Functional Properties of Cardiac L-Type Calcium Channels Transiently Expressed in HEK293 Cells

Roles of α_1 and β Subunits

MARÍA TERESA PÉREZ-GARCÍA, TIMOTHY J. KAMP, and EDUARDO MARBÁN

From the Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT The cardiac dihydropyridine-sensitive calcium channel was transiently expressed in HEK293 cells by transfecting the rabbit cardiac calcium channel α_1 subunit (α_{1C}) alone or in combination with the rabbit calcium channel β subunit cloned from skeletal muscle. Transfection with α_{1C} alone leads to the expression of inward, voltage-activated, calcium or barium currents that exhibit dihydropyridine sensitivity and voltage- as well as calcium-dependent inactivation. Coexpression of the skeletal muscle β subunit increases current density and the number of high-affinity dihydropyridine binding sites and also affects the macroscopic kinetics of the current. Recombinant $\alpha_{1C}\beta$ channels exhibit a slowing of activation and a faster inactivation rate when either calcium or barium carries the charge. Our data suggest that both an increase in the number of channels as well as modulatory effects on gating underlie the modifications observed upon β subunit coexpression.

INTRODUCTION

Voltage-sensitive calcium channels play an important role in a variety of cell functions by regulating calcium entry in many cell types. These membrane-spanning proteins consist of several transmembrane subunits (Takahashi, Seagar, Jones, Reber, and Catterall, 1987; Catterall, 1988) and have been classified into multiple types on the basis of their functional properties including gating behavior, single-channel conductance, pharmacology and cellular distribution (Tsien, Ellinor, and Horne, 1991). Purification of the dihydropyridine (DHP)-sensitive, L-type Ca channel from skeletal muscle (Tanabe, Takeshima, Mikami, Flockerzi, Takahashi, Kangawa, Kojima, Matsuo, Hirose, and Numa, 1987) led to the isolation of a complex of five different subunits: α_1, α_2-δ, β, and γ. Expression of different cloned L-type α_1 subunits in *Xenopus* oocytes (Mikami, Imoto, Tanabe, Niidome, Mori, Takeshima, Narumiya, and

Address correspondence to Dr. Eduardo Marbán, Department of Medicine, Division of Cardiology, 844 Ross Building, The Johns Hopkins University School of Medicine, 720 North Rutland Avenue, Baltimore, MD 21205.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/95/02/0289/18 $2.00
Volume 105 February 1995 289–306

Numa, 1989; Biel, Ruth, Bosse, Stühmer, Flockerzi, and Hofmann, 1990), dysgenic myotubes (Tanabe, Beam, Powell, and Numa, 1988), murine L cells (Pérez-Reyes, Kim, Lacerda, Horne, Wei, Rampe, Campbell, Brown, and Birnbaumer, 1989) and CHO cells (Bosse, Bottlender, Kleppisch, Hescheler, Welling, Hofmann, and Flockerzi, 1992) has shown that this subunit is the voltage-sensitive and pore-forming component of the channel, which contains the binding site for DHPs and other organic calcium channel blockers. Diversity among Ca channels arises from multiple genes encoding α_1 subunits as well as alternative splicing (Snutch, Leonard, Gilbert, Lester, and Davidson, 1990; Pérez-Reyes, Wei, Castellano, and Birnbaumer, 1990; Tsien et al., 1991; Snutch and Reiner, 1992). The L-type Ca channel α_1 subunits are encoded by three different genes that are expressed in a tissue-specific manner (Zhang, Randall, Ellinor, Horne, Shater, Tanabe, Schwarz, and Tsien, 1993). With regard to the β subunit, multiple isoforms exist in skeletal muscle, heart, brain, and possibly smooth muscle (Hullin, Singer-Lahat, Freichel, Biel, Dascal, Hofmann, and Flockerzi, 1992), although tissue specificity has not been demonstrated. A similar pattern probably holds for the γ subunit, whereas the α_2/δ complex is highly conserved in all excitable tissues (Krizanova, Diebold, Lory, and Schwartz, 1993).

Transient expression studies of DHP-sensitive Ca channels have been carried out mostly in *Xenopus* oocytes. The presence in this preparation of large calcium-activated chloride currents (Barish, 1983) has biased these studies to the use of Ba^{2+} as the charge carrier, precluding the analysis of the roles of the different subunits in the Ca^{2+}-dependent modulation of the current. *Xenopus* oocytes also contain endogenous Ca channels (Singer, Biel, Lotan, Flockerzi, Hofmann, and Dascal, 1991) which might interfere with the study of the expressed Ca channel subunits. It has been reported that DHP-insensitive Ca currents arising from an endogenous α_1 subunit can be stimulated in oocytes injected with only the β subunit (Castellano, Wei, Birnbaumer, and Peréz-Reyes, 1993*a*). The utilization of a different expression system that circumvents these complications can complement studies in *Xenopus* oocytes and contribute to a better understanding of the structure-function relationship of recombinant L-type Ca channels. In the present work, we transiently expressed the α_1 subunit of the L-type cardiac Ca channel in a mammalian cell line, human embryonic kidney cells (HEK293), alone or in combination with the skeletal muscle β subunit. In our experimental preparation, the use of Ca^{2+} as the charge carrier allows us to study the kinetics of the DHP-sensitive Ca channel with its physiological permeant ion. Thus, it is possible to investigate the relative contributions of α_1 and β subunits to the fundamental electrophysiological properties of L-type Ca channels, particularly the interplay between voltage- and Ca^{2+}-dependent inactivation.

Our results show that transfection of HEK cells with the α_1 subunit of the L-type cardiac Ca channel leads to the expression of a Ca current with L-type pharmacological and kinetic properties, including the characteristic voltage- and Ca^{2+}-dependent inactivation (Brehm and Eckert, 1978; Lee, Marbán, and Tsien, 1985; Gutnick, Lux, Swandulla, and Zucker, 1989). Cotransfection of the α_1 subunit with the β subunit dramatically enhances the efficiency of expression of functional channels, as reflected by the increase in the number of DHP binding sites and in the magnitude of the

current. In addition, kinetic analysis reveals significant changes in gating when β subunit is coexpressed with α_1.

METHODS

HEK293 Cell Transfections

HEK293 cells were purchased from American Type Culture Collection (Rockville, MD) and maintained in DMEM supplemented with 10% fetal calf serum (GIBCO-BRL, Gaithersburg, MD), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Cells were grown as a monolayer and plated on 35-mm Petri dishes at a density of 2–4 × 10^5 cells/dish the day before transfection. Transient transfections were performed using the calcium-phosphate method (Wigler, Pellicer, Silverstein, and Axel, 1978) with 2–3 μg of plasmid DNA encoding the rabbit cardiac Ca channel α_{1C} subunit (Slish, Engle, Varadi, Lotan, Singer, Dascal, and Schwartz, 1989; Lory, Varady, Slish, Varadi, and Schwartz, 1993) alone or in combination with 1–2 μg of plasmid DNA encoding the rabbit skeletal muscle β subunit (Pragnell, Sakamoto, Jay, and Campbell, 1991) in a molar ratio 1:1 of (α_1:β). The β subunit cloned from skeletal muscle (β_1) shows extensive sequence homology with that cloned from heart (β_2) and functionally associates with α_{1C} in a similar manner (Shater, Tanabe, Zhang, Mori, Adams, and Tsien, 1993). Because most previous subunit coexpression studies have used β_1, we chose this particular isoform in order to facilitate comparison with results obtained in other heterologous expression systems. The α_{1C} subunit was subcloned in two different mammalian expression vectors, pGW1H (British Biotechnology Ltd., Oxford, UK) and pRcRSV (Invitrogen, San Diego, CA), and β subunit was subcloned in pRcRSV. In all cases, 1 μg of RSVβ-gal in pBR322, a β-galactosidase expression plasmid, was included to permit transfection efficiency estimates by histochemical staining using X-gal (GIBCO-BRL). When the pGW1Hα_{1C} construct was used, 0.8 μg of pTag, a plasmid encoding T antigen, was also added to the DNA transfection mixture to enhance the expression.

