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Properties of the α_1-β Anchoring Site in Voltage-dependent Ca^{2+} Channels*

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In voltage-dependent Ca^{2+} channels, the β subunit interacts with the α_1 subunit via a cytoplasmic site. A biochemical assay has been developed to quantitatively describe the interaction between both subunits. *In vitro* synthesized ^{35}S-labeled β subunits specifically bind to a glutathione *S*-transferase (GST) fusion protein containing the α_{1A} interaction domain (AID_A, located between the amino-acids 383 and 400 of the cytoplasmic loop between the hydrophobic domains I and II). Kinetic analysis demonstrates that the association of ^{35}S-labeled β_{1b} subunit to the AID_A GST fusion protein occurs with a fast rate constant at 4 °C. The binding is almost irreversible as demonstrated by the absence of dissociation observed after an 8-h incubation with an 18-amino acid synthetic AID_A peptide. The α_1-β binding site does not seem to be a target for cytoplasmic regulation. The interaction is mostly unaffected by changes in ionic strength, pH, and Ca^{2+} concentration or by protein kinase C phosphorylation. The specificity of subunit interaction in voltage-dependent Ca^{2+} channels was also followed by saturation analyses. The data obtained show that the AID_A GST fusion protein binds to a single site on the β_{1b} with an apparent K_d of 5 nM. The affinities of AID_A GST fusion protein for various β subunits was measured and demonstrate that β subunits associate with different affinities to each α_1 interaction domain. The rank order of AID_A affinity for each β subunit is as follows: $\beta_4 > \beta_{2a} > \beta_{1b} \gg \beta_3$. The binding of the β subunit to α_1 subunit can be inhibited *in vitro* by the AID_A synthetic peptide with an apparent K_i of 285 nM. This interaction can also be prevented in heterologous Ca^{2+} channels by the injection of the AID_A GST fusion protein into *Xenopus* oocytes. Our results demonstrate that the site of interaction between AID and β subunit is responsible for anchoring the β subunit to the α_1 subunit and thus allowing the β subunit to modify Ca^{2+} channel activity.

Voltage-dependent calcium channels are a primary pathway for Ca^{2+} entry into cells, thereby allowing the activation of numerous cellular processes (1). Several classes of Ca^{2+} channels have been distinguished based upon their functional and pharmacological properties (2). N-type and P-type Ca^{2+} channels are found in numerous central and peripheral neurons and play a key role in the control of neurotransmitter release (3). L-type Ca^{2+} channels are involved in excitation-contraction coupling in skeletal and cardiac muscle, whereas T-type Ca^{2+} channels are implicated in pacemaker activity. Two subtypes of these channels have been purified, the high voltage-activated skeletal muscle L-type channel (4) and the brain ω-conotoxin GVIA-sensitive N-type channel (5). Both Ca^{2+} channels are a complex of three structurally conserved (α_1, $\alpha_2\delta$, and β) and one more variable (γ or 95-kDa protein) subunits. The α_1 subunit contains the pore of the channel and constitutes the receptor of most drugs and toxins that regulate channel activity. The $\alpha_2\delta$ subunit is a 160-kDa glycosylated disulfide-linked complex of two proteins (α_2 and δ) that are associated via disulfide bonds. β subunits are smaller proteins of about 52–76 kDa (6–8). They are entirely cytoplasmic and, like the α_1 subunit, are substrates for various protein kinases (9). Previous biochemical analyses of these Ca^{2+} channels have demonstrated a very tight interaction between the α_1 and β subunits. It was found that both subunits are co-localized in the transverse tubular membrane of skeletal muscle (10), co-immunoprecipitate (7), and also co-purify from skeletal muscle (4) and from brain (5).

Ca^{2+} channel subunits have been separated into distinct classes based on molecular differences in α_1 (classes S, A, B, C, D, and E) and β (β_1, β_2, β_3, and β_4) subunits (6). Additional molecular diversity is introduced by alternative splicing from these genes (11–13). In most cases, expression of these α_1 subunits alone is sufficient to form calcium-selective and voltage-dependent channels. However, the coexpression of both $\alpha_2\delta$ and β subunits is likely required for the membrane targeting and/or the proper conformation of the α_1 subunit, thereby facilitating its functional expression (14–15). Both of these ancillary subunits also affect the channel gating by regulating the voltage-dependence and kinetics of its activation and inactivation (16–21). Interestingly, despite the extreme functional and molecular diversity of voltage-dependent Ca^{2+} channels, it was recently demonstrated that the structural and functional importance of subunit interaction sites is well conserved among these channels. Analysis of the β subunit regulation of voltage-dependent α_1 subunits has led to the identification of a primary interaction site between both subunits (20–21). The β subunit binds to a cytoplasmic sequence that is highly conserved and located on the cytoplasmic linker between repeats I and II of all functionally distinct α_1 subunits (Fig. 1*a*). To facilitate the description of this site, we have called it the α_1 subunit interaction domain or AID.[1] AID represents an 18-amino acid sequence of which only 9 amino acids are conserved among α_1 subunits (Fig. 1*b*). Thus, the 9 nonconserved residues make the AID sequence unique to each α_1 subunit isoform. In addition, the corresponding interacting domain on the β subunit was

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[1] The abbreviations used are: AID, α_1 subunit interaction domain; BID, β subunit interaction domain; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline.

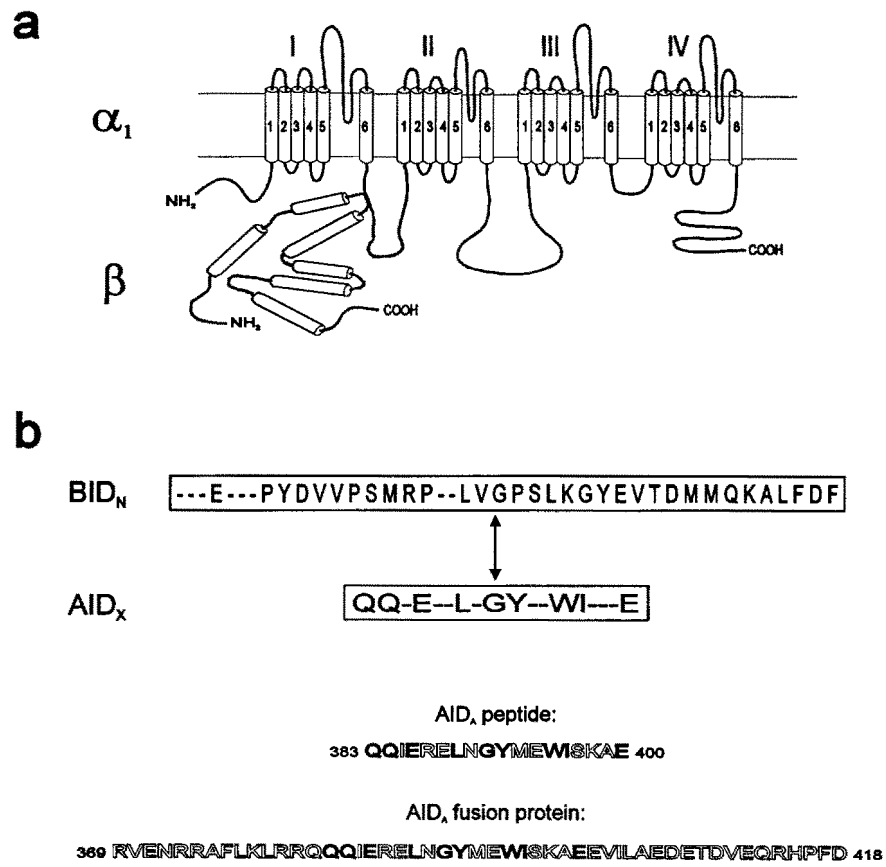

FIG. 1. **Diagram of α_1-β interaction site.** *a*, transmembrane folding models and interaction site of the Ca^{2+} channel α_1 and β subunits as determined by primary structure analyses. *Cylinders* represent predicted α-helical segments in the transmembrane regions of the α_1 subunit and in the peripherally associated β subunit. *b*, consensus α_1 (AID_X) and β (BID_{Ny}) interaction sites. The AID_X and BID_{Ny} sites describe the region of α_{1X}-β_{Ny} interaction where *X* is a capital letter (S, A, B, C, D, or E) that identifies the genes from which the α_1 subunit originates, *N* is a number (1, 2, 3, or 4) that identifies the genes from which β subunit originates, and *y* is the splice variant of that gene. The sequences of two AID proteins used throughout this manuscript are reproduced; the 18-amino acid AID_A peptide (amino acids 383–400 of the α_{1A} subunit) and the 50-amino acid AID_A GST fusion protein (amino acids 369–418). The conserved amino acids are in *filled lettering*.

