Multiple Modulation Pathways of Calcium Channel Activity by a β Subunit

DIRECT EVIDENCE OF β SUBUNIT PARTICIPATION IN MEMBRANE TRAFFICKING OF THE α1C SUBUNIT*

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In order to study the precise mechanisms of α1 subunit modulation by an auxiliary β subunit of voltage-dependent calcium channels, a recombinant β2a subunit fusion protein was produced and introduced into oocytes that express the human α1C subunit. Injection of the β2 subunit protein rapidly modulated the current kinetics and voltage dependence of activation, whereas massive augmentation of peak current amplitude occurred over a longer time scale. Consistent with the latter, a several-fold increase in the amount of the α1C subunit in the plasma membrane was detected by quantitative confocal laser-scanning microscopy after β2a subunit injection. Pretreatment of oocytes with bafilomycin A1, a vacuolar type H+ -ATPase inhibitor, abolished the increase of the α1C subunit in the plasma membrane, attenuated current increase, but did not affect the modulation of current kinetics and voltage dependence by the β2a subunit. These results provide clear evidence that the β subunit modifies the calcium channel complex in a binary fashion; one is an allosteric modulation of the α1 subunit function and the other is a chaperoning of the α1 subunit to the plasma membrane.

Voltage-gated calcium channels are heteromultimeric protein complexes that consist of at least three (α1, α2/δ, and β) subunits and play a central role in diverse biological functions such as excitation-contraction coupling, excitation-secretion coupling, neurotransmitter release, and regulation of gene expression. The α1 subunit accommodates the channel pore, voltage sensor, and binding sites for various channel-modifying compounds. It has also been shown that the basic characteristics of the different channel types (L-, N-, P-, Q-, and R-type) are carried by the corresponding α1 subunits (1). Auxiliary subunits (α2/δ, β, and γ) modulate calcium channel characteristics, such as current amplitude, voltage dependence, and kinetics of activation and inactivation as well as sensitivity to calcium channel antagonists (2–4).

The modulatory effects of the β subunit on the Ca2+ channel complex have been extensively studied using coexpression of cDNAs in heterologous systems. It is well established that coexpression of the α1 subunit with a β subunit results in an increase of peak current density (5), acceleration of activation and inactivation kinetics, a leftward shift of the current-voltage relationship, and increased dihydropyridine (DHP)1 binding activity (6–12). However, there have been inconsistencies in the reported mechanism(s) by which these effects occur. Varadi et al. (6) reported a 10-fold increase in the number of DHP-binding sites by coexpression of the α1 subunit with the β subunit, suggesting an increase in available channels within the plasma membrane. In contrast, an increase in current amplitude without affecting charge movement by β subunit coexpression was shown by Neely et al. (8). These same authors later reported two modes of activation of the α1C subunit (13). Coexpression of a β subunit potentiated current by an increase of the fast-activating component, an acceleration of the slow component, and a larger proportion of long openings. An increase in DHP binding and current density without a change in the amount of α1 subunit protein in the plasma membrane was reported by Nishimura et al. (10). These reports suggest that the β subunit modulates channel properties by “assisting” the α1 subunit in establishing a proper conformation suitable for a functional Ca2+ channel, rather than affecting expression, trafficking, or stability of the α1 subunit (reviewed by Catterall (14)).

By using immunocytochemical methods, Chien et al. (15) showed that the β2a subunit acts as a chaperone-like molecule to facilitate membrane targeting of the α1C subunit, without affecting the total amount of expressed α1C subunit in human embryonic kidney cells. Coexpression of the α1C subunit with β2a resulted in a marked increase in localization of the α1C subunit to the plasma membrane. These observations were confirmed by Brice et al. (16) who coexpressed the α1A with α2/δ and several different β subunits. By using Xenopus oocytes as an expression system, Shistik et al. (17) reported opposite results in that no change in the amount of α1 subunit protein was found in the plasma membrane when coexpressed with a β subunit. Interestingly, an increase in charge movement has been reported when α1C and β subunits were coexpressed in a mammalian cell system (18, 19). These discrepancies have been attributed to differences in expression systems employed (20). Taken together, the mechanisms by which the β subunit modulates calcium channel activity remain unclear. The limitation

