Folding of Active Calcium Channel β_{1b}-Subunit by Size-exclusion Chromatography and Its Role on Channel Function*

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Voltage-gated calcium channels mediate the influx of Ca^{2+} ions into eukaryotic cells in response to membrane depolarization. They are hetero-multimer membrane proteins formed by at least three subunits, the pore-forming α_{1}-subunit and the auxiliary β- and α_{2}δ-subunits. The β-subunit is essential for channel performance because it regulates two distinct features of voltage-gated calcium channels, the surface expression and the channel activity. Four β-subunit genes have been cloned, β_{1-4}, with molecular masses ranging from 52 to 78 kDa, and several splice variants have been identified. The β_{1b}-subunit, expressed at high levels in mammalian brain, has been used extensively to study the interaction between the pore forming α_{1C} and the regulatory β-subunit. However, structural characterization has been impaired for its tendency to form aggregates when expressed in bacteria. We applied an on-column refolding procedure based on size exclusion chromatography to fold the β_{1b}-subunit of the voltage-gated-calcium channels from *Escherichia coli* inclusion bodies. The β_{1b}-subunit refolds into monomers, as shown by sucrose gradient analysis, and binds to a glutathione S-transferase protein fused to the known target in the α_{1C}-subunit (the α-interaction domain). Using the cut-open oocyte voltage clamp technique, we measured gating and ionic currents in *Xenopus* oocytes expressing cardiac α_{1C}-subunit (α_{1C}) co-injected with folded-β_{1b}-protein or β_{1b}-eRNA. We demonstrate that the co-expression of the α_{1C}-subunit with either folded-β_{1b}-protein or β_{1b}-eRNA increases ionic currents to a similar extent and with no changes in charge movement, indicating that the β_{1b}-subunit primarily modulates channel activity, rather than expression.

Changes in the intracellular calcium concentration regulate a variety of cellular functions such as neurotransmission, muscle contraction, hormone secretion, and gene expression. High threshold voltage-activated calcium channels are the main route for calcium entry in electrically excitable cells. They are membrane protein complexes composed of at least three non-homologous subunits, the α_{1C}, β_{1}, and the α_{2}δ-subunit. Through molecular cloning, at least 10 genes encoding mammalian α_{1C}-subunits (α_{1A.1} and α_{1B}) have been identified in different cell types (1). Although the α_{1C}-subunit encompasses all the structural elements of a functional voltage-activated calcium channel, such as the ion-conduction pathway, the voltage sensor, and drug-binding sites, the β-subunit seems to be essential for channel performance (2) and to be acting at two levels: (i) channel expression by interfering with the α_{1C}-subunit endoplasmic reticulum (ER)^{1}-retention signal to facilitate intracellular trafficking (3, 4), and (ii) channel activity by modifying the electrophysiological properties of the channel (5–7).

Two highly conserved sequences have been identified as the primary interaction site between the α_{1C} and the β-subunit, the α_{1C}-subunit interaction domain (AID) that lies within the cytoplasmic I-II loop of the α_{1C}-subunit (8), and the β-subunit interaction domain that lies within the second conserved sequence domain of the β-subunit (9). Four β-subunit isoforms (β_{1-4}) with molecular masses between 52–78 kDa from four non-allelic genes have been cloned, each encoding multiple splice variants. Sequence analysis of all β-subunits reveals five homology domains, two of them highly conserved among all β-subunits and known to be important for channel function (10). Structural modeling based on homology searches has been carried out for one of the β-subunit isoforms, the β_{1b}. This study proposes a modular structure of the protein containing a PDZ-like, an Src homology 3, and a guanylate kinase domain (11). However, the functional relevance of these structural domains is yet to be established. Direct biochemical and structural characterization has been hampered by the lack of appropriate expression and purification protocols to produce soluble and stable protein in sufficient amounts. The β_{1b}-subunit has a length of 597 amino acids and a calculated molecular mass of 65,679 Da, and although it expresses at high levels in *Escherichia coli*, the majority of the recombinant protein accumulates in inclusion bodies. We describe here a procedure to purify and refold the β_{1b}-subunit from inclusion bodies using size-exclusion chromatography (SEC). Sedimentation profile in a sucrose density gradient is consistent with a monomeric state of the folded protein. This protein is stable in solution up to 5–6 mg/ml for about 2 weeks and displays binding affinity for the AID site of the cardiac isoform of the α_{1C}-subunit (α_{1C}). We investigated the biological function of the β_{1b}-subunit, either