Electrophysiology

Functional expression of membrane currents was evaluated using the whole-cell configuration of the patch clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981). HEK cells with no contact with surrounding cells were studied 1–5 d after transfection. A petri dish was mounted on the stage of an inverted microscope and the cells were bathed in a solution containing (in millimolar): 40 $BaCl_2$ or 40 $CaCl_2$, 80 CsCl, and 10 HEPES (pH 7.4 with CsOH). When DHP agents were added to this bath solution, the final concentration of ethanol from the drug stocks was always <0.1%. The patch clamp pipettes were pulled with a multistage micropipette puller (Sutter Instrument Co., Novato, CA) and heat-polished to obtain resistances between 5–10 MΩ when filled with the internal solution. The composition of this solution was (in millimolar): 120 CsCl, 3 MgATP, 10 EGTA, 10 HEPES (pH 7.2 with CsOH). The bath solution was connected to ground via a 3M KCl agar bridge and a Ag-AgCl electrode. Whole-cell currents were recorded using a patch clamp amplifier (Axopatch 200, Axon Instruments, Inc., Foster City, CA), sampled at 10 kHz and filtered at 2 kHz (−3 dB, 4-pole Bessel filter). After establishing the whole-cell configuration, capacity transients were analogue-compensated, and the series resistance was routinely compensated by 60–70%. Currents elicited in response to depolarizing steps to potentials ranging from −60 to +60 mV were recorded from a holding potential of −80 mV. Data were leak subtracted on line by a P/4 protocol. Acquisition and analysis of the data were performed with custom software.

Pooled data are expressed as mean ± standard error of the mean. Statistical comparisons were performed with the two-tailed *t* test for paired or unpaired data where appropriate, and

values of $P < 0.05$ were considered statistically different. The analysis of the differences between two groups of data when comparing more than one variable was carried out with a fully factorial analysis of variance ((M)ANOVA) using commercial software (SYSTAT, SYSTAT, Inc., Evanston, IL). All the experiments were done at room temperature (20–22°C).

Binding Experiments

HEK293 cells were harvested 3–4 d after transfection, and 2–4 × 10^5 cells were incubated in 1 ml of buffer containing (in millimolar) 140 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$ and 10 HEPES (pH 7.4 with KOH) with 0.01 to 1 nM of ^{3}H-PN200-110 (New England Nuclear, Boston, MA). After 90 min the cells were filtered over Whatman GF/C filters using a Brandel cell harvester (Brandel Corp., Gaithersburg, MD). Specific ^{3}H-PN200-110 activity was defined as the binding in absence of nitrendipine minus the binding in the presence of 1 μM nitrendipine. Data were fit by a single-site binding model using nonlinear regression analysis.

RESULTS

Expression of α_1 Subunit Alone

Transfection of HEK cells with the cardiac Ca channel α_{1C} subunit alone led to the expression of inward currents carried by either Ca^{2+} or Ba^{2+}. Fig. 1 *A* shows the current-voltage relationships for the two permeant ions obtained in one cell transfected with only α_{1C}. In both cases, the current begins to turn on at ~ −20 mV; the peak current amplitude occurred at a membrane potential of +20 mV for the Ba currents and at +30 mV for the Ca currents. The overall shape of the curves as well as the voltage and time dependence of the currents resemble those of native L-type cardiac Ca channels (e.g., Lee et al., 1985). The figure reveals that current amplitude increases dramatically with Ba^{2+} as the charge carrier. The average increase in peak amplitude when we compared Ca^{2+}-versus Ba^{2+}-carried inward current in α_{1C}-transfected cells was 3.65 ± 0.4-fold ($n = 5$; $P < 0.005$). The inset shows a representative example of the currents obtained in both conditions in the same cell at a test potential of +30 mV. It is clear that recombinant channel inactivation is faster when Ca^{2+} (*upper trace*) rather than Ba^{2+} (*lower trace*) is the charge carrier. Over the duration of the long (500 ms) depolarizing pulses shown in this figure, Ba currents inactivate ~30%, whereas there is virtually no Ca current at the end of the pulse. These data indicate that, like native channels, Ca channels formed with α_{1C} subunit alone inactivate via voltage- as well as Ca^{2+}-dependent pathways (Lee et al., 1985; Hadley and Hume, 1987; cf. Zong, Zhou, and Tanabe, 1994). Fig. 1 *B* plots the current-voltage relationship obtained in HEK cells transfected with the same amount of plasmid vector DNA but without the α_{1C} insert. Even in the presence of 40 mM Ba^{2+}, there is no measurable inward current at any potential. The inset shows a current record obtained during a 500-ms step to +30 mV. These data are representative of more than 50 mock-transfected HEK cells studied electrophysiologically and indicate the absence of endogenous voltage-activated Ca currents in these cells.

Dihydropyridine Sensitivity of the α_{1C}-only Currents

Although the electrophysiological properties of the expressed currents are consistent with those of L-type Ca channels, Fig. 2 confirms that the barium currents expressed

in cells transfected with α_{1C} are sensitive to DHP agonists and antagonists. Fig. 2 *A* demonstrates the response of a representative cell to 1 μM BAY K 8644, which increased the current at all test potentials and also shifted the peak of the current-voltage relationship towards more negative potentials and accelerated current decay. All these modifications are typical DHP agonist effects on L-type cardiac Ca channels (Sanguinetti, Krafte, and Kass, 1986). After washing out the BAY K 8644, the addition of the DHP antagonist nitrendipine (10 μM) completely blocked the inward current. The inhibitory effect of nitrendipine was strongly dependent on the membrane holding potential, being more potent at more depolarized voltages (not shown), as is typical for DHP block of L-type channels (Bean, 1984; Sanguinetti