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* The abbreviations used are: DHP, dihydropyridine; CLSM, confocal laser-scanning microscopy; V-ATPase, vacuolar type H+ -ATPase; IPTG, isopropyl β-d-thiogalactopyranoside; GST, glutathione S-transferase; AID, α1 interaction domain; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

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of previous studies are that they were all done using coexpression of the α1 and β subunits, which makes it difficult to answer the question as to which level of the biosynthesis of the channel complex is the β subunit working, i.e., translation, trafficking, and/or direct binding to the membrane-incorporated α1 subunit.

We address these questions by injecting a recombinant β subunit protein into Xenopus oocytes expressing the α1C subunit and observing the time course of modulation. We found that changes in voltage dependence of activation and kinetics occurred at an earlier stage in the time course, compared with increases in current amplitude which required a substantially longer time to reach plateau. This suggests that the effects are functionally uncoupled and occur by distinct mechanisms. By using confocal laser-scanning microscopy (CLSM), we found that the amount of the α1 subunit in the plasma membrane was substantially increased by the β subunit. Furthermore, pre-treatment of oocytes with bafilomycin A1, a vacuolar type H+-ATPase (V-ATPase) inhibitor (21), inhibited the β subunit on current amplitude, abolished the increase in channel amount in the plasma membrane, but did not influence the β effects on voltage dependence and current kinetics.

**EXPERIMENTAL PROCEDURES**

**Bacterial Production and Purification of β Subunit Fusion Protein**—The human calcium channel β1c DNA clone (22) was subcloned into pBluescript SK- (+) between the HindIII and BamHI sites. This plasmid was cleaved with HindIII, and the protruding end was filled in with T4 DNA polymerase and then cut with BamHI to liberate the β1c-coding fragment. The pET15b(+) vector (Novagen) was cleaved with NdeI, filled in with T4 DNA polymerase, and then cut with BamHI. Finally, the β1c fragment was ligated into the blunt end/BamHI sites. This strategy has generated an in-frame cloning of the β1c protein with the His6-tag sequence and resulted in a fusion product that had the following peptide fused to the C-terminal sequence of the human β1c protein: MGSSHHHHHSSGLVPRGSHKLDP. The sequence of the construct was verified by sequencing through the junction regions.

*Escherichia coli* BL21(AD3) (Novagen) cells were transformed with the above construct and used for mass production of the His6-tagged β1c fusion protein. The cells were cultured in 500 ml of LB medium in the presence of 100 μg/ml ampicillin, oocytes were injected with 50 nl of the β1c subunit fusion protein solution (90 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, 10 mM EDTA, pH 7.4, with Tris) or vehicle (same composition except protein). The final concentrations in the oocytes were 300 mM β1c subunit fusion protein, 4.5 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, 0.5 mM EDTA, assuming the volume of oocytes was 1 μl.

**Electrophysiology—Xenopus laevis oocytes (stage V–VI)** were prepared as described previously (26). Capped cRNA was synthesized from XboI-linearized human heart L-type calcium channel α1 subunit DNA template (23) and injected into the oocytes (50 nl, 0.2 μg/μl). After 4–5 days of incubation at 19 °C in the solution containing (in mM) 96 NaCl, 2 KCl, 1 MgCl2, 1.8 CaCl2, 5 HEPES, 2.5 sodium pyruvate, 0.5 theophylline, pH 7.5, supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin, oocytes were injected with 50 nl of either β1c subunit fusion protein solution (90 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, 10 mM EDTA, 340 ng/ml protein, pH 7.4, with Tris) or vehicle (same composition except protein). The final concentrations in the oocytes were 300 mM β1c subunit fusion protein, 4.5 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, 0.5 mM EDTA, assuming the volume of oocytes was 1 μl. We found this was a substantial amount of protein since in our preliminary experiments, higher concentrations did not result in greater effects. In the experiments indicated, oocytes were preincubated in a solution containing 500 mM N-ethylmaleimide for 2–3 h prior to the injection of the protein. After injection, they were further incubated in the presence of N-ethylmaleimide until currents were measured. N-ethylmaleimide was dissolved in dimethyl sulfoxide as a 1 M stock solution. The final concentration of dimethyl sulfoxide was 0.05%, which had no effect on the currents or the β effect.

