Functional Characterization of Ca\textsubscript{\textalpha}2\delta Mutations Associated with Sudden Cardiac Death*

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Background: Missense mutations in Ca\textsubscript{\textalpha}2\delta1, an auxiliary subunit of cardiac L-type Ca\textsubscript{\textalpha}1.2 channels, are associated with arrhythmias.

Results: The reduction in the cell surface density of Ca\textsubscript{\textalpha}2\delta1 D550Y/Q917H was sufficient to impair Ca\textsubscript{\textalpha}1.2 currents.

Conclusion: Defects in the cell surface trafficking of Ca\textsubscript{\textalpha}2\delta1 mutants down-regulate L-type currents.

Significance: CACNA2D1 genetic variants may trigger arrhythmias by reducing L-type Ca\textsuperscript{2+} currents. L-type Ca\textsuperscript{2+} channels play a critical role in cardiac rhythmicity. These ion channels are oligomeric complexes formed by the pore-forming Ca\textsubscript{\textalpha}1 with the auxiliary Ca\textsubscript{\textbeta} and Ca\textsubscript{\textalpha}2\delta subunits. Ca\textsubscript{\textalpha}2\delta increases the peak current density and improves the voltage-dependent activation gating of Ca\textsubscript{\textalpha}1.2 channels without increasing the surface expression of the Ca\textsubscript{\textalpha}1 subunit. The functional impact of genetic variants of CACNA2D1 (the gene encoding for Ca\textsubscript{\textalpha}2\delta), associated with shorter repolarization QT intervals (the time interval between the Q and the T waves on the cardiac electrocardiogram), was investigated after recombinant expression of the full complement of L-type Ca\textsubscript{\textalpha}1.2 subunits in human embryonic kidney 293 cells. By performing side-by-side high resolution flow cytometry assays and whole-cell patch clamp recordings, we revealed that the surface density of the Ca\textsubscript{\textalpha}2\delta wild-type protein correlates with the peak current density. Furthermore, the cell surface density of Ca\textsubscript{\textalpha}2\delta mutants S755T, Q917H, and S956T was not significantly different from the cell surface density of the Ca\textsubscript{\textalpha}2\delta wild-type protein expressed under the same conditions. In contrast, the cell surface expression of Ca\textsubscript{\textalpha}2\delta D550Y, Ca\textsubscript{\textalpha}2\delta S709N, and the double mutant D550Y/Q917H was reduced, respectively, by 

The reduction in the cell surface density of Ca\textsubscript{\textalpha}2\delta1 D550Y/Q917H was sufficient to impair Ca\textsubscript{\textalpha}1.2 currents. Polymorphic ventricular tachycardia is one of the leading causes of sudden cardiac death in children and young adults (1). These cardiac arrhythmias, which are reported in the absence of structural heart defects, coronary artery disease, or heart failure, are detected in a noninvasive fashion by measuring changes in the QT\textsuperscript{3} interval on the electrocardiogram. Either excessive prolongation (LQT) or shortening of the QT (SQT) intervals are associated with an increased risk of sudden cardiac death (2). Inherited Mendelian long QT syndrome (LQTS) and short QT syndrome (SQTS) originate from mutations in genes encoding ion channels or channel-interacting proteins (3). Genome-wide linkage studies of families with LQTS have reported 13 LQTS susceptibility genes with 75% of LQTS cases stemming from mutations in KCNQ1 (LQT1), KCNH2 (LQT2), and SCN5A (LQT3) (4). Only recently have mutations of genes encoding for subunits forming the L-type Ca\textsuperscript{2+} channel been linked to inherited arrhythmogenic diseases caused by LQTS or SQTS (5). The molecular mechanism underlying the cardiac dysfunction remains unknown in many cases.

The CACNA1C gene encodes the L-type Ca\textsubscript{\textalpha}1.2 channel that carries the vast majority of the L-type calcium current in the adult heart (6, 7) which in turn initiates the coordinated contraction of the cardiac ventricles (8). Its unique role is substantiated by the observation that homozygous knock-out of the CACNA1C gene is lethal (9, 10). In addition to initiating the coordinated contraction of the cardiac ventricles, Ca\textsubscript{\textalpha}1.2 channels are critical to the heart’s normal rhythmic activity (8). During the cardiac action potential, Ca\textsuperscript{2+} enters the cell through the voltage-dependent L-type Ca\textsubscript{\textalpha}1.2 generating an inward Ca\textsuperscript{2+} current that contributes to the plateau phase of the action potential (11). The kinetic properties of this channel must be properly timed so that depolarization and contraction are synchronized during the systolic-diastolic cycle of the heart. Even a slight disruption of Ca\textsuperscript{2+} cycling can have a profound impact on action-potential duration and trigger early after depolarizations, ultimately cumulating in lethal

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\textsuperscript{3} The abbreviations used are: QT, time interval between the Q and the T waves on the cardiac electrocardiogram; Hekt, human embryonic kidney 293 cell; LQTS, long QT syndrome; MFI, mean fluorescent intensity; SQTS, short QT syndrome; pF, picofarad.
arrhythmias (5, 12), including torsade de pointes and ventricular fibrillation (2). Prolonged inward Ca\(^{2+}\) current during the plateau phase of the cardiac action potential leads to delays in ventricular myocyte repolarization, a subsequent prolonged QT repolarization interval on the electrocardiogram and a highly arrhythmogenic and potentially lethal substrate. On the other end of the spectrum, any significant decrease in inward currents or significant increase in outward currents may lead to lethal arrhythmias associated with shorter than normal QT repolarization intervals (3).

The L-type Ca\(_{\alpha}1.2\) channel belongs to the molecular family of high voltage activated Ca\(_{\alpha}\) channels. High voltage-activated Ca\(_{\alpha}1.2\) channels are hetero-oligomers formed by the main pore-forming Ca\(_{\alpha}\alpha1\) subunit in a complex with the cytoplasmic Ca\(_{\alpha}\beta\) auxiliary subunit, the EF-hand protein calmodulin constitutively bound to the C terminus of Ca\(_{\alpha}\alpha1\), and the mostly extracellular Ca\(_{\alpha}\alpha2\delta\) subunit (13–18). The full complement of auxiliary subunits is required to produce high voltage-activated Ca\(_{\alpha}1.2\) channels with the properties of the native channels. Ca\(_{\alpha}\beta\) promotes the cell surface density of Ca\(_{\alpha}1.2\) channels (19) in part by preventing its degradation by the ubiquitin/proteasome system (20). Co-expression of Ca\(_{\alpha}\alpha2\delta\) subunit with Ca\(_{\alpha}\beta\)-bound Ca\(_{\alpha}\alpha1\) increases peak current density and hyperpolarizes the voltage of activation of the L-type Ca\(_{\alpha}1.2\) (19, 21–23).