identified as a 30-amino acid sequence in the N terminus of the second highly conserved domain of the β subunit (21). This site is responsible for the anchoring of the β subunit to AID and is required for current stimulation and hyperpolarizing shift in activation. We have named this second site the β interaction domain or BID. BID is even more conserved among β subunits, with a minimum of 76% amino acid identity than AID is among α_1 subunits. The overall amino acid identity of BID was, in fact, lowered by the discovery of more variability in the N-terminal sequence of a housefly β subunit (22). Both AID and BID are required for the binding of the β subunit to the α_1 subunit and for current regulation. The identification of these two domains in both α_1 and β subunits therefore provides a unique opportunity to characterize the biochemical properties of this subunit interaction. The results reported here establish the basis of a detailed analysis of the α_1-β interaction, which is aimed at resolving the structural determinants involved in the specificity of subunit recognition in voltage-dependent Ca^{2+} channels.

EXPERIMENTAL PROCEDURES

Reagents—TNT® coupled reticulocyte lysate system was purchased from Promega. The peptide corresponding to the amino acid sequence between 383 and 400 of the rabbit brain α_{1A} subunit (23) was synthesized at the biopolymers facility of the Howard Hughes Medical Institute (University of Texas Southwestern Medical Center). Isopropyl-1-thio-β-D-galactopyranoside was from Life Technologies, Inc.; reduced glutathione was from U. S. Biochemical Corp. [^{35}S]Methionine was from Amersham Corp., and glutathione-Sepharose 4B and protein G-Sepharose were from Pharmacia Biotech Inc. All other chemicals were of reagent grade.

In Vitro Translation of β Subunits—The ^{35}S-labeled β subunit probes were synthesized by coupled *in vitro* transcription and translation using the TNT system (Promega). Four cDNA clones were used: rat brain β_{1b} (GenBank accession number X61394), rabbit heart β_{2a} (X64297), rabbit heart β_3 (M88751), and rat brain β_4 (L02315). The concentration of the probe in the lysate after synthesis was determined by trichloroacetic acid protein precipitation in the presence of 2% casamino acids followed by scintillation counting. Control experiments demonstrate that between 6.3 and 8.4% of the free radioactive [^{35}S]methionine is incorporated into newly synthesized β subunits.

Overlay Experiments—The glutathione *S*-transferase (GST) fusion protein epitope of the α_{1A} subunit (23) was constructed and induced as described previously (21). Crude *Escherichia coli* DH5α cell lysate containing the α_{1A} GST fusion protein was pelleted and resuspended in equal volume of phosphate-buffered saline (PBS; 150 mM NaCl, 50 mM sodium phosphate (pH 7.4)). Samples of the lysate were electrophoretically separated on 3–12% gradient SDS-polyacrylamide gel and electrotransferred to nitrocellulose. The blots were blocked for 30 min with 5% nonfat dry milk in PBS. The probes were overlaid in PBS (1 μl/ml) and supplemented with 5% bovine serum albumin and 0.5% nonfat dry milk at different concentrations and times. The transfers were washed 1 h with 5% bovine serum albumin in PBS at room temperature, air-dried, and exposed to film (Kodak X-Omat AR) for specified times.

Immunoprecipitation of ^{35}S-labeled β Subunit—mAb $VD2_1$ ascites (7) were incubated in PBS overnight at 4 °C with protein G-Sepharose beads. These beads were washed 3 times with PBS and equilibrated in immunoprecipitation buffer containing 0.3 M NaCl, 20 mM HEPES, 1 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride (pH 7.4). *In vitro* translated ^{35}S-labeled β subunits (1–2 μl/ml) were incubated with saturating concentrations of $VD2_1$ protein G-Sepharose beads. After a variable amount of incubation time at 4 °C, the beads were centrifuged and washed 4 times with immunoprecipitation buffer, and the radioactivity bound to the beads was analyzed by liquid scintillation counting. Nonspecific binding was determined by measuring the radioactivity bound to control protein G-Sepharose beads.

Purification of GST Fusion Proteins—Large bacteria cultures containing the plasmids coding for control GST and α_1 GST fusion proteins (wild-type and mutant α_1 sequences containing the amino acid motif that binds β subunits) were inoculated with small overnight cultures, grown at 37 °C for 1 h, and induced for 4 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were pelleted and resuspended in PBS containing 1% Triton X-100 and sonicated twice for 15 s each. The sonicated material was centrifuged (12,000 rpm for 12 min on Beckmann JA-17 rotor). At this stage, the procedure differed for the control GST fusion protein and the wild-type or mutant α_{1A} GST fusion proteins. The supernatant of the control GST fusion protein was incubated with glutathione-Sepharose beads. In contrast, because more than 90%

of the wild-type or mutant α_{1A} GST fusion proteins remained in inclusion bodies (thus were largely insoluble), the initial supernatants were discarded, and the pellets were resuspended in 10 ml of PBS containing 1% sarcosyl. After a 30-min incubation at 4 °C, the insoluble material was removed by centrifugation as stated above. Triton X-100 was added to the supernatants to a final concentration of 2% and, as with the control GST fusion protein, the supernatants were then incubated with glutathione-Sepharose beads for 30 min at 4 °C. The beads that bound either the control GST, wild-type, or mutant α_{1A} GST fusion proteins were placed in columns and extensively washed with PBS. The GST fusion proteins were then eluted with 10 mM glutathione in 50 mM Tris (pH 8.0). Some batches of purified GST fusion proteins were also concentrated (Amicon) and dialyzed (Spectrum, 8000 molecular weight cut-off) against PBS at 4 °C to remove the glutathione that would compete for the reassociation of the GST fusion proteins to glutathione-Sepharose beads.

Covalent Coupling of Purified Fusion Proteins to Sepharose Beads—The purified control GST and α_{1A} GST fusion proteins were covalently coupled to cyanogen bromide (CNBr)-activated Sepharose beads in 100 mM $NaHCO_3$, 500 mM NaCl (pH 8.3) for 3 h at room temperature and at a protein concentration of 0.5 mg·ml^{-1}. The resins were extensively washed with PBS, and the remaining active groups were blocked with 0.2 M glycine (pH 8.0) for 2 h at room temperature.

Binding of the AID_A GST Fusion Protein to ^{35}S-Labeled β Subunits—Control and α_1 binding assays were performed in 1 ml of PBS containing 1–3 μl of *in vitro* translated ^{35}S-labeled β subunit and either 30 μl of control GST or α_{1A} GST fusion protein covalently coupled to CNBr-Sepharose beads or variable concentrations of purified control GST or α_{1A} GST fusion proteins noncovalently coupled to 30 μl of Sepharose beads. Reaction time and probe concentrations were varied independently as defined in the figure legends. After incubation, the beads were centrifuged and washed 4 times with PBS and counted by liquid scintillation. Nonspecific counts were determined by measuring the radioactivity bound to control glutathione-Sepharose beads. Control experiments demonstrate that these nonspecific counts are mainly due to the association of free [^{35}S]methionine (not ^{35}S-labeled β subunits) to glutathione-Sepharose beads. Also, the nonspecific binding of [^{35}S]methionine or ^{35}S-labeled β subunits to control GST fusion protein itself (up to 10 μM) was negligible. Thus, changing the concentration of GST fusion proteins bound to glutathione-Sepharose beads did not affect the amount of nonspecific counts. Also, because in most assays the concentration of the radioactive probe was not changed (thus also of [^{35}S]methionine), the nonspecific counts remained constant. Determination of the total amount of nonspecific counts showed that it represented between 10 and 20% of the total binding (nonspecific counts of [^{35}S]methionine binding to Sepharose beads + specific binding of ^{35}S-labeled β subunits to AID_A GST fusion protein). All of the experiments were performed in triplicate and the mean values are shown with ± S.D.

