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Reverse engineering the L-type Ca^{2+} channel α_{1c} subunit in adult cardiac myocytes using novel adenoviral vectors

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Abstract

The α_{1c} subunit of the cardiac L-type Ca^{2+} channel, which contains the channel pore, voltage- and Ca^{2+} -dependent gating structures, and drug binding sites, has been well studied in heterologous expression systems, but many aspects of L-type Ca^{2+} channel behavior in intact cardiomyocytes remain poorly characterized. Here, we develop adenoviral constructs with E1, E3 and fiber gene deletions, to allow incorporation of full-length α_{1c} gene cassettes into the adenovirus backbone. Wild-type ($\alpha_{1c\text{-wt}}$) and mutant ($\alpha_{1c\text{-D}}$) Ca^{2+} channel adenoviruses were constructed. The $\alpha_{1c\text{-D}}$ contained four point substitutions at amino acid residues known to be critical for dihydropyridine binding. Both $\alpha_{1c\text{-wt}}$ and $\alpha_{1c\text{-D}}$ expressed robustly in A549 cells (peak L-type Ca^{2+} current (I_{CaL}) at 0 mV: $\alpha_{1c\text{-wt}}$ -9.94 ± 1.00 pA/pF, $n = 9$; $\alpha_{1c\text{-D}}$ -10.30 pA/pF, $n = 12$). I_{CaL} carried by $\alpha_{1c\text{-D}}$ was markedly less sensitive to nitrendipine (IC_{50} 17.1 μM) than $\alpha_{1c\text{-wt}}$ (IC_{50} 88 nM); a feature exploited to discriminate between engineered and native currents in transduced guinea-pig myocytes. 10 μM nitrendipine blocked only $51 \pm 5\%$ ($n = 9$) of I_{CaL} in $\alpha_{1c\text{-D}}$ -expressing myocytes, in comparison to $86 \pm 8\%$ ($n = 9$) of I_{CaL} in control myocytes. Moreover, in 20 μM nitrendipine, calcium transients could still be evoked in $\alpha_{1c\text{-D}}$ -transduced cells, but were largely blocked in control myocytes, indicating that the engineered channels were coupled to sarcoplasmic reticular Ca^{2+} release. These α_{1c} adenoviruses provide an unprecedented tool for structure-function studies of cardiac excitation–contraction coupling and L-type Ca^{2+} channel regulation in the native myocyte background.

Keywords

Dihydropyridine; Excitation–contraction coupling; Gene transfer; Adenovirus; Ion channel

The cardiac L-type Ca^{2+} channel ($\text{Ca}_v1.2a$) is a multisubunit complex of five proteins located on the surface membrane of the cardiac myocyte. The largest component is the pore-forming α_1 subunit (~220 kDa), the critical determinant of most functional properties of the channel, including voltage-dependent gating, Ca^{2+} permeability, Ca^{2+} -dependent inactivation [1,2], and inhibition by the three principal classes of Ca^{2+} channel antagonists [3–5].

While many of the biophysical properties of the channel have been extensively investigated in heterologous expression systems, important features of L-type Ca^{2+} channel regulation are not well reproduced outside native cardiac myocytes. For example, the prominent increase in L-type Ca^{2+} current (I_{CaL}) amplitude observed in cardiac myocytes in response to PKA-mediated phosphorylation is difficult to reproduce in heterologous expression systems [6]. Additionally, while structural interactions between the L-type Ca^{2+} channel and the ryanodine receptor have been proposed [7,8], no model system is currently available to reproduce the native spatial organization of the dyadic space, rendering exploration of this issue impossible.

Since adult cardiomyocytes are largely resistant to standard transfection methods with plasmid DNA, adenoviral gene transfer of the $\alpha_{1\text{c}}$ subunit would provide an ideal method for exploring these questions [9]. However, the 6.6 kb size of the $\alpha_{1\text{c}}$ subunit cDNA has previously precluded its successful incorporation into the standard E1, E3-deleted adenoviral backbone. Helper-dependent adenoviruses, which have the capacity for a larger transgene up to 30 kb, are unable to produce virus at high enough titer for effective cardiomyocyte gene transfer [9,10]. While able to accommodate inserts up to 10 kb [11], adenoviruses expressing multidomain α subunits of voltage-gated channels have also been difficult to produce using other commercially available systems.

To overcome these problems, we produced novel adenoviral constructs containing deletions of the E1, E3, and for the first time, the fiber gene. The additional deletion provides the physical space to accommodate full length $\alpha_{1\text{c}}$ subunit gene cassettes, with the fiber gene product provided in trans by cell lines engineered to stably express the fiber gene.

Here, we demonstrate that this method can be used to construct $\alpha_{1\text{c}}$ subunit adenoviruses and show that they are expressed and functionally coupled to Ca^{2+} release in adult cardiomyocytes. By engineering dihydropyridine-insensitive $\alpha_{1\text{c}}$ mutant channels, the adenoviral constructs could be clearly discriminated from the native L-type Ca^{2+} channels, opening the door for further exploration of the structural requirements of Ca^{2+} channel regulation and the trigger mechanism of cardiac excitation–contraction coupling.

Experimental procedures

$\alpha_{1\text{c-D}}$ plasmid preparation

The full-length rabbit $\alpha_{1\text{c}}$ subunit (with the alternatively spliced domain in IVS3, as previously described [12]) was ligated into pAdEcd, an adenovirus shuttle vector containing an ecdysone inducible promoter [13], to make pAdEcd- $\alpha_{1\text{c-wt}}$.

A dihydropyridine-insensitive mutant ($\alpha_{1\text{c-D}}$, pAdEcd- $\alpha_{1\text{c-D}}$) was engineered using site-directed mutagenesis to create four amino acid substitutions in the dihydropyridine binding pocket. Mutagenesis was performed using the QuikChangeXL kit (Stratagene) according to the manufacturer's instructions. This DHP-insensitive mutant contained mutations at the critical residues Thr1066 in IIIS5 [4,14,15], as well as Phe 1463, Met1464, and Iso1471 in IVS6 [3–5]. These mutations have previously been shown to decrease the affinity of $\alpha_{1\text{c}}$ for dihydropyridines by over 100-fold, without significant effects on the biophysical properties of the channel [3].

Virus construction

To create recombinant viruses deleted for the fiber gene, it was first necessary to engineer a cell line to propagate them in and a cell line that allowed Cre mediated recombination into the E1 region. The vector pDV67, which contains a fiber expression cassette and a gene for zeocin resistance (a kind gift from Dr. von Seggern) [16], was linearized and transfected into both Cre8 [17] cells and 911 cell [18]. Cells were selected for resistance to zeocin and resulting

clones were screened for their ability to complement the fiber defect in the virus Ad5.GFPDF [16] and designated CRE-FB and 911-FB, respectively. The hybrid virus was created by co-transfecting the left end of the virus ψ 5 and the right end of the virus Ad5.GFPDF into 911-FB cells. Resulting viruses were first screened for a lack of GFP expression and then for their inability to form plaques on non-fiber expressing cells. One plaque (designated ψ FB6) conformed to these requirements and was expanded. DNA was isolated and analyzed by cutting with *Eco*RI, the resulting restriction pattern (Fig. 1B) is what would be predicted for a hybrid between the two parent viruses. In order to assess its functionality, ψ FB6 DNA was co-transfected with shuttle plasmids pADCGI [19] or pAdEcd- α_{1c-wt} , or pAdEcd- α_{1c-D} , into CRE-FB cells as previously described [13]. The resulting viruses were analyzed by plaque assays on 911-FB cells or by patch-clamp analysis of A549 cells as described below.

Heterologous expression

Heterologous expression studies were performed in A549 cells (ATCC CCL-185), maintained in DMEM with 10% FBS, 1% penicillin–streptomycin (5% CO₂, 37 °C). Briefly, 24 h prior to transduction, confluent A549 cells were trypsinized and plated on glass coverslips. The cells were transduced with either α_{1c-wt} or α_{1c-D} adenoviruses at $\sim 5 \times 10^8$ PFU/mL, along with a β_{2a} -GFP fusion protein adenovirus at $\sim 8 \times 10^8$ PFU/mL and the ecdysone receptor virus AdDBEcR [19] at $\sim 3 \times 10^8$ PFU/mL. After a 2 h incubation, the virus-containing medium was replaced with fresh DMEM, with the addition of 10 μ M ponasterone-A (H101-01, Invitrogen). After 24 h, the media were again replaced with fresh DMEM and ponasterone.

Adult cardiomyocyte 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 adult guinea pig ventricular cardiac myocytes were obtained by enzymatic digestion as previously described [20,21]. Freshly isolated cardiomyocytes were plated on laminin-coated glass coverslips and bathed in DMEM supplemented with 5% FBS. After allowing 1–2 h for the cells to adhere to the coverslips, virus was added in the same media, in identical concentrations as the A549 experiments. The cardiac myocytes were transduced with α_{1c-D} , β_{2a} -GFP, and AdDMEcR viruses, or β_{2a} -GFP and AdDBEcR alone. Inclusion of the β_{2a} -GFP virus facilitated the maintenance of Ca²⁺ current densities in myocytes cultured for 24–48 h to levels similar to that found in freshly isolated myocytes. The adenovirus-containing medium was replaced with fresh DMEM with 10 μ M ponasterone-A after 2–4 h, with a second change of media after 24 h.

Electrophysiological recordings

Electrophysiological recordings of both A549 cells and cardiac myocytes were performed 36–48 h following transduction using the whole-cell patch-clamp technique. Data were acquired at room temperature, using an Axopatch amplifier. For basic current recordings, cells were superfused with a cesium-containing external solution comprised of (mM): NaCl 140, CsCl 5, MgCl₂ 1, Hepes 10, CaCl₂ 2, and D-glucose 10. The pipette solution contained (mM): CsCl 110, MgCl₂ 0.4, D-glucose 5, Hepes 10, TEA 20, and BAPTA 5. Voltage pulses were applied and data were recorded with custom software and analyzed offline. Leak currents were corrected by subtraction of Cd²⁺-sensitive current at the end of each experiment. Nitrendipine (RBI, Natick, MA) was dissolved in DMSO to make a 100 mM stock on the day of the experiment and diluted to its final concentration in external solution. DMSO concentration did not exceed 0.1%.

Excitation–contraction coupling experiments

For calcium transient experiments, the external solution was a modified Tyrode's solution similar to that described above, but containing 5 mM KCl in place of CsCl: the pipette solution used was 130 K-glutamate, KCl 9, NaCl 10, MgCl₂ 0.4, Mg-ATP 5, and Hepes 10, with the addition of 100 μM indo-1 (pentapotassium salt, Molecular Probes), as previously described [22,23]. A correction was made for a liquid-junction potential of ~−17 mV. The indo-1 was excited with a xenon arc lamp at 365 nm, and emitted fluorescence was acquired with a two-channel photomultiplier tube at 405 and 495 nm. After correcting for autofluorescence, the Ca²⁺ transient was calculated according to the following equation:

$$Ca_i^{2+} = \beta \times K_d \times [(R - R_{\min}) / (R_{\max} - R)], \text{ with } R_{\min} = 0.7, R_{\max} = 4, \beta = 2, \text{ and the } K_d \text{ of indo-1 } 844 \text{ nM [22,23].}$$

Statistical analysis

All data are presented as means ± SEM. Statistical significance was calculated using an unpaired Student's *t* test, and differences were considered significant at *p* < 0.05.

Results

Recombinant viruses

A schematic of the virus construction is shown in Fig. 1A, while Fig. 1B shows the restriction analysis of ψFB6 and the two parental viruses. Co-transfection of ψFB6 with pAd-CGI produced recombinants in which the GFP cassette was efficiently inserted into the E1 region such that all of the plaques were GFP positive following three passages on Cre-FB cells. Likewise co-transfection of pAdEcd-α_{1c-wt} or pAdEcd-α_{1c-D.} with ψFB6 resulted in viruses which produced functional Ca²⁺ channels upon infection of A549 cells as described below.

Adenoviral expression of L-type Ca²⁺ channels in A549 cells

To assess the ability of these novel adenoviruses to form viable L-type Ca²⁺ channels, we co-expressed in A549 cells, either α_{1c-wt} or α_{1c-D.}, with a β_{2a}-GFP adenovirus [24], and an ecdysone-receptor adenovirus, AddMEcR [25]. Both α_{1c-wt} and α_{1c-D.} adenoviruses formed functional L-type Ca²⁺ channels in A549 cells (Figs. 2A,B). To test the relative nitrendipine sensitivity, we used 50 ms test pulses to 0 mV from a holding potential of −80 mV, pulsing at a frequency of 0.1 Hz. The test pulses were preceded by a 400 ms prepulse to +100 mV, to enhance the voltage-dependent binding of nitrendipine [1,2]. In our experiments, we used the neutral dihydropyridine antagonist nitrendipine, which acts in a more strictly voltage-dependent, rather than use-dependent, manner [2].

The concentration dependence of nitrendipine block of *I*_{CaL} carried by α_{1c-D.} and α_{1c-wt} is shown in Fig. 2C. As expected, α_{1c-D.} was less sensitive to nitrendipine block (IC₅₀ 17.1 μM) than α_{1c-wt} (IC₅₀ 88 nM). This nearly 200-fold difference in IC₅₀ was sufficient to allow pharmacological discrimination between native and exogenous α_{1c} subunits in the cardiac myocyte setting (below). Additionally, the observed current density was similar in α_{1c-D.} and α_{1c-wt}-transduced cells. There was no detectable shift in the voltage dependence of activation of α_{1c-D.} L-type Ca²⁺ channels, as compared with α_{1c-wt}.

Expression of α_{1c-D.} in cardiac myocytes

We next tested the efficiency of expression of engineered L-type Ca²⁺ channels in adult guinea pig cardiomyocytes infected with the α_{1c-D.} adenovirus. In these experiments, we co-expressed the rat β_{2a} subunit (fused to GFP), as it has previously been demonstrated that β subunit co-expression is a limiting factor for L-type Ca²⁺ current density in cultured adult cardiomyocytes [24,26,27]. In preliminary experiments, we also observed that *I*_{CaL} declined markedly over 48

h in culture in the absence of β subunit over-expression (data not shown). Therefore, myocytes infected with β_{2a} subunit alone were used as controls for comparison with α_{1c-D} + β_{2a} -infected cells.

Cultured adult guinea pig cardiomyocytes typically exhibited large I_{CaL} densities in the presence of β_{2a} -GFP, consistent with previous reports [24,26,27]. However, cells transduced with α_{1c-D} , in addition to β_{2a} -GFP, showed a larger I_{CaL} density than those infected with β_{2a} -GFP alone (Fig. 3). This increase in current density was not associated with any changes in the voltage dependence of activation or inactivation, suggesting that the increase in current density was related to an increase in the number of functional channels at the sarcolemma.

To assess the relative contribution of native L-type Ca^{2+} channels and engineered α_{1c} subunits to total I_{CaL} , we measured L-type Ca^{2+} currents in the presence of various concentrations of nitrendipine. The concentration–response curve for myocytes transduced with β_{2a} -GFP alone was similar to that observed in A549 cells (Fig. 2B). Nitrendipine block of I_{CaL} in myocytes transduced with α_{1c-D} differed markedly from controls, showing complex binding behavior with a substantially decreased sensitivity to nitrendipine.

We found that 10 μ M nitrendipine blocked $51 \pm 5\%$ ($n = 9$) of I_{CaL} in α_{1c-D} transduced myocytes, in comparison to $86 \pm 8\%$ ($n = 9$) of I_{CaL} in myocytes transduced with β_{2a} -GFP alone (Fig. 3C). The ability to pharmacologically discriminate between native and exogenous L-type current allows this DHP-insensitive α_{1c} subunit adenovirus to be used as a backbone for structure–function studies of L-type Ca^{2+} channel regulation in cardiac myocytes.

α_{1c-D} L-type current elicits SR Ca^{2+} release in adult cardiomyocytes

One of the goals of this approach is to develop vectors useful for the study of excitation–contraction coupling in intact cells. In addition, the ability to initiate Ca^{2+} release in α_{1c-D} expressing cells serves as an indirect indicator of the appropriate trafficking of the channel to the dyad. We measured Ca^{2+} transients elicited with an identical voltage protocol as above, applied at a frequency of 0.5 Hz, in myocytes with K^+ -containing internal and external solutions, in the absence and presence of 20 μ M nitrendipine. The application of nitrendipine largely blocked the rapidly rising phase of the intracellular Ca^{2+} transient in β_{2a} -GFP transduced cells, as a consequence of blocking most of the L-type Ca^{2+} current trigger. However, in α_{1c-D} transduced cells, the evoked Ca^{2+} transient retained a rapid release phase and was only partially inhibited in amplitude following the application of nitrendipine (Fig. 4B). This suggests that exogenously transduced viruses are not only effectively expressed in cardiomyocytes, but that the α_{1c-D} adenoviral construct is a useful backbone for structure–function studies of excitation–contraction coupling.

Discussion

We have developed novel adenoviral constructs, capable of expressing transgenes with larger sizes than standard methods. For the first time, we demonstrate that L-type Ca^{2+} channel α_{1c} subunits can be expressed using adenoviruses. In our experiments, these viruses are robustly expressed in both the A549 cell and in adult guinea pig cardiac myocytes. Ca^{2+} current resulting from these exogenously expressed channels is also capable of eliciting SR Ca^{2+} release in adult guinea pig cardiac myocytes in culture.

This “reverse engineering” approach represents an important proof-of-principle that will facilitate the exploration of a number of key questions in L-type Ca channel biology, by permitting the expression of α_{1c} subunits in the native cell environment. Further, the addition of minimal substitutions in the dihydropyridine-binding domain enables differentiation of the mutant channels from native L-type Ca^{2+} currents. Infection of cardiomyocytes with the

α_{1c-D} - virus yielded nitrendipine inhibition curves that had lower slope fractions, indicating that there was a mixed population of native and mutant α_1 subunits expressed. If the nitrendipine inhibition curves for wild-type and α_{1c-D} channels, expressed individually in A549 cells, are compared with the data obtained in α_{1c-D} infected adult cardiomyocytes, we estimate that more than half of the Ca^{2+} channels in the membrane are mutants, since approximately 50% of the current remained in the presence of 10 μM nitrendipine. A significant degree of replacement of the L-type Ca^{2+} channel population would be expected, due to competition between native and mutant mRNAs for the translation machinery. α_{1c} -infected cells only had ~40–50% higher current densities than those infected with β alone, this was likely due to the judicious use of an inducible promoter which limits the level of channel expression to within a range that is not harmful to the cell.

The molecular determinants of many features of L-type Ca^{2+} channel regulation in the native cell environment remain largely uncharacterized. Using heterologous expression systems, prior structure–function studies have extensively characterized regions of the channel involved in selectivity, permeation, gating, and Ca^{2+} /calmodulin-dependent inactivation, but many fundamental questions remain regarding the behavior of the Ca^{2+} channel in the native environment of the dyad. A key example is whether modulation of I_{CaL} by cyclic AMP-dependent protein kinase requires direct phosphorylation of the α_1 subunit [6,28,29], the β subunit, or a combination of both. Similarly, interactions between L-type Ca^{2+} channel domains and the ryanodine receptor, or other dyadic proteins have been proposed [7,30], but there has been no direct method for testing these hypotheses. The present findings importantly demonstrate that excitation–contraction coupling between the transduced α_1 subunit and the SR Ca^{2+} release channel was reconstituted. This suggests that the engineered L-type Ca^{2+} channel was inserted into the sarcolemma in close apposition to the ryanodine receptor.

The present work has implications beyond the structure–function analysis of the L-type Ca^{2+} channels. The expanded capacity of the fiber-deleted adenoviral backbone opens the door for the expression of other multidomain ion channels or large proteins, as well as the possibility to express multiple transgenes in the same vector. This will extend the use of adenoviruses to express new genes of interest in primary cell types which are otherwise resistant to in vivo or in vitro transfection. Ultimately, development of such vectors may permit targeted gene therapy for diseases involving impaired ion channel function [31–33] in the future.

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Fig. 1.

Creation of the fiber deleted $\psi 5$ virus. Schematic showing the two parental viruses that were used to create novel recombination compatible fiber deleted virus, FB6. Purified viral DNA was cut with *EcoRI* and run on a 0.6% agarose gel. Lane 1, DNA size marker; lane 2, $\psi 5$ DNA; lane 3, $\psi FB6$ DNA; and lane 4, GFP-F DNA. The presence of bands * and ** in $\psi FB6$ is consistent with the formation of a hybrid virus from $\psi 5$ and GFP-F parent viruses.

Fig. 2.

α_{1c-D-} and α_{1c-wt} adenoviruses are robustly expressed in A549 cells. (A,B) Example records shown for α_{1c-wt} and α_{1c-D-} adenoviruses, each co-expressed in A549 cells with β_{2a} -GFP and AddMEcR adenoviruses, following voltage steps from -50 to 0 mV. (C) Dose-response curves for α_{1c-D-} and α_{1c-wt} adenoviruses, each co-expressed in A549 cells with β_{2a} and AddMEcR adenoviruses, following voltage steps from -50 to 0 mV, in sequentially increasing concentrations of the dihydropyridine antagonist, nitrendipine.

Fig. 3.

α_{1c-D-} adenoviruses are expressed in cardiac myocytes (A,B): individual current traces obtained before and after the administration of 10 μ M nitrendipine, during voltage steps from -50 to 0 mV, in (A) β_{2a} transduced adult guinea pig cardiomyocytes and (B) α_{1c-D-} and β_{2a} transduced cardiomyocytes. (C) Current to voltage relationships plotted for α_{1c-D-} adenovirus, co-expressed in myocytes with β_{2a} and AddMEcR adenoviruses (D) dose-response curves for α_{1c-D-} adenoviruses, each co-expressed in adult guinea pig cardiac myocyte with β_{2a} and AddMEcR adenoviruses, following voltage steps from -50 to 0 mV, in sequentially increasing concentrations of the dihydropyridine antagonist, nitrendipine. As a control, the effect of nitrendipine on myocytes transduced with β_{2a} and AddMEcR alone is shown.

Fig. 4.

$\alpha_{1c-D.}$ adenoviruses expressed in cardiac myocytes are able to elicit SR Ca release. Examples of steady state $[Ca]_i$ transients are shown, during stimulation with 50 ms depolarizing steps from -50 to 0 mV, before and after the administration of 20μ M nitrendipine, in (A) β_{2a} transduced adult guinea pig cardiomyocytes and (B) $\alpha_{1c-D.}$ and β_{2a} transduced cardiomyocytes.