The currents were recorded 1–4 h after injection with the β protein or vehicle using the standard two-electrode voltage-clamp technique at room temperature (20–21 °C). One oocyte was used for only one recording. The recording medium contained (in mM): 40 Ba(OH)2, 50 N-methyl-D-glucamine, 2 KOH, 5 HEPES, 0.5 niphoside, pH 7.4, with methanesulfonic acid. The voltage recording electrode and the current injection electrode were filled with 3 M KCl and had resistances of 0.5–1 MΩ. Currents were recorded using an Axoclamp-2A (Axon Instruments) amplifier. Pulses were applied from a holding potential of −80 mV every 15 s. Whole cell leakage and capacitative currents were digitally subtracted using the P4 protocol. Data were filtered at 1 kHz, sampled at 10 kHz and stored on a KDH disk. Batch analysis showed significant (>20 nA) endogenous Ca2+ channel current were excluded from the analysis. The unpaired t test and analysis of variance were performed for the statistical analyses.

**Immunofluorescence Studies**—DNA sequence complementary to the YYPDVFDDYA epitope sequence of influenza virus hemagglutinin (HA) (27), which is recognized by the 12CA5 mouse monoclonal antibody, was
introduced to the N terminus immediately after the initiator methionine of human heart L-type calcium channel α1C subunit DNA by PCR-based mutagenesis. The cRNA of the epitope-tagged α1 subunit was transcribed and injected into X. laevis oocytes to express tagged channels. The functional characteristics of HA epitope-tagged α1 subunit were tested in a separate set of experiments, and we found that the expressed currents and modulation of them by the β3 subunit were indistinguishable from those of wild type (data not shown). After 4–5 days of incubation, oocytes were injected with the β3 subunit fusion protein as described above. Four hours after injection, oocytes were fixed with 3.7% formaldehyde, 0.25% glutaraldehyde, and permeabilized with 0.2% Triton X-100 at room temperature. Oocytes were then post-fixed in 1% methanol at −20 °C overnight, incubated with 2% bovine serum albumin for 1 h at room temperature, followed by incubation with 10 μg/ml fluorescein-conjugated anti-HA monoclonal antibody (Boehringer Mannheim) at 4 °C overnight. Oocytes were then washed extensively in PBS and examined by CLSM.

RESULTS

Production and Purification of a Recombinant Human β3 Subunit Fusion Protein—The human β3 subunit fusion protein was purified from the soluble fraction of a bacterial culture induced with IPTG at 23 °C for 8 h. The soluble fraction was then applied to a Ni2+–agarose column and elution profile of proteins. B, purity of the human β3-chromatographic fractions. Lanes 1 and 8, molecular weight markers; lane 2, P12 fraction; lane 3, P12; lane 4, S fraction; lane 5, flow-through; lane 6, wash with 60 mM imidazole; lane 7, pure β3 eluted with 1 M imidazole + 0.5 M NaCl. 5 μl of fractions were separated on 10–20% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue.

