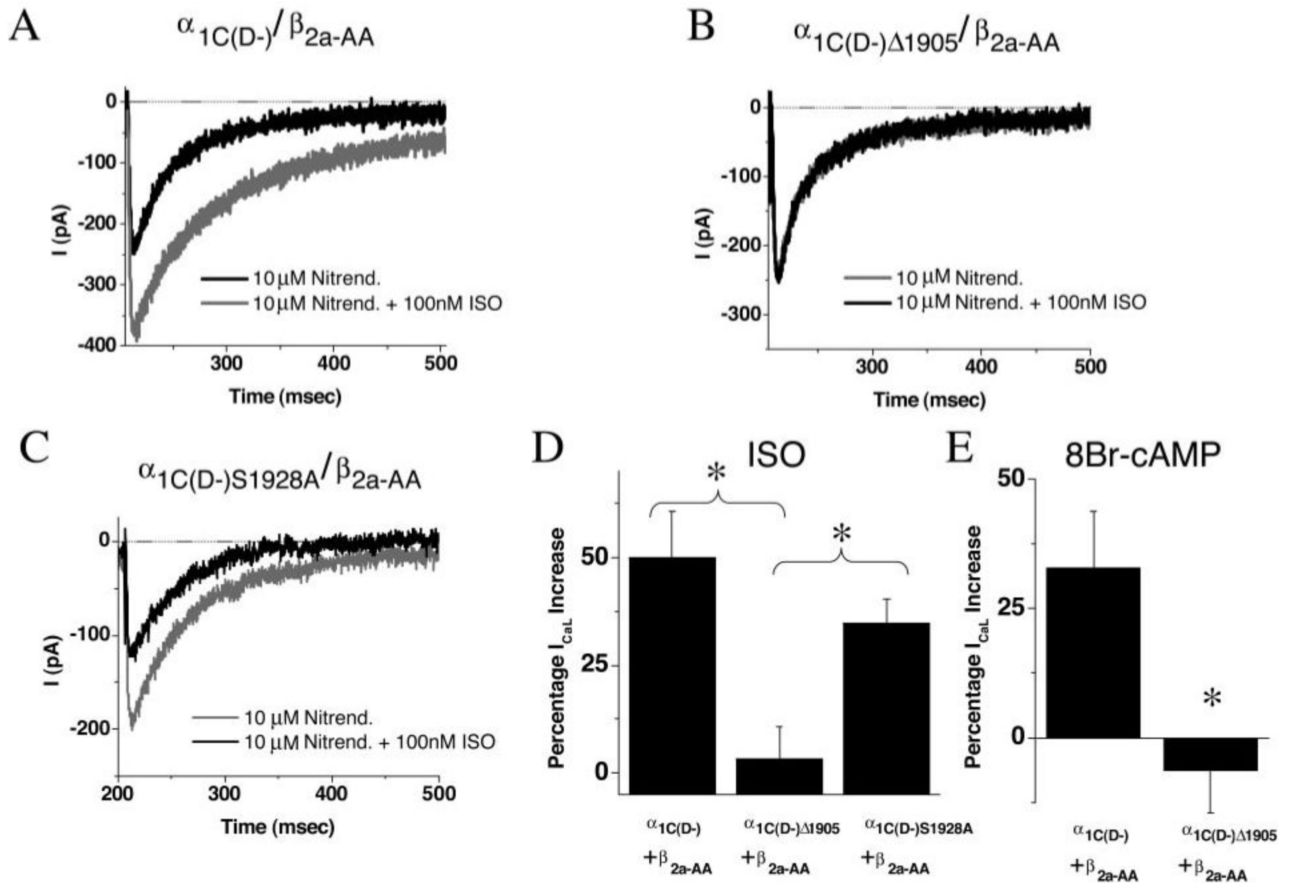

Figure 3.