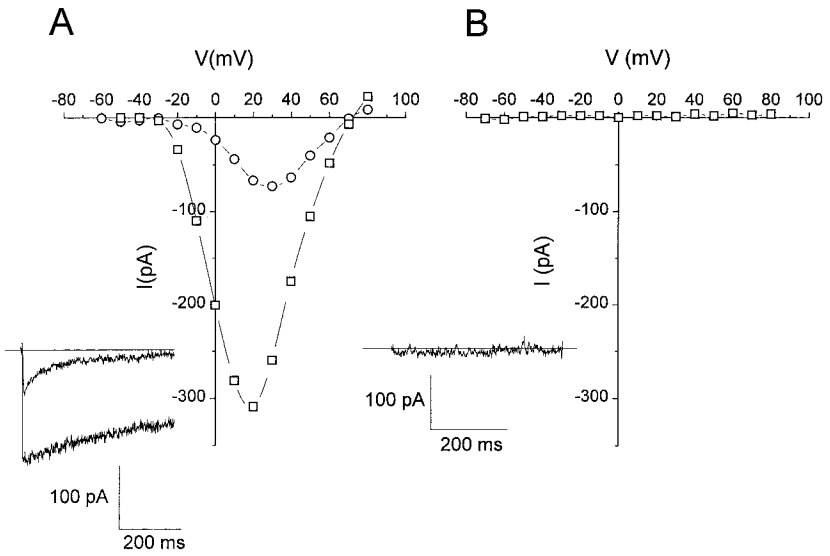

FIGURE 1. (*A*) Current-voltage relationships obtained in one cell transfected with α_{1C} in the presence of 40 mM Ca^{2+} (○) or 40 mM Ba^{2+} (□) in the external solution. The peak current amplitude obtained in steps from a holding potential of −80 mV is plotted as a function of the pulse potential. The curves are fitted by eye. The inset shows representative Ca^{2+} (*upper trace*) and Ba^{2+} currents from the same cell during steps to +30 mV. (*B*) Current-voltage relation in 40 mM Ba^{2+} from a mock-transfected cell using the same protocol as in *A*. The inset shows the current elicited by a depolarizing step to +30 from −80 mV.

and Kass, 1984). The average effect of BAY K 8644 on whole-cell conductance for seven cells transfected with α_{1C} alone is shown in Fig. 2 *B*. Application of BAY K 8644 resulted in an increase in the maximal conductance of 3.59 ± 0.64-fold and a shift in the $V_{1/2}$ for activation from 3 ± 1 to −20 ± 4 mV.

Transfection Efficiency and Detection Frequency of Expressed Currents with and without β Subunit Coexpression

Expression of the cardiac α_1 subunit in other heterologous systems can be modulated by coexpression of other subunits of the Ca channel. We initiated the study of the

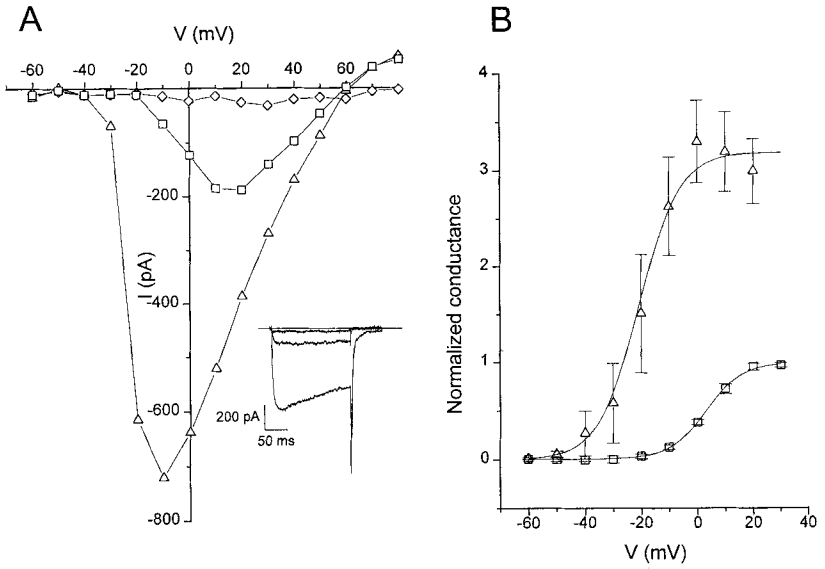

FIGURE 2. Dihydropyridine sensitivity of the recombinant Ca channels. (*A*) Peak current vs voltage relation from an α_{1C}-expressing cell in 40 mM Ba^{2+} before (□) and after addition of 1 μM BAY K 8644 (△) or 10 μM nitrendipine (◇). (*Inset*) Inward currents recorded in the three conditions during steps to 0 mV from a holding potential of −80 mV. (*B*) Averaged conductance versus voltage relationships of 7 α_{1C}-transfected cells in 40 mM Ba^{2+} (□) and during exposure to 1 μM BAY K 8644 (△). In each cell, the conductances in the absence and presence of the drug were normalized relative to the maximal conductance under control conditions. Data were fitted to a Boltzmann function. The average G_{max} in control conditions was 3.36 ± 1 nS.

regulation by other subunits in our preparation by examining the effects of cotransfection of the skeletal muscle β subunit with α_{1C}. Table I summarizes the results of our screening in the different experimental conditions tested, comparing in each situation the percentage of cells with current and the transfection efficiency. In a total of 50 transfections, the efficiency of transfection as measured by histochemical staining for β galactosidase ranged between 20 and 60% (43 ± 2%). Cells that take up the exogenous DNA appear to express channels efficiently: when the current amplitude was optimized (i.e., when Ba^{2+} was the permeant ion), the percentage of cells with detectable currents was close to the average percentage of cells positive for β galactosidase, even though the techniques used in each case (electrophysiological

TABLE I

Efficiencies of Current Expression and Transfection

Transfection condition	Divalent	Cells screened	Cells with current	Percent of cells with current	Transfection efficiency
α_{1C}	40 mM Ba^{2+}	56	20	36%	41%
$\alpha_{1C}\beta_1$	40 mM Ba^{2+}	47	15	32%	39%
α_{1C}	40 mM Ca^{2+}	225	18	8%	39%
$\alpha_{1C}\beta_1$	40 mM Ca^{2+}	77	8	10%	37%

recording versus histochemical staining) are very different. In 40 mM Ba^{2+}, the presence of β subunit did not increase the percentage of cells with measurable currents as compared with α_{1C}-only cells, despite a large enhancement of the peak current amplitude (see below). In Ca^{2+}-containing extracellular solutions, the percentage of cells with measurable currents was smaller than in Ba^{2+}, reflecting in all likelihood our inability to resolve very small currents (< 15 pA). The maximal open state probability of L-type Ca channels is very low (< 0.06; Rose, Balke, Wier, and Marbán, 1992) with Ca^{2+} as the permeant ion, particularly at room temperature in the absence of agonist drugs; thus, the values in Table I for the percentage of cells that express Ca currents must be considered lower-limit estimates.

Binding of ^{3}H-PN200-110 to Transfected and Nontransfected Cells

To characterize the DHP-binding affinity of the α_{1C}-transfected cells and its possible modulation by β subunit coexpression, we performed equilibrium binding experiments with the DHP antagonist ^{3}H-PN200-110 (Fig. 3). The transfected cells exhibit

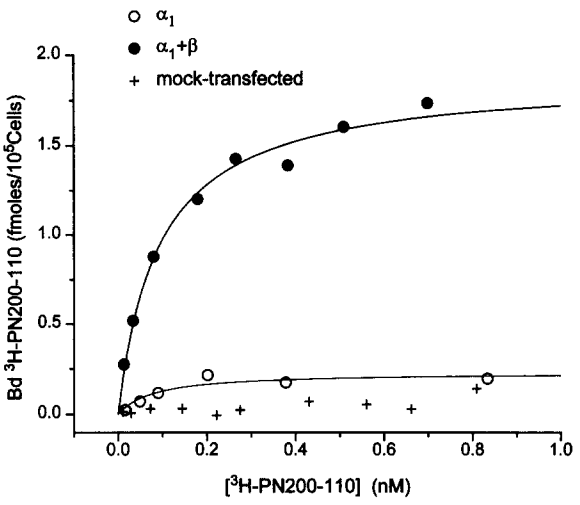

FIGURE 3. Equilibrium binding curves for the DHP antagonist ^{3}H-PN200-110 in mock-transfected HEK cells (+) or cells transfected with α_{1C} (○) or $\alpha_{1C}\beta$ (●) Ca channel subunits. The binding of ^{3}H-PN200-110 expressed as fmoles/10^5 cells (ordinate) is plotted as a function of its concentration (abscissa). The data for each group are fitted to a single binding site model.

a single class of saturable, high-affinity binding sites. When comparing cells transfected with α_{1C} (n = 4) versus $\alpha_{1C}\beta$ (n = 3) there were no differences in binding affinity (0.136 ± 0.036 nM versus 0.112 ± 0.017 nM respectively), but a large increase of B_{max} (0.305 ± 0.047 fmol/10^5 cells versus 3.45 ± 0.814 fmol/10^5 cells). There was no detectable high-affinity binding to nontransfected cells, consistent with the absence of measurable endogenous Ca currents.