Gain-of-function mutations in the pore-forming Ca\(_{\alpha}\alpha1\) subunit of the L-type Ca\(^{2+}\) channel lead to the highly arrhythmogenic Timothy syndrome. Timothy syndromes 1 and 2 are rare variants of the long QT syndrome (LQT8) (24, 25) characterized by extreme QT-prolongation and gain-of-function mutations in the pore-forming subunit of Ca\(_{\alpha}1.2\) (24–28). Mutations in the auxiliary subunits forming the L-type Ca\(_{\alpha}1.2\) channel have also been identified among probands diagnosed with Brugada syndrome, idiopathic ventricular fibrillation, and early repolarization syndrome associated with short QT interval (QTc ≤360 ms) (29–31). According to these studies, the shorter QT interval might result from loss-of-function mutations in the Ca\(_{\alpha}1\) genes coding for Ca\(_{\alpha}\alpha1\) (CACNA1C), Ca\(_{\alpha}\beta\) (CACNB2), or Ca\(_{\alpha}\alpha2\delta\) (CACNA2D1). Missense mutations D550Y, S709N, S755T, Q917H, and S956T in the Ca\(_{\alpha}\alpha2\delta\) protein have been associated with congenital arrhythmias (referred to as short QT syndrome 6 (SQT6) (29–31)), but the molecular mechanism(s) underlying the change in function remains to be established (32).

In this work, we have characterized the Ca\(_{\alpha}\alpha2\delta\) mutations associated with arrhythmogenic activity in regard to their functional impact on the cardiac L-type Ca\(_{\alpha}1.2\) currents. Using a fluorescence-labeled and extracellularly tagged Ca\(_{\alpha}\alpha2\delta\) subunit, we show here that missense mutations D550Y, S709N and D550Y/Q917H significantly altered the cell surface density of Ca\(_{\alpha}\alpha2\delta\). Decreasing the cell surface density of Ca\(_{\alpha}\alpha2\delta\) D550Y/Q917H was found to profoundly decrease L-type peak current densities as compared with currents measured with Ca\(_{\alpha}\alpha2\delta\) wild type (WT). Altogether, our data support a model where Ca\(_{\alpha}\alpha2\delta\) modulates channel function without altering the trafficking of Ca\(_{\alpha}\alpha1\) and establishes the cell surface density of Ca\(_{\alpha}\alpha2\delta\) relative to the pore-forming Ca\(_{\alpha}\alpha1\) as the single most important determinant in the stimulation of Ca\(_{\alpha}1.2\) currents by Ca\(_{\alpha}\alpha2\delta\).
cells (90% confluence) were transiently transfected with similar amounts of DNA (4 μg each for a total of 8 μg per 10^6 cells) as follows: CaV_1.2 WT-HA (Figs. 1 and 2), CaV_1.2 WT (Figs. 3–11), CaV_α2δ WT, CaV_α2δ-HA, or empty vector in 10 μl of Lipofectamine 2000 (Invitrogen) using a DNA/lipid ratio of 1:2.5 as described elsewhere (19). In some experiments, the amount of DNA for CaV_α2δ was decreased in the 0.2–4.0-μg range to decrease the CaV_α2δ/CaV_1.2 expression ratio, but the total DNA amount was kept constant at 8 μg by using a mock vector.

**Antibodies and Reagents**—The following primary antibodies were used for Western blots: anti-CaV_1.2 (Alomone Labs, Jerusalem, Israel) (1:5000); anti-CaV_α2δ (Alomone Labs) (1:500); anti-CaV_β3 (Alomone Labs) (1:5000); anti-mCherry (Biovision, Milpitas, CA) (1:20,000); anti-HA (Covance Biotechnology, Quebec, Canada) (1:1000); and anti-GAPDH (Sigma) (1:10,000). Cycloheximide was used at 100 μg/ml for up to 48 h (Sigma). For the flow cytometry assays, the HA tag was detected using the FITC-conjugated mouse monoclonal anti-HA (Sigma) (5 μg/ml). We used the mouse IgG1 isotype control antibody conjugated to FITC (Sigma) to help assess the level of background staining in the flow cytometry experiments (5 μg/ml).

**Flow Cytometry Experiments**—Flow cytometry experiments were conducted as described elsewhere (19, 35). To determine the cell surface expression level of the tagged proteins, cells were harvested 24 h after transfection, washed in a PBS 1× buffer, and stained with the FITC-labeled anti-HA epitope tag antibody at 5 μg/ml (Clone HA-7, Sigma) at 4 °C for 30 min. To determine the density of both intracellular and extracellular expression of the tagged proteins, cells were fixed and permeabilized using BD Cytofix/Cytoperm™ fixation/permeabilization solution kit (BD Biosciences, 554714) according to the manufacturer’s instructions. Briefly, cells were washed with the 1× BD Perm/Wash buffer containing both FBS and saponin and stained 30 min at 4 °C with the FITC-labeled anti-HA epitope tag antibody. A maximum of 10,000 cells were counted using a FACSAria III® special order research product flow cytometer (BD Biosciences) at the flow cytometry facility located at the Department of Microbiology, Université de Montréal. Relative expression of CaV_α2δ was calculated based on the mean fluorescence intensity (ΔMFI) for each fluorophore (mCherry or FITC) rather than computing the number of fluorescent cells (see below) (19). Three control conditions were always carried out for each series of experiments as follows: (a) untransfected HEKT cells with the murine IgG1-FITC isotype control (5 μg/ml); (b) transfected HEKT cells without the anti-HA FITC antibody but with the murine IgG1-FITC isotype control (5 μg/ml); (c) HEKT cells transfected with the wild-type HA-tagged construct. No fluorescence was detected with the IgG1-FITC isotype control murine (5 μg/ml) nor in untransfected cells with the anti-HA FITC conjugated antibody (5 μg/ml) (data not shown). The fluorescence intensity of each construct transfected in any given condition was measured using six distinct cell dishes for an average of 60,000 cells for each condition. Flow cytometry experiments performed at 36 h to mimic as closely as possible the experimental conditions used for the patch clamp experiments (see below), yielded quantitatively similar results as experiments performed at 24 h (data not shown).

**Quantification of Cell Surface Expression**—Flow cytometry data were analyzed, and figures were produced using the FlowJo software, version 10 (TreeStar, Ashland, OR). Dead cells were excluded based on forward scatter/side scatter profiles. The FITC-positive cells gate (P2) and the FITC negative cells gate (P3) were set manually. The FITC fluorescence intensity within the region delineated by the P2 and P3 gate was displayed as cell count versus FITC fluorescence intensity (histograms in Figs. 4, 5, and 8). The ΔMFI for FITC was calculated by subtracting the FITC fluorescence density of the FITC-negative cells (P3) from the fluorescence density of the FITC-positive cells (P2). ΔMFI was used as an index of the cell surface density of HA-tagged proteins (either HA-CaV_1.2 or pmCherry-CaV_α2δ-HA) in intact nonpermeabilized cells or the total expression (cell surface and intracellular protein density) of HA-tagged proteins in permeabilized cells. The two HA-tagged proteins were never expressed together. Because cellular autofluorescence levels are altered by the change in the cell medium, the actual ΔMFI values in arbitrary units cannot be compared between intact nonpermeabilized and permeabilized cells. In Figs. 5 and 9, ΔMFI were pooled and normalized to the average value obtained for the pmCherry-CaV_α2δ-HA WT construct that was expressed under the same conditions and quantified under the same experimental conditions (see above).