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*The abbreviations used are: ER, endoplasmic reticulum; AID, α_{1C}-subunit interaction domain; SEC, size-exclusion chromatography; α_{1C}, cardiac isoform of the α_{1C}-subunit; DTT, dithiothreitol; GST, glutathione S-transferase.*
Experimental Procedures

Protein Preparation—The cDNA encoding the β1b-subunit (GenBank™ accession number X61394) residues 24–597 was subcloned between the BamHI and EcoRI sites by conventional PCR methods into pRSETB vector containing a polyhistidine (His6) at the N-terminal end (Invitrogen). The entire cDNA was sequenced after being subcloned in pRSETB vector, and seven amino acid substitutions were detected (F158L, S348F, R417S, V435A, V449A, W492R, and V511A) when compared with the sequence reported by Pragnell et al. (13). (Swiss-Prot accession number P54263). The His6-β1b-tagged protein was used for Western blotting analysis with anti-His antibodies according to the manufacturer’s instructions (Qiagen). E. coli BL-21 transformed with the β1b-pRSET vector were grown at 25 °C to an optical density of 0.7 and induced 90 min with 0.5 mM isopropyl-1-thiogalactopyranoside (IPTG) until 2 h. Cells were harvested by centrifugation and stored at −20 °C until use.

After lysis by sonication and centrifugation, the pellet fraction was collected and stored at 4 °C until use. The protein concentration was measured by absorbance at 280 nm using the absorbance coefficient of 1.3 (1-cm cell path length). The cDNA encoding the I-II loop residues 422–597 of the α1C calcium channel subunit (GenBank™ accession number X61394) was subcloned between the BamHI and EcoRI sites by conventional PCR methods into the pGEX-4T-1 vector containing a glutathione S-transferase (GST) gene fusion pGEX vector (Amersham Biosciences). E. coli BL-21 pLyS3 transformed with the pGEX GST-β1b vector were grown at 23 °C to an optical density of 0.7 and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 2 h. Cells were harvested by centrifugation and stored at −80 °C until use.

The GST-β1b fusion protein was purified from the cleared cell lysate by glutathione-affinity chromatography (glutathione-Sepharose 4 Fast Flow, Amersham Biosciences), according to the manufacturer’s instructions, except that 10 mL DTT and 1% reduced Triton X-100 (Sigma) was added to the buffers throughout the whole purification procedure, and the Tris-HCl elution buffer was replaced by phosphate-buffered saline buffer. The eluted fractions containing GST-β1b were verified by SDS-PAGE, pooled, concentrated to 3–6 mg/mL, and stored at 4 °C until use.

The GST-β1b fusion protein was eluted from the column by 10 mM DTT and 1% reduced Triton X-100 at a flow rate of 0.5 mL/min. The fractions containing the protein were verified by SDS-PAGE, pooled, concentrated to 3 mg/mL, and stored at 4 °C until use.

The protein concentration was measured by absorbance at 280 nm using the absorbance coefficient of 1 mg/mL = 1.3 (1-cm cell path length). The cDNA encoding the I-II loop residues 422–597 of the α1C calcium channel subunit (GenBank™ accession number X61394) was subcloned between the BamHI and EcoRI sites by conventional PCR methods into the pGEX-4T-1 vector containing a glutathione S-transferase (GST) gene fusion pGEX vector (Amersham Biosciences). E. coli BL-21 pLyS3 transformed with the pGEX GST-β1b vector were grown at 23 °C to an optical density of 0.7 and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 2 h. Cells were harvested by centrifugation and stored at −80 °C until use.

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Sucrose Density Gradient Centrifugation—Sucrose gradients 0–15% in buffer A were generated by a gradient mixer to a final volume of 4 mL. 20 μL of purified β1b-subunit, together with protein markers or on separate gradients were layered on top of the gradient and centrifuged in a Beckman SW-56 rotor at 100,000 × g for 16 h at 4 °C. After sedimentation, individual fractions were fractionated bottom-to-top by drop-wise collection into 16 tubes (250 μL each). Aliquots of each fraction were analyzed by SDS-PAGE, and UV-absorbance was measured at 280 nm (Ultrascan 2000, Pharmacia Biotech).

Sucrose and Protein Preparations

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remove contaminants (Fig. 1A, lane 7), and the purified inclusion bodies were solubilized in denaturing buffer containing 6 M GdmCl. After the refolding procedure was completed (see below), the \( \beta_{1b} \) purified from the inclusion bodies was analyzed by Western blotting using anti-His antibody (Fig. 1B).