Effect of β Subunit on Macroscopic Kinetics of the Ca Currents

Virtually all of the available data regarding the expression of Ca channels in heterologous systems and the modulatory role of the different subunits have been obtained using Ba^{2+} as the charge carrier. For this reason, it is still unknown if the effects of coexpressing other subunits in combination with α_1 are comparable with the physiological permeant ion. Fig. 4 compares the currents expressed in HEK cells transfected with α_{1C} alone or with $\alpha_{1C}\beta$ when Ca^{2+} is the permeant ion. The

representative records in *A* show that cotransfection with β subunit enhanced current amplitude at all voltages, as confirmed by the averaged current-voltage relationships in panel B. The peak current increased from −67 ± 13 pA in the α_{1C}-transfected cells to −217 ± 75 pA in the presence of β subunit. The upper panels show representative records of membrane current obtained during 200-ms steps to four different potentials in each condition. Inactivation is clearly faster in α_{1C}β-cotransfected cells as compared with α_{1C} alone.

Previous studies of DHP-sensitive Ca channel α_1 subunit expression in other heterologous systems (*Xenopus* oocytes and L cells) have shown that coexpression of β subunit not only enhances the magnitude of the currents but also affects their kinetics (Lacerda, Kim, Ruth, Pérez-Reyes, Flockerzi, Hofmann, Birnbaumer and Brown,

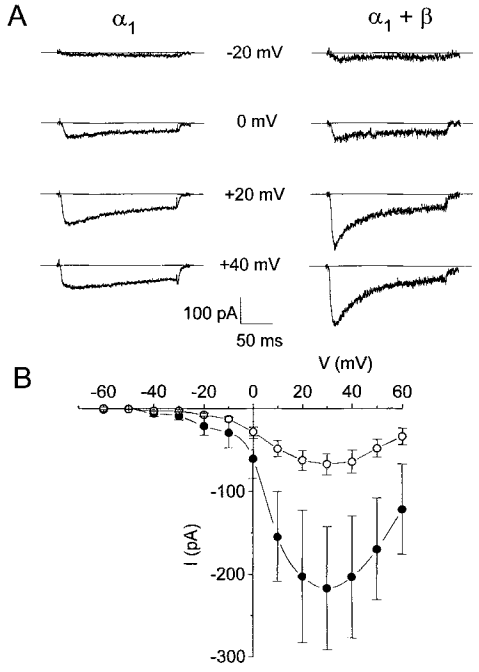

FIGURE 4. (*A*) Current through calcium channels carried by Ca^{2+} in HEK cells transfected with α_{1C} (*left*) or α_{1C}β (*right*). Currents are the result of depolarizations to the membrane potential indicated next to each record from a holding potential of −80 mV. (*B*) Averaged peak current vs voltage relationships of α_{1C} alone (○) or α_{1C}β expressing cells (●). Each point represents five to seven cells. The curves were drawn by eye.

1991; Varadi, Lory, Schultz, Varadi, and Schwartz, 1991; Singer, Biel, Lotan, Flockerzi, Hofmann, and Dascal, 1991; Wei, Pérez-Reyes, Lacerda, Schuster, Brown, and Birnbaumer, 1991; Hullin et al., 1992; Pérez-Reyes, Castellano, Kim, Bertrand, Baggstrom, Lacerda, Wei, and Birnbaumer, 1992). For this reason, we have analyzed in more detail the modulation by β subunit of the macroscopic kinetics of the currents. Fig. 5 plots the voltage dependence of activation and inactivation with 40 mM Ca^{2+} in the external solution. Activation was quantified as the time required to reach 70% of peak amplitude (*A*), while the time course of inactivation was gauged from the percentage of current remaining 50 ms into the depolarizing pulse (*B*). Activation was faster in α_{1C}-transfected cells than in the α_{1C}β-cotransfected cells at positive test potentials. In contrast, the presence of β subunit speeds up inactivation

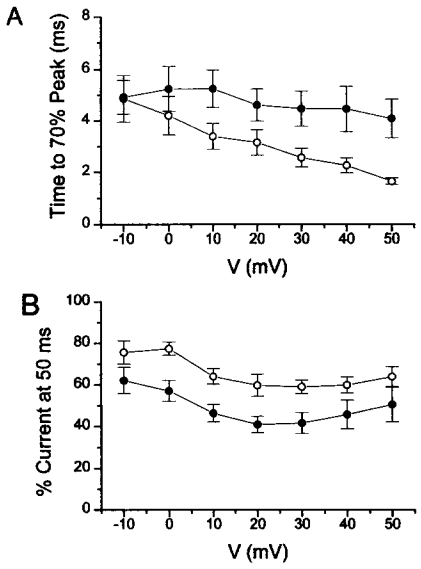

FIGURE 5. Comparison of the macroscopic kinetics of the Ca currents expressed in α_{1C}- (○) or $\alpha_{1C}\beta$-transfected cells (●). (*A*) Voltage dependence of activation. Activation is expressed as the time to 70% of the peak amplitude from the beginning of the depolarizing step and is plotted as a function of the membrane potential. The values with α_{1C} alone are different from those with $\alpha_{1C}\beta$ ($P < 0.05$ by MANOVA). (*B*) Voltage dependence of inactivation. The percentage of current remaining 50 ms into the depolarizing pulse is plotted at various membrane potentials. The two groups differed by MANOVA ($P < 0.02$; n = five to six cells per point).

at all the voltages tested. In both α_{1C}- and $\alpha_{1C}\beta$-transfected cells, the voltage dependence of inactivation is not monotonic but exhibits an "U" shape that may reflect a contribution of Ca^{2+}-dependent inactivation (Eckert and Chad, 1984).