**Patch Clamp Experiments in HEKT Cells**—Whole-cell voltage clamp recordings were performed on isolated cells 30–38 h after transfection using the methods described above in the presence of the pcGFP vector (0.2 μg) as a control for transfection. In all cases, the experiments were carried out under optimal transfection conditions after assessment of the mCherry fluorescence of the mCherry-CaV_α2δ constructs. Patch clamp experiments were carried out with the Axopatch 200-B amplifier (Molecular Devices, Union City, CA). Electrodes were filled with a solution containing (in mM) 140 CsCl, 0.6 NaGTP, 3 MgATP, 10 EGTA, 10 HEPES, titrated to pH 7.3 with NaOH. Pipette resistance ranged from 2 to 4 meqohms. Cells were bathed in a modified Earle’s saline solution (in mM) as follows: 135 NaCl, 20 tetraethylammonium chloride, 2 CaCl_2, 1 MgCl_2, 10 HEPES, titrated to pH 7.3 with KOH. PClamp software Clampex 10.2 coupled to a Digidata 1440A acquisition system (Molecular Devices) was used for on-line data acquisition and analysis. Pipette and cell capacitance cancellation and series resistance compensation were applied (up to 80%) using the cancellation feature of the amplifier. Cellular capacitance was estimated by measuring the time constant of current decay evoked by a 10-mV depolarizing pulse applied to the cell from a holding potential of −100 mV. A series of 450-ms voltage pulses were applied from a holding potential of −100 mV at a frequency of 0.2 Hz, from −60 to +70 mV at 5-mV intervals. Unless stated otherwise, data were sampled at 5 kHz and filtered at 1 kHz. Experiments were performed at room temperature (20–22 °C). Activation parameters were estimated from the peak I-V curves obtained for each channel combination and are reported as the mean of individual measurements ± S.E. as described elsewhere (37). Briefly, the I-V relationships were normalized to the maximum amplitude and were fitted to a
Boltzmann equation with $E_{0.5, \text{act}}$ being the mid-potential of activation. The free energy of activation was calculated using the mid-activation potential shown in Equation 1,

$$
\Delta G_{\text{act}} = z \cdot F \cdot E_{0.5, \text{act}}
$$

(Eq. 1)

where $z$ is the effective charge displacement during activation, and $F$ is the Faraday constant (38). The $r100$ ratio is defined as the ratio of peak whole-cell currents remaining after a depolarizing pulse of 100 ms ($I_{100 \text{ ms}}/I_{\text{peak}}$) and was used as an indicator of the inactivation kinetics.

Unless specified otherwise (as in Fig. 1), patch clamp experiments were performed with the untagged version of pCMV-CaV1.2 WT transfected with pmCherry-CaV1.2-HA WT or mutant in stable CaV1.2 cells. Each novel pmCherry-CaV1.2-HA mutant was always tested alongside the control condition (pCMV-CaV1.2 WT + pmCherry-CaV1.2-HA WT in stable CaV1.2β3 cells) to assess for internal consistency. Experiments performed under the same conditions yielded peak current densities $\pm 20\%$ between samples and between series of experiments. All experiments were pooled and biophysical properties are reported in Table 1.

**Statistics**—Results were expressed as mean $\pm$ S.E. Tests of significance were carried out using the unpaired analysis of variance test embedded in the Origin 7.0 analysis software (OriginLab Corp., Northampton, MA). Data were considered statistically significant at $p < 0.05$.

**RESULTS**

HA-tagged CaV1.2β3 Stimulates CaV1.2 Currents—Co-expression of CaV1.2 and CaV2.2 with the auxiliary CaV1.2β3 subunit was shown to stimulate whole-cell currents (19, 39). Functional modulation of CaV1.2 by the various CaV1.2β3 constructs was studied after recombinant expression in HEK cell stably transfected with CaV1.2β3 (Fig. 1A) as explained earlier (19). The immunoreactivity and the integrity of the constructs were verified by Western blotting (data not shown). As shown in Fig. 1A,
whole-cell currents, recorded in the presence of a saline solution containing a physiological concentration of $\text{Ca}^{2+}$ (2 mM), were significantly larger when measured in the presence of the Ca$\gamma$α$\delta$ confirming that Ca$\gamma$α$\delta$ stimulates whole-cell currents of Ca$\gamma$1.2/Ca$\gamma$3 (19). Peak current densities increased from $-5 \pm 1 \text{ pA/F} (n = 26)$ (no insert in the pmCherry vector) to $-56 \pm 3 \text{ pA/F} (n = 23)$ in the presence of Ca$\gamma$α$\delta$ WT (Fig. 1B). The increase in peak current densities was associated with a $-15$-mV leftward shift in the activation potential of Ca$\gamma$1.2 from $E_{\text{act}} = 5 \pm 2 \text{ mV} (n = 26)$ (no Ca$\gamma$α$\delta$) to $E_{\text{act}} = -10.1 \pm 0.5 \text{ mV} (n = 23)$ (with Ca$\gamma$α$\delta$). Whole-cell currents recorded with untagged Ca$\gamma$1.2 WT + mCherry Ca$\gamma$α$\delta$-HA or with HA-tagged Ca$\gamma$1.2 + mCherry Ca$\gamma$α$\delta$-HA were not significantly different from currents recorded with untagged Ca$\gamma$1.2 WT + mCherry Ca$\gamma$α$\delta$ WT. This result validates the constructions and confirms that the HA epitopes inserted in extracellular loops of both Ca$\gamma$α$\delta$ and Ca$\gamma$1.2 are not occluding each other. The free energy of activation ($\Delta G_{\text{act}}$) measured in the presence of Ca$\gamma$α$\delta$ was well described by a Gaussian distribution centered around $-0.86 \text{ kcal mol}^{-1}$ (Fig. 1C), whereas the $\Delta G_{\text{act}}$ measured in the absence of Ca$\gamma$α$\delta$ displayed a broader distribution centered at a value close to $\approx 0 \text{ kcal mol}^{-1}$. These results are compatible with a model whereby Ca$\gamma$α$\delta$ stimulates peak current density by setting Ca$\gamma$1.2 channels in a conformational state very close to the open state (40).

Ca$\gamma$α$\delta$ Improves Total but Not Surface Expression of Ca$\gamma$1.2—To evaluate the impact of Ca$\gamma$α$\delta$ on the protein expression of Ca$\gamma$1.2, the HA-tagged version of Ca$\gamma$1.2 was expressed in HEKT cells and in stable Ca$\gamma$3 cells in the presence or absence of Ca$\gamma$α$\delta$ (Fig. 2). Flow cytometry assays were carried out in the presence of the FITC-conjugated anti-HA in intact and in permeabilized cells. Control experiments carried out with a pCMV-Ca$\gamma$1.2 control construct that was not HA-

