The Voltage-dependent Calcium Channel β Subunit Contains Two Stable Interacting Domains*

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Voltage-dependent calcium channels selectively enable Ca\textsuperscript{2+} ion movement through cellular membranes. These multiprotein complexes are involved in a wide spectrum of biological processes such as signal transduction and cellular homeostasis. α\textsubscript{1} is the membrane pore-forming subunit, whereas β is an intracellular subunit that binds to α\textsubscript{1}, facilitating and modulating channel function. We have expressed, purified, and characterized recombinant β\textsubscript{3} and μ\textsubscript{2a} using both biochemical and biophysical methods, including electrophysiology, to better understand the β family’s protein structural and functional correlates. Our results indicate that the β protein is composed of two distinct domains that associate with one another in a stable manner. The data also suggest that the polypeptide regions outside these domains are not structured when β is not in complex with the channel. In addition, the β structural core, comprised of just these two domains without other sequences, binds tightly to the α interaction domain (AID) motif, a sequence derived from the α\textsubscript{1} subunit and the principal anchor site of β. Domain II is responsible for this binding, but domain I enhances it.

Voltage-dependent calcium channels (VDCCs)\textsuperscript{1} permit the flow of Ca\textsuperscript{2+} ions through cellular membranes as a function of membrane potential. These protein complexes are central components in a variety of physiological systems of organisms, ranging from yeast to human. They play pivotal roles in signal transduction and homeostasis processes. Functional roles for these channels vary based on cell type. In muscle, both skeletal and cardiac, the predominant VDCCs (Ca\textsubscript{1.1} and Ca\textsubscript{1.2}) cause release of Ca\textsuperscript{2+} into the cytosol from intracellular stores, thereby initiating contraction (1, 2). In neurons and endocrine cells, neurotransmitter or hormone secretion requires VDCC activity. In addition, electrical activity, specifically the action potential in cardiac myocytes, is regulated by VDCCs. Finally, calcium influx and concentration controlled by VDCCs plays a significant role in neuronal gene expression (3). Pathways have been elucidated, where for one example, VDCC activity gives rise to phosphorylation of CREB, a transcription factor (4), thereby activating transcription of a myriad of target genes.

The VDCC comprises four distinct polypeptides: α\textsubscript{1}, α\textsubscript{δ}, β, and γ\textsubscript{5}. α\textsubscript{1} is the membrane pore-forming subunit and numbers between 1800 and 2400 residues in length. Its sequence exhibits repeats comprising four transmembrane modules or domains, akin to the tetrameric architecture of potassium channels. Each module contains the canonical transmembrane arrangement for voltage-gated ion channels i.e. six transmembrane segments. Modules are connected by linkers that are located in the intracellular milieu, as are both the N and C termini. The high voltage-activated channel subunits, Ca\textsubscript{1.1}x and Ca\textsubscript{1.2}x (α\textsubscript{1}), numbering seven in total, share a high degree of sequence similarity but nevertheless encode distinct electrophysiological activities.

The β subunit was first isolated biochemically in tight association with α\textsubscript{1} (6). Four isoforms have been discovered, ranging in size from 52 to 78 kDa (7). The lack of a transmembrane region suggested that β very likely interacts with the channel through one of the intracellular domains of α\textsubscript{1}. Campbell and coworkers (8) found such a region in the linker between repeats I and II of the α\textsubscript{1} subunit. The data indicate that α\textsubscript{1} and β subunits interact directly with each other with a stoichiometry of 1:1 (9). β also interacts with other regions of α\textsubscript{1} (10–14), depending on the isoform, but the region discovered by Campbell and coworkers appears to be the primary, high affinity site of interaction.

The electrophysiological function of the β subunit is a modulatory one (15–17). It increases VDCC peak current amplitude from 10- to 18-fold depending on the channel type. Furthermore, β accelerates channel activation, modifies inactivation kinetics, and shifts the voltage-dependence of the channel (18). It has been convincingly shown that β acts by both chaperoning VDCCs to the membrane and modulating gating (19–21). The diverse β isoforms have quantitatively different effects on the various electrophysiological parameters. These isoform differences suggest a mechanism for the cellular specificity of VDCC activity through combinatorial permutations of different β isoform subunits associating with different α\textsubscript{1} isoforms. Nevertheless, the molecular mechanism of the β subunit remains unclear. Protein-protein interactions, which have been mapped to a minimum of 18 residues in the α\textsubscript{1} module I–II linker described above, dubbed the AID (α interaction domain), and a 30-residue sequence in VDCC β, dubbed the BID (β interaction domain), play a critical role (8, 22).