This analysis of the inactivation kinetics does not take into account the differences in current magnitude when comparing α_{1C}- versus $\alpha_{1C}\beta$-expressing cells and consequently does not enable us to elucidate whether the changes in inactivation are simply due to increased Ca^{2+}-dependent inactivation. Fig. 6 addresses this point by plotting the percentage of current remaining at 50 ms in α_{1C}- and $\alpha_{1C}\beta$-transfected cells as a function of the current amplitude at two representative voltages. The data suggest that the increased current amplitude observed in the β-cotransfected cells might not be the only factor involved in their faster inactivation because when the magnitude of the currents is comparable, there is still a tendency towards an increase

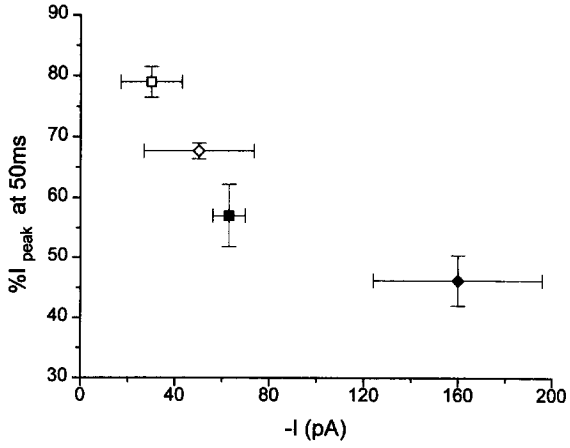

FIGURE 6. Current dependence of the inactivation of Ca currents. The graph shows the percentage of current remaining 50 ms after the onset of a depolarizing step to 0 mV (□, ■) or +10 mV (◇, ◆) as a function of the peak current amplitude in cells transfected with α_{1C} alone (*open symbols*) or $\alpha_{1C}\beta$ (*filled symbols*). Pooled data from four to five cells.

in the speed of inactivation of the $\alpha_{1C}\beta$-coexpressing cells. Intrinsic effects of β subunit coexpression on gating might be more evident with Ba^{2+} as the charge carrier, so this was our next approach.

β Subunit Modulation of Ba Currents

Using Ba^{2+} as the permeant ion, the role of current-dependent inactivation is minimized because the kinetics of the currents are not affected by their absolute magnitude (Lee et al., 1985). Fig. 7 *A* shows representative Ba currents obtained at different potentials in α_{1C} and $\alpha_{1C}\beta$ cells; *B* shows the pooled current-voltage relationships in both conditions. The addition of β subunit leads to an increase in the

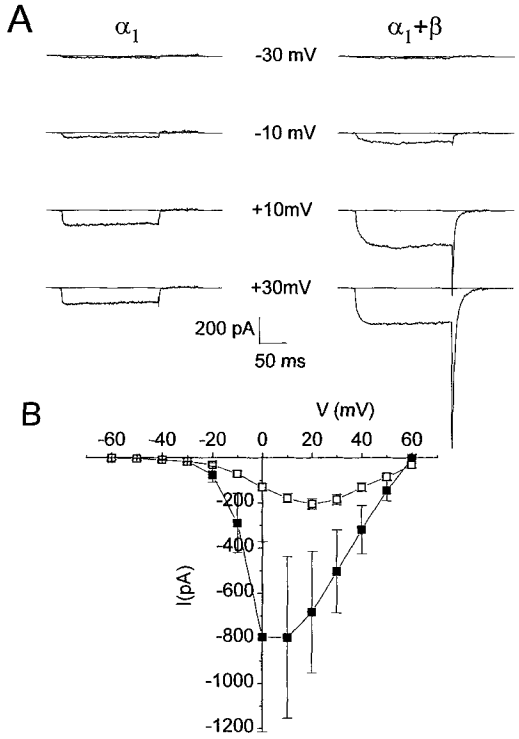

FIGURE 7. (*A*) Time course of the barium currents during 200-ms depolarizing pulses to the indicated membrane potentials through α_{1C} or $\alpha_{1C}\beta$ recombinant Ca channels. (*B*) Peak current amplitude is plotted as a function of the step membrane potential for α_{1C} alone (□) or $\alpha_{1C}\beta$ expressing cells (■). Curves were fitted by eye; each point represents data from five to eight cells.

current amplitude of a magnitude similar to that observed when Ca^{2+} is the permeant ion. There is also a shift of the current-voltage relationship in the hyperpolarizing direction, consistent with previous studies using *Xenopus* oocytes (Wei et al., 1991; Pérez-Reyes et al., 1992; Castellano et al., 1993*a;* Neely, Wei, Olcese, Birnbaumer, and Stefani, 1993). It is noteworthy that Ba currents in the $\alpha_{1C}\beta$-cotransfected cells showed consistently slower decay of the tail currents. Such currents were not resolved in the absence of BAY K 8644 in cells transfected with α_{1C} alone. The observation of tail currents presumably indicates a slowing of channel deactivation and suggests that modulation of gating may be one of the mechanisms involved in the effects of β subunit on current amplitude.

With regards to the DHP sensitivity of the recombinant Ba currents, β subunit coexpression has been reported to reduce or even to eliminate the agonist effect of BAY K 8644 in oocytes and L cells (Varadi et al., 1991; Singer et al., 1991; Hullin et al., 1992), whereas other studies report no modification of the response to BAY K 8644 (Wei et al., 1991; Pérez-Reyes et al., 1992). In our preparation, the application of 1 μM BAY K 8644 to the $\alpha_{1C}\beta$-cotransfected cells produced a smaller increase in current amplitude as compared to that observed in cells transfected with α_{1C} alone. When the effect of BAY K 8644 is expressed as normalized maximal conductance, the drug elicited a 1.53 ± 0.23-fold increase in $\alpha_{1C}\beta$- versus 3.59 ± 0.64-fold in

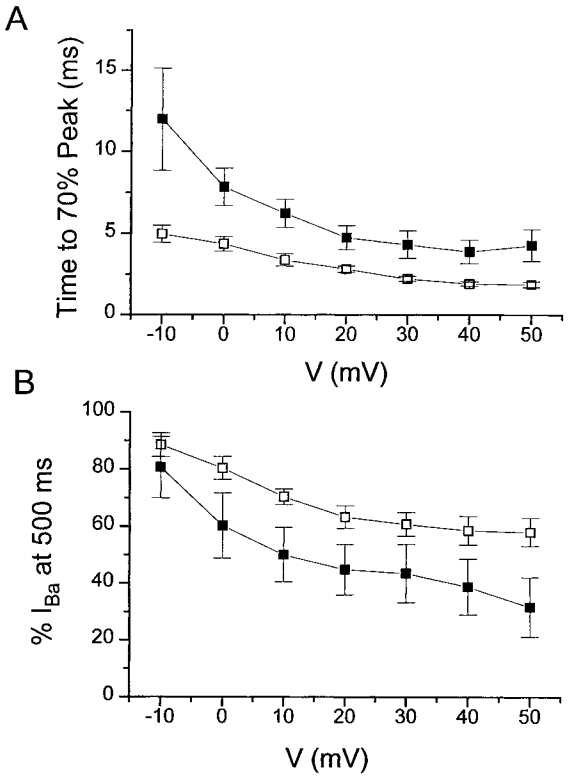

FIGURE 8. Voltage dependence of activation and inactivation of Ba currents. (*A*) The time to 70% of the peak amplitude of the currents expressed in α_{1C} (□) or $\alpha_{1C}\beta$-transfected cells (■) is plotted as a function of the step voltage. Analysis of variance showed a significant difference between the two groups ($P < 0.002$). (*B*) The percentage of current remaining 500 ms into the depolarizing pulse is plotted as a function of membrane potential. The differences between the two conditions were also statistically significant ($P < 0.05$; n = five to eight cells per point).

α_{1C}-transfected cells (mean ± SEM of six and seven cells, respectively). This difference is statistically significant ($P < 0.02$) and supports our previous suggestion that the presence of β subunit might induce changes in basal gating behavior that make the channels appear to be less responsive to DHP agonists.