**FIGURE 2. Ca$\gamma$α$\delta$ stimulates the total protein expression of Ca$\gamma$1.2 in HEKT cells.** HA-tagged Ca$\gamma$1.2 WT was co-expressed transiently either in HEKT cells (A and B, top rows) or in stable Ca$\gamma$3 cells (A and B, bottom rows) with or without pCMV-Ca$\gamma$α$\delta$. Cell surface expression of Ca$\gamma$1.2-HA WT was determined in intact nonpermeabilized cells (A) or after cell permeabilization by flow cytometry using the anti-HA FITC conjugate antibody (B). All histograms display live cells. ∆MFI was used as an index of the cell surface density of Ca$\gamma$1.2 in intact nonpermeabilized cells or the total expression (cell surface and intracellular protein density) of Ca$\gamma$1.2 in permeabilized cells. As seen, the surface expression of Ca$\gamma$1.2 was not significantly improved in the presence of Ca$\gamma$α$\delta$ in contrast to the robust stimulation observed with Ca$\gamma$3. The total protein expression of Ca$\gamma$1.2 was improved after co-expression with Ca$\gamma$α$\delta$, but stronger stimulation was observed in the combined presence of Ca$\gamma$3 with Ca$\gamma$α$\delta$. C, predicted secondary structure of the HA-tagged Ca$\gamma$1.2 construct used in the figure. Please note that Ca$\gamma$1.2 was not tagged. D, bar graph summarizing the results shown in A and B with the values for the cell surface density in light gray bars and the total protein density in dark gray bars. Each experimental condition was quantified in triplicate. The fluorescence intensity is shown in arbitrary units. The statistical analysis was performed by comparing the ∆MFI values measured in all experimental groups versus ∆MFI values measured for Ca$\gamma$1.2-HA alone in intact versus permeabilized cells respectively. **, $p < 0.01$; ***, $p < 0.001$. 
tagged, confirmed the specificity of the FITC antibody in these series of experiments (data not shown). Given that the HA epitope is located in the extracellular portion of the protein, the fluorescence intensity for FITC obtained in the presence of intact cells reflects the cell surface density of CaV1.2. Fluorescence for FITC was measured after cell permeabilization to confirm the accessibility of the HA epitope. The fluorescence intensity for FITC obtained in the presence of intact cells reflects the cell surface density of CaV1.2. Fluorescence histograms are reported in Fig. 2, A and B, and the averaged mean fluorescence intensities are shown in Fig. 2D. CaVα2δ alone improved total protein expression of CaV1.2 without any significant change in the cell surface density of CaV1.2. This contrasts with the impact of Cavβ (Fig. 2D) that improves both cell surface and total protein density of CaV1.2 (20). In particular, CaVα3 increased by ~200% the cell surface density of CaV1.2, whereas CaVα2δ barely stimulated cell surface density by 2 ± 3% (n = 3). Co-expression with both CaVα3 and CaVα2δ did not increase cell surface detection of CaV1.2, although total protein stability was improved (Fig. 2, A–D). The former results contrast with confocal imaging data showing that co-expression with both CaVα2δ and CaVβ1b is required to achieve the maximal cell surface staining for CaV1.2 (39). We had previously reported similar results with an HA-tagged CaV1.2 construct bearing its HA epitope in domain I (19).

The flow cytometry data were validated by cycloheximide chase analysis (Fig. 3, A–C). CaVα3 increased the protein expression of CaV1.2 in total lysates, whereas CaVα2δ alone did not significantly alter protein density of CaV1.2. As seen in flow cytometry assays (Fig. 2D), total protein expression of CaV1.2 was also increased in the combined presence of CaVβ3 and CaVα2δ auxiliary subunits (Fig. 3, D and E), as also reported by others (20).

CaV1.2/CaVα3 Stabilizes the Expression of CaVα2δ—CaVβ Subunits Promote the Cell Surface Density of CaV1.2, but little is known about their role on the trafficking of CaVα2δ. To investigate the trafficking of CaVα2δ, the pmCherry-CaVα2δ-HA...
**Congenital Mutations in the Cardiac L-type Channel**

**Methodology and Results:**

- **A.** Intact Non-Permeabilized (NP) and Permeabilized (P)

- **Ac.** Representative two-dimensional plots of mCherry versus FITC fluorescence are shown for each condition as stated in the left panels. The vertical line indicates the median fluorescence intensity for FITC to facilitate the visual comparison between the different experimental groups. As seen, the cellular autofluorescence levels increased after permeabilization, which prevents comparison of the absolute fluorescence values between intact nonpermeabilized and permeabilized cells. B, predicted secondary structure of the pmCherry-CaV_\(\alpha_2\delta\)-HA construct used in the figure. Please note that CaV_1.2 was not tagged either in the N or C termini. C, bar graph shows the ΔMFI measured in the presence of FITC in intact cells for each experimental condition. Experiments were conducted in triplicate, and each bar is the mean ± S.E. of ΔMFI in arbitrary units. Under these conditions, ΔMFI measured for FITC reflects the relative cell surface protein expression of CaV_\(\alpha_2\delta\). NP, nonpermeabilized; P, permeabilized cells. D, bar graph shows the ΔMFI measured for FITC in permeabilized cells for each experimental condition. Experiments were conducted in triplicate, and each bar is the mean ± S.E. of ΔMFI in arbitrary units. Under these conditions, ΔMFI measured for FITC reflects the total protein expression of CaV_\(\alpha_2\delta\). *p < 0.05; **p < 0.01; ***p < 0.001.

**Discussion:**

The protein expression of CaV_\(\alpha_2\delta\) was transiently transfected with a control pCMV plasmid in HEK T cells; Ab with CaV_1.2 (untagged) in HEK T cells; Ac, in stable CaV_3 β3 cells; and Ad with CaV_1.2 (untagged) in stable CaV_3 β3 cells. The HA tag of CaV_\(\alpha_2\delta\)-HA was detected using an anti-HA FITC-conjugated antibody. Dead cells were excluded based on the forward scatter/side scatter profiles. Representative two-dimensional plots of mCherry versus FITC fluorescence are shown for each condition as stated (left panels). The vertical line indicates the median fluorescence intensity for FITC to facilitate the visual comparison between the different experimental groups. As seen, the cellular autofluorescence levels increased after permeabilization, which prevents comparison of the absolute fluorescence values between intact nonpermeabilized and permeabilized cells. B, predicted secondary structure of the pmCherry-CaV_\(\alpha_2\delta\)-HA construct used in the figure. Please note that CaV_1.2 was not tagged either in the N or C termini. C, bar graph shows the ΔMFI measured in the presence of FITC in intact cells for each experimental condition. Experiments were conducted in triplicate, and each bar is the mean ± S.E. of ΔMFI in arbitrary units. Under these conditions, ΔMFI measured for FITC reflects the relative cell surface protein expression of CaV_\(\alpha_2\delta\). NP, nonpermeabilized; P, permeabilized cells. D, bar graph shows the ΔMFI measured for FITC in permeabilized cells for each experimental condition. Experiments were conducted in triplicate, and each bar is the mean ± S.E. of ΔMFI in arbitrary units. Under these conditions, ΔMFI measured for FITC reflects the total protein expression of CaV_\(\alpha_2\delta\). *p < 0.05; **p < 0.01; ***p < 0.001.