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\textsuperscript{1} The abbreviations used are: VDCC, voltage-dependant calcium channel; AID, α interaction domain; BID, β interacting domain; IPTG, isopropyl-1-thio-D-galactopyranoside; TEV, tobacco etch virus; PME, β-mercaptoethanol; HPLC, high-pressure liquid chromatography; CD, circular dichroism; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; PB, protein buffer; WT, wild type.

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Although, considerable attention has been paid to VDCC β2, especially on the functional level, little experimental data is available concerning the protein’s tertiary structure. Cloning and sequencing of the various β isoforms from a spectrum of species showed that two sequence regions were fairly conserved (Fig. 1). Based on the identification of these regions, structure-function analyses have shown that much of the functional activity associated with VDCC β resides in these two regions (22). Subsequently, bioinformatic analysis, including molecular modeling studies, proposed that these two regions have structural similarity to SH3 and guanylate kinase folds (23). Here, we describe experimental studies of the VDCC β3 protein, which give insight into the domain structure of the protein and the interaction between those domains, as well as correlate this data with functional biochemistry and electrophysiology.

EXPERIMENTAL PROCEDURES

Subcloning, Expression, and Purification of VDCC β3 Subunit—Recombinant rat VDCC β3 subunit (M88751) was expressed in Escherichia coli strain BL-21 Tuner (Novagen), containing the “RIL” Codon Plus™ plasmid (Stratagene), using a modified pET21 (Novagen) vector (an His, and TEV protease site were engineered between the NcoI and BamHI sites of pET21d, a gift of Dr. Sean Juo). The protein was purified by sequential metal-chelate, ion-exchange, and hydroxylapatite chromatography.

PCR was used to engineer EcoRI and BamHI restriction sites into the β3 gene. The oligonucleotide primers used for amplification of the β3 gene from the original plasmid were the following: sense primer A, 5’-GCCGGATCCATATGACGACTCTACAGTCUCUC; antisense primer B, 5’-GCCGGATCCATATGACGACTCTACAGTCGGCC. PCR product was ligated into doubly digested (EcoRI and BamHI) pET21d vector. Positive clones were identified by restriction analysis and subsequently sequenced.

Transformed Tuner cells were grown for 3–4 h at 37 °C in 10 liters of LB media, containing 100 μg/ml ampicillin, and 34 μg/ml chloramphenicol. Upon reaching an A_{600} of 0.3, the temperature was lowered to 16 °C, and growth continued until the culture reached an A_{600} of 0.6. Protein expression was induced with 200 μM IPTG. Cells were harvested after 14 h by centrifugation, frozen, and suspended in 100 ml of lysis buffer, buffer L (300 mM NaCl, 50 mM sodium phosphate (pH 7), 1 mM phenylmethylsulfonyl fluoride, 5 mM MgCl2, 0.2% Triton X-100, 1 mM EDTA, 10 mg of lysozyme, 1 mg of DNase). After lysis by French pressure cell (Aminco), cell debris was removed by centrifugation at 20,000 g. The soluble fraction was loaded onto a pre-equilibrated metal chelate “Talon” (Clontech) column (buffer A: 300 mM NaCl, 50 mM sodium phosphate, pH 7), at a flow rate of 1.5 ml/min. The column was washed with buffer A, containing 5 mM imidazole, until a stable base line was achieved. After elution with buffer A, supplemented with 150 mM imidazole, the protein eluate was then diluted 3-fold with water and loaded onto a pre-equilibrated Source-Q (Amersham Biosciences) column (buffer Q: 70 mM NaCl, 20 mM sodium phosphate, pH 7). The column was then washed with buffer Q, and fractions were eluted with a shallow linear gradient of buffer Q, containing 70–300 mM NaCl. VDCC β3-containing fractions were pooled (130–150 mM NaCl) and diluted 2-fold in 600 mM NaCl to buffer H concentrations (10 mM phosphate buffer (pH 7) and 300 mM NaCl) and subjected to TEV protease treatment.