Electrophysiological Properties of Expressed Human α1C Subunit in Xenopus Oocytes and Time-dependent Effects of Injected Human β3 Subunit Fusion Protein—Oocytes were injected with human α1C subunit cRNA and incubated for 4–5 days. The control (α1C subunit alone) Ca2+ channels expressed in oocytes showed peak barium currents of 149 ± 14 nA (n = 14) when depolarized from a holding potential of −80 mV to test potentials between −30 and +60 mV. The control current exhibited a slow activation and very little inactivation (Fig. 2A). In addition, these results indicate that the β3 subunit did not interact with the control GST, II–III, and the III–IV loops. A sample of the eluate was run on an 4–15% SDS-PAGE gel and stained with Coomassie Brilliant Blue to determine whether the purified fusion protein could also be eluted from the glutathione-Sepharose. All four GST fusion proteins were purified and present in the binding assay eliminating the possibility of nonspecific interaction with other protein components (Fig. 2B). These experiments confirm that the His6-tagged β3 subunit maintains its ability to interact with the I–II loop of the α1C subunit in vitro.

FIG. 1. Purification of human β3 subunit fusion protein. A, purification on Ni2+–agarose column and elution profile of proteins. B, purity of the human β3-chromatographic fractions. Lanes 1 and 8, molecular weight markers; lane 2, P12 fraction; lane 3, P12; lane 4, S fraction; lane 5, flow-through; lane 6, wash with 60 mM imidazole; lane 7, pure β3 eluted with 1 M imidazole + 0.5 M NaCl. 5 μl of fractions were separated on 10–20% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue.

In Vitro Binding of the β3 Subunit to the I–II Intracellular Loop—To determine whether the recombinant His6-tagged β3 subunit was able to interact with the calcium channel intracellular I–II loop, as identified by Pragnell et al. (28), we created GST fusion proteins of the I–II, II–III, and III–IV loops of the α1C subunit and screened with an in vitro transcribed and translated, 35S-labeled His6-tagged β3 subunit. The data from these experiments clearly show that the His6-tagged β3 subunit is able to interact with the I–II loop in a highly specific manner (Fig. 2A). In addition, these results indicate that the β3 subunit did not interact with the control GST, II–III, and the III–IV loops. A sample of the eluate was run on an 4–15% SDS-PAGE gel and stained with Coomassie Brilliant Blue to determine whether the purified fusion protein could also be eluted from the glutathione-Sepharose. All four GST fusion proteins were purified and present in the binding assay eliminating the possibility of nonspecific interaction with other protein components (Fig. 2B). These experiments confirm that the His6-tagged β3 subunit maintains its ability to interact with the I–II loop of the α1C subunit in vitro.

FIG. 2. Determining human β3 subunit interaction with the calcium channel intracellular loops. Elution of GST-bound fusion proteins from glutathione-Sepharose resulted in a sample that was split in half and run simultaneously on separate gels. The first gel was used for autoradiography, and the other was stained with Coomassie Brilliant Blue. A, shown is the autoradiogram of a 4–15% SDS-PAGE gel run with samples of elute from GST fusion proteins screened with an in vitro transcribed and translated 35S-labeled β3 subunit. Lane 1, aliquot of 35S-labeled β3; lane 2, GST control; lane 3, I–II loop; lane 4, II–III loop; lane 5, III–IV loop. B, displays a 4–15% SDS-PAGE gel stained with Coomassie Brilliant Blue. Lane 2, GST-control; lane 3, I–II loop, lane 4, II–III loop, lane 5, III–IV loop.
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in activation and inactivation kinetics occurred in a time-dependent manner (Fig. 3A). After injection of the \( \beta_3 \) subunit fusion protein the activation threshold shifted to hyperpolarizing potentials between \(-30\) and \(-20\) mV, and the current peaked between \(+20\) and \(+30\) mV. The current-voltage (I–V) relationships corresponding to the current traces depicted in Fig. 3A are superimposed in Fig. 3B. Most notably, the shift of the I–V curve occurs within 2 h, whereas the increase in current amplitude requires a longer time scale.