**Further Analysis:**

Channel Modulation Depends upon the Cell Surface Expression of CaV_\(\alpha_2\delta\)—The correlation between the protein expression of CaV_\(\alpha_2\delta\) and the modulation of CaV_1.2 channels was quantified by co-expressing different DNA ratios of CaV_\(\alpha_2\delta\) and CaV_1.2. The ΔMFI values for FITC measured in intact and permeabilized cells increased steeply in the range from 1:20 to a 1:2 DNA ratio. Both the cell surface and the total protein densities followed a similar pattern (Fig. 5). Under our experimental conditions, the 1:1 DNA ratio yielded an additional 20% increase in the protein density, but raising further the relative amount of DNA coding for CaV_\(\alpha_2\delta\) was found to impair trans-
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A. 
Intact Non-Permeabilized (NP) 
Permeabilized (P)

B. 
Ca\(\alpha_{2}\delta\) : Ca\(\gamma_{1.2}\)

C. 
normalized \(\Delta MFI\) FITC

FIGURE 5. Cell surface expression of Ca\(\alpha_{2}\delta\1\) as a function of DNA ratios. A, pmCherry-Ca\(\alpha_{2}\delta\1-HA\) WT (in variable amounts) was transiently transfected with Ca\(\gamma_{1.2}\) in stable Ca\(\beta_{3}\) cells. Protein expression was assessed in flow cytometry assays using the gating strategy explained in Fig. 4. Representative two-dimensional plots of mCherry versus FITC fluorescence are shown for each Ca\(\alpha_{2}\delta\1-HA/Ca\(\gamma_{1.2}\) DNA ratio as follows: ratio 1:8 (0.5 \(\mu\)g for Ca\(\alpha_{2}\delta\1-HA\); ratio 1:4 (1 \(\mu\)g); ratio 1:2 (2 \(\mu\)g); and ratio 1:1 (4 \(\mu\)g) (left panels). Histogram plots show the distribution of fluorescence intensity of the anti-HA FITC-conjugated antibody staining in intact nonpermeabilized (NP) cells (middle panels) or in permeabilized (P) cells (right panels). B, overlay representation of the data is shown to facilitate visualization of the differences in fluorescence intensity as follows: Ratio 1:8 (filled line); ratio 1:4 (dotted line); ratio 1:2 (dashed line); and ratio 1:1 (full line). C, bar graph shows the normalized \(\Delta MFI\) measured in the presence of FITC in intact nonpermeabilized (dark gray bars) and permeabilized (hatched bars) cells for Ca\(\alpha_{2}\delta\1-HA\) expressed in the combined presence of Ca\(\gamma_{1.2}\) and Ca\(\beta_{3}\). All \(\Delta MFI\) values were normalized using the \(\Delta MFI\) measured in the presence of a Ca\(\gamma_{2}\delta\1-HA/Ca\(\gamma_{1.2}\) DNA ratio of 1:1. As seen, the cell surface and total protein expression of Ca\(\alpha_{2}\delta\1WT both increase steeply up to 1.5 to 2 \(\mu\)g of cDNA coding for Ca\(\alpha_{2}\delta\1-HA\) WT (ratios 1:3 to 1:2).

Section Text:

...activation gating of Ca\(\gamma_{1.2}\) by promoting the channel activation subunits forming the Ca\(\gamma_{1.2}\) L-type calcium channel... (29) discovered 23 rare missense variants in three genes encoding at more negative potentials.

...intact nonpermeabilized (NP) cells (middle panels) or in permeabilized (P) cells (right panels). B, overlay representation of the data is shown to facilitate visualization of the differences in fluorescence intensity as follows: Ratio 1:8 (filled line); ratio 1:4 (dotted line); ratio 1:2 (dashed line); and ratio 1:1 (full line). C, bar graph shows the normalized \(\Delta MFI\) measured in the presence of FITC in intact nonpermeabilized (dark gray bars) and permeabilized (hatched bars) cells for Ca\(\alpha_{2}\delta\1-HA\) expressed in the combined presence of Ca\(\gamma_{1.2}\) and Ca\(\beta_{3}\). All \(\Delta MFI\) values were normalized using the \(\Delta MFI\) measured in the presence of a Ca\(\gamma_{2}\delta\1-HA/Ca\(\gamma_{1.2}\) DNA ratio of 1:1. As seen, the cell surface and total protein expression of Ca\(\alpha_{2}\delta\1WT both increase steeply up to 1.5 to 2 \(\mu\)g of cDNA coding for Ca\(\alpha_{2}\delta\1-HA\) WT (ratios 1:3 to 1:2).

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...transfection efficiency (data not shown). Patch clamp experiments established that increasing the cell surface expression of Ca\(\alpha_{2}\delta\) improves peak current density and activation gating of Ca\(\gamma_{1.2}\) (Fig. 6). Peak current density increased as a function of the cell surface density of Ca\(\alpha_{2}\delta\) within the range of our experimental conditions (Fig. 7). This suggests that increasing the cell surface density of Ca\(\alpha_{2}\delta\) improves the macroscopic activation gating of Ca\(\gamma_{1.2}\) by promoting the channel activation at more negative potentials.

Cell Surface Density Is Impaired in Ca\(\alpha_{2}\delta\) Genetic Variants—To gain further insight into the molecular mechanism underlying channel modulation by Ca\(\alpha_{2}\delta\), we turned to mutations of Ca\(\alpha_{2}\delta\) associated with cardiac arrhythmias. Burashnikov et al. (29) discovered 23 rare missense variants in three genes encoding subunits forming the Ca\(\gamma_{1.2}\) L-type calcium channel in 205 patients diagnosed with “\(J\)-wave syndromes.” Four genetic variants were identified in the CACNA2D1 gene encoding Ca\(\alpha_{2}\delta\1. The mutation S709N was found in two unrelated patients. Two mutations (D550Y and Q917H) were identified in the same individual (29), but their expression profile has yet to been fully characterized (32). Ca\(\alpha_{2}\delta\) missense mutants D550Y, S709N, Q917H, S956T, the double D550Y/Q917H (29), as well as Ca\(\alpha_{2}\delta\) S755T mutant associated with SQTS6 (31) were expressed alone or in combination with Ca\(\gamma_{1.2}\) and Ca\(\beta_{3}\), the same subunit composition we used for functional characterization in patch clamp experiments. These six Ca\(\alpha_{2}\delta\) mutations were co-expressed with Ca\(\gamma_{1.2}\) WT (no HA) in the maximum 1:1 ratio in stable Ca\(\beta_{3}\) cells. The transfection efficiency (as assessed by the number of fluorescent cells) was found to be not significantly different between the HA-tagged Ca\(\alpha_{2}\delta\) WT and the HA-tagged Ca\(\alpha_{2}\delta\) mutant constructs. Overall, the total protein density of all mutants estimated from permeabilized cells or from the constitutive mCherry fluorescence (data not shown) significantly increased in the combined presence of Ca\(\gamma_{1.2}\) and Ca\(\gamma_{1.3}\). As shown in Figs. 8 and 9, the \(\Delta MFI\) for FITC of Ca\(\alpha_{2}\delta\) S755T, Q917H, and S956T mutants in intact cells under these conditions was similar to the \(\Delta MFI\) measured for Ca\(\alpha_{2}\delta\)-HA WT suggesting that the cell surface density of Ca\(\alpha_{2}\delta\) was not affected by these single mutations. In contrast, missense mutations D550Y and S709N and more significantly the double mutant D550Y/Q917H impaired the cell surface targeting of Ca\(\alpha_{2}\delta\)-HA with \(\approx\)30% reduction for the former mutants and \(\approx\)60% decrease for the latter. Doubling the amount of cDNA used for transfection did not improve cell surface expression because the transfection efficiency decreased with larger DNA concentrations. The surface density of D550Y/Q917H proteins remained on average 60% lower than the one measured for the Ca\(\alpha_{2}\delta\)-HA WT protein at each DNA ratio tested from 1:20 (data not shown) to 1:1. Flow cytometry assays carried out 36 h after transfection yielded similar results suggesting that a 24-h culture time is sufficient to observe the optimal protein expression (data not shown). The fluorescence intensities for FITC in permeabilized cells and for the constitutive mCherry were only decreased by \(\approx\)30% when compared with the signals measured for
inactivation kinetics measured after co-expression of CaV