FIG. 1. Schematic of the rabbit VDCC β2a primary sequence, a representative of the β family, and the various constructs prepared in this study. The lightly stippled box denotes the first conserved domain, domain I, and the darkly stippled box denotes the second conserved domain, domain II. The BID, a sequence responsible for binding to VDCC α1, is located as shown in the beginning of domain II.
proteins were purified as described for \( \beta_p \). Purification was as follows. The soluble fraction of the crude extract was loaded on a pre-equilibrated Ni-CAM column (Sigma) (buffer B: 50 mM sodium phosphate (pH 8) and 300 mM NaCl) and washed with buffer B, containing 150 mM imidazole, diluted 6-fold, followed by loading onto a Q-Sepharose (Amersham Pharmacia) column (buffer C: 20 mM sodium phosphate (pH 8), 40 mM NaCl, 5 mM \( \betaME \)). Fractions were eluted with a linear gradient of buffer C containing 150–200 mM NaCl and subjected to TEV proteolysis for 12 h. In the absence of the \( \beta_p \) gene the following primers were used: sense primer C, 5'-GGCGGGATCCCCTGAGGGCCTCCGTCCGCGC-3'; antisense primer D, 5'-GGCGGAATTCTCCCTAGGTTGAGGATTG-3'. Subsequent subcloning was as described for VDCC \( \beta_p \). The VDCC \( \beta_p \) subunit was expressed as for \( \beta_p \). Purification was as follows. The soluble fraction of the crude extract was loaded on a pre-equilibrated Talon column (buffer A) and washed with buffer A, containing 75 mM imidazole. The protein was then eluted with buffer A, containing 150 mM imidazole, pooled, and subjected to TEV proteolysis for 12 h. The protein was then diluted 3-fold with 300 mM NaCl, loaded onto a pre-equilibrated (buffer H) hydroxylapatite column, and eluted with a linear gradient of H buffer (10–250 mM potassium phosphate) at about 120 mM potassium phosphate. Pooled fractions were diluted 4-fold and subjected for 12 h to TEV proteolysis. 10 mM \( \betaME \) was added to the sample and loaded onto a pre-equilibrated gel filtration column (G buffer). Protein was eluted with buffer G. The pooled fractions were processed as above.

Limited Proteolysis of VDCC \( \beta_p \) and \( \beta_S \) Subunits—Papain (20 \( \mu \)g/ml) (Sigma-Aldrich) was activated for 30 min in activation buffer (1.25 mM EDTA, 62.5 mM cysteine, 62.5 mM \( \betaME \), at pH 7) and added to 3 \( \mu \)g/ml of \( \beta_p \) in a 120-fold dilution. The final ratio of papain to protein was 1:3000. Trypsin (60 \( \mu \)g/ml) was added to 3 \( \mu \)g/ml \( \beta_p \) in a 1:200 dilution, giving a final ratio of protease to protein of 1:1000. Reactions were performed on ice. Proteolysis progress (at different time intervals) was monitored by SDS-PAGE. Proteolysis products were purified for further analysis by HPLC reverse phase chromatography using a C4 column (Vydac) with a shallow acetonitrile gradient 30–80% (both solvents were supplemented with 0.05% trifluoroacetic acid).

AID Peptide Binding Assays—Fluorescence polarization was used to determine the equilibrium dissociation constant (\( K_D \)) for the interaction between a fluorescein-labeled AID peptide and various purified VDCC constructs. The synthetic peptide was purified by HPLC reverse phase chromatography using a C18 column (Vydac) with a shallow acetonitrile gradient 20–80% (both solvents were supplemented with 0.05% trifluoroacetic acid). Its sequence is derived from the AID motif of the Cx40.1.1-II linker and is as follows: fluorescein-GGGQLELDGRGNSWITQGE-COOH. A mutant peptide, fluorescein-GGGQLELDGRGNSWITQGE-GO, was prepared in the following: the peptide was incubated with 0.5 mM labeled peptide for 10 min in the dark at room temperature. Polarization measurements were taken with an IKS K2 fluorescence spectrophotometer at excitation and emission wavelengths of 492 and 520 nm, respectively, at 20 °C, maintained at a temperature-controlled water bath. Polarization measurements were made with integration times on the order of 20 s, achieving a standard deviation of 5% of signal. Binding isotherms for the various samples were measured three independent times. Binding data were analyzed in SigmaPlot (SPSS) by nonlinear regression used to fit a binding function as defined by the following equation,