Our attempts to refold the protein using conventional methods for denaturant removal such as dialysis or fast dilution failed, and therefore, we investigated a new strategy to refold the \( \beta_{1b} \)-subunit from the inclusion bodies (16, 17). The method uses SEC to exchange the buffer, remove aggregates, and promote folding in a single step during elution with refolding buffer. The solubilized inclusion bodies were incubated for 10 mins at 95 °C under reducing conditions (10 mM DTT), loaded onto a Superdex-200 26/60 column equilibrated with refolding buffer (buffer A), and eluted at a flow rate of 2.5 ml/min at 12 °C. The eluted fractions were monitored at 280 nm and further analyzed by reducing SDS-PAGE. The elution profile shows three peaks (Fig. 2A) containing pure \( \beta_{1b} \), as judged by SDS-PAGE (data not shown). One peak eluted within the column void volume (\( V_0 \)), indicating the presence of higher aggregates of \( \beta_{1b} \). Two distinct peaks (peaks I and II) eluted within the column included volume, suggesting that the \( \beta_{1b} \)-subunit may have been refolded into at least two relatively discrete states. When the higher molecular weight peak (peak I) was recovered from the column, concentrated to 1.5 mg/ml, and reloaded onto the column, a significant portion eluted in the void volume and became the predominant peak within a couple of days (data not shown), indicating that the protein aggregates over time. In contrast, when peak II was recovered and concentrated to 3.7 mg/ml, it eluted predominantly as a single peak with a minor shoulder (Fig. 2B). This profile remained essentially unchanged upon storage for about 2 weeks, even at higher protein concentrations, indicating that the protein was stable and likely in a fully folded conformation. Protein isolated from peak II was, therefore, referred to as folded-\( \beta_{1b} \), and used for further studies. The integrity of the folded-\( \beta_{1b} \) was confirmed by amino acid analysis.

To optimize the buffer conditions for the folded-\( \beta_{1b} \), a 1 mg/ml protein stock solution was diluted six times in each different test buffer and loaded onto the Superdex 200 10/30 equilibrated column with the same dilution buffer. The SEC elution profiles for the folded-\( \beta_{1b} \) were superimposable at pH 8.0 and above and at salt concentrations higher than 200 mM NaCl (data not shown). However, at lower salt concentrations or at pH below 6.2 no peak was detected. Removal of EDTA from buffer A did not alter the elution curve.

A SEC calibration curve prepared with a set of globular protein standards yielded an apparent molecular mass of 188 kDa for the folded-\( \beta_{1b} \) (Fig. 2C), more than twice the molecular mass predicted by the amino acid sequence (66 kDa). This finding may indicate either formation of oligomers (dimers or trimers) or alternatively, an anomalous SEC behavior due to molecule asymmetry, because the molecular mass of native proteins estimated by SEC depends highly upon the shape of the molecule (18).

**Sucrose Gradient Analysis**—We used sucrose gradient analysis to resolve independently from SEC whether or not the folded-\( \beta_{1b} \) forms multimers. The folded protein was subjected to 0–15% sucrose gradient centrifugation either in a separate gradient or along with two protein markers, albumin that has a comparable molecular mass (67 kDa) and catalase that has a higher molecular mass (232 kDa) (Fig. 3). Analysis of the different sucrose gradient fractions by reducing SDS-PAGE and UV-absorbance at 280 nm showed that the folded-\( \beta_{1b} \) sediments at one position along the gradient (Fig. 3A), indicating the presence of a homogenous protein population in a defined conformation. This analysis also shows that folded-\( \beta_{1b} \) co-sediments with albumin while it separates from catalase (Fig. 3B). Albumin and catalase sediment at the same position of the gradient fraction when run alone (data not shown). It is readily apparent from Figs. 2C and 3B that, although catalase behaves as expected from the calculated
molecular mass in SEC and sucrose gradient analysis, it migrates faster in SDS-PAGE. The same fast migrating band in molecular mass in SEC and sucrose gradient analysis, it migrates faster than predicted in reducing SDS-PAGE. Binding in Vitro of the Folded-β1b to the AID Site—Because refolded proteins may not necessarily adopt their native conformation with retention of biological activity, we investigated the ability of the folded-β1b to bind to its natural target on the α1 pore-forming subunit, the highly conserved AID site. The I-II loop of the α1C-subunit (residues Gly422-Arg534) bearing the AID site was fused to GST (GST-AID\textsubscript{α1C}) and used for \textit{in vitro} binding assays, as described under “Experimental Procedures.” The folded-β1b bound to the GST-AID\textsubscript{α1C} fusion protein (Fig. 4A) but not to GST protein alone, even at a 4-fold excess over GST-AID\textsubscript{α1C} (Fig. 4B) or to GST fused to an unrelated sequence, such as the carboxyl-terminal end of the hCIC1 channel protein (Fig. 4C, GST-CIC\textsubscript{126}). These results show that the folded-β1b binds specifically to the AID\textsubscript{α1C} site.