Scatchard Analysis—0.8–1.2 pM of *in vitro* translated ^{35}S-labeled β subunits (receptor R*) were incubated for 15 h at 4 °C in PBS with varying concentrations (50 pM to 2.5 μM) of α_1 GST fusion proteins (ligand L) that were noncovalently coupled to glutathione-Sepharose beads. Total binding and nonspecific counts were assessed following the procedure described under "Binding of the ^{35}S-Labeled β Subunit to the α_1 Subunit." Specific binding (complex LR*) was calculated by subtracting the nonspecific counts from the total binding. The values were then normalized with respect to their maximal and minimal counts. The affinity of the fusion protein for the β subunit was calculated according to the function $[LR^*]/[L_F] = -1/K_d \cdot [LR^*] + [LR^*]_{max}/K_D$ where K_D is the dissociation constant, $[L_F]$ is the free ligand concentration at equilibrium, and $[LR^*]_{max} = 1$ is the normalized maximum specific binding.

Preparation of Xenopus laevis Oocytes and Injections of cRNAs and GST Fusion Proteins—Stage V and VI oocytes were prepared as described previously (21) and maintained in a defined nutrient oocyte medium (24). cRNAs were transcribed *in vitro* using T7 (β_{1b} subunit) or SP6 polymerase (pSPCBI-2 cDNA). 50 nl of various subunit compositions were injected into each oocyte at the following concentrations: 0.7 μg·μl^{-1} (α_{1A} subunit) and 0.2 μg·μl^{-1} (β_{1b} subunit). Some batches of oocytes were also coinjected with control GST or α_{1A} GST fusion proteins at approximately 0.5 μM concentration in the oocyte. Prior to their injection, these fusion proteins were purified and dialyzed against PBS to remove any contaminating glutathione.

Electrophysiological Recordings and Data Analysis—Two-electrode voltage-clamps were performed using a Dagan TEV-200 amplifier. Voltage and current electrodes were filled with 3 M KCl and had resistances between 0.5 and 2 megaohms. The bath solution was clamped to 0 mV, which served as reference potential. The recording solution had the following composition: 40 mM $Ba(OH)_2$, 50 mM NaOH, 2 mM KCl, 1 mM niflumic acid, 0.1 mM EGTA, 5 mM HEPES (pH 7.4) (adjusted with methanesulfonic acid). Records were filtered at 0.2–0.5 kHz and sampled at 1–2 kHz. Leak and capacitance currents were subtracted on-line by a P/6 protocol. Voltage pulses were delivered every 10 s from a holding potential of −90 mV to various test pulses to determine the peak current. Data were analyzed using PCLAMP version 5.5 (Axon Instruments). All mean values are shown with ± S.E.

RESULTS AND DISCUSSION

In Vitro Binding Assay for the α_1-β Interaction—In order to investigate the α_1-β interaction in a quantitative manner, we developed an *in vitro* binding assay. The α_{1A} GST fusion protein coupled to CNBr-activated Sepharose beads (AID_A CNBr-Sepharose beads) can be used as a ligand for *in vitro* translated ^{35}S-labeled β_{1b} subunit. Fig. 2*a* demonstrates that ^{35}S-labeled β_{1b} subunit binds specifically to AID_A CNBr-Sepharose beads, whereas no specific binding is detected with an equivalent amount of control GST fusion protein coupled to CNBr-activated Sepharose beads (GST CNBr-Sepharose beads). As a control, we found that no binding occurs when the β subunit is denatured by boiling the probe before use (data not shown). The identity of the ^{35}S-labeled β_{1b} subunit recognized by AID_A CNBr-Sepharose beads is confirmed by its immunoprecipitation by $VD2_1$, a monoclonal antibody directed against the skeletal β_{1a} subunit, an alternative splice variant that shares strong sequence identity with the β_{1b} subunit (25). Finally, the AID_A CNBr-Sepharose beads fail to bind the *in vitro* translated ^{35}S-labeled $\alpha_2\delta_b$ subunit. We confirmed that both the AID and BID sequences were specifically required for the interaction between AID-Sepharose beads and β subunits by using previously characterized mutants of these domains (20–21). We found that, compared with the wild-type AID_A GST fusion protein, there is a 15-fold reduction in the maximum binding of 1 μM of AID_{Y392S}-Sepharose beads to ^{35}S-labeled β_{1b}, a noninteracting AID_A mutant in an overlay assay (20) (Fig. 2*b*). Also 1 μM mutant AID_{E400A}, which shows a significant degree of structural and functional interaction, bound 58.4 ± 1.7% (n = 3), only 1.7 times less than the maximum binding by 1 μM wild-type AID_A GST fusion protein. These values are thus in close agreement with a 9- and 1.3-fold reduction in current amplitude stimulation by β_{1b} subunit observed upon coexpression of the full-length mutants $Y392S_{\alpha 1A}$ and $E400A_{\alpha 1A}$ subunits, respectively (20). Also, consistent with the results of the AID mutations, we found that AID_A Sepharose beads binds 95.6 ± 16.3% (n = 3) of the ^{35}S-labeled β_{1b} that can be immunoprecipitated by the monoclonal antibody $VD2_1$. In contrast, AID_A Sepharose beads bound 0 ± 2% (n = 3) of ^{35}S-labeled β_{P237R}, a noninteracting BID mutant in overlay and expression experiments (21), and 101.6 ± 6.9% (n = 3) of ^{35}S-labeled β_{P221R}, an interacting BID mutant (Fig. 2*c*). Overall, these results demonstrate that, in this *in vitro* bead assay, the binding of β subunits to AID_A-Sepharose beads occurs specifically via the AID and BID sequences.

Association and Dissociation Kinetics of the AID_A GST Fusion Protein to β Subunit—In order to determine association kinetics, we measured the amount of ^{35}S-labeled β_{1b} subunit bound to AID_A-Sepharose beads over time as the binding reaction approached equilibrium (Fig. 3*a*). Fitting the data with a hyperbolic function revealed that the AID_A association was monophasic. The calculated association rate constant is k_a = 0.1 min^{-1}·μM^{-1} for the binding of ^{35}S-labeled β_{1b} to AID_A-Sepharose beads. At 500 nM AID_A GST fusion protein, the concentration used in this experiment, this rate constant corresponds to a half-time $t_{1/2}$ of about 20 min. The kinetics of ^{35}S-labeled β_{1b} subunit binding to a saturating concentration of $VD2_1$ protein G-Sepharose was also measured (Fig. 3*b*). With a half-time of 61 min, the association to the antibody was thus 3-fold slower than to AID_A. In both cases, the binding was