Effects of β Subunit Coexpression on the Macroscopic Kinetics of Ba Currents

Fig. 8 summarizes the macroscopic gating properties of the Ba currents expressed in α_{1C}- or in $\alpha_{1C}\beta$-transfected cells. Activation is clearly voltage dependent when Ba^{2+} is the permeant ion (Fig. 8 *A*), regardless of the transfection conditions, being faster at

more depolarized potentials. Activation in 40 mM Ba^{2+} becomes slower upon coexpression of β subunit. This effect is most pronounced at the more negative potentials, although the difference was statistically significant at all the voltages tested. Inactivation was quantified by measuring the percentage of current remaining at 500 ms (Fig. 8 *B*) instead of the 50 ms chosen for the study of inactivation when Ca^{2+} carried the charge. In both cases, the proportion of current that inactivated at the different voltages was similar. In 40 mM Ba^{2+}, the inactivation process exhibits a monotonic voltage dependence, and the presence of β subunit produces a significant acceleration in inactivation at positive voltages. These data indicate that cotransfection with β subunit modulates the kinetics of the recombinant channels and lead us to conclude that the modifications of inactivation kinetics observed for Ca currents upon β subunit coexpression involve a direct effect on gating in addition to a simple enhancement of Ca^{2+}-dependent inactivation.

DISCUSSION

Effects of Cardiac Ca Channel α_1 Subunit Expression

Transient transfection of Ca channels in HEK cells enabled us to analyze the macroscopic currents of individual cells expressing α_{1C} or $\alpha_{1C}\beta$. Previous studies indicated the absence of detectable levels of α_1, α_2 or β_1 mRNAs in HEK293 cells by Northern hybridization (Brust, Simerson, McCue, Deal, Schoonmaker, Williams, Veliçelebi, Johnson, Harpold, and Ellis, 1993) or detectable levels of α_{1C} protein by Western blot analysis (M. M. Hosey, personal communication). In addition, we did not find any measurable Ba or Ca currents in mock-transfected HEK cells. Because the currents observed in the transfected cells are not likely to be modulated by any endogenous subunit, expression of α_1 subunit in HEK cells allows us to determine which characteristics of the L-type Ca channels have their basis in this subunit, and coexpression with other Ca channel subunits provides reliable information about their interactions. In agreement with previous reports, we found that α_1 is the channel-forming subunit and contains the binding sites for DHPs (Figs. 1 and 2) and other organic channel blockers (data not shown). In addition, our data indicate that Ca^{2+}-dependent inactivation of the L-type Ca channel is also an intrinsic property of the α_{1C} subunit. This conclusion provides a structural basis for recent electrophysiological data reporting that the high local concentrations of Ca^{2+} required for Ca^{2+}-dependent inactivation can be reached only at short distances from the mouth of the channel (Imredy and Yue, 1992; Shirokov, Levis, Shirokova, and Ríos, 1993), consistent with studies of cardiac Ca channels in lipid bilayers (Rosenberg and Haack, 1994). Welling, Kwan, Bosse, Flockerzi, Hofmann, and Kasset (1993) also found that the α_1 subunit of the vascular smooth muscle L-type Ca channel, stably transfected in CHO cells, sufficed to confer Ca^{2+}-dependent inactivation.

Effects of β Subunit Coexpression on Current Amplitude

Although the α_1 subunit embodies the essential properties of a Ca channel, coexpression with small subunits alters the functional properties of the current. This modulatory role of the non-α_1 subunits in channel activity is universally accepted, but the literature is full of discrepancies regarding the contribution of each of these

subunits to the biophysical properties of the channel. It is likely that most of the variability in the reported results arises from the use of different heterologous expression systems and/or different sources of the subunit-encoding genes. Effects of coexpression of the β subunit with the DHP-sensitive α_1 subunit have been studied using mainly *Xenopus* oocytes (Singer et al., 1991; Wei et al., 1991; Hullin et al., 1992; Pérez-Reyes et al., 1992; Castellano et al., 1993*a*), although there are some studies in mammalian cell lines (Lacerda et al., 1991; Varadi et al., 1991; Lory et al., 1993; Nishimura, Takeshima, Hofmann, Flockerzi, and Imoto, 1993). In all cases, coexpression of any β subunit with α_{1C} results in an increase in the amplitude of the barium currents, within a broad range (from threefold to more than 100-fold). Increases of more than two orders of magnitude were observed when the expression of α_{1C} alone was barely resolvable and probably overestimated the genuine effect of adding β subunit. In our system we observed an increase in the peak current amplitude that is at the low end of the range of those previously reported and was equivalent using either Ca^{2+} or Ba^{2+} as the charge carrier (3.2 and 3.1 times, respectively), even though the absolute magnitude of the currents was about fourfold larger in the presence of Ba^{2+}.

Possible Mechanisms Underlying the Effects of β Subunit Coexpression

The mechanism by which β subunit modulates Ca channel activity is still not clear. Changes in current density can be achieved either by increasing the number of functional Ca channels in the cell surface (by altering membrane trafficking, post-translational maturation or metabolic degradation) or by modulation of the gating of those channels that do reach the cell surface. Coexpression of α_{1C} with β results in a significant increase in the total number of DHP binding sites (B_{max}) without altering the apparent dissociation constant. These results, together with the increase in the magnitude of the macroscopic currents, are consistent with the hypothesis that an increase in the number of functional channels represents one mechanism mediating the effects of β subunit cotransfection. Nevertheless, recent electrophysiological data in *Xenopus* oocytes by Neely et al. (1993) suggest that β subunit does not alter the surface membrane density of α_{1C}. These authors showed that potentiation by β subunit of the ionic current induced by α_{1C} is due to an increase in the open probability of the channel and not to a change in the number of expressed channels (as gauged by gating current density). In support of this interpretation, Nishimura et al. (1993) failed to detect by immunoblotting an increase in the expression of α_{1C} by cotransfection with β subunit in stably transfected CHO cells, even though they found large increases in high-affinity DHP binding sites and in current amplitude in the presence of β subunit. These two groups of workers speculated that β subunit coexpression may induce a shift in the equilibrium of α_1 between two DHP-binding states, one of which has such a low binding affinity that it is not detectable experimentally. Although our data raise the possibility that coexpression of β subunit increases the number of functional Ca channels in HEK cells, some of the experimental findings clearly reveal a modulatory effect of β subunit on the gating of the channel. In this context, the slow decay of the Ba tail currents in the α_{1C}β-cotransfected cells (Fig. 7) may reflect an increase in the number of channels that exhibit the gating mode characterized by long-lasting openings and

brief closures ("mode 2" of Hess, Lansman, and Tsien, 1984). If so, the decreased effectiveness of BAY K 8644 in $\alpha_{1C}\beta$-cotransfected cells may simply reflect a higher proportion of mode 2 activity under drug-free conditions, since the DHP agonists act by favoring this mode of gating (Hess et al., 1984).