In the presence of nitrendipine, carboxyl-terminal truncation mutant shows diminished response to ISO, but S1928A remains responsive. Whole-cell current recordings were obtained in adult myocytes before and after 100 nmol/L ISO, for myocytes transduced with $\alpha_{1C(D\text{-})}$ (A), $\alpha_{1C(D\text{-})\Delta1905}$ (B), or $\alpha_{1C(D\text{-})S1928A}$ (C) viruses. Current records are for repeated pulses to 0 mV in the continuous presence of 10 μmol/L nitrendipine, to block I_{CaL} carried by native channels. The fractional response to 100 nmol/L ISO is shown in D. ISO (100 nmol/L) elicited a robust increase in I_{CaL} in $\alpha_{1C(D\text{-})}$ cells in the presence of the blocking dose of nitrendipine (A and D) but failed to elicit I_{CaL} upregulation in $\alpha_{1C(D\text{-})\Delta1905}$ cells (B and D), indicating that the carboxyl terminus of α_{1C} is necessary for β-adrenergic modulation of $Ca_v1.2$ channels in cardiac myocytes. ISO also elicited a robust increase in I_{CaL} in $\alpha_{1C(D\text{-})S1928A}$ cells, indicating that S1928 phosphorylation is not essential for β-adrenergic modulation of L-type Ca^{2+} current. E, 8-Br-cAMP (1 mmol/L) elicited an increase in I_{CaL} in $\alpha_{1C(D\text{-})}$ cells in the presence of 10 μmol/L nitrendipine but failed to elicit I_{CaL} upregulation in $\alpha_{1C(D\text{-})\Delta1905}$ cells. Values are mean ±SEM. *P<0.05, **P<0.01.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