Peak current density was normalized to the mean value measured with 4

large number of equations, but it is shown as a linear regression to facilitate

2862

CaV

density of CaV

experimental variation, there is a positive correlation between the cell surface

FIGURE 6. Modulation of L-type CaV1.2 currents parallels the cell surface expression of CaVα2δ1. A, pCMV-CaV1.2 (untagged) was co-expressed in stable CaVβ3 cells with variable pmCherry-CaVα2δ1-HA DNA ratios for CaVα2δ1/CaV1.2 as follows: Aa, ratio 1:8 [0.5 μg]; Ab, ratio 1:4 (1 μg); Ac, ratio 1:2 (1.5 μg); Ad, ratio 1:2 (2 μg); and Ae, ratio 1:1 (4 μg). Typical whole-cell current traces were recorded in a 2 mM Ca2+ solution from a holding potential of −100 mV. The pmCherry-CaVα2δ1-HA construct used for the patch clamp experiments was identical to the constructs studied in the flow cytometry assays. Time scale is 100 ms throughout. Unless specified otherwise, the current density scale is 10 pA/pF. B, peak current densities increased from −5 ± 1 pA/pF (n = 26) (no insert in the pmCherry vector) to −67 ± 3 pA/pF (n = 163) in the presence of 4 μg of cDNA (ratio 1:1) coding for pmCherry-CaVα2δ1-HA WT. The number in parentheses indicates the number of independent patch clamp recordings. C, histogram reporting the distribution of the individual ΔGact values (kilocalories/mol) for each concentration of cDNA coding for CaVα2δ1. As seen, the ΔGact are significantly shifted to the left even when CaV1.2 is co-expressed with CaVα2δ1 in a 1:1 ratio. D, inactivation kinetics of the CaV1.2 currents expressed with CaVα2δ1 in a 1:2 ratio (gray hatched bars) were in general 20% slower (p < 0.05) than the inactivation kinetics measured after co-expression of CaVα2δ1 in a 1:1 ratio (white striated red bars) (p < 0.05).

FIGURE 7. Correlation between the cell surface density of CaVα2δ1-HA WT and the changes in ΔGact and peak current density of CaV1.2 currents. Peak current density was normalized to the mean value measured with 4 μg of cDNA for CaVα2δ1-HA (ratio 1:1); ΔGact was calculated relative to the value of ΔGact = 0.48 ± 0.09 kcal mol−1 for currents measured in the absence of CaVα2δ1; the cell surface density was computed using the normalized ΔMFI measured for FITC in intact nonpermeabilized cells. Despite the large experimental variation, there is a positive correlation between the cell surface density of CaVα2δ1 and the two other parameters (peak current density and ΔGact of macroscopic currents). The variation of ΔGact with the surface density of CaVα2δ1 suggests that CaVα2δ1 influences the fraction of ion channels active in a given gating mode. The correlation could be described by a large number of equations, but it is shown as a linear regression to facilitate visualization.

CaVα2δ-HA WT. These data indicate the following: (a) HA tag remained accessible in the double mutant; (b) the cell and the total protein density were both affected by the D550Y/Q917H double mutation albeit to a different extent. Altogether, these data suggest that the double mutation impairs the trafficking of CaVα2δ to a greater extent than protein stability.

Channel Modulation Is Altered in the Presence of CaVα2δ Genetic Variants—The functional impact of the missense CaVα2δ mutations was characterized by electrophysiology after co-expression of CaV1.2 and CaVα2δ in stable CaVβ3 cells. In the presence of a 1:1 ratio, the five single point mutations, including D550Y and S709N, increased CaV1.2 peak current densities by 10–13-fold with a negative shift in the voltage-dependent gating activation (ΔGact ≈ −1 kcal mol−1) in a fashion reminiscent of CaVα2δ WT (Table 1). Hence, CaVα2δ D550Y and S709N mutations boosted the peak current densities despite a 30% decrease in the cell surface expression of CaVα2δ. These results are in agreement with Fig. 7 showing that an ≈30% decrease in the cell surface density of CaVα2δ is not sufficient to significantly prevent the up-regulation of macroscopic CaV1.2 currents. In contrast, co-expression of CaVα2δ-HA D550Y/Q917H produced currents that were 35% lower than CaVα2δ-HA WT with an average of −40 ± 10 pA/pF (n = 10) compared with −67 ± 3 pA/pF (n = 163) (Fig 10). The experimental variation, however, limits the statistical significance to p < 0.5. Nonetheless, there seems to be a trend toward a decreased function that was also reported by the authors of the original paper (29). We hypothesized that the decrease in the cell surface density of the CaVα2δ D550Y/Q917H mutant could become more significant when expressing CaVα2δ/CaV1.2 in a 1:20 ratio. Patch clamp experiments
were thus carried out with cDNA ratios of 1:1, 1:8, and 1:20 CaV1.2/CaV2.2 (0.2:4 μg of DNA) (Fig 11). As shown, the behavior of the double mutant departed more significantly from the wild-type protein at lower cDNA concentrations. It is interesting to note that the decrease in peak current density was accompanied by a rightward shift in the activation potentials. Altogether, our results suggest that some CaV1.2 arrhythmo-

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DISCUSSION
CaV1.2δ Improves the Stability of CaV1.2-CaVβ Complexes—
It is generally agreed that recombinant expression of CaV1.2δ boosts peak current density of CaV1.2 in recombinant cells (19)

FIGURE 8. Cell surface and total protein expression of CaV1.2δ/D550Y/Q917H mutant is significantly decreased, pmCherry-CaV1.2-HA WT and mutants were transiently transfected with pCMV-CaV1.2 (untagged) in stable CaVβ3 cells. Fluorescence was computed according to the gating strategy described under “Experimental Procedures” and in Fig. 4. Representative two-dimensional plots of mCherry versus FITC fluorescence (left panels) are shown for each CaV1.2δ mutant as follows: D550Y, S709N, S755T, Q917H, S956T, and D550Y/Q917H (from top to bottom). Histogram plots show the distribution of fluorescence intensity for the anti-HA FITC conjugated antibody staining in intact nonpermeabilized (NP) cells (middle panels) or in permeabilized (P) cells (right panels). The median fluorescence intensity of FITC is indicated by a straight line on the histograms to help comparison between the different mutants. Increasing the DNA amount to 8 μg (CaV1.2δ/CaV1.2 DNA ratio of 2:1) did not increase further the surface expression of D550Y/Q917H.
As well as in native mouse cardiomyocytes (21). Whether this modulation is conveyed through an increase in the cell surface density of Cav1.2 remains debated (19, 20, 42) in part because the macroscopic peak current density is the product of three parameters as follows: the number of channels in the plasma membrane, the open channel probability, and the single-channel conductance. We have opted to investigate changes in the macroscopic peak current density, the number of channels in the plasma membrane, and the single-channel conductance. We have opted to investigate changes in the macroscopic peak current density using a sensitive and high throughput flow cytometry fluorescence-based assay. In this assay, the cells sample go through a gating process that excludes dead cells, debris, and aggregates such that the fluorescence intensity reflects protein expression in intact cells of similar morphological properties. This approach required the insertion of a 9-residue HA epitope in the extracellular face of the Cav1.2 pore-forming subunit. Such manipulation may interfere with protein expression and/or interaction with other subunits as shown very elegantly in Ref. 39. Indeed, we have tested many constructs before identifying one that preserves the up-regulation of Cav1.2 currents by Cavα2δ. As shown in the first series of experiments, whole-cell currents measured with the HA-tagged version of Cav1.2 were functionally up-regulated by the tagged and the untagged versions of Cavα2δ. Using the untagged version of Cavα2δ, we evaluated the changes in the surface and the total protein expression of HA-Cav1.2 from the relative fluorescence of the FITC-conjugated antibody in intact and permeabilized cells. Total protein density was also measured using Western blotting. Altogether, our results show the following: (a) Cavα2δ alone, unlike Cavβ (20, 35), does not prevent the degradation or improve the cell surface density of Cav1.2 proteins; and (b) in the presence of Cavβ3, Cavα2δ improves the stability and/or the total protein expression of Cav1.2 proteins. Hence, we show that Cavα2δ up-regulates Cav1.2 currents by improving the channel activation gating without significantly increasing the number of Cav1.2 proteins at the cell surface.