Modulatory Effects of β Subunit on Macroscopic Kinetics

The inactivation of L-type Ca channels involves at least two mechanisms, including a fast Ca^{2+}-dependent component and a slower voltage-dependent component (Lee et al., 1985; Hadley and Hume, 1987). In native L-type Ca channels, Ba^{2+} does not substitute for Ca^{2+} in supporting Ca^{2+}-dependent inactivation. Our results with both Ca^{2+} and Ba^{2+} as permeant ions reveal a significant increase in the rate of inactivation over a broad range of voltages. Heterologous expression of $\alpha_{1C}\beta$ in *Xenopus* oocytes does not alter the rate of inactivation of Ba currents as compared with α_{1C} alone (Wei et al., 1991; Singer et al., 1991). However, when the neuronal class C α_1 subunit, which appears to arise from the same gene encoding the cardiac and smooth muscle α_1 subunits, is coexpressed with the skeletal muscle β subunit in oocytes there is an increase in the speed of inactivation without a change in voltage dependence (Tomlinson, Stea, Bourinet, Charnet, Nargeot, and Snutch, 1993). The only study available reporting some effect on the inactivation kinetics when β subunit is coexpressed with α_{1C} in a mammalian cell line was carried out in L cells (Lory et al., 1993); an increase in the rate of inactivation was described, albeit in the presence of BAY K 8644 which complicates the kinetic analysis of the data.

Our findings regarding activation kinetics disagree with previous studies using different expression systems. When comparing the activation kinetics of α_{1C} versus $\alpha_{1C}\beta$ in *Xenopus* oocytes, either no effect (Singer et al., 1991) or an increase in the speed of the activation has been reported, the latter effect in the presence of BAY K 8644 (Wei et al., 1991). Such an increase in the rate of activation in BAY K 8644-containing solutions was also reported in L cells cotransfected with $\alpha_{1C}\beta$ (Lory et al., 1993). Nevertheless, coexpression of α_{1C} with the three other isoforms of the β subunit in oocytes is able to produce an acceleration of activation in DHP-free solutions (Pérez-Reyes et al., 1992; Hullin et al., 1992; Castellano et al., 1993*a*, Castellano, Wei, Birnbaumer, and Peréz-Reyes, 1993*b*). All these studies were carried out using Ba^{2+} as the permeant ion, and in this condition we found a significant *slowing* in the time course of activation at all the voltages tested. The same effect is observed when Ca^{2+} carries the charge.

Taking these results together, it is likely that the difference in the genetic background of the heterologous system used for expressing the Ca channel subunits (*Xenopus* oocytes versus a mammalian cell line such as HEK cells) can explain most of the differences between our study and previous reports on the kinetics of the recombinant Ca channel using the same subunits. For example, the difference in the kinetics of the currents recorded upon expression of α_{1C} alone is remarkable. Whereas in HEK cells the time courses of activation and inactivation are close to those reported in native channels, in *Xenopus* oocytes they are anomalously slow in all the studies. When β subunit is coexpressed, and especially if α_2 is also present, there is a dramatic acceleration of both activation and inactivation in oocytes, which tends to normalize the kinetics of these recombinant channels. Chinese hamster ovary

(CHO) cells can stably and robustly express L-type calcium channels whose properties resemble those of native channels, but so far they have proven to be of little utility in transient expression studies. Comparison of HEK cells with other mammalian expression systems used to study transiently expressed recombinant Ca channels (mainly L cells) shows that they are able to express Ca channels in a more efficient way, thus enabling resolution of the kinetics of currents carried by Ca^{2+} or Ba^{2+} in solutions free of DHP agonists.

The authors thank Melissa Appel for excellent technical assistance. We thank the following individuals for providing cDNAs: T. Tanabe (α_{1C}), D. Slish and A. Schwartz (α_{1C}), K. Campbell (β), and T. Quertermous (β-gal).

This work was supported by grants from NIH (RO1 HL 36957 to E. Marbán), the Howard Hughes Medical Institute (fellowship to T. J. Kamp) and the Fundación Ramón Areces (fellowship to M. T. Pérez-García).

Original version received 26 May 1994 and accepted version received 14 October 1994.

REFERENCES

Barish, M. E. 1983. A transient calcium-dependent chloride current in the inmature *Xenopus* oocyte. *Journal of Physiology.* 342:309–325.

Bean, B. P. 1984. Nitrendipine block of cardiac calcium channels: high affinity binding to the inactivated state. *Proceedings of the National Academy of Science, USA.* 81:6388–6392.

Biel, M., P. Ruth, E. Bosse, W. Stühmer, V. Flockerzi, and F. Hofmann. 1990. Primary structure and functional expression of a high voltage activated calcium channel from rabbit lung. *FEBS Letters.* 269:409–412.

Bosse, E., R. Bottlender, T. Kleppisch, J. Hescheler, A. Welling, F. Hofmann, and V. Flockerzi. 1992. Stable and functional expression of the calcium channel α_1 subunit from smooth muscle in somatic cell lines. *EMBO Journal.* 11:2033–2038.

Brehm, P., and R. Eckert. 1978. Calcium entry leads to inactivation of calcium channel in *Paramecium. Science.* 202:1203–1206.

Brust, P. F., S. Simerson, A. F. McCue, C. R. Deal, S. Schoonmaker, M. E. Williams, G. Veliçelebi, E. C. Johnson, M. M. Harpold, and S. B. Ellis. 1993. Human neuronal voltage-dependent calcium channels: studies on subunit structure and role in channel assembly. *Neuropharmacology.* 32:1089–1102.

Castellano, A., X. Wei, L. Birnbaumer, and E. Peréz-Reyes. 1993*a*. Cloning and expression of a third calcium channel β subunit. *Journal of Biological Chemistry.* 268:3450–3455.

Castellano, A., X. Wei, L. Birnbaumer, and E. Peréz-Reyes. 1993*b*. Cloning and expression of a neuronal calcium channel β subunit. *Journal of Biological Chemistry.* 268:12359–12366.

Catterall, W. A. 1988. Structure and function of voltage-sensitive ion channels. *Science.* 242:50–61.

Eckert, R., and J. E. Chad. 1984. Inactivation of Ca channels. *Progress in Biophysics and Molecular Biology.* 44:215–267.

Gutnick, M. J., H. D. Lux, D. Swandulla, and H. Zucker. 1989. Voltage-dependent and calcium-dependent inactivation of calcium channel current in identified snail neurones. *Journal of Physiology.* 412:197–220.

Hadley, R. W., and J. R. Hume. 1987. An intrinsic potential-dependent inactivation mechanism associated with calcium channels in guinea-pig myocytes. *Journal of Physiology.* 389:205–222.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recordings from cells and cell-free membrane patches. *Pflügers Archiv.* 391:85–100.

Hess, P., J. B. Lansman, and R. W. Tsien. 1984. Different modes of Ca^{2+} channel gating behavior favored by dihydropyridine Ca^{2+} agonists and antagonists. *Nature.* 311:538–544.

Hullin R., D. Singer-Lahat, M. Freichel, M. Biel, N. Dascal, F. Hofmann, and V. Flockerzi. 1992. Calcium channel β subunit heterogeneity: functional expression of cloned DNA from heart, aorta and brain. *EMBO Journal.* 11:885–890.

Imredy, J. P., and D. T. Yue. 1992. Submicroscopic Ca^{2+} diffusion mediates inhibitory coupling between individual Ca channels. *Neuron.* 9:197–209.

Krizanova, O., R. Diebold, P. Lory, and A. Schwartz. 1993. Molecular aspects and diversity of voltage-dependent calcium channels. *Circulation.* 84(Suppl. VII):VII44–VII48.

Lacerda, A. E., H. S. Kim, P. Ruth, E. Perez-Reyes, V. Flockerzi, F. Hofmann, L. Birnbaumer, and A. M. Brown. 1991. Normalization of current kinetics by interaction between α_1 and β subunits of the skeletal muscle dihydropyridine-sensitive Ca^{2+} channel. *Nature.* 352:527–530.