Effect of the Folded-β1b on α1C-Calcium Channels Expressed in Xenopus laevis Oocytes—To prove further that the β1b-subunit folds to an active form and to investigate the functional effects of this β-subunit isoform on calcium channel activity, we examined the effect of the β1b-subunit originated from cRNA and the folded protein on α1C expressing \textit{Xenopus} oocytes. We used the cut-open oocyte voltage-clamp technique to simultaneously record ionic and gating currents. The ability to measure gating and ionic currents in the same oocyte allow us to discriminate between the effect of the β-subunit on channel expression from the one on channel activity as follows. The movement of charged residues in the channel protein, as it undergoes conformational changes, gives rise to gating currents reflected as small outward transients preceding ionic currents at the onset of depolarizing pulses (19). The integral of these gating currents yields the charge movement during channel activation and is a function of the number of channels present in the membrane and the number of charged residues per channel that move within the electric field during activation (20). On the other hand, the amplitude of ionic currents, carried by Ba\textsuperscript{2+} (\(I_{\text{Ba}}\)) in our case, is a function of the number of channels, the single-channel conductance, and the probability of channel opening. Thus, changes in charge movement report alteration in channel expression, whereas Ba\textsuperscript{2+} currents, when normalized by charge movement, reveal changes in channel activity. Using this approach, it has been shown that in \textit{Xenopus} oocytes injected with cRNA for the pore-forming α1C-subunit alone or together with cRNA encoding the β1b-subunit, a similar number of α1C-subunits reach the membrane, but co-expression of β1b-subunit yields larger ionic currents (6). This increase was also observed when co-injecting cRNA for the β1b-subunit (15).

Here, we injected \textit{Xenopus} oocytes with α1C-DN60-cRNA alone or together with β1b-cRNA or folded-β1b-protein and measured gating and ionic currents (Fig. 5A). Currents recorded in the absence of exogenous β1b-subunit showed a rapid outward transient corresponding to gating currents, followed by a small non-inactivating current (Fig. 5A, upper panel). In contrast, oocytes co-injected with β1b-cRNA displayed inward currents that are larger in amplitude than gating currents (Fig. 5A, middle panel). Likewise, injection of the folded-β1b protein to α1C-DN60-expressing oocytes also leads to an increase in ionic current amplitude relative to the gating currents (Fig. 5A, lower panel). Another property shared by calcium channel β-subunits is their ability to shift the voltage-dependence of the activation curve toward more hyperpolarized potentials. Fig. 5D shows the voltage dependence of averaged current amplitudes, and Fig. 5E shows the voltage-dependence of the normalized conductance from oocytes expressing α1C-DN60 alone (A), co-injected with β1b-cRNA (●), or with folded-β1b-protein (○). From these measurements, \(G_{\text{max}}\) for α1C-DN60 (2.2 ± 0.3 μS; \(n = 9\)) compares to 2.4 ± 0.3 μS (\(n = 15\)) and 2.1 ± 0.3 μS (\(n = 15\)) measured in the presence of β1b-cRNA or folded-β1b-protein respectively. On the other hand, the mid-
point of the activation, $V_{1/2}$ shifts from 26 ± 1 mV to 4 ± 1 mV after the injection of folded-$\beta_{1b}$, which compares to the $V_{1/2}$ measured in oocytes co-expressing $\beta_{1b}$-cRNA (−1 ± 1 mV).

These results show that the folded-$\beta_{1b}$ protein is nearly as effective as the $\beta_{1b}$-subunit derived from cRNA in modulating channel activity, and that the expressed $\alpha_{1C}$-calcium channels are fully available to interact with injected $\beta_{1b}$-protein. Injection of folded-$\beta_{1b}$ previously heated at 100 °C for 15 min produces no apparent changes in the relationship between ionic and gating currents (data not shown).