Effects of \( \beta_3 \) Subunit Fusion Protein on Peak Current Amplification

Fig. 3. Effects of \( \beta_3 \) subunit fusion protein on the Ba\(^{2+}\) current through the expressed \( \alpha_{1C} \) subunit. A, the representative whole cell current traces for the control (\( \alpha_{1C} \) subunit alone) and after the injection of \( \beta_3 \) subunit fusion protein or vehicle are shown. All traces in this figure were recorded from the same batch of oocytes. Currents were elicited by 1000-ms command pulses from a holding potential of \(-80\) mV to indicated potentials. B, current-voltage relationship corresponding to the traces shown in A. The peak Ba\(^{2+}\) current amplitude was plotted against each test potential. The curve for vehicle-injected oocyte is not shown in order to keep clarity.
However, these data were not statistically different (versus Bafiments. Error bars of Currents were elicited by depolarizing pulses from a holding potential reaching a plateau at 3 h (6.5- and 6.6-fold for 3 and 4 h after injection, respectively). The effect of the β3 subunit on the current amplitude is clearly slower than the effect on the voltage dependence of activation (cf. Fig. 5). The time of the half-maximal effect for the increase in current amplitude was between 2 and 3 h. Injection of the vehicle had no significant effect on peak current amplitude, although after 4 h the current amplitude appeared to be smaller in some cases. We attribute this to a nonspecific mechanistic disruption by the injection itself.

It has been established that the V-ATPase is responsible for the maintenance of the luminal acidic environment within cell organelles including the Golgi complex, lysosomes, and endosomes (29). Inhibition of the V-ATPase by bafilomycin A1 impairs the intracellualar transportation of glycoproteins via alkalinization of the organelles (30). Thus, if the β3 subunit assists the translocation of the α1 subunit to the plasma membrane, inhibition of this pathway may be imposed by consequences on one or all of the auxiliary β subunit modulatory function(s).

Therefore, we pretreated oocytes with bafilomycin A1 to determine whether the β subunit participates in the intracellular translocation of the α1C subunit. Pretreatment of oocytes expressing the α1C subunit with bafilomycin A1 for 2–3 h showed a slight decrease in the control (α1C subunit alone) current (114 ± 14 nA, n = 7) compared with the untreated control. However, these data were not statistically different (p = 0.13 versus untreated control). The effect of the β2 subunit protein on peak current amplitude was effectively blocked by preincubation with bafilomycin A1 prior to subunit injection. Only 1.5-, 1.8-, 1.85-, and 1.7-fold current increase was observed at 1–4 h after injection of the β3 subunit protein, respectively.

**Effects of β3 Subunit Protein on the Voltage Dependence of Activation**—Steady-state activation curves were derived from I–V relationships, and the β3 subunit effect on the voltage dependence of activation was further analyzed (Fig. 5A). Control currents showed a shallow activation curve with a half-activation potential of 29.9 ± 1.5 mV (n = 14). Injection of the β3 subunit protein caused a leftward shift of the curve and an increase in the slope. Most dramatic changes occurred within 1 h, and no significant changes were observed between 2, 3, and 4 h after injection. As shown in Fig. 5B, ~70% of the negative shift in the half-activation potential occurred within 1 h after injection, reaching a plateau at 2 h. The time of the half-maximal effect was less than 1 h. The vehicle had no effect on the voltage dependence of activation. Bafilomycin A1 treatment did not influence the ability of the β3 subunit to shift the half-activation potential in the negative direction (Fig. 5B).

**Effects of the β3 Subunit Protein on Current Kinetics**—We measured the time to half-peak current as a parameter of the macroscopic activation kinetics. As shown in Fig. 5A, the control (α1C subunit alone) current showed slow activation. When depolarized to +30 mV from a holding potential of −80 mV, the time to half-peak was 19.2 ± 1.0 ms (n = 9) (Fig. 6A). The injection of oocytes with the β subunit protein rapidly facilitated activation kinetics, reaching a plateau within 2 h, with the time of the half-maximal effect occurring within 1 h. The vehicle did not change activation kinetics. Pretreatment of

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Fig. 6. Time-dependent effect of β3 subunit fusion protein on the current kinetics. A, time courses of the β subunit effect on the activation kinetics. Time to half-peak current was measured in current traces that were induced in response to the depolarizing pulses from −80 to 30 mV. Averaged value from 4 to 9 experiments are shown. Error bars show S.E. Baf, bafilomycin A1-treated group. *, p < 0.05 versus vehicle injected group. B, time courses of the β subunit effect on the inactivation kinetics. Inactivated fraction at 1 s after the beginning of the test pulse (−80 to 30 mV) was measured. Data points are mean values of 4–16 experiments. Error bars show S.E. Baf, bafilomycin A1-treated group. *, p < 0.05 versus vehicle injected group.