Recombinant Cavα2δ Is Strongly Expressed at the Membrane—The trafficking of Cavα2δ was studied using a similar fluorescence-based assay, but surface and total protein density were measured in the presence of FITC in intact cells for each pmCherry-Cavα2δ-HA construct (WT and mutants) expressed alone (hatched bars) and in the presence of Cav1.2 (untagged) in stable Cavβ3 cells (dark gray bars). As seen, the relative cell surface density measured in intact cells decreased by 29 ± 2, 31 ± 3, and 63 ± 1% for Cavα2δ mutants D550Y, S709N, and the double mutant D550Y/Q917H (shown as DY/QH), respectively, when expressed alone (hatched bars) or in stable Cavβ3 cells with Cav1.2 (filled dark gray bars). Values were normalized as compared with the ΔMFI for FITC measured for Cavα2δ-HA WT under the same experimental conditions (p < 0.001). NP, nonpermeabilized. B, bar graph shows the ΔMFI measured for FITC in permeabilized (P) cells for each Cavα2δ-HA construct (WT and mutants) expressed alone (hatched bars) or in stable Cavβ3 cells with Cav1.2 (filled gray bars). All ΔMFI values were normalized as compared with the average ΔMFI measured with Cavα2δ-HA WT expressed alone in HEK cells. ΔMFI for FITC measured in permeabilized cells reflects the total protein expression of Cavα2δ-HA constructs. As seen, the total protein density decreased by 20 ± 1, 21 ± 2, and 24 ± 11% for Cavα2δ-HA D550Y, S709N, and D550Y/Q917H, respectively (p < 0.05), when compared with the wild-type construct. This small decrease in total protein density of these mutants could account in part for their decreased cell surface density. This decrease was also observed for the constitutive fluorescence of mCherry (data not shown). For all mutants, please note that the total protein density of all Cavα2δ-HA constructs nearly doubled when co-expressed with Cavα2δ under the same Cavβ3 cells. * p < 0.05; ** p < 0.001.

| TABLE 1 Biophysical properties of Cav1.2/Cavβ3 channels with and without Cavα2δ |
|---------------------|---------------------|---------------------|---------------------|---------------------|
| Electrophysiological properties |  |  |  |  |
| Cavα2δ WT in Cavβ3 stable HEK cells with 2 mm Ca2+ |  |  |  |  |
| F0.95, act | ΔGact | Peak density | r100 at +5 mV |  |
| mV | kcal mol⁻¹ | pA/pF |  |
| No Cavα2δ-HA (pmCherry-no insert) | 4 ± 1 (26) | 0.5 ± 0.1 (26) | −5.4 ± 0.7 (26) | ND |
| + Cavα2δ-WT no HA (1:1 ratio) | −10.1 ± 0.5 (23) | −0.86 ± 0.05 (23) | −56 ± 3 (23) | 0.31 ± 0.02 (23) |
| + Cavα2δ-HA WT (1:1 ratio) | −10.6 ± 0.2 (163) | −0.90 ± 0.03 (163) | −67 ± 3 (163) | 0.38 ± 0.02 (163) |
| + Cavα2δ-HA D550Y (1:1 ratio) | −10.9 ± 0.7 (10) | −0.9 ± 0.1 (10) | −61 ± 9 (10) | 0.33 ± 0.01 (10) |
| + Cavα2δ-HA S709N (1:1 ratio) | −11.3 ± 0.6 (10) | −0.9 ± 0.1 (10) | −49 ± 10 (10) | 0.37 ± 0.02 (10) |
| + Cavα2δ-HA S755T (1:1 ratio) | −11.4 ± 0.4 (20) | −1.0 ± 0.0 (20) | −73 ± 10 (20) | 0.29 ± 0.02 (20) |
| + Cavα2δ-HA Q917H (1:1 ratio) | −11.4 ± 0.7 (10) | −1.0 ± 0.1 (10) | −69 ± 9 (10) | 0.37 ± 0.02 (10) |
| + Cavα2δ-HA S956T (1:1 ratio) | −10.8 ± 0.6 (10) | −0.9 ± 0.1 (10) | −84 ± 9 (10) | 0.33 ± 0.02 (10) |
| + Cavα2δ-HA D550Y/Q917H (1:1 ratio) | −9 ± 2 (10) | −0.8 ± 0.2 (10) | −40 ± 10 (10) | 0.35 ± 0.03 (10) |

FIGURE 9. A, bar graph shows the normalized ΔMFI measured in the presence of FITC in intact cells for each pmCherry-Cavα2δ-HA construct (WT and mutants) expressed alone (hatched bars) and in the presence of Cav1.2 (untagged) in stable Cavβ3 cells (dark gray bars). As seen, the relative cell surface density measured in intact cells decreased by 29 ± 2, 31 ± 3, and 63 ± 1% for Cavα2δ mutants D550Y, S709N, and the double mutant D550Y/Q917H (shown as DY/QH), respectively, when expressed alone (hatched bars) or in stable Cavβ3 cells with Cav1.2 (filled dark gray bars). Values were normalized as compared with the ΔMFI for FITC measured for Cavα2δ-HA WT under the same experimental conditions (p < 0.001). NP, nonpermeabilized. B, bar graph shows the ΔMFI measured for FITC in permeabilized (P) cells for each Cavα2δ-HA construct (WT and mutants) expressed alone (hatched bars) or in stable Cavβ3 cells with Cav1.2 (filled gray bars). All ΔMFI values were normalized as compared with the average ΔMFI measured with Cavα2δ-HA WT expressed alone in HEK cells. ΔMFI for FITC measured in permeabilized cells reflects the total protein expression of Cavα2δ-HA constructs. As seen, the total protein density decreased by 20 ± 1, 21 ± 2, and 24 ± 11% for Cavα2δ-HA D550Y, S709N, and D550Y/Q917H, respectively (p < 0.05), when compared with the wild-type construct. This small decrease in total protein density of these mutants could account in part for their decreased cell surface density. This decrease was also observed for the constitutive fluorescence of mCherry (data not shown). For all mutants, please note that the total protein density of all Cavα2δ-HA constructs nearly doubled when co-expressed with Cav1.2 in stable Cavβ3 cells. * p < 0.05; ** p < 0.001.
tracked by the external HA epitope and the intracellular mCherry tag, respectively. The constitutive mCherry fluorescence was used as an index of Ca_{\text{v}}\alpha_{2\delta} expression under all conditions for the wild-type protein as well as the Ca_{\text{v}}\alpha_{2\delta} mutants (see below). Patch clamp experiments carried out with the untagged version of Ca_{\text{v}}1.2 but using the HA-tagged mCherry Ca_{\text{v}}\alpha_{2\delta} constructs (WT and mutants) confirmed the latter construct was functional. It is essential to stress that fluorescence cell sorting experiments (protein expression) and whole-cell recordings (channel function) were carried out with cells grown under the same conditions. These data validated the double modification, insertion of the HA epitope and the mCherry protein, did not prevent functional modulation of Ca_{\text{v}}1.2 currents expressed with the Ca_{\text{v}}\alpha_{2\delta} mutants (see below). Nonethe-