Lee, K. S., E. Marbán, and R. W. Tsien. 1985. Inactivation of calcium channels in mammalian heart cells: joint dependence on membrane potential and intracellular calcium. *Journal of Physiology.* 364:395–411.

Lory, P., G. Varady, D. Slish, M. Varadi, and A. Schwartz. 1993. Characterization of β subunit modulation of a rabbit cardiac L-type Ca^{2+} channel α_1 subunit as expressed in mouse L cells. *FEBS Letters.* 315:167–172.

Mikami, A., K. Imoto, T. Tanabe, T. Niidome, Y. Mori, H. Takeshima, S. Narumiya, and S. Numa. 1989. Primary structure and functional expression of the cardiac dihydropyridine-sensitive calcium channel. *Nature.* 340:230–233.

Neely, A., X. Wei, R. Olcese, L. Birnbaumer, and E. Stefani. 1993. Potentiation by the β subunit of the ratio of the ionic current to the charge movement in the cardiac calcium channel. *Science.* 262:575–577.

Nishimura, S., H. Takeshima, F. Hofmann, V. Flockerzi, and K. Imoto. 1993. Requirement of the calcium channel β subunit for functional conformation. *FEBS Letters.* 324:283–286.

Pérez-Reyes, E., A. Castellano, H. S. Kim, P. Bertrand, E. Baggstrom, A. E. Lacerda, X. Wei, and L. Birnbaumer. 1992. Cloning and expression of a cardiac/brain β subunit of the L-type calcium channel. *Journal of Biological Chemistry.* 267:1792–1797.

Pérez-Reyes, E., H. S. Kim, A. E. Lacerda, W. Horne, X. Wei, D. Rampe, K. P. Campbell, A. M. Brown and L. Birnbaumer. 1989. Induction of calcium currents by the expression of the α_1 subunit of the dihydropyridine receptor from skeletal muscle. *Nature.* 340:233–236.

Pérez-Reyes, E., X. Wei, A. Castellano, and L. Birnbaumer. 1990. Molecular diversity of L-type calcium channels. *Journal of Biological Chemistry.* 265:20430–20436.

Pragnell, M., J. Sakamoto, S. D. Jay, and K. P. Campbell. 1991. Cloning and tissue-specific expression of the brain calcium channel β-subunit. *FEBS Letters.* 291:253–258.

Rose, W. C., C. W. Balke, W. G. Wier, and E. Marbán. 1992. Macroscopic and unitary properties of physiological ion flux through L-type Ca^{2+} channels in guinea-pig heart cells. *Journal of Physiology.* 456:267–284.

Rosenberg, R. L. and J. A. Haack. 1994. Calcium-dependent inactivation of L-type calcium channels in planar lipid bilayers. *Biophysical Journal.* 66:1051–1060.

Sanguinetti, M. C., and R. S. Kass. 1984. Voltage-dependent block of calcium channel current in the calf cardiac Purkinje fiber by dihydropyridine calcium channel antagonists. *Circulation Research.* 55:336–346.

Sanguinetti, M. C., D. S. Krafte, and R. S. Kass. 1986. Voltage-dependent modulation of Ca^{2+} channel current in heart cells by BAY K8644. *Journal of General Physiology.* 88:369–392.

Shater, W. A., T. Tanabe, J.-F. Zhang, Y. Mori, M. E. Adams, and R. W. Tsien. 1993. Distinctive biophysical and pharmacological properties of class A (BI) calcium channel α_1 subunits. *Neuron.* 11:291–303.

Shirokov, R., R. Levis, N. Shirokova, and E. Ríos. 1993. Ca^{2+}-dependent inactivation of cardiac L-type Ca^{2+} channels does not affect their voltage sensor. *Journal of General Physiology.* 102:1005–1030.

Singer, D., M. Biel, I. Lotan, V. Flockerzi, F. Hofmann, and N. Dascal. 1991. The roles of the subunits in the function of the calcium channel. *Science.* 253:1553–1557.

Slish, D. F., D. B. Engle, G. Varady, I. Lotan, D. Singer, N. Dascal, and A. Schwartz. 1989. Evidence for the existence of a cardiac specific isoform of the α_1 subunit of the voltage dependent calcium channel. *FEBS Letters.* 250:509–514.

Snutch, T. P., and P. B. Reiner. 1992. Ca^{2+} channels: diversity of form and function. *Current Opinion in Neurobiology.* 2:247–253.

Snutch, T. P., J. P. Leonard, M. M. Gilbert, H. A. Lester, and N. Davidson. 1990. Rat brain expresses an heterologous family of calcium channels. *Proceedings of the National Academy of Science, USA.* 87:3391–3395.

Takahashi, M., M. J. Seagar, J. F. Jones, B. F. X. Reber, and W. A. Catterall. 1987. Subunit structure of dihydropyridine-sensitive calcium channels from skeletal muscle. *Proceedings of the National Academy of Science, USA.* 84:5478–5482.

Tanabe, T., K. B. Beam, J. A. Powell, and S. Numa. 1988. Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. *Nature.* 336:134–139.

Tanabe, T., H. Takeshima, A. Mikami, V. Flockerzi, H. Takahashi, K. Kangawa, M. Kojima, H. Matsuo, T. Hirose, and S. Numa. 1987. Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature.* 328:313–318.

Tomlinson, W. J., A. Stea, E. Bourinet, P. Charnet, J. Nargeot, and T. P. Snutch. 1993. Functional properties of a neuronal class C L-type calcium channel. *Neuropharmacology.* 32:1117–1126.

Tsien, R. W., P. T. Ellinor, and W. A. Horne. 1991. Molecular diversity of voltage-dependent Ca^{2+} channels. *Trends in Pharmacological Sciences.* 12:349–354.

Varadi, G., P. Lory, D. Schultz, M. Varadi, and A. Schwartz. 1991. Acceleration of activation and inactivation by the β subunit of the skeletal muscle calcium channel. *Nature.* 352:159–162.

Wei, X., E. Perez-Reyes, A. E. Lacerda, G. Schuster, A. M. Brown, and L. Birnbaumer. 1991. Heterologous regulation of the cardiac Ca^{2+} channel α_1 subunit by skeletal muscle β and γ subunits. *Journal of Biological Chemistry.* 266:21943–21947.

Welling, A., Y. W. Kwan, E. Bosse, V. Flockerzi, F. Hofmann, and R. S. Kass. 1993. Subunit-dependent modulation of recombinant L-type calcium channels. *Circulation Research.* 73:974–980.

Wigler, M., A. Pellicer, S. Silverstein, and R. Axel. 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. *Cell.* 14:725–731.

Zhang, J. F., A. D. Randall, P. T. Ellinor, W. A. Horne, W. A. Shater, T. Tanabe, T. L. Schwarz, and R. W. Tsien. 1993. Distinctive pharmacology and kinetics of cloned neuronal Ca^{2+} channels and their possible counterparts in mammalian CNS neurons. *Neuropharmacology.* 32:1075–1088.

Zong, S., J. Zhou, and T. Tanabe. 1994. Molecular determinants of calcium-dependent inactivation in cardiac L-type calcium channels. *Biochemical and Biophysical Research Communications.* 201:1117–1123.