We also investigated the kinetics of inactivation for macroscopic Ba2+ currents. The rate of inactivation was quantitated as the inactivated fraction of Ba2+ current 1 s after the application of a depolarizing test pulse. In control oocytes, the current elicited by the α1 subunit alone showed very little or no inactivation (see Fig. 3A). After injection with the β3 subunit protein the current exhibited a time-dependent faster inactivation (Fig. 6B), similar to the time-dependent changes of voltage dependence and activation kinetics. Again, changes occurred maximally within 2 h, with the time of the half-maximal effect being less than 1 h. The vehicle did not significantly affect the inactivation of Ba2+ current. In addition, pretreatment of oocytes with bafilomycin A1 did not influence the control current nor the effect of the β subunit protein on inactivation kinetics (Fig. 6B), suggesting this mechanism of modulation is independent of protein translocation.

β Subunit Protein Increases the Amount of α1, Subunit in the Plasma Membrane—We tested whether the injection of the β3 subunit protein into oocytes promoted α1C protein delivery to the plasma membrane. The HA epitope-tagged α1C subunit was expressed in Xenopus oocytes that were then injected with the β3 subunit protein. We found no difference in either the characteristics of Ba2+ current or effects of the β3 subunit protein between the wild-type channels and epitope-tagged channels. The epitope-tagged α1C subunits were detected in the plasma membrane immunocytochemically using CLSM and quantitated by measuring the pixel intensity. When the epitope-tagged α1C was expressed alone in Xenopus oocytes, we observed very little fluorescence in the plasma membrane. In the presence of the β3 subunit protein, the amount of the α1C subunit in the plasma membrane increased severalfold (Fig. 7). Furthermore, pretreatment of oocytes with bafilomycin A1 completely abolished this increase, strongly suggesting that this process is dependent on protein translocation. We obtained similar results in 10–15 oocytes for each group over two different batches of oocytes.

DISCUSSION

Modulation of calcium channel activity by auxiliary subunits has been extensively studied using many different recombinant expression systems. These studies have clearly demonstrated that coexpression of the β subunit alters calcium channel characteristics by increasing current density, shifting the voltage dependence of activation and accelerating channel kinetics. Taken together, these modulatory alterations are believed to impart the inherent current characteristics observed in native preparations. An issue that remains unresolved is whether the β subunit increases the amount of the α1 subunit in the plasma membrane or modifies calcium channel functions solely through an allosteric pathway. Experimental observations to date present conflicting results. When the α1 subunit was coexpressed with the β subunit in Xenopus oocytes, no change in channel expression was observed, when measured as a function of gating charge movement (8). Moreover, in the same experimental system, the amount of 35S-labeled α1 subunit in the plasma membrane did not change (17). Furthermore, Nishimura et al. (10) have also shown no change in the α1 subunit content of membrane fractions by immunoblotting analysis of cells transfected with the β subunit. In contrast, several studies using mammalian cells have shown an increase in charge movement when transfected with β (18, 19) and the involvement of the β subunit in translocation of the α1 subunit to the membrane (15, 16). However, the biochemical mechanism(s) responsible for this modulation have remained unclear due to insufficient experimental methods to monitor time-dependent biological events occurring intracellularly. In an effort to overcome this limitation, and to resolve apparent conflicting data, we expressed the L-type calcium channel α1 subunit in oocytes, and after injecting a highly purified recombinant β3 subunit protein, we examined the time-dependent changes to peak current, voltage dependence, and kinetics. Our results demonstrate for the first time a time-dependent uncoupling of β3 subunit modulation of voltage dependence and kinetics from enhancement of peak current density. Moreover, these results suggest that β subunit modulation occurs via an allosteric mechanism and through facilitation of protein translocation.