To resolve the role of the $\beta_{1b}$-subunit in channel expression and/or activity, we estimated the number of channels contributing to the measured currents by integrating the first 2 ms of gating currents evoked by a pulse from −80 to −30 mV ($Q_{on}$) for the three subunit combinations ($\alpha_{1C}$, $\alpha_{1C}^{beta_1}$RNA, and $\alpha_{1C}^{beta_1}$ protein, Fig. 5B). At this membrane potential, the ionic current component is minimal and is delayed by about 4 ms with respect to the gating currents. Because the voltage- and time-dependence of the charge movement during the initial 2 ms is not affected by the presence of the $\beta$-subunit (6), $Q_{on}$ is expected to be proportional to the number of $\alpha_{1C}$ channels present in the recorded membrane (21). From these measurements, we found that there is no significant difference in expression levels among the different subunit combinations (Fig. 5B: 17.1 ± 3.0 pC for $\alpha_{1C}$, 17.7 ± 5.7 pC for $\alpha_{1C}$-cRNA, and 19.5 ± 3.9 pC for $\alpha_{1C}$-cRNA/$\beta_{1b}$-protein). A, ratios of ionic currents at 0 mV versus $Q_{on}$. Oocytes expressing $\alpha_{1C}$ alone transport 0.26 ± 0.06 nA/pC, which is significantly smaller ($p < 0.01$) than when combined with $\beta_{1b}$ injected as cRNA (5.25 ± 0.28 nA/pC) or as folded protein (3.28 ± 1.09 nA/pC). A, current-voltage plot for the different subunit combinations, oocytes expressing $\alpha_{1C}$-cRNA alone ($n = 9$), $\alpha_{1C}$-cRNA/$\beta_{1b}$-RNA ($n = 14$), and $\alpha_{1C}$-cRNA/$\beta_{1b}$-protein ($n = 15$), measured at the end of a 60-ms pulse in 10 mV increments. E, voltage-dependence of the normalized conductances obtained from the current-voltage measurements (discussed under “Experimental Procedures”). At least three batches of Xenopus oocytes were analyzed for each subunit combination.

Fig. 5. Gating and ionic currents from oocytes injected with $\alpha_{1C}$-cRNA alone or combined with $\beta_{1b}$-cRNA or folded-$\beta_{1b}$-protein. A, representative gating and ionic traces from oocytes expressing three different subunit combination during 60-ms voltage pulse to −30, 0, and +30 mV from a holding potential of −80 mV (upper traces). Horizontal bars indicate the 30 ms displayed on an expanded scale as inset for the pulse to −30 mV. The initial 2 ms under the outward transient corresponding to gating currents was integrated to obtain $Q_{on}$ (highlighted as the gray shaded area). Vertical bars in the inset correspond to 10 nA. B, average $Q_{on}$ from oocytes expressing the different subunit combination. Similar values were obtained: 17.1 ± 3.0 pC ($n = 16$) for oocytes expressing $\alpha_{1C}$-cRNA alone; 17.7 ± 5.7 pC ($n = 9$) for $\alpha_{1C}$-beta1-cRNA, and 19.5 ± 3.9 pC ($n = 14$) for $\alpha_{1C}$-cRNA/$\beta_{1b}$-protein. C, ratios of ionic currents at 0 mV versus $Q_{on}$. Oocytes expressing $\alpha_{1C}$ alone transport 0.26 ± 0.06 nA/pC, which is significantly smaller ($p < 0.01$) than when combined with $\beta_{1b}$ injected as cRNA (5.25 ± 0.28 nA/pC) or as folded protein (3.28 ± 1.09 nA/pC). A, current-voltage plot for the different subunit combinations, oocytes expressing $\alpha_{1C}$-cRNA alone ($n = 9$), $\alpha_{1C}$-beta1-cRNA ($n = 14$), and $\alpha_{1C}$-cRNA/$\beta_{1b}$-protein ($n = 15$), measured at the end of a 60-ms pulse in 10 mV increments. E, voltage-dependence of the normalized conductances obtained from the current-voltage measurements (discussed under “Experimental Procedures”). At least three batches of Xenopus oocytes were analyzed for each subunit combination.

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Fig. 6 shows the time course of the charge movement alone and the ionic current versus charge movement ratio after injection of
folded-β₁b. During the initial 30 min after injection of folded-β₁b, we did not observe changes with respect to the recordings in the absence β₁b (Fig. 6A). After this time, ionic current versus charge movement ratio increased gradually until it reached a plateau at 2 h (Fig. 6C). We did not observe any secondary increase for up to 7 h after protein injection, and during this time period, there was no sign of an increase in the number of functional channels, as assessed by the Qon, values at −30 mV (Fig. 6B), which indicate that most of the changes arise from the modulation of channels pre-existing in the membrane.