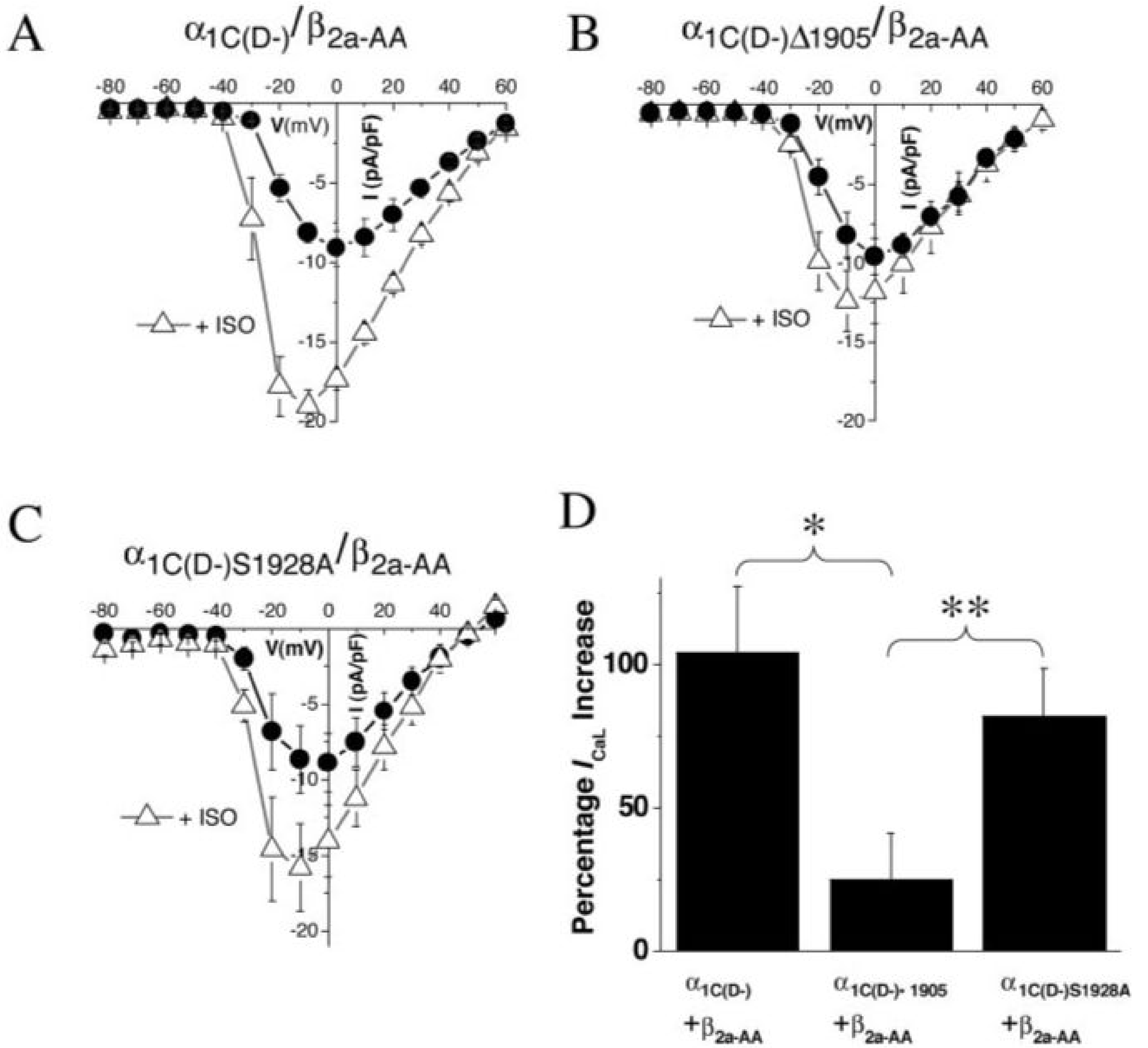

Figure 4.

Carboxyl-terminal truncation mutant shows diminished response to ISO, but S1928A remains responsive. To ensure that the results were not a consequence of the presence of nitrendipine, whole-cell current recordings were obtained from myocytes before and after 100 nmol/L ISO in the absence of DHP. Current-voltage relationships before (black circles) and after (open triangles) 100 nmol/L ISO are plotted for myocytes transduced with $\alpha_{1C\ (D\text{-})}/\beta_{2a\text{-}AA}$ (A), $\alpha_{1C(D\text{-})\Delta 1905}/\beta_{2a\text{-}AA}$ (B), or $\alpha_{1C(D\text{-})S1928A}/\beta_{2a\text{-}AA}$ (C) adenoviruses. The β-adrenergic response of I_{CaL} was markedly attenuated in $\alpha_{1C(D\text{-})\Delta 1905}/\beta_{2a\text{-}AA}$ cells (B and D), as it was in the presence of nitrendipine (Figure 3). However, the β-adrenergic enhancement of I_{CaL} was preserved in $\alpha_{1C(D\text{-})S1928A}/\beta_{2a}$ myocytes (C and D). The fractional increase in response to 100 nmol/L ISO of the peak I_{CaL} is shown in D. Values are mean±SEM. *P<0.05, **P<0.025.

NIH-PA Author Manuscript

Voltage Dependence of Activation ($V_{1/2}$) of L-Type Ca^{2+} Channels in Cardiac Myocytes

	$V_{1/2}$	$V_{1/2}$ (100 nmol/L ISO)
Control	-7.9±0.1	-20.4±2.2*
$\beta_{2a\text{-}AA}$	-12.70±2.4	-24.20±2.6†
$\alpha_{1C(D-)}/\beta_{2a\text{-}AA}$	-16.95±1.9	-25.21±1.9‡
$\alpha_{1C(D-)1905}/\beta_{2a\text{-}AA}$	-14.52±2.4	-19.60±2.4§
$\alpha_{1C(D-)S1928A}/\beta_{2a\text{-}AA}$	-13.5±3.3	-23.1±1.4¶

*P<0.05 vs control

†P<0.05 vs β2a-AA

‡P<0.05 vs α1C(D-)/β2a-AA

§P=NS vs α1C(D-)Δ1905/β2a-AA

¶P<0.05 vs α1C(D-)S1928A/β2a-AA