β3 Subunit Binding to the I–II Intracellular Loop—In order to determine whether the His6-tagged β3 subunit interacts with the α1 interaction domain (AID) of the intracellular I–II loop, as described by Pragnell et al. (28), we screened GST fusion proteins containing each of the three intracellular loops I–II, II–III, and III–IV with an 35S-labeled His6-tagged β3 subunit. We found that the β3 subunit was able to interact with the I–II loop, suggesting that the presence of six histidine residues at N terminus does not interfere with the binding of this subunit to its intracellular binding site. Since Walker and
co-workers (31) have shown that the modulatory functions of the β subunit are largely dependent on this interaction, and we have shown that the His6-tagged β3 subunit can interact with the I–II loop, the β3 subunit should modulate calcium channel function when injected into oocytes as a purified fusion protein. It was also clear from the results of these experiments that the β3 subunit did not interact with either the intracellular II–III or the III–IV loops. Although our in vitro experiment showed highly specific interaction between the β3 subunit and the α1C I–II loop, we cannot exclude possible weak or transient interactions with other intracellular regions.

Injection of Oocytes Expressing the Ca2+ Channel α1 Subunit with a Recombinant β Subunit Fusion Protein Induces Effects Comparable to α1-β Coexpression—Effects of β subunit coexpression on the α1C subunit have been studied using Xenopus oocytes (7, 8, 17, 32, 33) as well as in mammalian cells (6, 9, 10, 15, 18). It is now generally accepted that coexpression of the β subunit results in a 2-fold to more than 100-fold increase in peak current amplitude, a hyperpolarizing shift of the voltage dependence of activation, and acceleration of kinetics of the current (to a different extent, depending on the different types of β subunits). Since our results showed comparable effects of the β subunit on these parameters (e.g., ~6.5-fold increase in current amplitude, ~16 mV hyperpolarizing shift of half-activation potential, and acceleration of activation and inactivation kinetics) within 3 h of β subunit protein injection, it is unlikely that the β subunit is enhancing protein synthesis of α1 subunits in the endoplasmic reticulum. Employing our experimental conditions, it takes at least 3 days from the time of co-injection of the α1C and β subunit cRNAs and at least 4 days for the α1C subunit cRNA alone to get measurable current through these expressed channels. Therefore, even if we assume that the presence of the β subunit somehow facilitates protein synthesis of the α1 subunit, its contribution to the observed effects should be minimal, since our recording time scale is shorter. Thus, we believe that the effects of the β subunit are exerted mainly on mechanism(s) downstream of protein synthesis.

The Time Course of the β Subunit Effects Can Be Categorized in Two Distinct Patterns—In our present study, we analyzed the time course of four parameters after injection of the β subunit protein as follows: 1) peak Ba2+ current amplitude; 2) voltage dependence of activation; 3) activation kinetics; and 4) inactivation kinetics. Among these, only the peak current amplitude increased within a slow time framework; it took ~3 h to reach plateau and the time of the half-maximal effect was between 2 and 3 h. The other three parameters behaved very similarly to each other and changed with a faster time course, reaching a steady level in 2 h, and the time of the half-maximal effect was less than 1 h. According to the in vitro binding study by De Waard et al. (24), AID and the β1b subunit bound at a rate constant of 0.1 min−1 μM−1, and when using 500 nM AID, the rate constant corresponded to a half-time of about 20 min.
Since our “faster” time course falls within the same time range as their results, we believe that the faster time course indicates direct binding of the injected β subunit protein to the α1C subunits that already exist in the plasma membrane. We do not know the exact time it takes for the injected β subunit protein to diffuse, reach the plasma membrane, and build to a saturated concentration. However, since an excess amount of β subunit protein was injected (final concentration in the oocyte cytoplasm was 300 nM, which is about 5.4 times higher than the reported K_{D} of AID and β subunit by De Waard et al. (24), 55.1 nM), we assume it takes less than 1 h. Taken together, it seems that the binding of the injected β subunit protein to the α1C subunits in the plasma membrane reaches equilibrium within 2 h. Bafilomycin A_{1} treatment did not influence the β effects that are categorized with a faster time course but did abolish the increase of α1 subunit by the β subunit. This finding also supports the concept that allosteric modulation of α1 subunits in the plasma membrane by the β subunit is responsible for the faster effects, whereas the “slower” component implies the existence of a distinct mechanism for modulation. In order to clarify this, we addressed the question whether the β subunit modulation of current amplitude occurs during protein translocation.