**DISCUSSION**

We used size-exclusion chromatography to fold the β₁b-subunit from voltage-activated calcium channels into active monomers. The β₁b-subunit with 65 kDa is the largest protein so far refolded by this method that was initially applied to fold smaller proteins with molecular masses between 14–20 kDa (17, 22, 23). The successful use of this method for the β₁b-subunit might be associated to its multi-domain structure (11) and, whether the SEC-based refolding procedure can be generally applied to larger proteins lacking such a feature, can not be anticipated from this work. The aggregation reaction during folding was not totally inhibited, because a peak eluting within the void volume of the column was consistently observed. During the on-column folding reaction, two main conformational states, represented by peaks I and II, were detected. Peak II corresponds to the native-like state of the protein as judged by the stability and functional activity, whereas peak I includes a mixture of more expanded states that aggregate fast over time. The average recovery of active β₁b-subunit from the Superdex 200 column was 50%. The folded protein is stable at pH 8–10, with salt concentrations above 200 mM NaCl, and is independent of the presence of divalent cations. The folded-β₁b interacts specifically with the highly conserved AID site because it binds to GST-AID₀₁C fusion protein but not to GST alone or GST-CIC₁₂₆. It also interacts with the α₁C-subunit of the voltage-gated-calcium channels expressed in Xenopus oocytes, showing that folded-β₁b retains biological activity.

When injected into α₁C-subunit-expressing oocytes, the folded-β₁b shifts the activation curve toward more negative potentials and increases significantly the ratio of ionic current versus charge movement, whereas charge movement itself remains more or less invariant. Oocytes co-injected with α₁C- and β₁b-cRNA behave similarly. These results show that β₁b modulates function through an interaction site that is equally accessible to β₁b synthesized from cRNA or to folded-β₁b protein, and that channel density, as assessed by the gating current measurements, is not affected by the presence of exogenous β₁b-subunit. The increase in ionic current versus charge movement ratio becomes apparent after 30 min elapsed from the injection of the folded-β₁b, and it develops slowly to a single plateau 2 h later. The charge movement remains constant over the whole period of time after protein injection. Consequently, the effect of the β₁b-subunit on channel activity must be a separate action from the effect on channel expression. This conclusion is also consistent with the recent finding that in spherical vesicles derived from frog and mouse skeletal muscle plasma membranes, ionic currents through calcium channels are increased without changes in charge movement by β₁b purified from COS-transfected cells (24). On the other hand, the lack of effect of the β₁b-subunit on channel expression observed here might be explained by sufficient amount of endogenous β₁b-subunit (β₁bₑ) that was available to replace the α₁C-subunit from the ER, bypassing the need of exogenous β₁b-subunit. An alternative scenario to explain the increase in ionic current with no increase in channel density is that the folded-β₁b-subunit binds to the α₁C retained in the ER and gives rise to functional channels with increased ionic current versus charge movement. In such a case, and to maintain Qon, the α₁C/β₁b₀- channels should replace virtually all α₁C- or α₁C/β₁bₑ- channels within the first 2 or 3 h after protein injection. Although it seems unlikely, specific experiments need to be carried out to fully address this possibility.

Two models have been proposed for the double action of the β-subunit (25): (i) the β-subunit interacts sequentially with the same subunit binding at different stages of channel cycle to modify channel expression and later function while maintaining a one-to-one stoichiometry (one-site model); and (ii) the different functions result from binding at two different sites (two-site model). The second hypothesis implies that binding of the first β-subunit in the ER facilitates expression of the α₁C-subunit and that binding of a second β-subunit modifies channel activity, as proposed by Tareilus et al. (26). Although our experiments cannot discard a two-to-one stoichiometry, they clearly show that the binding surface responsible for the functional changes is available to the folded-β₁b protein despite the presence of an endogenous β, and thus, binding to this surface occurs downstream to the release from the ER, which favors the idea of a sequential binding.

In summary, we have successfully refolded the β₁b-subunit of the voltage-gated calcium channel from *E. coli* inclusion bodies using SEC. The folded-β₁b subunit is monomer in solution and binds in vitro to the AID₀₁C domain. Our data are consistent with the β₁b-subunit acting primarily on calcium channels pre-existing in the plasma membrane to regulate the ion conductive properties of the channel.

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