FIGURE 10. Ca_{\text{v}}\alpha_{2\delta} short QT mutants functionally modulate the L-type Ca_{\text{v}}1.2 currents. A, pCMV-Ca_{\text{v}}1.2 was co-expressed in stable Ca_{\text{v}}\beta_3 stable cells with the following: Ca_{\text{v}}\alpha_{2\delta}1WT (Ad); Ca_{\text{v}}\alpha_{2\delta}1 D550Y (Ab); (Ac) Ca_{\text{v}}\alpha_{2\delta}1 S709N; Ca_{\text{v}}\alpha_{2\delta}1 S755T (Ad); and Ca_{\text{v}}\alpha_{2\delta}1 D550Y/Q917H (Ae). Typical whole-cell current traces were recorded in a 2 mM Ca^{2+} solution from a holding potential of −100 mV. The pmCherry-Ca_{\text{v}}\alpha_{2\delta}1-HA constructs were identical to the constructs used for the flow cytometry assays. Time scale is 100 ms throughout. Unless specified otherwise, the current density scale is 10 pA/pF. B, peak current densities increased from −5 ± 1 pA/pF (n = 26) (no insert in the pmCherry vector) to −67 ± 3 pA/pF (n = 163) in the presence of pmCherry-Ca_{\text{v}}\alpha_{2\delta}1-HA WT (p < 10^{-6}). Co-expression with Ca_{\text{v}}\alpha_{2\delta}1 D550Y/Q917H significantly increased peak current densities of Ca_{\text{v}}1.2 as compared with the currents obtained in the absence of Ca_{\text{v}}\alpha_{2\delta}1 and did not produce currents significantly different from Ca_{\text{v}}\alpha_{2\delta}1 WT (p > 0.1) with a value of 40 ± 10 pA/pF (n = 10). The properties of Ca_{\text{v}}\alpha_{2\delta}1 mutants were estimated from 10 to 15 independent patch clamp recordings. C, histogram reporting the distribution of the individual ΔG_act values (kcal/mole) for each Ca_{\text{v}}\alpha_{2\delta}1 mutant. As seen, every single Ca_{\text{v}}\alpha_{2\delta}1 mutant negatively shifted the activation energy of Ca_{\text{v}}1.2 with a distribution of ΔG_act values that superimposed with the ΔG_act values of the Ca_{\text{v}}\alpha_{2\delta}1 WT. D, bar graph shows the r100 values (the ratio of peak whole-cell currents remaining at the end of a 100-ms depolarization) measured at membrane potentials from −10 to +10 mV. Faster inactivation kinetics yield lower r100 values. Inactivation kinetics of the Ca_{\text{v}}1.2 currents expressed with the Ca_{\text{v}}\alpha_{2\delta}1 mutants S709N and Q917H were not significantly different from the ones measured in the presence of Ca_{\text{v}}\alpha_{2\delta}1 WT. The r100 values of Ca_{\text{v}}\alpha_{2\delta}1 mutants D550Y and S755T were 15% smaller suggesting faster inactivation kinetics for these Ca_{\text{v}}\alpha_{2\delta}1 mutants. *, p < 0.05.
Congenital Mutations in the Cardiac L-type Channel

from these disease susceptibility genes have significantly advanced our understanding of the pathophysiology of these syndromes and have paved the way to the development of new treatment strategies. Over the last few years, single missense mutations and/or genetic variants of CaVα2δ have been identified in patients experiencing cardiac arrhythmias associated with repolarizing QT interval (SQT) shorter than normal. Short QT arrhythmias are generally associated with gain-of-function outward currents (potassium currents) or loss-of-function of inward (sodium or calcium) currents. At the time of their identification, the missense mutations in CaVα2δ have been labeled loss-of-function mutations and were proposed to alter L-type CaV1.2 peak current density through a change in the membrane expression of CaV1.2 (29). Having established a reliable experimental model to define the surface expression and the function of the different components of the L-type CaV1.2 channel, we explored the impact of CaVα2δ mutations associated with sudden cardiac death. Recombinant expression in HEK293 cells of CaVα2δ single mutants S755T, Q917H, and S956T associated with shorter QT intervals did not cause any significant change in the cell surface expression of CaVα2δ or in the function of CaVα2δ currents when expressed using a 1:1 cDNA ratio. This is true also for the mutation CaVα2δ S755T that was reported by others to prevent the up-regulation of CaV1.2 currents by CaVα2δ in HEK cells (31). In contrast, we have observed a ~30% decrease in the cell surface density of CaVα2δ D550Y and S709N single mutants accompanied with an ~15% in the total protein density that remained unchanged up to 36 h after transfection. Despite causing a reduction in the cell surface expression of CaVα2δ, the single mutants D550Y and S709N did not significantly impair the modulation of CaV1.2 currents when co-expressed in a 1:1 cDNA ratio. Combining two mutations in CaVα2δ with D550Y/Q917H significantly reduced the cell surface density of CaVα2δ (60%) under the same conditions. Although both the cell surface and the total protein density of the double mutant were reduced, the decrease in the cell surface...
expression was more important than the reduction in the total protein density. Altogether, these data suggest that the trafficking of CaV_1.2δ was significantly disrupted by the double mutation. The decrease in the expression of CaV_1.2δ caused a significant 40% reduction in the peak current density of CaV_1.2 when co-expressed with CaV_1.2δ WT in stable CaV_3δ3 cells. Hence, the combination of two variants that are relatively silent polymorphisms on their own could significantly impair the trafficking of CaV_1.2δ and consequently reduce L-type CaV_1.2 currents.

Conclusion—Current genetic studies are placing the homeostasis of Ca_{2+} as a central modulator of cardiac repolarization with genes such as CACNA1C as well as ATP2A2, PLN, PRKCA, SRL, and SLCA8J (2). Genetic studies have reported a decreased expression of CaV_1.2δ transcripts in patients suffering from atrial (43) and ventricular fibrillation (44). Our current confirm results that mutations in CACNA2D1 could be a contributing factor in cardiac sudden death associated with a short QT interval. It is important to note that the strongest reduction in the cell surface density of CaV_1.2δ was observed by combining two genetic variants that had little impact when tested individually. Hence, the role of polymorphisms in CACNA2D1 is not to be neglected. Missense and/or truncation mutations of CaV_1.2δ could severely impair CaV_1.2 currents providing that the said mutation significantly decreases the cell surface protein expression of CaV_1.2δ.

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