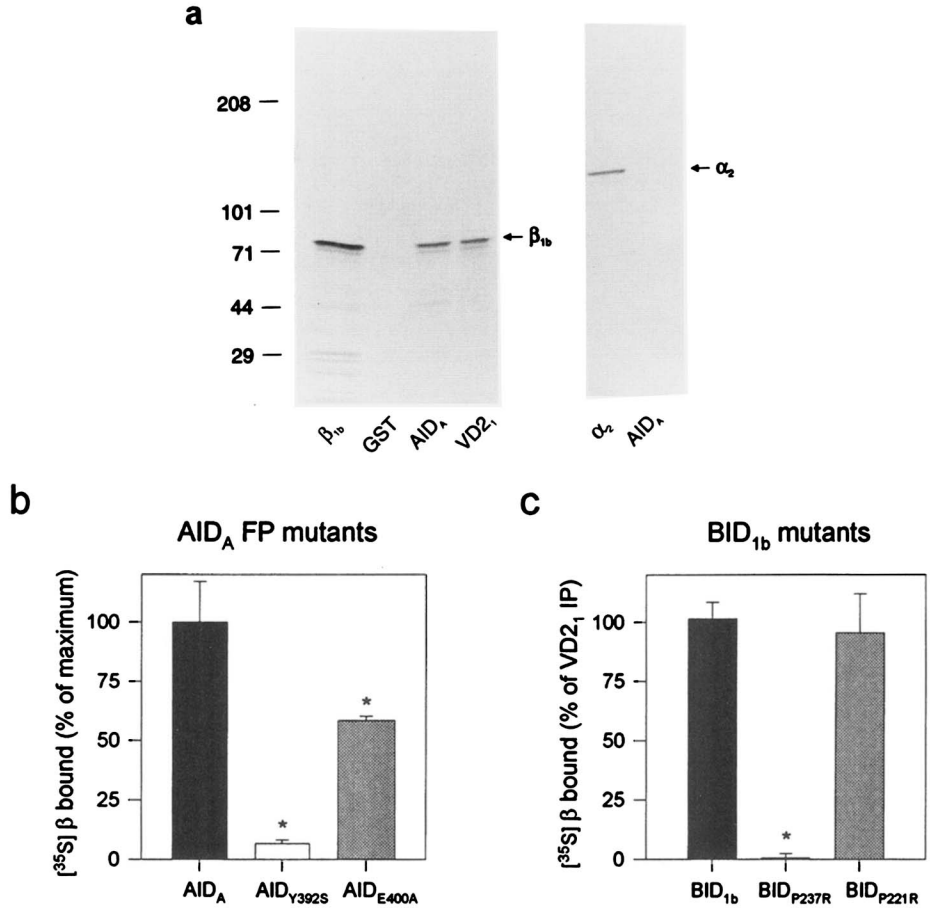

FIG. 2. **Probing the α_1-β interaction with an *in vitro* binding assay.** *a*, autoradiogram of a polyacrylamide gel with 4 μl of lysate containing 1.3 nM *in vitro* translated ^{35}S-labeled β_{1b} (β_{1b}), ^{35}S-labeled β_{1b} bound to control GST CNBr-Sepharose beads (*GST*), ^{35}S-labeled β_{1b} bound to AID_A CNBr-Sepharose beads (AID_A), immunoprecipitation of ^{35}S-labeled β_{1b} by a saturating concentration of $VD2_1$ protein G-Sepharose beads ($VD2_1$), 4 μl of lysate containing 0.8 nM *in vitro* translated ^{35}S-labeled $\alpha_2\delta_b$ (α_2) and ^{35}S-labeled $\alpha_2\delta_b$ bound to AID_A CNBr-Sepharose beads (AID_A). *b*, ^{35}S-labeled β_{1b} subunit bound to 1 μM AID_{Y392S} and AID_{E400A}-Sepharose beads expressed as a percentage of ^{35}S-labeled β_{1b} bound to 1 μM wild-type AID_A-Sepharose beads (overnight reaction times). *c*, wild-type and mutant ^{35}S-labeled β_{1b} bound to 1 μM AID_A-Sepharose beads expressed as a percentage of wild-type or mutant ^{35}S-labeled β_{1b} subunit immunoprecipitated (*IP*) by $VD2_1$ (overnight reaction times). Probe concentration in *b* and *c* was 0.38 pM.

stable for the duration of the experiment once it reached its equilibrium value.

With respect to the functional importance of β subunits in the regulation of current properties, we further analyzed the effects of different agents that may affect the conformation of the β_{1b} subunit and its ability to associate with AID_A CNBr-Sepharose beads. We found that the maximum association of ^{35}S-labeled β_{1b} was not affected by changes in Ca^{2+} concentration (1 nM to 1 mM), ionic strength (0 to 2 M NaCl), or phosphorylation by protein kinase C (data not shown). Large variations in pH (4 to 10) could decrease the maximal association by 42%. This interaction was even totally abolished at pH 12. However, small variations of pH in physiological range (6.9–7.5) were without effects. These results are therefore consistent with previous observations that the purified N-type Ca^{2+} channel complex remains intact at pH 10 (5, 26). Overall, these data demonstrate that the AID-BID interaction site is an unlikely target for cell inhibitory regulation of the channel activity such as those implicated in Ca^{2+} dependent inactivation.

The dissociation of the ^{35}S-labeled β_{1b}-AID_A-Sepharose bead complex was monitored after the binding reactions reached equilibrium (with 500 nM AID_A GST fusion protein). The beads were washed twice with PBS to eliminate the free [^{35}S]methionine and the residual unbound probe. They were then resuspended in 1 ml of PBS, and the dissociation was triggered by the addition of an excess (500 μM) of the competing AID_A peptide. This concentration of the peptide is sufficient to fully inhibit the association of ^{35}S-labeled β_{1b} to AID_A CNBr-Sepharose beads (see Fig. 7). After various times, the beads were washed twice again to remove the probe dissociated from AID_A-Sepharose beads, and the amount of ^{35}S-labeled β_{1b} that remained bound was measured. At 4 °C, the interaction between AID_A and β_{1b} was almost irreversible, as no measurable decrease in the amount of β subunit bound to the beads was observed up to 8 h (Fig. 3*c*). A similar result was obtained with AID_A-CNBr-Sepharose beads in the absence of AID_A peptide (data not shown). Also, this result is consistent with the observation that injection of the AID_A peptide or AID_A GST fusion protein did not induce dissociation of preformed voltage-dependent Ca^{2+} channels expressed in *Xenopus* oocytes (data not shown).

Four Different β Subunits Can Interact with the Same AID Sequence—^{35}S-Labeled *in vitro* translated β subunits (β_{1b}, β_{2a}, β_3, and β_4) were incubated overnight with AID_A GST fusion protein from *E. coli* lysates separated on SDS-polyacrylamide gels and transferred to nitrocellulose (Fig. 4). The data demonstrate that all four β subunits were capable of interacting with the same AID sequence. Thus these overlay experiments prove that sequence variability among and beyond the BID sequences of these β subunits does not prevent their interaction with a single AID sequence. These results confirm recent expression experiments demonstrating the functional interaction of several β subunit types with the full-length α_{1A} or α_{1C} subunits by amplitude stimulation and changes in the voltage-dependence and kinetics of the current (21, 27). They are also comparable with the results showing that a single BID sequence can interact with multiple AID sequences (see Fig. 6*b*). Altogether, these observations suggest the possible existence of a cross-reactivity between various α_1 and β subunit isoforms. If these interactions are confirmed *in situ*, such a cross-reactivity would have important consequences on the functional diversity of voltage-dependent Ca^{2+} channels. However, preliminary results based on the characterization of the subunits that compose the skeletal muscle L-type and the neuronal N-type Ca^{2+} channels have suggested the existence of a rather strong specificity in the interaction between α_1 and β subtypes. In these Ca^{2+}

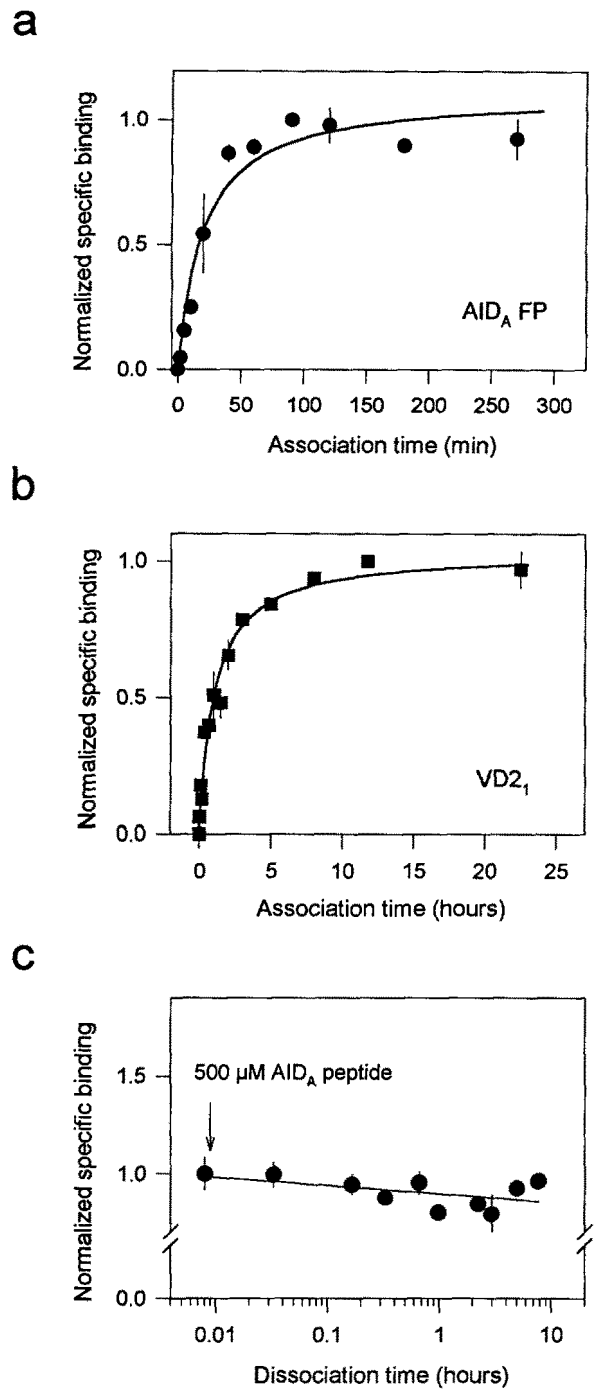

FIG. 3. **Time course of AID$_A$ GST fusion protein association and dissociation to [^{35}S]β_{1b} subunit.** *a*, binding to AID$_A$-Sepharose beads. 500 nM of AID$_A$ GST fusion protein coupled to 40 μl of Sepharose beads were incubated at 4 °C for various times with 0.32 pM of *in vitro* translated ^{35}S-labeled β_{1b} probe in a 1-ml reaction volume. The beads were then washed 4 times with cold PBS, and the association of the probe was measured by counting. Nonspecific counts increased linearly with time by 22%/hour (not shown). The highest nonspecific was 15.7 ± 1.4% (n = 3) of maximum total binding. For each time point, the specific binding was normalized to the maximum specific binding. The data were fitted with a hyperbolic function $f = a \cdot t/(t_{½} + t)$ where t is the association time, a = 1.109 (the asymptotic maximum), and $t_{½}$ = 20 min (the time of half-association). *b*, binding to VD2$_1$ monoclonal antibody coupled to protein G-Sepharose beads. 40 μl of beads were incubated at 4 °C for various times with 0.34 pM ^{35}S-labeled β_{1b} in 1 ml of PBS. Nonspecific counts were 6.8 ± 2.2% (n = 3) of maximum total binding. The asymptotic maximum is a = 1.03, and the half-association time is $t_{½}$ = 63 min. *c*, the association was allowed to reach equilibrium by overnight incubation of the reactants under similar concentrations than those described in *a*. The dissociation was then measured at 4 °C in the presence of 500 μM AID$_A$ peptide. Nonspecific counts remained fairly constant with a linear decrease of 4%/hour (not shown). The highest nonspecific binding was 16.1 ± 1.9% (n = 3) of maximum total binding. A semilogarithmic plot was used to represent the data and the linear regression was fit to the data.

channels, the α_{1S} subunit (28) interacts specifically with the β_{1a} subunit (29), whereas the α_{1B} subunit interacts with the β_3 subunit (5). In an attempt to resolve some of these experimental contradictions and in order to get a better understanding of the possible molecular basis for the specificity of α_1-β interaction seen in purified Ca^{2+} channels, we have analyzed the affinity of various β subunits for the AID$_A$ site.

Ligand Binding Properties—Analysis of the binding of AID$_A$ to ^{35}S-labeled β_{1b} subunit demonstrates that the specific binding is saturable and occurs on a single binding site (Fig. 5). We calculated an apparent K_d of 5.8 nM for the binding of AID$_A$ GST fusion protein to β_{1b} subunit. It is, however, likely that the real affinity between α_1 and β subunits is in fact higher than reported here, considering the use of a fusion protein as the ligand instead of the full-length α_1 subunit. Scatchard analysis of the binding of AID$_A$ GST fusion protein to other β subunits confirms the observations of the overlay experiment of Fig. 4 demonstrating that AID$_A$ is able to interact with all four classes of β subunits. However, this analysis provided more detailed information than the overlay data. Despite the strong sequence homology in the respective BID sequence of these β subunits (87% amino acid identity), the binding of AID$_A$ GST fusion protein to all four β subunits occurs with different apparent affinities. For instance, the binding of AID$_A$ GST fusion protein to the β_3 subunit occurs with a K_d of about 55 nM, which is at least 1 order of magnitude lower than the high affinity state observed for β_{1b}, β_{2a}, and β_4 subunits. This result strongly points to the importance of the conformation of β subunits in determining the affinity of the α_1-β interaction. Also, two binding sites can be seen with β_{2a} and β_4 subunits. The presence of the lower affinity site cannot be due to proteolytic fragments of the fusion protein since the same purified material was used for all four Scatchard experiments described in Fig. 5. The low and high affinity sites seen with β_{2a} and β_4 subunits were therefore inherent to the β subunit themselves. Expression experiments with truncated forms of the β subunits have suggested that sequence deletion may induce a lower affinity of the β subunit for the α_{1A} channel expressed in *Xenopus* oocytes (21). Therefore, it is likely that the binding of partially proteolyzed forms of β subunits accounts for the component of lowest affinity. Consistent with this interpretation are the observations that (i) β_{2a} and β_4 were the most sensitive to proteolysis (Fig. 4*a*), (ii) synthetic peptides containing the BID$_b$ sequence (*i.e.* β_{1b} 213–245) could not bind the AID$_A$ GST fusion protein (data not shown), and (iii) very proteolyzed forms of the β subunit could not interact with AID$_A$ GST fusion protein beads (Fig. 2*a*). Alternatively, we cannot rule out that the second site of lowest affinity resulted from misfolding of a fraction of the *in vitro* synthesized β subunits. Finally, the Scatchard analyses provide a rank order of binding affinity of each β subunit to AID$_A$ GST fusion protein that is $\beta_4 > \beta_{2a} > \beta_{1b} \geqslant \beta_3$ when taking into account the component of highest affinity. With the exception of β_{2a}, this is also the order in which β subunits can be classified for their potency of α_{1A} current stimulation in oocytes, suggesting that the affinity between α_1 and β, and the β current stimulation efficiencies may be correlated (21). Incidentally, these results also confirm several predictions suggesting that the β_4 subunit is probably associated with a class A α_1 subunit in native Ca^{2+} channels expressed in the cerebellum (30–31). The development of this quantitative assay capable of analyzing the interaction of var-

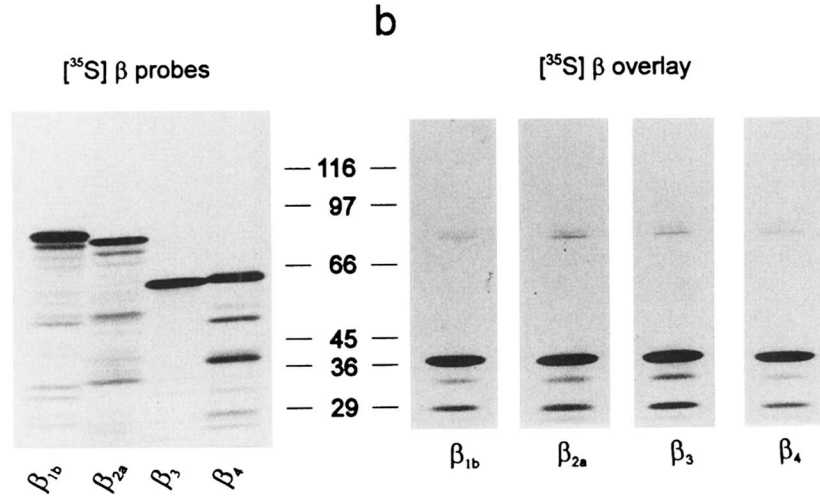

FIG. 4. **Four different β subunit gene products interact with AID_A expressed as a GST fusion protein.** *a*, autoradiogram of SDS-polyacrylamide gel of *in vitro* translated [^{35}S]methionine-labeled β_{1b}, β_{2a}, β_3, and β_4 subunits. 5 μl of each translation reaction were run per lane. The film was exposed for 2 h. *b*, autoradiogram of ^{35}S-labeled β subunit overlays on AID_A GST fusion protein immobilized on nitrocellulose. The probe concentrations were between 0.3 and 0.6 pM. The overlays were exposed for 3 h. Molecular weight standards ($\times 10^{-3}$) are indicated in the *middle*.

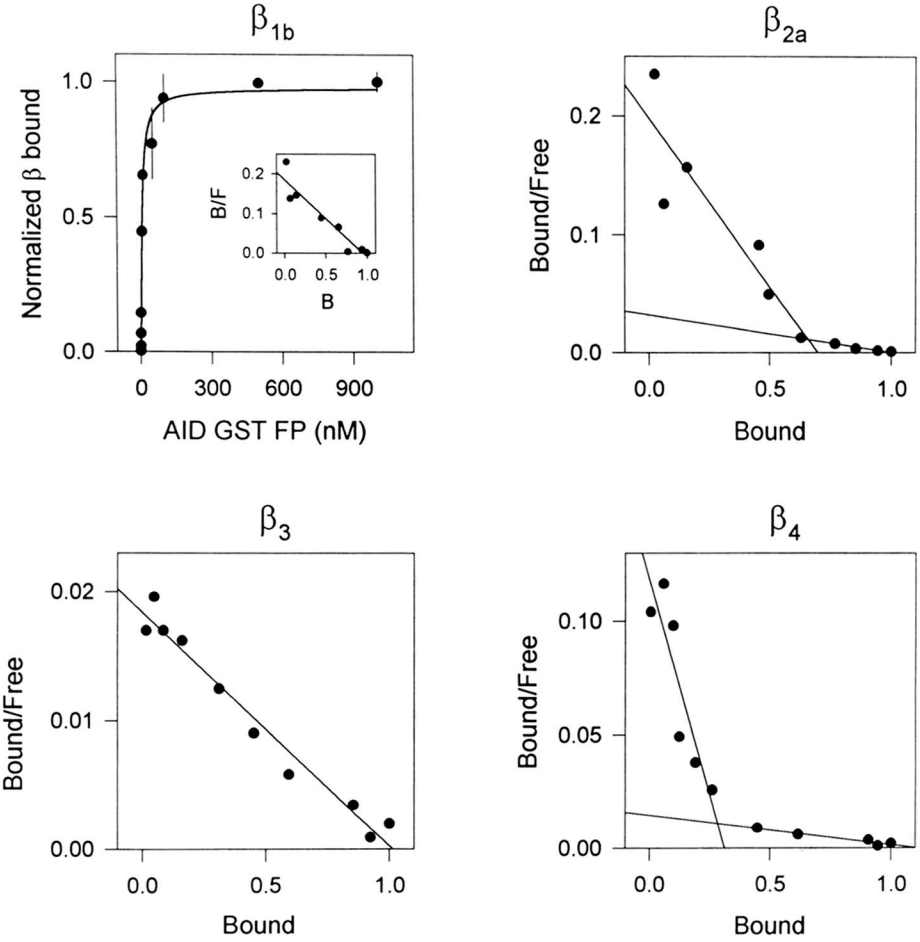

FIG. 5. **Scatchard analysis of AID_A GST fusion protein binding to various β subunits.** Various concentrations of AID_A GST fusion protein (50 pM to 1 μM) were precoupled for 1 h to glutathione-Sepharose beads and then incubated for 15 h at 4 °C with *in vitro* translated ^{35}S-labeled β subunits (0.7 to 1.2 pM) as described under "Experimental Procedures." A saturation curve is shown for β_{1b} and Scatchard representation for all β subunits (see *inset* for β_{1b}). Linear regression fitting of the data yielded apparent K_d values of 5.8 nM (β_{1b}), 3.5 and 36 nM (β_{2a}), 55.1 nM (β_3), and 2.6 and 76 nM (β_4).

ious BID sequences with a given AID sequence represents therefore a unique way to test the affinity of several β subunits for a given α_1 subunit. This assay combined with an extensive mutagenesis of the AID and BID sequences, will ultimately prove helpful to the understanding of the molecular determinants implicated into the specificity of subunit interaction of native voltage-dependent Ca^{2+} channels (4–5).

Competition of the β-AID_A GST Fusion Protein Interaction by a Synthetic AID_A Peptide—Competition experiments were performed with the 18-amino acid synthetic AID_A peptide. Four hours of preincubation of the ^{35}S-labeled β_{1b} probe with 100 μM AID peptide was sufficient to entirely prevent the subsequent association of ^{35}S-labeled β_{1b} with AID_S, AID_A, AID_B, and AID_C GST fusion proteins immobilized on nitrocellulose (Fig. 6). Similar results were obtained by using 1 μM AID_S GST fusion protein instead of 100 μM AID_A peptide (data not shown). In contrast, the same concentration of a control unrelated peptide had no effect. These experiments demonstrate that (i) the AID sequence is by itself able to bind to β subunits without requiring N and C termini flanking sequences and (ii) the binding of the peptide is also irreversible. This 18-amino acid sequence is therefore sufficient by itself to prevent the association between α_1 and β subunits. The AID_A GST fusion protein differs from the AID_A peptide by the presence of additional 14- and 18-amino acid sequences at the N and C termini of the α_{1A} sequence. In order to understand the role of these nonbinding

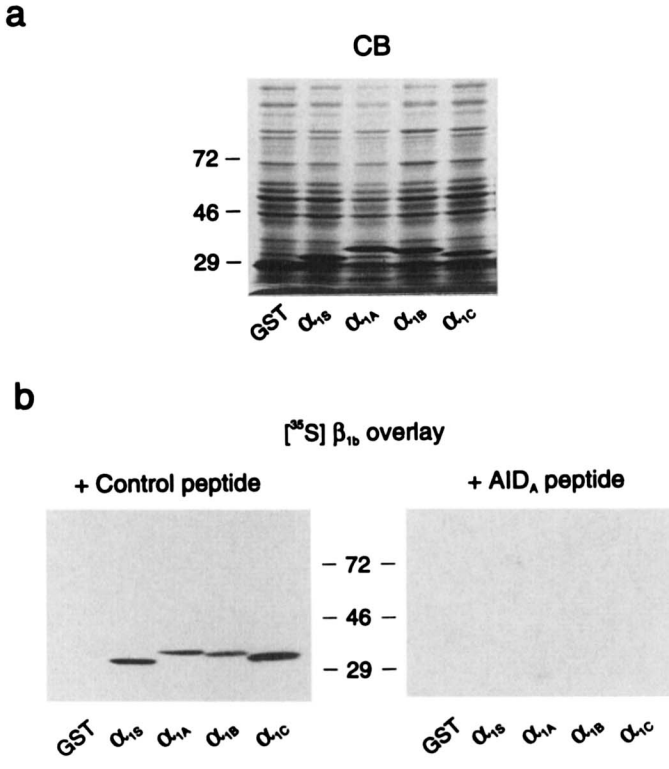

FIG. 6. **Peptide inhibition of α_1-β association.** *a*, crude *E. coli* lysates (70 μl) of control GST, and AID_S, AID_A, AID_B, and AID_C GST fusion proteins were analyzed on Coomassie Blue-stained 3–12% SDS-polyacrylamide gel. The sequence of these fusion proteins were described elsewhere (20). *b*, autoradiograms of the corresponding overlays of these fusion proteins with 0.5 pM ^{35}S-labeled β_{1b} subunit probe. The probe was preincubated for 4 h with 100 μM of a control 12-amino acid peptide (CCPNVPSRPQAM) of the C-terminal dystrophin related protein (33) (*left*) or 100 μM 18-amino acid AID_A peptide (*right*). The overlays were exposed for 2 h. The positions of the molecular weight standards ($\times 10^{-3}$) are shown on the *left* of the Coomassie Blue-stained gel and between the two overlays.

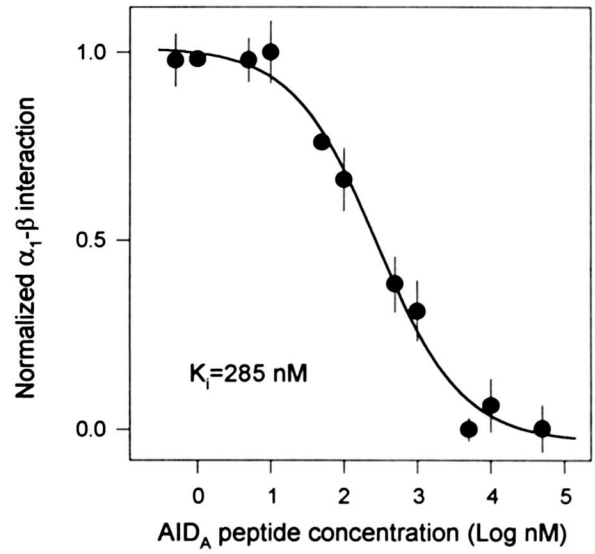

FIG. 7. **AID_A peptide inhibition of AID_A GST fusion protein binding to [^{35}S]labeled β_{1b} subunit.** The β_{1b} probe (0.32 pM) was preincubated for 6 h with various concentrations of the AID_A peptide and then incubated with AID_A CNBr-Sepharose beads. Nonspecific counts were 18 ± 2.2% (n = 3) of total maximum binding. The data were fitted with a four-parameter logistic function $f([AID]) = (a - c)/(1 + ([AID]/K_i)^b) + c$ where a = 1.013 and c = −0.03 are the asymptotic maximum and minimum respectively, b = 0.75 is the slope parameter, K_i = 285.7 nM is the inhibition constant at the inflection point, and [AID] is the concentration of AID_A peptide.

sequences in the AID-β interaction, we compared the relative affinities of the AID_A peptide and AID_A GST fusion protein to bind to the ^{35}S-labeled β_{1b} subunit.

The ^{35}S-labeled β_{1b} probe was incubated for 6 h with increasing concentrations of AID_A peptide (0.5 nM to 100 μM). The mixture was then incubated overnight with AID_A CNBr-Sepharose beads, and the amount of ^{35}S-labeled β_{1b} able to bind to the AID_A CNBr-Sepharose beads was measured by scintillation counting. The results demonstrate that the binding of the AID_A peptide to ^{35}S-labeled β_{1b} prevents the association of the probe to the AID_A CNBr-Sepharose beads with a K_i of 285 nM (Fig. 7). Complete inhibition of ^{35}S-labeled β_{1b} association to AID_A CNBr-Sepharose beads by AID_A peptide occurs at concentrations above 10 μM. These data show that the association of AID_A peptide to the β subunit occurs with a 57-fold lower affinity than the association of the AID_A GST fusion protein to the β subunit. The results suggest therefore that the interaction of the AID sequence with the β subunit is greatly facilitated by flanking noninteracting sequences. This probably occurs by the determination of a more favorable conformation of the AID sequence. Because of the ability of the AID sequence to prevent the interaction between the β subunit and the AID GST fusion protein, we further tried to determine whether the synthetic AID_A peptide or the AID_A GST fusion protein were also capable of inhibiting the native interaction between the β subunit and the full-length α_1 subunit in cells.

Injection of AID_A GST Fusion Protein into Xenopus Oocytes Prevents the Native Interaction between α_{1A} and β_{1b} Subunits—Both AID and BID are essential structural elements to the functional regulation of voltage-dependent Ca^{2+} entry by β subunits. We have previously demonstrated that the interaction of these two domains is required for (i) the subunit assembly of the Ca^{2+} channel and (ii) the biophysical and pharmacological changes by β subunits (21). In the case of the α_{1A} subunit, the biophysical regulation by various β subunits include a dramatic stimulation in current amplitude, hyperpolarizing displacements of the voltage-dependence of activation, and inactivation and modulations in the kinetics of inactivation (32). Injection of 0.5 μM AID_A GST fusion protein into *Xenopus* oocytes sustainably decreased the amplitude of the current carried by the α_{1A} subunit (Fig. 8*a*). There was an average 9.2 ± 2.7-fold (n = 3) decrease in current amplitude stimulation by the β_{1b} subunit that lasted for at least 5 days after injection of the cRNAs encoding the α_{1A} and the β_{1b} subunits (Fig. 8*b*). In comparison, the control GST fusion protein only reduced the β current stimulation by a nonsignificant factor of 1.09 ± 0.36-fold (n = 3) over the same time period. Also, the AID_A GST fusion protein induces a depolarizing shift in the current-voltage relationship of activation (Fig. 8*c*). The amplitude of the shift equaled 11 mV at the peak of the current amplitude. Finally, the AID_A GST fusion protein induced a depolarizing shift in the voltage dependence of inactivation (data not shown). None of these depolarizing shifts were detected with the injection of an identical concentration of control GST fusion protein, demonstrating the implication of the AID_A sequence. All of these effects (decrease in current amplitude and depolarizing shifts in activation and inactivation) go into directions that are opposite to the effects of β subunits on the α_{1A} current (21, 32). These results demonstrate, therefore that the AID_A GST fusion protein is able to durably prevent the association between full-length α_{1A} and β_{1b} subunits in native conditions. In contrast, this association was not prevented by the AID_A peptide, probably because of its lower affinity for the β subunit or because of a reduced stability in oocytes (data not shown). The Scatchard analyses of the interaction between AID_A and

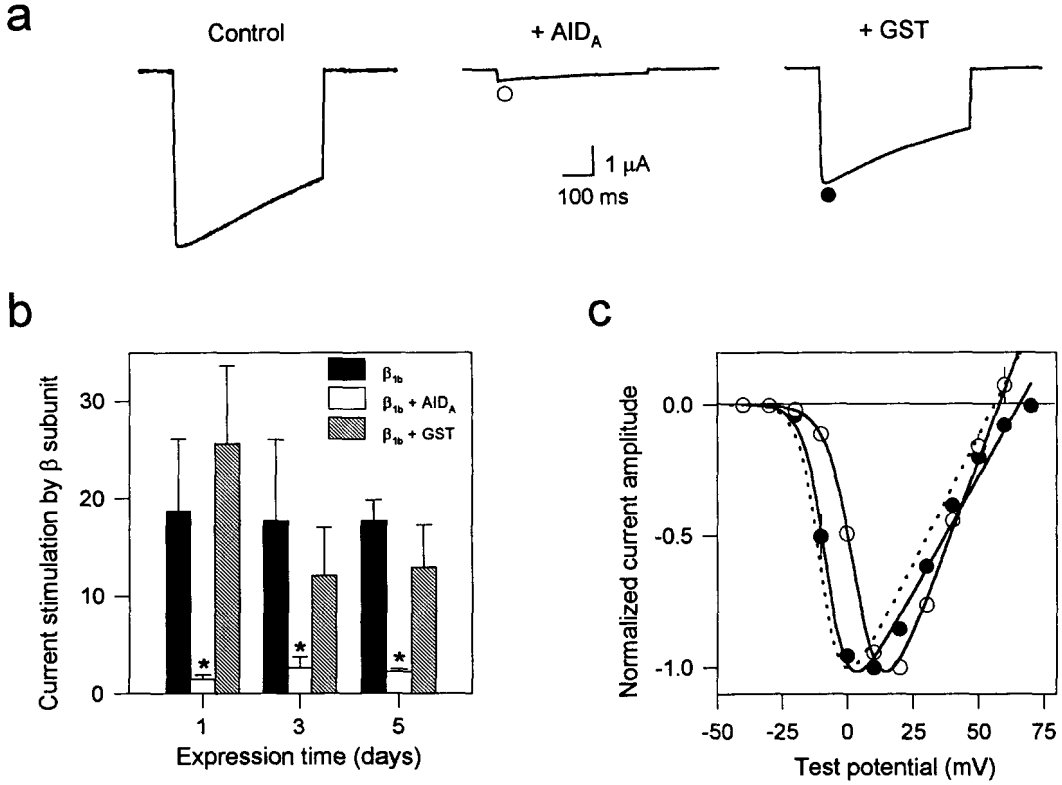

FIG. 8. **Injection of AID$_A$ fusion protein blocks the interaction between full-length α_1 and β subunits in oocytes.** *a*, Ba^{2+} current traces obtained 2 days after expression of α_{1A} and β_{1b} subunits without (*control*) or with (*GST* or *AID$_A$*) coinjection of 0.5 μM control GST or AID$_A$ GST fusion protein. The holding potential is −90 mV, and the test potential is 10 mV (*control* and *GST*) or 20 mV (*AID$_A$*) to induce the maximum inward current. *b*, average Ba^{2+} current stimulation induced by β_{1b} subunit in control oocytes and AID$_A$- or GST-injected oocytes as a function of expression time. Data are the average of 64 oocytes. *c*, average normalized current-voltage relation for $\alpha_{1A}\beta_{1b}$ + control GST (*filled circle*) or $\alpha_{1A}\beta_{1b}$ + AID$_A$ (*empty circle*). The current-voltage relationship for $\alpha_{1A}\beta_{1b}$ is given as a *dashed line*. Data were fitted with a modified Boltzmann function with $I_{Ba} = (g(TP - E))/(1 + \exp(-(TP - V_{½})/k))$ where g = 79.1 microsiemens (control GST) or 17.9 microsiemens (AID$_A$) is the conductance, E = 65 (GST) or 58 mV (AID$_A$) is the reversal potential, TP is the test potential, and k = 4.3 (GST) or 5.2 mV (AID$_A$) is the range of potential for an e-fold change around $V_{½}$ = −7.6 (GST) or 4.1 mV (AID$_A$) oocytes. The data and the curve resulting from the fit of the data were normalized with respect to the maximum current amplitude reached in each experimental condition.

various β subunits suggest that probably all AID sequences are likely able to inhibit the regulatory function of β subunits by diverting their binding activity. However, because of differences in AID-BID affinities, it is also likely that the AID GST fusion protein inhibition of the α_1-β coupling can be greatly facilitated by the use of the appropriate AID sequence.

In conclusion, expression in various cell types of proteins containing the AID sequence should prove useful to investigate the functional importance of β subunits in several important Ca^{2+} channel functions such as excitation-contraction coupling or excitation-secretion coupling. Disruption of the anchoring of β subunits to α_1 subunits in native Ca^{2+} channels may contribute to the identification of the molecular targets of various channel regulators, such as G proteins or endogenous kinases. Finally, the *in vitro* method described here for the study of the α_1-β interaction will also be useful to the screening of drugs capable of modulating the anchoring/regulation of β subunits to/of Ca^{2+} channels.

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REFERENCES

1. Pietrobon, D., Di Virgilio, F., and Pozzan, T. (1990) *Eur. J. Biochem.* **193,** 599–622
2. Miller, R. (1992) *J. Biol. Chem.* **267,** 1403–1406
3. Takahashi, T., and Momiyama, A. (1993) *Nature* **366,** 156–158
4. Flockerzi, V., Oeken, H.-J., Hofmann, F., Pelzer, D., Cavalie, A., and Trautwein, W. (1986) *Nature* **323,** 66–68
5. Witcher, D. R., De Waard, M., Sakamoto, J., Franzini-Armstrong, C., Pragnell, M., Kahl, S. D., and Campbell, K. P. (1993) *Science* **261,** 486–489
6. Birnbaumer, L., Campbell, K. P., Catterall, W. A., Harpold, M. M., Hofmann, F., Horne, W. A., Mori, Y., Schwartz, A., Snutch, T. P., Tanabe, T., and Tsien, R. W. (1994) *Neuron* **13,** 503–506
7. Leung, A. T., Imagawa, T., Block, B., Franzini-Armstrong, C., and Campbell, K. P. (1988) *J. Biol. Chem.* **263,** 994-1001
8. Pragnell, M., Sakamoto, J., Jay, S. D., and Campbell, K. P. (1991) *FEBS Lett.* **291,** 253–258
9. Jahn, H., Nastainczyk, W., Röhrkasten, A., Schneider, T., and Hofmann, F. (1988) *Eur. J. Biochem.* **178,** 535–542
10. Jorgensen, A. O., Shen, A. C.-Y., Arnold, W., Leung, A. T., and Campbell, K. P. (1989) *J. Cell Biol.* **109,** 135–147
11. Perez-Reyes, E., Wei, X., Castellano, A., and Birnbaumer, L. (1990) *J. Biol. Chem.* **265,** 20430–20436
12. Powers, P. A., Liu, S., Hogan, K., and Gregg, R. G. (1992) *J. Biol. Chem.* **267,** 22967–22972
13. Snutch, T. P., Tomlinson, W. J., Leonard, J. P., and Gilbert, M. M. (1991) *Neuron* **7,** 45–57
14. Neely, A., Wei, X., Olcese, R., Birnbaumer, L., and Stefani, E. (1993) *Science* **262,** 575–578
15. Nishimura, S., Takeshima, H., Hofmann, F., Flockerzi, V., and Imoto, K. (1993) *FEBS Lett.* **324,** 283–286
16. Lacerda, A. E., Kim, H. S., Ruth, P., Perez-Reyes, E., Flockerzi, V., Hofmann, F., Birnbaumer, L., and Brown, A. M. (1991) *Nature* **352,** 527–530
17. Singer, D., Biel, M., Lotan, I., Flockerzi, V., Hofmann, F., and Dascal, N. (1991) *Science* **253,** 1553–1557
18. Soong, T. W., Stea, A., Hodson, C. D., Dubel, S. J., Vincent, S. R., and Snutch, T. P. (1993) *Science* **260,** 1193–1136
19. Varadi, G., Lory, P., Schultz, D., Varadi, M., and Schwartz, A. (1991) *Nature* **352,** 159–162
20. Pragnell, M., De Waard, M., Mori, Y., Tanabe, T., Snutch, T. P., and Campbell, K. P. (1994) *Nature* **368,** 67–70
21. De Waard, M., Pragnell, M., and Campbell, K. P. (1994) *Neuron* **13,** 495–503
22. Grabner, M., Wang, Z., Mitterdorfer, J., Rosenthal, F., Charnet, P., Savchenko, A., Hering, S., Ren, D., