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Adenoviral-mediated expression of dihydropyridine-insensitive L-type calcium channels in cardiac ventricular myocytes and fibroblasts

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Abstract

Cardiac voltage-gated Ca^{2+} channels regulate the intracellular Ca^{2+} concentration and are therefore essential for muscle contraction, second messenger activation, gene expression and electrical signaling. As a first step in accessing the structural versus functional properties of the L-type Ca^{2+} channel in the heart, we have expressed a dihydropyridine (DHP)-insensitive $Ca_V1.2$ channel in rat ventricular myocytes and fibroblasts. Following isolation and culture, cells were infected with adenovirus expressing either LacZ or a mutant $Ca_V1.2$ channel ($Ca_V1.2DHP_i$) containing the double mutation (T1039Y & Q1043M). This mutation renders the channel insensitive to neutral DHP compounds such as nisoldipine. The whole-cell, L-type Ca^{2+} current (I_{Ca}) measured in control myocytes was inhibited in a concentration-dependent manner by nisoldipine with an IC_{50} of 66 nM and complete block at 250 nM. In contrast, I_{Ca} in cells infected with $AdCa_V1.2DHP_i$ was inhibited by only 35% by 500 nM nisoldipine but completely blocked by 50 μM diltiazem. In order to study $Ca_V1.2DHP_i$ in isolation, myocytes infected with $AdCa_V1.2DHP_i$ were incubated with nisoldipine. Under this condition the cells expressed a large I_{Ca} (12 pA/pF) and displayed Ca^{2+} transients during field stimulation. Furthermore, addition of 2 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine (IBMX), to stimulate protein kinase A, strongly increased I_{Ba} in the $AdCa_V1.2DHP_i$-infected cells. A Cd^{2+}-sensitive I_{Ba} was also recorded in cardiac fibroblasts infected with $AdCa_V1.2DHP_i$. Thus, expression of $Ca_V1.2DHP_i$ will provide an important tool in studies of cardiac myocyte and fibroblast function.
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1. Introduction

Voltage-gated Ca^{2+} (Ca_V) channels are multimeric protein complexes consisting of at least four subunits. The major component of the channels is the pore-forming α_1 subunit, which contains the binding site for Ca^{2+} channel blockers, the voltage-sensor and the selectivity filter (Catterall, 2000; Moosmang et al., 2005). The $Ca_V1.2$ channel (α_{1C}) is the primary isoform found in the heart and gives rise to high-voltage-activated, L-type Ca^{2+} currents (I_{Ca}) (Catterall, 2000; Moosmang et al., 2005). $Ca_V1.2$ interacts with auxiliary channel subunits including $Ca_V\beta$ subunits, which exert important modulatory effects on the voltage-dependence of channel activation and the inactivation kinetics (Perez-Reyes et al., 1992; Singer et al., 1991; DeWaard et al., 1994). In addition, $Ca_V\beta$ subunits control the targeting of the α_1 subunit to the sarcolemmal membrane (Chien et al., 1995; Yamaguchi et al., 2000; Colecraft et al., 2002) and enhance the open probability of the channel (Costantin et al., 1998).

Almost all information concerning the structural versus functional properties of $Ca_V1.2$ has come from studies in which the channel is expressed in heterologous cell lines (Catterall, 2000). However, these cell lines lack native cardiac cell constituents that are needed for normal channel function. For example, the strong regulatory action of protein kinase A (PKA) on the cardiac I_{Ca} is not fully reproduced when $Ca_V1.2$ is expressed in cell lines (Gao et al., 1997a; Bünemann et al., 1999; Hulme et al., 2003). Furthermore, these studies provide limited information on the specific role of the channel in cardiac

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excitation–contraction (EC) coupling. The reintroduction of Ca^{2+} channels into skeletal myotubes from dysgenic mice, which lack the endogenous skeletal muscle $Ca_v1.1$ channel, has provided novel insights into the interaction of the channel with the sarcoplasmic reticulum ryanodine receptor/Ca^{2+} release channel (Tanabe et al., 1988; Tanabe et al., 1990a; Tanabe et al., 1990b). Recently, to address this problem, a recombinant adenovirus was used to introduce a dihydropyridine (DHP)-insensitive $Ca_V1.2$ channel into cardiac ventricular myocytes (Ganesan et al., 2005). However, these investigations provided limited information on the biophysical and pharmacological properties of the expressed channel in the absence of the endogenous I_{Ca}. In addition, it has not been determined if adenoviral-mediated expression of the $Ca_V1.2$ channel can be utilized in other cardiac cell types (fibroblasts, endothelial cells, etc.).

In this study neonatal rat ventricular myocytes and fibroblasts were infected with a virus expressing a mutant $Ca_V1.2$ channel ($AdCa_V1.2DHP_i$) containing the double mutation (T1039Y & Q1043M). This mutation renders the channel insensitive to neutral DHP compounds such as nisoldipine (Hockerman et al., 1997; He et al., 1997). Myocytes infected with $AdCa_V1.2DHP_i$ displayed a large, DHP-insensitive I_{Ca} that was inhibited by the benzothiazepine Ca^{2+} channel blocker diltiazem, but displayed activation and inactivation properties that were different from the endogenous current. In myocytes expressing $Ca_V1.2DHP_i$, Ca^{2+} transients evoked by field stimulation were not significantly reduced by nisoldipine. In addition, cardiac fibroblasts infected with $AdCa_V1.2DHP_i$ expressed a Cd^{2+}-sensitive I_{Ca} and displayed an altered intracellular Ca^{2+} response to anigotensin II. Thus, expression of $Ca_V1.2DHP_i$ will provide an important tool in Ca^{2+} channel structure versus function studies in cardiac muscle.

2. Materials and methods

2.1. Isolation and culture of cardiac ventricular myocytes and fibroblasts

Neonatal rat ventricular myocytes and fibroblasts were isolated and cultured as described previously (Walsh and Parks, 2002; Simpson et al., 1994). In brief, heart ventricles were removed from neonatal pups (days 3–4 in age), minced into 1 mm^3 pieces and subjected to collagenase (type B, Boehringer Mannheim Biochemicals) dissociation (Walsh and Parks, 2002; Simpson et al., 1994). Cells were separated using selective attachment procedures and cultured in DMEM (Gibco), supplemented with 10% horse serum (Flow Laboratories). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Adenovirus construction, cell infection and transfection

Myocytes (day one of culture) and fibroblasts (passage two) were infected with adenovirus expressing either β-galactosidase adenovirus (AdLacZ) or a mutant $Ca_V1.2$ channel ($AdCa_V1.2DHP_i$) containing the double mutation (T1039Y & Q1043M). This mutation renders the channel insensitive to neutral DHP Ca^{2+} channel blockers such as nisoldipine (Hockerman et al., 1997; He et al., 1997). Viruses were constructed using the pAdEasy system (Stratagene). The $Ca_V1.2DHP_i$ cDNA (original $Ca_V1.2$ clone provided by Dr. Terry Snutch, University of British Columbia), with the entire 5′-untranslated region removed, was subcloned into the pShuttle-CMV vector between the Kpn 1 and Not 1 restriction sites. This construct was used to transfer the $Ca_V1.2DHP_i$ cDNA into the pAdEasy-1 vector via homologous recombination (per the manufacturer's instructions). Viral titer was determined on all stocks using the Adeno-X kit (BD Biosciences). Myocytes were infected at matched multiplicities of infection (moi) of 10 or 20 and expression of $Ca_V1.2DHP_i$ confirmed by immunoblot analysis. Fibroblasts were infected at a moi of 50 and treated with phorbol 12-myristate 13-acetate (PMA, 100 nM) for 48 h prior to experimentation to activate the adenovirus CMV promoter (Maass et al., 2003). Patch clamp recordings, intracellular Ca^{2+} transients and Western blot analysis were performed 2 to 3 days following infection.

A plasmid expressing the β_{2b} subunit fused to green fluorescent protein (β_{2b}-GFP) was generously supplied by Dr. Henry Colecraft (John Hopkins University) (Colecraft et al., 2002). Cells previously infected with $AdCa_V1.2DHP_i$, were transiently transfected with 10 μg of β_{2b}-GFP using lipofectamine (Invitrogen). GFP positive cells were then identified using a fluorescent microscope.

2.3. Recording procedure and measurement of voltage-gated Ca^{2+} currents

The patch clamp method (Hamill et al., 1981) was used to record the whole-cell, L-type calcium current (I_{Ca}) using L/M EPC-7 (Adams & List Associates) and Axopatch 200 (Axon Instruments) amplifiers. Our procedure for the measurement and analysis of this current has been described (Walsh and Parks, 2002; Walsh and Cheng, 2004). Pipettes were made from Prism glass capillaries (Dagan Corp.) and had resistances of 1–2 MΩ when filled with internal solution. All experiments were conducted on isolated, non-coupled cells at room temperature (22–24 °C). For the measurement of $I_{Ca,}$ cells were placed in an external Tyrode's solution consisting of (in mM); 132 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 dextrose, 5 HEPES, pH 7.4 (with NaOH) (280 mOsm.). I_{Na} was blocked with tetrodotoxin (10 μM) and the Na^+ channels inactivated by maintaining the myocytes at a holding potential of −40 mV. External $CaCl_2$ was replaced with $BaCl_2$ in the PKA regulatory experiments. Substitution of Ca^{2+} with Ba^{2+} was necessary in order to prevent Ca^{2+}-dependent inhibitory effects on adenylyl cyclase and other signaling molecules that diminish β-adrenergic actions on the Ca^{2+} channel (Yu et al., 1993; Walsh and Cheng, 2004). The normal internal solution consisted of (in mM); 60 CsCl, 50 Cs-Aspartate, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 3 ATP, 10 HEPES, pH 7.3 (with CsOH) (280 mOsm.). Percent inhibition of I_{Ca} by nisoldipine and diltiazem was determined in each experiment as that relative to the block produced by 100 μM $CdCl_2$. Flat lines were drawn to the holding current (−40 mV) in the I_{Ca} traces (Fig. 1, 2, 4 and 6).

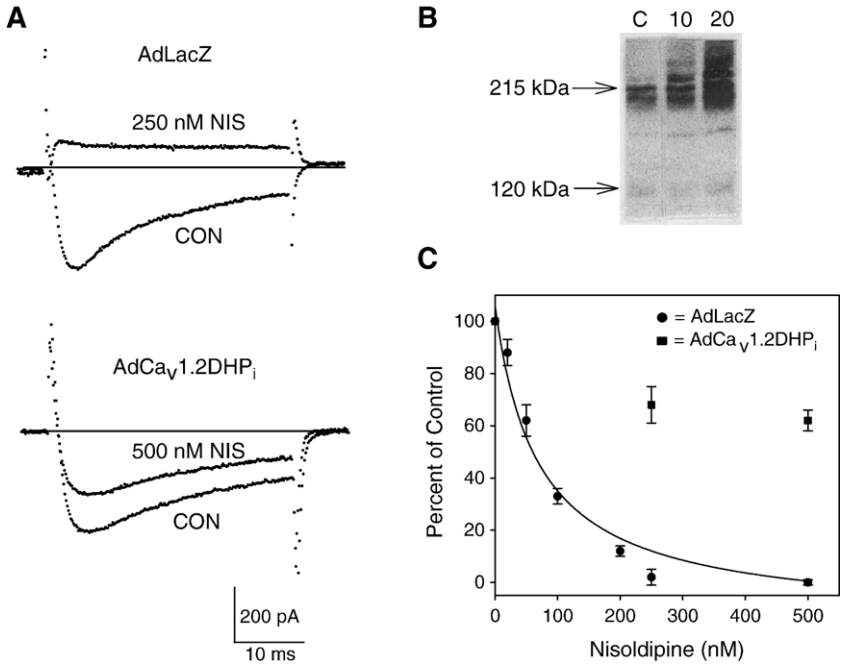

Fig. 1. Expression of $Ca_V1.2DHP_i$ in rat cardiac ventricular myocytes. Cells were infected with adenovirus expressing either LacZ (AdLacZ) or a mutant $Ca_V1.2$ channel containing the double mutation (T1039Y & Q1043M) ($AdCa_V1.2DHP_i$). A: I_{Ca} recorded during a voltage step applied from −40 mV to 0 mV in the presence or absence of the Ca^{2+} channel blocker nisoldipine (NIS). B: Immunoblots for $Ca_V1.2$ in myocytes infected with either control virus or $AdCa_V1.2DHP_i$ at multiplicities of infection of 10 and 20. C: Dose versus response curve for nisoldipine block of I_{Ca}. Nisoldipine inhibited the control currents with a 50% inhibitory concentration (IC_{50}) of 66 nM. Even at a concentration of 500 nM, nisoldipine caused only partial block of I_{Ca} in cells expressing $Ca_V1.2DHP_i$. Each point represents the mean±S.E.M. inhibition measured in three to six cells.