\[
\Delta P = \frac{P_{\text{max}} \cdot X}{K_D \cdot X} \tag{1}\]

where \( X \) is the concentration of free ligand, \( \Delta P \) is the change in fluorescence polarization of the fluorescent probe (the baseline polarization of the labeled peptide alone was subtracted), \( P_{\text{max}} \) is the maximum change in polarization upon saturation, and \( K_D \) is the concentration of ligand required to reach halfmaximal binding. VDCC \( \beta_p \) and \( \beta_S \) subunit domains were expressed by subcloning into the following plasmids: the mammalian expression vectors: \( pCMV_{\text{His}} \) for the \( \beta_p \) subunit domain I and domain II encoding fragments. The oligonucleotide primers used for amplification of the \( \beta_p \) domain I fragment from the original \( \beta_p \) plasmid were the following: sense primer E, 5'-CGCGGAATTC ATTCTGTTCTC GTCATGTCGTAG-T3'; antisense primer J, 5'-CGCGGAATTC ATTCTGTTCTC GTCATGTCGTAG-T3'. The oligonucleotide primers used for amplification of the \( \beta_p \) domain II fragment from the original \( \beta_p \) plasmid were the following: sense primer K, 5'-GGCGGAATTC ATTCTGTTCTC GTCATGTCGTAG-T3'; antisense primer L, 5'-TTATACGACG CGCTCAAAAGGA GAGGTTGGAGGATT-3'. Subsequent subcloning and restriction analysis yielded the following constructs. The synthetic peptide was purified by HPLC reverse phase chromatography using a C4 column (Vydac) with a shallow acetonitrile gradient 30–80% (both solvents were supplemented with 0.05% trifluoroacetic acid). Its sequence is derived from the AID motif of the Cx40.1.1-II linker and is as follows: fluorescein-GGGQLELDGRGNSWITQGE-COOH. A mutant peptide, fluorescein-GGGQLELDGRGNSWITQGE-GO, was prepared in the following: the peptide was incubated with 0.5 mM labeled peptide for 10 min in the dark at room temperature. Polarization measurements were taken with an IKS K2 fluorescence spectrophotometer at excitation and emission wavelengths of 492 and 520 nm, respectively, at 20 °C, maintained at a temperature-controlled water bath. Polarization measurements were made with integration times on the order of 20 s, achieving a standard deviation of 5% of signal. Binding isotherms for the various samples were measured three independent times. Binding data were analyzed in SigmaPlot (SPSS) by nonlinear regression used to fit a binding function as defined by the following equation,
purification step). Producing a distinct mobility shift; lane 7, metal chelate column eluate fraction (first major purification step); lane 6, protein after TEV digestion, which removes a 26-residue N terminus, including the histidine tag, producing a distinct mobility shift; lane 5, metal chelate column flowthrough fraction; lane 3, metal chelate column eluate fraction (second major purification step); lane 4, ion-exchange column flowthrough fraction; lane 5, ion-exchange column eluate fraction. We subjected both \( \beta_{2a} \) and \( \beta_3 \) recombinant full-length proteins to such an analysis using both papain, a highly nonspecific protease, and trypsin, a more specific protease. The time-course results for limited digestion by papain are shown in Fig. 3. Early in the time course, a stable polypeptide emerged that runs as a fragment of ~40 kDa. Subsequently, this fragment is further digested such that after 16 h, two stable fragments remain, one of higher mobility and another of medium mobility. Both isoforms basically follow the same pattern (\( \beta_{2a} \) seems to have an intermediate fragment). Having established a set of stable polypeptides, we sought to identify them. Preparative limited proteolyses of VDCC \( \beta_3 \) were performed, and at the appropriate times the reaction was stopped by flash freezing with liquid N\(_2\). The samples were then applied to a reverse phase C4 HPLC column and separated by an acetonitrile gradient. The separated fragments were sent for mass spectrometric and N-terminal peptide sequencing analyses.

**Limited Proteolysis**—An important and classic method of probing protein structure is limited proteolysis (for a review see Ref. 28). The goal here is determination of domain structure. The premise of the method assumes that flexible and exposed regions of the protein are available to the protease and thus subject to hydrolysis.

We subjected both \( \beta_{2a} \) and \( \beta_3 \) recombinant full-length proteins to such an analysis using both papain, a highly nonspecific protease, and trypsin, a more specific protease. The time-course results for limited digestion by papain are shown in Fig. 3. Early in the time course, a stable polypeptide emerged that runs as a fragment of ~40 kDa. Subsequently, this fragment is further digested such that after 16 h, two stable fragments remain, one of higher mobility and another of medium mobility. Both isoforms basically follow the same pattern (\( \beta_{2a} \) seems to have an intermediate fragment). Having established a set of stable polypeptides, we sought to identify them. Preparative limited proteolyses of VDCC \( \beta_3 \) were performed, and at the appropriate times the reaction was stopped by flash freezing with liquid N\(_2\). The samples were then applied to a reverse phase C4 HPLC column and separated by an acetonitrile gradient. The separated fragments were sent for mass spectrometric and N-terminal peptide sequencing analyses.

**Expression and Purification**—Using a T7 expression system, an *E. coli* BL-21 derivative cell line that allows for fine tuning IPTG induction by better controlling IPTG concentration, and low temperature growths, we obtained overexpression of soluble, full-length rat VDCC \( \beta_3 \). In addition, we subcloned and expressed the rabbit \( \beta_{2a} \) isoform in the same system. The expression vectors encode a histidine tag on the N terminus, followed by a TEV protease site and the ensuing desired coding sequence. We have found that VDCC \( \beta_{2a} \) manifests robust expression. \( \beta_3 \) expression is not exceptional.

Our purification schemes for the VDCC \( \beta \) isoforms vary. However, all utilize as a first step subsequent to lysis, metal chelate resin chromatography to isolate the His-tagged target proteins. This first step usually produces protein that is greater than 80–85% homogeneous. Histidine tags are removed by cleavage with TEV protease. The TEV protease is highly specific and does not cleave other sites on the protein. Full-length VDCC \( \beta_3 \) is highly sensitive to proteolysis. Therefore, efficient and rapid chromatographic processing has proven essential and has been applied systematically. The full-length proteins are shown in Fig. 2. The bacterial expressed recombinant proteins are both electrophysiologically and biochemically active (see below).
domains are derivatives of this early fragment as discerned by the time-course results, requiring them to be equal or smaller than the early fragment. We conclude that the large fragment is a core protein. Further support for this structural division, i.e. two domains with a flexible connecting linker, came from limited proteolysis of a recombinant protein, whose ends were engineered based on homology of the VDCC β family (see Fig. 1, predicted core). This limited proteolysis resulted in two fragments of the same size as the long term proteolyses of the full-length proteins (data not shown).

Domain Analysis—Having defined two stable domains of β, we chose to determine whether they interacted or rather were inert with regards to each other and behaved as pearls on a string. Constructs expressing His-tagged β2a domain I alone and domain II as a C-terminal fusion protein with NusA were prepared (see Fig. 1). His-tagged domain I was mixed with domain II, which had been isolated away from the fusion partner NusA and shown to bind in a pull-down assay, indicating stable association of the two domains (Fig. 4A).

In addition, chromatographic experiments suggest that β2a domains I and II stably interact. Gel filtration analysis of domain I alone gives an elution volume of 93 ml on a Superdex 200 size-exclusion column. When purified removable linker core protein (Fig. 1) was digested by TEV so that the domains were no longer covalently linked, to give linkerless core, and then run out on the identical column, both domains I and II co-eluted at an elution volume of 83 ml (Fig. 4B). The significant shift in elution volume for domain I and coelution of both domains strongly supports association of domains I and II. The solution conditions, 200 mM NaCl and 10 mM βME, for this chromatography run rule out nonspecific or adventitious association.

Circular Dichroism Spectroscopy—We measured CD spectra of several VDCC β forms, and they are shown in Fig. 5. The spectra of the full-length β3 and β2a proteins are similar indicating comparable secondary structure. Furthermore, these spectra indicate that the proteins are of a mixed α-helix/β-sheet type, as seen by visual inspection of the curves as well as by deconvolution calculations. The calculations point to ~35% sheet and 15% helix. A comparison of β3 and the β2a linkerless core spectra shows a very strong resemblance between these two proteins. In addition, the difference spectrum between β2a full-length protein and the β2a linkerless core protein gives insight into the segments of protein outside of domains I and II, namely the N-terminal segment, the linker between domains, and the C-terminal segment. This spectrum points to a significant fraction of random coil structure as evidenced by the shift in minimum and magnitude toward 198 nm and diminution of the other minimum at 222 nm (25), consonant with the limited proteolysis results.

AID Binding Assay—To enable structure-function correlations regarding our domain analysis and to have a robust in vitro assay for further correlations with structural and electrophysiological experiments, we have developed a novel assay that measures binding of VDCC β proteins to an α1 I–II linker-derived AID peptide. The assay employs fluorescence polarization measurements. AID peptides of 18- to 20-residue length were synthesized, and some of them were labeled with fluorescein attached at the N terminus. Labeled peptide was then titrated with increasing concentrations of β, and the fluorescence polarization emitted by the labeled peptide was determined. Fluorescence emission polarization is proportional to the rotational correlation time (tumbling) of the labeled molecule. Tumbling, in part, depends on the molecular volume, i.e. larger molecules have larger volume and slower tumbling, which in turn gives rise to increased polarization of emitted light. If the peptide associates with β, its effective molecular volume greatly increases, as evidenced by values of fluorescence emissions polarization. Equilibrium isotherm titrations were performed with full-length β2a and linkerless core β2a. The binding curves are shown in Fig. 6 (A and B). They indicate high affinity single site binding on the order of 6–15 nM. The binding is specific, because addition of unlabeled AID peptide lowers the polarization to basal levels, i.e. it effectively competes with the labeled peptide (data not shown). The assay was further validated by testing a mutant AID peptide, which has a single amino acid change (Tyr to Ser). The same mutant AID bound less than 5% of WT previously, using another in vitro binding assay system (29). Using our fluorescence assay, no binding of the mutant AID was detected at concentrations of up to 350 nM β2a protein, where WT peptide has reached saturation binding.

We next used this binding assay to investigate which domains of β are required for association with the AID peptide (Fig. 6C). Our objective in this experiment was not to measure absolute affinities but rather to measure relative ones. First, binding of β2a domain I was assessed. As can be seen, binding was negligible in the estimated concentration regime tested. Next, the NusA-domain II fusion, which contains a TEV protease site separating NusA and domain II, was incubated with TEV protease and the labeled peptide for 1 h and then fluorescence polarization was measured. As observed, domain II demonstrated significant binding affinity for the AID. In stage three, the β2a domain I protein and the NusA-domain II fusion protein, again containing a TEV protease site separating NusA and domain II, were incubated with TEV protease and the labeled peptide for a period of 1 h, and then fluorescence polarization was assessed. The binding curve shows that the presumed domain I-domain II complex binds with even higher affinity. Because identical amounts of the various proteins were taken in the three stages of this experiment, the curves

![Fig. 3. Limited proteolyses of full-length VDCC β proteins. A, β1 was incubated with activated papain at a ratio of 3000:1 on ice in 10 mM Tris, pH 8, 200 mM NaCl, 5-10 mM βME. Aliquots were taken from the reaction at the indicated times, SDS sample buffer was added, and samples were boiled and analyzed later by SDS-PAGE, as shown. B, β2a incubated with activated papain and analyzed as for β1. Arrows indicate the protease-resistant fragments.](http://www.jbc.org/)
provide us with a reasonable relative measurement of binding for the different domains and their individual contributions. Thus, domain I does not bind by itself to the AID at high affinity, whereas domain II does. However, it is clear that domain I somehow contributes to the increased binding of the AID for the presumed domain I-domain II complex.

Electrophysiology—To assure that our bacterially expressed, recombinant proteins are physiologically active, we performed two-electrode voltage-clamp measurements of *Xenopus* oocytes expressing a and microinjected with our protein samples. In the I–V plots of Ca_{o,1.2} currents (Fig. 7), we show an increase of current amplitude and shift of the steady-state activation by injection of β proteins (except domain I), demonstrating the functional activity of the β proteins. Data values and statistical analyses are described in Table I. The current amplitude increase and change of steady-state activation are statistically significant in all experimental groups except a group injected with β_{2a} domain I protein.

Furthermore, the results show that the core protein electrophysiological activity corresponds well to full-length protein activity. In addition, the injection of purified domain I and domain II proteins prepared by proteolysis of full-length protein and subsequent purification, comparable to linkerless core protein from our *in vitro* experiments, still enabled significant changes in the current amplitude and activation shift in a qualitatively similar manner as native protein. It should be noted that the injected recombinant β_{2a} proteins were found subsequently to contain a mutation (P122R) due to the PCR subcloning. This mutation had no effect on the electrophysiological activity of the protein.
**FIG. 6.** Binding isotherms of VDCC β with a fluorescein-labeled AID peptide as measured by fluorescence polarization. 