**Influence of the Pretreatment with Bafilomycin A_{1}—Bafilomycin A_{1}, a V-ATPase inhibitor, inhibits intracellular glyceroen transport by impairing the acidification of organelles (30). In the presence of this compound the β subunit failed to increase the amount of α1 subunit in the plasma membrane. Bafilomycin A_{1} also significantly blocked the effect of the β subunit on current amplitude. The results strongly suggest that the effect of the β subunit protein on current amplitude is largely dependent on intracellular translocation of nascent α1 subunits. The β subunit elicited a −1.8-fold increase of current when oocytes were treated with bafilomycin A_{1}, despite a complete loss of increase of the α1 subunit in the plasma membrane. An attractive explanation for the current increase is that the injected β subunit allosterically modulates the population of α1 subunits that are already inserted in the membrane. This is in agreement with single channel analyses done by Wakamori et al. (33), in which the coexpression of the α_{1} subunit with the β subunit resulted in a 2-fold increase in the channel open probability.

In the present study, pretreatment with bafilomycin A_{1} slightly decreased the control (α1 subunit alone) current amplitude, without affecting other characteristics of the current. Assuming that there is turnover of channels in the plasma membrane (15), some breakdown of functional channels will occur and will be replaced by the translocation of new channels from the cytosolic region. Therefore, it seems reasonable that inhibition of protein translocation results in a decreased number of functional channels and, consequently, decreased current amplitude. The contribution of other mechanism(s), such as destabilization of membrane-incorporated channels, cannot be excluded.

**Possible Role of Xenopus Oocyte Endogenous β Subunit—** Recently Tareilus et al. (25) cloned an endogenous β subunit from Xenopus oocytes (β_{2}CA), which is highly homologous to the mammalian β subunit. Injection of Xenopus β antisense oligonucleotides significantly reduced the current through the expressed α_{1C} and α_{1B} subunit of the Ca^{2+} channel. Based on this, the authors proposed that the “α alone” channels are in fact forming an α1 subunit-endogenous β subunit complex (α1βCA). However, it is not possible at this time to determine whether the endogenous βCA was present in high enough concentration to complex with the α1 subunit in the absence of exogenous β. Thus, it seems likely that our observations incorporate the effects of exogenous β subunit on the α1βCA complex. However, the basic characteristics of the control (α1 alone) current in the present study, viz. slow activation, slow inactivation, smaller current amplitude, and a shifted voltage dependence to the depolarized direction, and the modulation of these parameters by the application of exogenous β subunit are in good agreement with previous studies using a variety of mammalian cells (6, 9, 10, 15, 19) in which no endogenous β subunits have been reported.

The strategy and molecular reagents used throughout these studies are thought to be required for efficient interaction among all calcium channel α1 and β subunits (25, 28, 34, 35), viz. the I-II intracellular connecting loop and C-terminal tail of α1 and a full-length β subunit. Therefore, it is reasonable to conclude that the mechanisms described in the present study should apply for all calcium channel α1 and β subunit interactions.

In summary, we have provided compelling evidence that the β subunit modulates the function of the α1 subunit of the voltage-dependent Ca^{2+} channel in two distinct modes, i.e. allosteric modulation and chaperoning of channels to the plasma membrane.

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