Membrane currents were recorded with 12-bit analog/digital converters (Axon Instruments). Unless otherwise indicated, data were sampled at 10 KHz and filtered at 2 KHz with a low pass Bessel filter (Frequency Devices). Voltage pulses were applied at 4–5 s intervals for 40 ms to voltages ranging from −60 to +50 mV. This allowed adequate time for Ca^{2+} channel recovery from inactivation. Series resistance was compensated to give the fastest possible capacity transient without producing oscillations. With this procedure >70% of the series resistance could be compensated. Averaged current values presented are means±S.E. Where appropriate, statistical significance was estimated using Student's *t* test for unpaired observations.

2.4. Intracellular Ca^{2+} transients

Ca^{2+} imaging was performed using either confocal fluorescent microscopy (myocytes) or spectrofluorometry (fibroblasts). Cells were incubated in a rotating water bath for 30 min in Tyrode's containing 10 μM Fluo-4 acetoxymethyl ester (Fluo-4/AM) and 0.01% Pluronic (Molecular Probes). The cells were then washed several times without the dye and incubated for 30 min to facilitate the de-esterification of the dye. Myocyte Ca^{2+} transients were obtained using an AttoFluor-CARV-1 confocal system (BD Biosciences) equipped with a Fluo-4 filter set and an ORCA ER 1394 12 bit CCD firewire camera. The AttoFluor system was interfaced to a Carl-Zeiss Axiovert 200 inverted microscope equipped with a 220 environmental chamber (Carl-Zeiss). Myocytes were stimulated at 0.5–1 Hz by applying 2 V for 5–10 ms using a pulse generator (Grass Instruments) through two platinum electrodes attached to both sides of a field stimulation chamber (Warner Instruments). Fluo-4 fluorescence was excited using an X-Cite (EFOS) metal-halide fluorescence illumination system (Photonic Solutions Inc.) with a GPF excitation/emission cube (492/520 nm) and imaged via a Zeiss 20X, NA 0.75 objective. Images were captured using Kinetic Imaging AQM6 software (Kinetic Imaging LTD) and intracellular Ca^{2+} determined as the average intensity of the gray pixels represented by the fluorescent signal. Fibroblast Ca^{2+} transients were measured with a FluoroMax-3 spectrofluorometer (Jobin Yvon) as described (Thomas et al., 1996). In brief, cultured fibroblasts were harvested by treatment with trypsin, centrifuged and resuspended in Tyrode's at a density of 0.5×10^6 cells/ml. The cell suspension (2 ml) was transferred to a cuvette and Fluo-4 fluorescence measured at an excitation wavelength of 485 nm and an emission wavelength of 516 nm.

2.5. Preparation of cardiac cell lysates and Western blot analysis

To prepare cell lysates for Western blot analysis, cardiac myocyte cultures were placed into a lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 50 mM dithio-threitol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethysulfonyl fluoride, 1 mM EGTA, 0.25% sodium deoxycholate, 2 μg/ml aprotinin and protease inhibitor cocktail (Roche)). Lysates were immediately transferred to pre-cooled microfuge tubes and sonicated to reduce viscosity. The protein

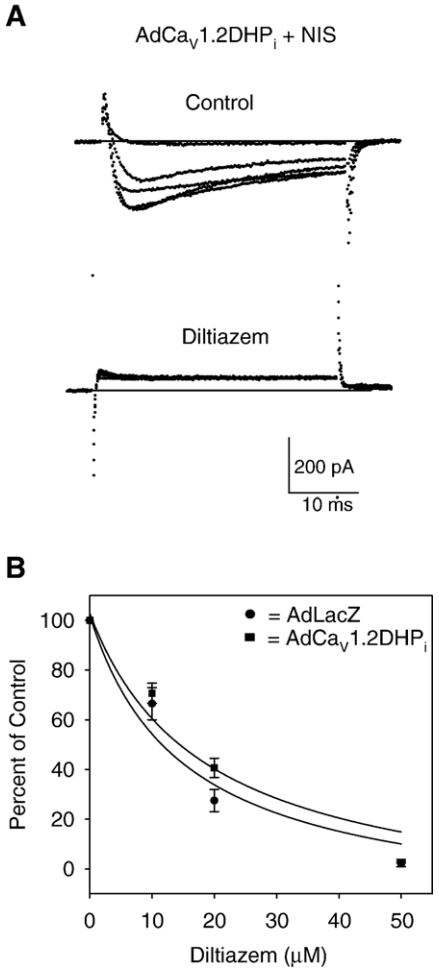

Fig. 2. Diltiazem block of $Ca_V1.2DHP_i$ expressed in ventricular myocytes. A: I_{Ca} recorded from a cell infected with $AdCa_V1.2DHP_i$ during voltage steps applied to −10 mV through +30 mV in 10 mV increments. Cells were pretreated for 5 min with 250 nM nisoldipine prior to the recording. I_{Ca} was measured in the presence or absence of the Ca^{2+} channel blocker diltiazem (50 μM). B: Dose versus response curves for diltiazem block of I_{Ca}. Diltiazem inhibited the currents with 50% inhibitory concentrations of 12±5 μM (AdLacZ) and 14±6 μM ($AdCa_V1.2DHP_i$). Each point represents the mean±S.E.M. inhibition measured in three to five cells.

content of the cell preparations was determined using a protein assay kit (Pierce). For Western blot analysis of the $Ca_V1.2$ channel, proteins were separated by electrophoresis on 6% SDS polyacrylamide gels using a mini PROTEAN cell (Bio-Rad). The running buffer contained 25 mM Tris, 193 mM Glycine, pH 8.3 and 0.1% SDS. Proteins were transferred to polyvinylidene difluoride membranes using a Trans-Blot apparatus (Bio-Rad). The transfer buffer contained 25 mM Tris, 192 mM glycine, pH 8.5 and 20% methanol. For immunodetection, membranes were first blocked in TBS containing 0.1% Tween-20, bovine serum albumin and 0.025% Na Azide for 60 min at room temperature. Antibodies to the $Ca_V1.2$ α_{1C} (1: 500) and β_2 subunits (Chemicon) were incubated with the membranes overnight at 4 °C. After primary antibody treatment, the membranes were washed with TBS-0.1% Tween-20 and incubated with a secondary antibody (HRP-conjugated mouse IgG) (Cell Signaling Technology). Immunoreactive bands were visualized on X-ray film (Kodak) using the enhanced chemiluminescence method (Pierce). Immunoblot results were confirmed on three separate myocyte cultures.

2.6. Drugs and chemicals

Diltiazem, forskolin, 3-isobutyl-1-methylxanthine (IBMX) and angiotensin II were purchased from Sigma Chemical Co. Miles Pharmaceuticals (New Haven, CT) generously supplied nisoldipine.

3. Results

3.1. Expression of $AdCa_V1.2DHP_i$ in neonatal rat ventricular myocytes

The left panel of Fig. 1 shows examples of the L-type Ca^{2+} current (I_{Ca}) recorded in neonatal rat ventricular myocytes infected with control adenovirus (AdLacZ). As expected, application of 250 nM of the dihydropyridine (DHP) Ca^{2+} channel blocker nisoldipine to myocytes infected with AdLacZ caused nearly complete block of I_{Ca}. Consistent with previous studies of the cardiac I_{Ca} (Kass et al., 1991; Welling et al., 1993), nisoldipine inhibited the current in a concentration-dependent manner with a 50% inhibitory concentration (IC_{50}) in the low nanomolar range (66 nM, at a holding potential of −40 mV) (Fig. 1, panel C).

As a first step in exploring the structural versus functional properties of the Ca^{2+} channel in the heart, myocytes were infected with a virus expressing a mutant $Ca_V1.2$ channel ($AdCa_V1.2DHP_i$). This α_1 subunit contains the double mutation (T1039Y & Q1043M), which renders the channel insensitive to neutral DHPs such as nisoldipine (Hockerman et al., 1997; He et al., 1997). Immunoblot analysis revealed that infection of the myocytes with $AdCa_V1.2DHP_i$ increased the expression of a protein in the range of 200–220 kDa (Fig. 1, panel B). This molecular weight corresponds to that reported for the full-sized α_{1C} subunit (De Jongh et al., 1996; Shistik et al., 1999). A minor band of approximately 180–190 kDa was also increased in cells infected with $AdCa_V1.2DHP_i$. The size of this band is consistent with that of a C-terminus truncated form of the channel (De Jongh et al., 1996; Shistik et al., 1999). Expression of $Ca_V1.2DHP_i$ in the myocytes was not associated with any change in the expression of the β_2 subunit (results not shown).

Currents recorded from cells expressing $Ca_V1.2DHP_i$ are shown in Fig. 1 (bottom, left panel). In the absence of nisoldipine the peak I_{Ca} amplitude measured in these cells was 16±2 pA/pF. In contrast to the results obtained for the currents in the control myocytes, I_{Ca} was only partially inhibited by 250 nM nisoldipine in cells expressing $Ca_V1.2DHP_i$. Even at a concentration of 500 nM, nisoldipine produced only a 35% block of I_{Ca} (Fig. 1).

The calcium channel blocker diltiazem binds to $Ca_V1.2$ at a site that is distinct from the DHP binding site (Hockerman et al., 2000). Fig. 2 displays I_{Ca} measured in a myocyte infected with $AdCa_V1.2DHP_i$ that was first treated with 250 nM nisoldipine

in order to block the endogenous Ca^{2+} channels. In the continued presence of nisoldipine, addition of 50 μM diltiazem lead to a complete block of $Ca_V1.2DHP_i$. The IC_{50} for diltiazem block of I_{Ca} was not significantly different between the cells infected with AdLacZ ($IC_{50}=12\pm5$ μM) and those infected with $AdCa_V1.2DHP_i$ that were pretreated with nisoldipine ($IC_{50}=14\pm6$ μM) (Fig. 2, panel B) ($P>0.05$). Both the endogenous Ca^{2+} channels and the expressed $Ca_V1.2DHP_i$ channels were also blocked by 100 μM $CdCl_2$ (results not shown).

3.2. Properties of $Ca_V1.2DHP_i$ expressed in ventricular myocytes

The results displayed in Figs. 1 and 2 indicate that expression of $Ca_V1.2DHP_i$ results in the appearance of a DHP-insensitive, diltiazem-sensitive I_{Ca}. In order to study $Ca_V1.2DHP_i$ in isolation, myocytes infected with $AdCa_V1.2DHP_i$ were incubated with 250 nM nisoldipine for 5–10 min prior to experimentation. In the presence of nisoldipine, myocytes expressing $Ca_V1.2DHP_i$ displayed a large I_{Ca} (12 ± 1 pA/pF) (Fig. 3). In contrast, no measurable I_{Ca} could be recorded under these conditions in uninfected or AdLacZ-infected myocytes (results not shown). The left panel of Fig. 3 displays the current versus voltage (I/V) relationship for cells infected with either AdLacZ or $AdCa_V1.2DHP_i$. Although there was no significant difference in the peak current density between myocytes expressing LacZ and $Ca_V1.2DHP_i$ (11 ± 1 and 12 ± 1 pA/pF, respectively, $P>0.05$), there was a rightward shift in the I/V relationship for I_{Ca} in the $AdCa_V1.2DHP_i$-infected cells.

In order to study the voltage-dependence of channel activation and inactivation in more detail, activation and steady-state inactivation curves were obtained for I_{Ca} (Fig. 3, panel B). Steady-state inactivation curves were generated using a two-pulse protocol with a prepulse to the indicated potentials. For the activation curves, the normalized conductance for the currents measured during the depolarization was plotted as a function of the test voltage. The continuous lines represent the best fits of the data points to the appropriate Boltzmann equations (see Fig. 3 legend). The half-maximal voltage required for inactivation was -21 ± 1 and -11 ± 1 mV in myocytes infected with AdLacZ and $AdCa_V1.2DHP_i$, respectively. As expected from the current versus voltage relationship, expression of $Ca_V1.2DHP_i$ was associated with a shift in the activation curve to more positive potentials with half-maximal activation voltages of -14 ± 1 and -4 ± 1 mV (for LacZ and $Ca_V1.2DHP_i$). These shifts in the voltage-dependence were significant ($P<0.05$).

When expressed along with the Ca^{2+} channel α_{1C} subunit, auxiliary Cavβ subunits produce a hyperpolarizing shift in the voltage-dependence of activation (Perez-Reyes et al., 1992;

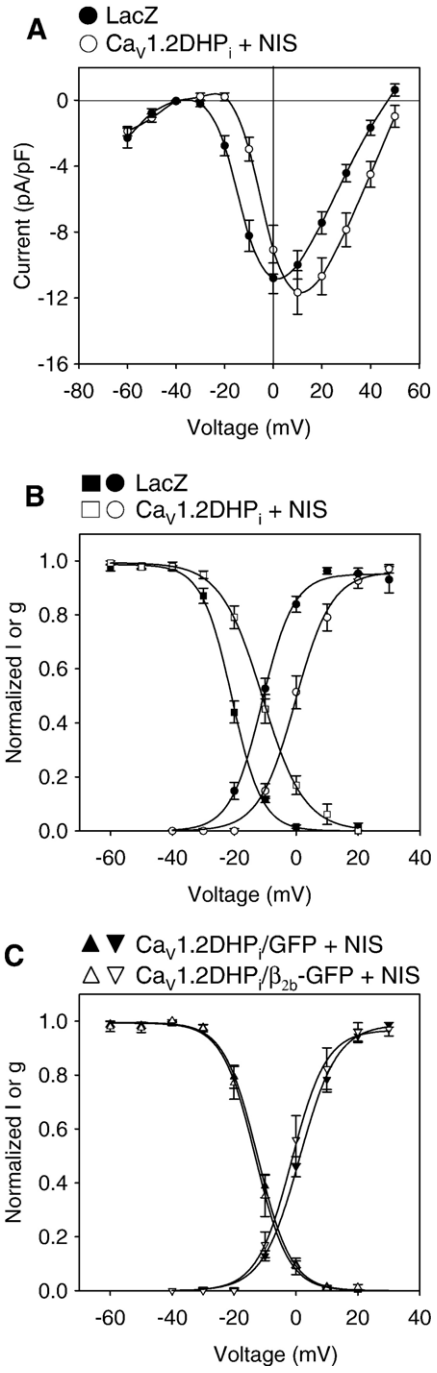

Fig. 3. Biophysical properties of $Ca_V1.2DHP_i$ expressed in ventricular myocytes. A: mean current versus voltage relationship for I_{Ca} measured in cells infected with control virus ($n=9$ cells) and $AdCa_V1.2DHP_i$ ($n=10$ cells). Cells expressing $Ca_V1.2DHP_i$ were pretreated with 250 nM nisoldipine prior to the recording. Currents were normalized to the cell membrane capacity. B: activation and inactivation curves obtained in the myocytes. For activation, conductance was determined by dividing the peak current amplitude at each potential by the driving force for Ca^+, (V_m-E_{Ca}). The continuous lines represent the best fits of Boltzmann equations to the data. The half-maximal voltage required for inactivation was -21 ± 1 and -11 ± 1 mV in myocytes infected with AdLacZ ($n=8$ cells) and $AdCa_V1.2DHP_i$ ($n=8$ cells), respectively. Expression of $Ca_V1.2DHP_i$ was also associated with a shift in the activation curve to more positive potentials with half-maximal activation voltages of -14 ± 1 (AdLacZ) ($n=8$ cells) and -4 ± 1 mV ($AdCa_V1.2DHP_i$) ($n=8$ cells). C: cells infected with $AdCa_V1.2DHP_i$ were transfected with either β_{2b}-GFP ($n=6$ cells) or GFP ($n=6$ cells) plasmid. The half-maximal voltages required for inactivation were -12 ± 1 ($AdCa_V1.2DHP_i$/GFP) and -13 ± 1 mV ($AdCa_V1.2DHP_i/\beta_{2b}$-GFP). The half-maximal voltages required for activation were 1 ± 1 ($AdCa_V1.2DHP_i$/GFP) and -2 ± 1 mV ($AdCa_V1.2DHP_i/\beta_{2b}$-GFP).

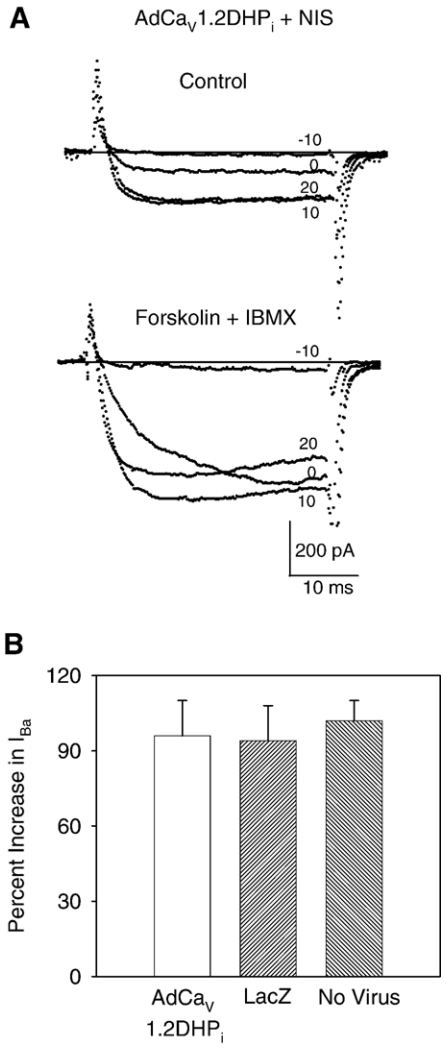

Fig. 4. Regulation of $Ca_V1.2DHP_i$ expressed in ventricular myocytes. A: Cells were infected with $AdCa_V1.2DHP_i$ and incubated with 250 nM nisoldipine. I_{Ba} recorded during voltage steps applied to −10 through +20 mV (as indicated for each trace) in the presence and absence of forskolin (2 μM) and IBMX (100 μM). B: Percent increase in peak I_{Ba} measured in the presence of forskolin and IBMX. Each bar represents the mean±S.E.M. increase obtained in seven to nine myocytes.

DeWaard et al., 1994; Singer et al., 1991). β subunits may also affect the steady-state inactivation of some α_1 subunits (DeWaard et al., 1994; Jones et al., 1998). One explanation for the rightward shift in the Ca^{2+} channel activation and inactivation curves in the myocytes expressing $Ca_V1.2DHP_i$ could be that levels of the endogenous β subunit are limited and easily saturated during over-expression of the α_1 subunits. Thus, a proportion of the $Ca_V1.2DHP_i$ channels may not be associated with β subunits. In order to test this hypothesis, myocytes infected with $AdCa_V1.2DHP_i$ were transiently transfected with β_{2b}-GFP. The β_{2b} subunit represents the major β subunit found in the rat heart (Colecraft et al., 2002). Despite an enhanced expression of the β_{2b} subunit in some of the myocytes, there was no significant change in the half-maximal voltages for channel activation or inactivation when compared with untransfected cells or cells transfected with a GFP plasmid alone (Fig. 3, panel C).

Cardiac L-type Ca^{2+} channels are regulated by β-adrenergic receptor agonists that act by stimulating protein kinase A (PKA). Since $Ca_V1.2DHP_i$ could be successfully expressed in the rat ventricular myocytes, it was important to determine if this channel could be regulated by PKA. As shown in Fig. 4, application of 2 μM forskolin, along with IBMX, to stimulate PKA, produced a strong increase in I_{Ba} in cells infected with $AdCa_V1.2DHP_i$ that were pretreated with nisoldipine. In 8 cells examined, addition of forskolin and IBMX augmented I_{Ba} by 96±14%. This increase was not significantly different from the increase measured in uninfected (102±8%, $n=8$) and AdLacZ-infected (94±12%, $n=8$) myocytes ($P>0.05$).

3.3. Excitation-contraction coupling in cells expressing $Ca_V1.2DHP_i$

As shown in Figs. 1–4, expression of $Ca_V1.2DHP_i$ results in the appearance of a DHP-insensitive, PKA-regulated I_{Ca}. In cardiac muscle, Ca^{2+} channels are localized to the t-tubular membrane in the area where the membrane abuts the junctional sarcoplasmic reticulum. If $Ca_V1.2DHP_i$ is correctly targeted to this region, expression of the channel should restore excitation–contraction (EC) coupling in the presence of nisoldipine. Fig. 5 displays Ca^{2+} transients measured from the myocytes during field stimulation. Application of 2 μM nisoldipine abolished the Ca^{2+} transient in the control myocytes. However, in cells infected with $AdCa_V1.2DHP_i$, field stimulation was still capable of evoking Ca^{2+} transients in the presence of nisoldipine.

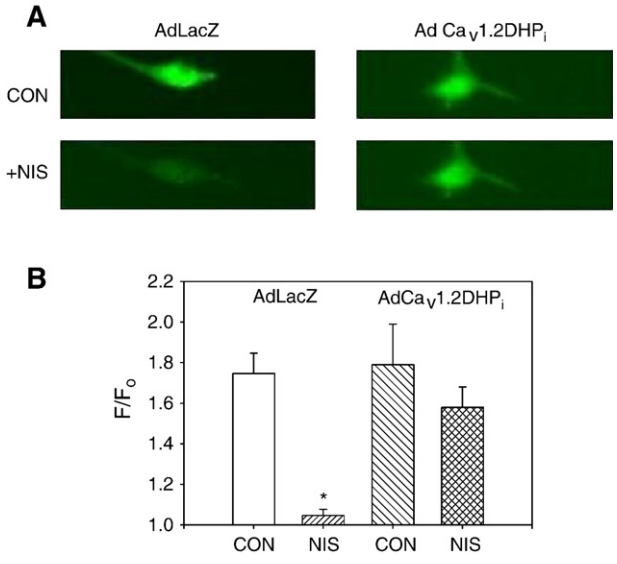

Fig. 5. Expression of $Ca_V1.2DHP_i$ restores excitation–contraction (EC) coupling in the presence of nisoldipine. A: Ca^{2+} transients measured during field stimulation in the presence (+NIS) and absence (CON) of 2 μM nisoldipine. Ca^{2+} transients were inhibited by nisoldipine in control cells, but not in cells infected with $AdCa_V1.2DHP_i$. B: Plot of the ratio of the whole-cell fluorescence intensity measured during stimulation (F) and rest (F_0). Each column represents the mean±S.E.M. intensity measured in three to six experiments. The asterisk indicates a significant difference ($P<0.05$) between the results in a column and the corresponding columns.

3.4. Expression of $AdCa_V1.2DHP_i$ in neonatal rat ventricular fibroblasts

In the final set of experiments it was determined if adenoviral-mediated expression of the $Ca_V1.2$ channel could be utilized in cardiac fibroblasts. Fig. 6 displays I_{Ba} measured in a fibroblast that was infected with $AdCa_V1.2DHP_i$, treated with PMA for 48 h to activate the CMV promoter (Maass et al., 2003), and incubated with nisoldipine. This current was not observed in uninfected fibroblasts or fibroblasts infected with AdLacZ that were treated with PMA ($n=12$ cells). Addition of the inorganic blocker Cd^{2+} (100 μM) caused a complete inhibition of I_{Ba}. The average I/V relationship ($n=6$ cells) for the $Ca_V1.2DHP_i$ channel expressed in the fibroblasts is shown in Fig. 6 (panel B). In order to determine if expression of $Ca_V1.2DHP_i$ alters the functional properties of the fibroblasts, angiotensin II-induced changes in intracellular Ca^{2+} were measured in cells infected with AdLacZ and $AdCa_V1.2DHP_i$. Application of 1 μM angiotensin II produced a strong, transient increase in the fibroblast intracellular Ca^{2+} signal (Fig. 6, panel C). Fibroblasts expressing $Ca_V1.2DHP_i$ displayed a more rapid decay of the Ca^{2+} transient when compared to the control cells. In three cell cultures examined, the time for 50% decay of the transient was 120±11 ms for the control fibroblasts and 51±7 ms for those expressing $Ca_V1.2DHP_i$.

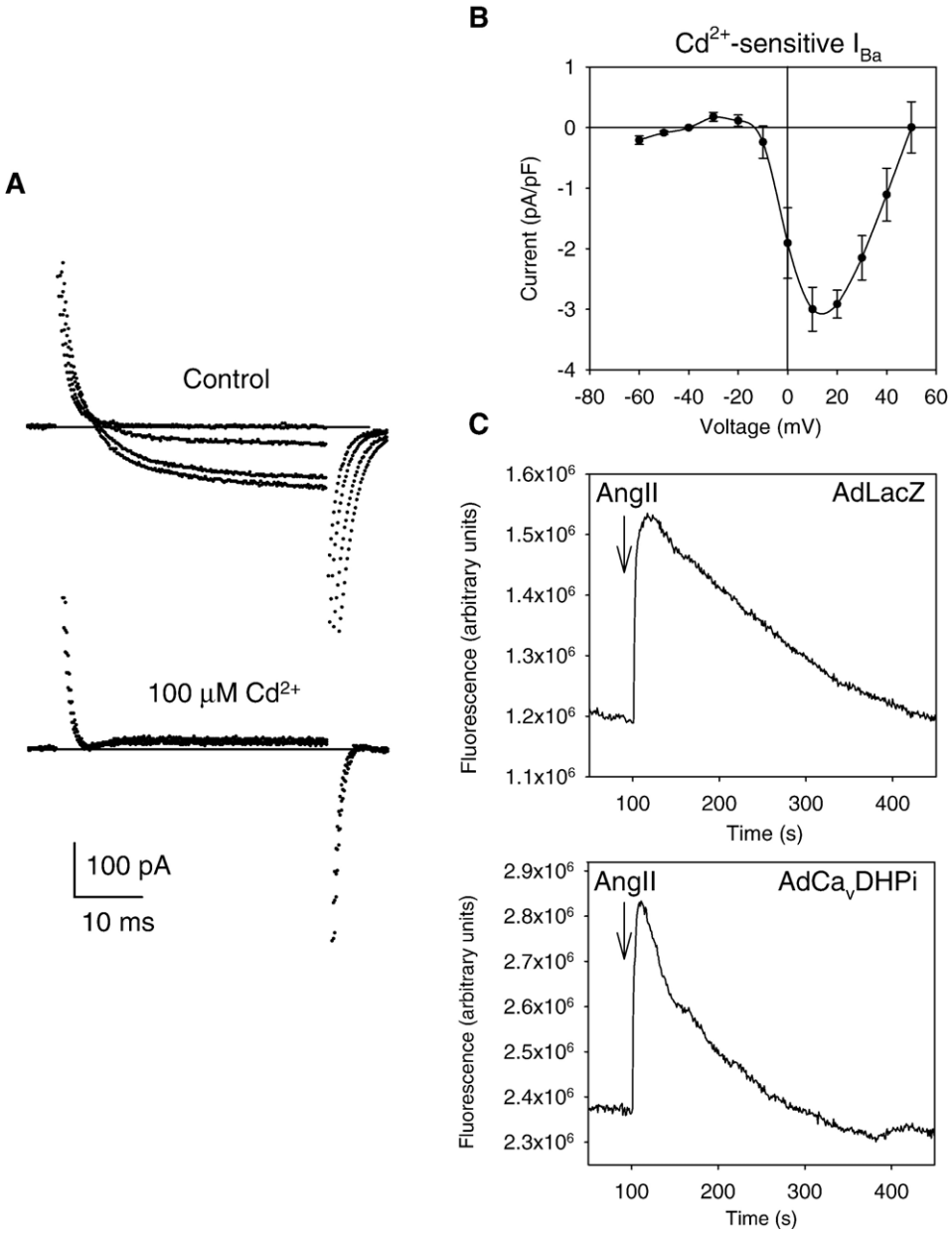

Fig. 6. Expression of $Ca_V1.2DHP_i$ in rat cardiac ventricular fibroblasts. A: I_{Ba} recorded from a cell infected with $AdCa_V1.2DHP_i$ during voltage steps applied to −10 mV through +20 mV in 10 mV increments. Cells were pretreated for 5 min with 250 nM nisoldipine prior to the recording. I_{Ba} was measured in the presence or absence of the Ca^{2+} channel blocker Cd^{2+} (100 μM). B: Mean current versus voltage relationship for I_{Ba} measured in cells infected $AdCa_V1.2DHP_i$ ($n=6$ cells). Fibroblasts expressing $Ca_V1.2DHP_i$ were pretreated with 250 nM nisoldipine prior to the recording. Currents were normalized to the cell membrane capacity. C: Ca^{2+} transients measured in fibroblasts infected with either $AdCa_V1.2DHP_i$ or AdLacZ and treated with 1 μM angiotensin II (AngII).

4. Discussion

The present study demonstrates that recombinant adenoviruses can be used to express the L-type Ca^{2+} channel in cultured cardiac ventricular myocytes and fibroblasts obtained from neonatal rats. Infection of the myocytes with $AdCa_V1.2DHP_i$ resulted in the expression of a DHP-insensitive I_{Ca} that was blocked by the benzothiazepine Ca^{2+} channel blocker diltiazem and Cd^{2+}. Of particular importance, the expressed current was augmented during stimulation of PKA by forskolin and IBMX. In addition, expression of $Ca_V1.2DHP_i$ restored field stimulation-evoked Ca^{2+} transients to the myocytes in the presence of nisoldipine. Thus, this study sets the groundwork for future function versus structure experiments that will explore how structural changes in the Ca^{2+} channel affect cardiac EC coupling.

For the most part, previous structure versus function studies of $Ca_V1.2$ have been carried out during expression of the channel in heterologous cell lines or in Xenopus oocytes. Structural regions of the protein that contribute to forming the pore, selectivity filter and gating components of the Ca^{2+} channel were identified in these studies (Catterall, 2000; Moosmang et al., 2005). In addition, this experimental approach was valuable for characterizing the binding sites for Ca^{2+} channel blockers such as the DHPs (Hockerman et al., 1997; He et al., 1997). However, these cell lines lack cardiac cell constituents that are needed for normal function. To examine the interaction of $Ca_V1.2$ with the cAMP response element binding protein (CREB) in cortical neurons, Dolmetsch et al. (Dolmetsch et al., 2001) expressed a DHP-insensitive $Ca_V1.2$ mutant (T1039Y). In the presence of the DHP nimodipine, as well as N and P/Q-type Ca^{2+} channel blockers, neurons expressing the mutant channel displayed normal Ca^{2+} transients and stimulation of CREB (Dolmetsch et al., 2001). A similar "knock-in" approach was recently utilized in guinea pig ventricular myocytes (Ganesan et al., 2005). Adenoviral-mediated expression of a mutant $Ca_V1.2$, containing four amino acid substitutions in the DHP binding pocket of the subunit, resulted in the appearance of Ca^{2+} channels with a reduced sensitivity to the DHP nitrendipine (Ganesan et al., 2005). However, five to ten-fold higher concentrations of the DHP compound were required to block the endogenous I_{Ca} in this study (Ganesan et al., 2005), compared with the present study (Fig. 1). In addition, the previous investigation provided limited information on the biophysical and pharmacological properties of the expressed $Ca_V1.2$ channel in the absence of endogenous current (Ganesan et al., 2005).

Although infection of the myocytes with $AdCa_V1.2DHP_i$ resulted in the over-expression of the $Ca_V1.2$ α_1 subunit, as determined by immunoblot analysis (Fig. 1), there was not a significant increase in I_{Ca} in cells expressing $Ca_V1.2DHP_i$ (in the presence of nisoldipine) compared with the control, LacZ cells (in the absence of nisoldipine) (Fig. 3). Since auxiliary subunits, such as the β_2 subunit, are required for the targeting of the $Ca_V1.2DHP_i$ α_1 subunit to the plasma membrane (Chien et al., 1995; Yamaguchi et al., 2000; Colecraft et al., 2002), it was possible that expression of these subunits was a limiting factor. To address this issue we transfected some of the myocytes with the β_{2b} subunit (Fig. 3). Co-expression of the β_{2b} subunit did not change the properties of the nisoldipine-insensitive currents (Fig. 3). However, expression of the β_2 subunit may not have been optimal under the conditions of our experiments. In addition, there may have been changes in the β subunit expression pattern as observed during constitutive cardiac over-expression of the human $Ca_V1.2$ α_1 subunit (Groner et al., 2004).

In cardiac ventricular cells, stimulation of PKA by the hormone epinephrine and the β-adrenergic receptor agonist isoproterenol augments the plateau phase of the action potential by stimulating I_{Ca} (Brum et al., 1984; Kameyama et al., 1986; Cachelin et al., 1983). Numerous studies have suggested that modulation of the L-type channel by PKA occurs subsequent to a direct phosphorylation of either the α_1 subunit or associated β_2 subunit of the protein (De Jongh et al., 1996; Mitterdorfer et al., 1996; Gao et al., 1997a). In a number of tissues, including the heart, the C-terminus of the Ca^{2+} channel is cleaved resulting in a smaller truncated form of the channel (Bünemann et al., 1999; Gao et al., 1997b). This truncated form of the α_{1C} subunit is not phosphorylated and is not regulated by PKA when expressed in heterologous cells (Bünemann et al., 1999) or cardiac myocytes (Ganesan et al., 2006). In contrast, the full-length α_{1C} subunit is phosphorylated by protein kinase A on single serine residue at position 1928 (De Jongh et al., 1996; Mitterdorfer et al., 1996). Mutation of this serine residue to an alanine reduces the responsiveness of I_{Ca} to PKA in heterologous cells transfected with α_{1C} (Perets et al., 1996; Gao et al., 1997a; Yoshida et al., 1992). However, as is the case with other transfection studies performed in non-cardiac cells, the extent of I_{Ca} regulation, when observed, is far less than that measured in native cardiac cells. For this reason, additional regulatory proteins, including the Ca^{2+} channel β_2 subunit, A kinase anchoring proteins (AKAPs) (Gray et al., 1998), and other endogenous cardiac components (Haase et al., 2005), have been postulated to be required for the full modulatory effects of PKA to occur. $Ca_V1.2DHP_i$ constructs containing PKA and protein kinase C phosphorylation site mutations will be very useful for evaluating the effect of α and β-adrenergic stimulation on Ca^{2+} channel function.

It was also demonstrated that infection of the cardiac myocytes with $AdCa_V1.2DHP_i$ restored EC coupling in the presence of nisoldipine. This indicates that Ca^{2+} influx through the expressed channels is capable of triggering sarcoplasmic reticulum (SR) Ca^{2+} release. Skeletal muscle in dysgenic mice lacks functional α_1 subunits (Cav1.1) and thus is defective in EC coupling (Tanabe et al., 1988). Myotubes isolated from these mice have provided an excellent model system for studying the role of the Ca^{2+} channel in EC coupling. Experiments performed with chimeric subunits, consisting of $Ca_V1.1$ and $Ca_V1.2$ subunits, identified the intracellular II–III linker of the skeletal muscle channel as an important determinant of EC coupling (Tanabe et al., 1990a). While comparable studies of $Ca_V1.2$ in cardiac muscle have been lacking, the present study provides a unique experimental approach for determining structural components of cardiac EC coupling. For example, it can be determined if the $Ca_V1.2$ II–III linker contributes in an analogous manner to cardiac SR Ca^{2+} release. In addition, it can be determined if other L-type Ca^{2+} channels, such as the $Ca_V1.1$ and $Ca_V1.3$ channels, substitute for $Ca_V1.2$ in initiating EC coupling.

Adenoviral-mediated expression of the $Ca_V1.2$ channel was also successfully demonstrated in cardiac fibroblasts. This is the first report of Ca_V channel expression in these cells. Consistent with a previous report (Chilton et al., 2005), an endogenous I_{Ca} was not measured in the fibroblasts. The voltage-dependence of activation of the expressed channel in the fibroblasts was consistent with that measured in the myocytes infected with $AdCa_V1.2DHP_i$. However, addition of PMA to activate the viral promoter in the infected fibroblasts may have altered the properties of the expressed channels. Since angiotensin II has previously been shown to mobilize intracellular Ca^{2+} release in cardiac fibroblasts (Thomas et al., 1996), we examined whether expression of $Ca_V1.2DHP_i$ might affect this release. As shown in Fig. 6, Ca^{2+} transients in fibroblasts expressing $Ca_V1.2DHP_i$ displayed an altered response to angiotensin II. This suggests that $Ca_V1.2$ channel expression, either by altering basal Ca^{2+} levels or signaling molecules within the cells, can regulate intracellular Ca^{2+} mobilization in the presence of angiotensin II. In a previous study, co-culture of cardiac myocytes with NIH 3T3 fibroblasts, expressing the voltage-gated K^+ channel Kv1.3, was found to reduce the electrical conduction velocity of the cultures (Feld et al., 2002). Thus, in addition to providing new information on hormonal signaling, expression of Ca_V channels in cardiac fibroblasts might be useful for modifying the physiological properties of heart muscle.

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