A, full-length VDCC β2a gives a $K_D$ of $16.1 \pm 3.8$ nM. 

B, linkerless core, i.e. domain I plus domain II, gives a $K_D$ of $6.6 \pm 1.3$ nM. 

C, relative binding curves: filled circles are domain I alone; empty circles are NusA-domain II fusion after incubation with TEV protease; filled triangles are domain I plus NusA-domain II fusion after incubation with TEV protease. Estimated protein concentration is in nanomolar units.
proteolysis of full-length protein and subsequent purification. In the presence of VDCC, consisted of mRNA and protein injection. Age clamp analysis of oocytes expressing VDCC by combination of activities of the purified proteins were examined by two-electrode voltammetry.

FIG. 7. Functional assay of purified β proteins. Physiological activities of the purified proteins were examined by two-electrode voltage clamp analysis of oocytes expressing VDCC by combination of mRNA and protein injection. A, VDCC consisted of α1,2 and β3 proteins. B, VDCC consisted of α1,2 and β3 proteins. PB, protein buffer; Pro, protein; DI, domain I; DI+II, domain I plus domain II prepared by proteolysis of full-length protein and subsequent purification.

DISCUSSION

Our limited proteolysis results, in conjunction with further analysis of the fragments by mass spectrometry and N-terminal sequencing, demonstrate experimentally for the first time the two-domain structure of the VDCC β family. The results correspond remarkably well with sequence analysis (Fig. 8), electrophysiological studies, and molecular modeling. Previous studies have divided the family into five domains, namely D1 corresponding to the N-terminal region, D2 (domain I in our work), D3 (the linker), D4 (domain II), and D5, the C-terminal region. Based on the studies described here, this division may be misleading, because D1, D3, and D5 do not likely represent authentic, independently folded domains for reasons elaborated below. Moreover, we posit that the domain structure applies to all members of the family, because at least two members, β2a and β3, exhibited parallel limited proteolysis results and because the domain ends coincide with sequence conservation boundaries. Buttingress this contention, the CD spectra of the two members have like features. These two structural domains seem to encode critical features of the functional activity of the β family, as summarized previously (18).

Studies of limited proteolysis have defined three major degradants of proteolytic susceptibility, namely exposure, flexibility, and sparse local interactions of the substrate protein (30). Protein regions with well defined secondary structure are rich in local interactions, preventing local unfolding and thus proteolysis (31). In light of the limited proteolysis results, we conclude that regions N-terminal to domain I and C-terminal to domain II are mostly likely not highly structured when β is alone in solution, i.e. when VDCC β is not bound to α1. This conclusion is supported by secondary structure prediction algorithms such as PredictProtein (32) and homology sequence analysis. In addition, the region linking domains I and II, dubbed the linker, was susceptible to digestion, suggesting that it too is not highly structured and quite flexible. Inspection of the linker’s sequences argues that sections of the linker are unlikely to be structured due to their low sequence complexity (very highly rich in serines, with a high fraction of prolines and glycines as well) (33). Here again, the CD difference spectrum (full-length β2a-linkerless core) is consistent with the notion that regions outside of domains I and II are mostly unstructured when not in complex with α1. This difference spectrum represents a polypeptide not present in the linkerless core, i.e. regions other than domains I and II. It is most easily interpreted as showing significant random coil conformation. Furthermore, we argue that the close correspondence of β2a-linkerless core and β3 spectra (see Fig. 5) emphasizes the nature of extra-domain regions. β2a contains 122 more residues than β3. These additions are located in the linker and C-terminal regions. On the other hand, β3 is the smallest member of the family with the shortest N-terminal, linker, and C-terminal regions. Thus, β3 most closely resembles a minimal version of the β family as represented by the engineered β2a-linkerless core, explaining the strong resemblance of the two spectra.

The domain analysis led us to propose two working models for the domain structure of β (Fig. 9). One model postulates that the domains do not associate and a flexible linker tethers them together. Alternatively, the domains associate with each other in a stable manner and a flexible linker connects them but is not required for the domain association. Biochemical results, i.e. the pull-down assay and size-exclusion chromatography, rule out the former possibility.

From the biochemical binding data for the various protein forms, it appears that binding determinants for the AID reside primarily in domain II, as noted previously (22). Nevertheless, domain I makes a contribution (Fig. 6C), either directly, by facilitating interactions between the AID and domain II, or indirectly, by stabilizing domain II as a folded protein entity due to domain I-domain II complexation. The contribution is also discernible in the electrophysiological data measured earlier (22) where current amplitude is increased upon injecting mRNA encoding the equivalent of β core versus β domain II (see Fig. 4 in Ref. 22). Notably, the linker clearly does not contribute to AID binding (Fig. 6B). Removing it appears to improve β-AID association. That the linkerless core binds the AID with maximal affinity, lends support to the model that domains I and II stably interact and that the linker is not necessary for the structural integrity of the protein. Indeed, due to its sequence composition it may encode a PEST determinant for degradation of the protein and thus is important for regulation of β, not its biochemical and electrophysiological activities. Supporting this hypothesis, analysis of the VDCC β2a sequence with PEST search algorithms (34) locates a sequence in the linker that is likely to be a good PEST candidate.

Two permutations arise in regard to AID binding: one, before complexation with the AID, β domains I and II are stably bound. Upon encountering the AID, a conformational change takes place and the two domains dissociate, with concomitant AID binding. Two, the domains remain stably bound together whether the AID associates or not. This second option does not rule out a conformational change on the part of β when complex formation with the AID occurs. Rather it negates a scenario...
ally interact in a stable manner. Extraneous sequences, includ-

ing VDCC domains do not dissociate upon AID binding (data not shown). 

ments in the presence of the AID peptide showed little differ-

istry, two secondary structure in VDCC domains. The refer-

ence sequence for numbering is rabbit main II. The re-

ference sequence for numbering is rabbit main I and II. 

These blocks correspond quite well with the biochemically defined do-

Conservation scores. Zero represents the average

values are presented as mean ± S.E.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Current amplitude (A)</th>
<th>Steady-state activation parameters</th>
<th>V(V) (mV)</th>
<th>n</th>
<th>Properties</th>
<th>Current amplitude (A)</th>
<th>Steady-state activation parameters</th>
<th>V(V) (mV)</th>
<th>n</th>
</tr>
</thead>
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<tr>
<td>I at 20 mV</td>
<td>0.15 ± 0.01</td>
<td>10</td>
<td>22.60 ± 4.22</td>
<td>10</td>
<td>0.15 ± 0.01</td>
<td>10</td>
<td>3.68 ± 0.33</td>
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<tr>
<td>I at 30 mV</td>
<td>0.16 ± 0.01</td>
<td>11</td>
<td>10.67 ± 1.95</td>
<td>11</td>
<td>0.49 ± 0.06</td>
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<td>8.48 ± 0.53</td>
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<tr>
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<td>9.08 ± 0.36</td>
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</tbody>
</table>

* I, current; V, potential; V_{50}, membrane potential for half-maximal activation; k, slope factor; n, cell number; Pro., protein; d, domain. 

FIG. 9. Schematic of alternative models for VDCC β domain configurations. 

wherein such a conformational switch results in dissociation between the two β domains. Interestingly, pull-down experiments in the presence of the AID peptide showed little difference from assays performed in its absence, arguing that the two domains do not dissociate upon AID binding (data not shown).

In summary, we have delineated, based on protein biochem-

stry, two bona fide folded domains with mixed helix/sheet secondary structure in VDCC β proteins. The domains mutually interact in a stable manner. Extraneous sequences, including the linker between domains, would appear to be highly flexible, solvent-exposed, and possibly unstructured. They are unnecessary for β protein binding to the primary anchor site in VDCC α1 subunits. Although domain II contains the function of current amplitude amplification and the voltage activation shift attributed previously to β function, domain I potentiates this facility (22). We speculate that these domains provide a stable structural center for β functionality. The remaining sequences (N terminus, C terminus, and linker) of the β subunit will then be positioned by way of the attachment of the core to the AID to interact with other surfaces on α1 (10, 11), influencing other important functional activities such as activation and inactivation kinetics (18). In addition, we have introduced a novel and robust in vitro binding assay for VDCC β and the AID, which may be readily used for screening compounds that inhibit or modulate this critical interaction.

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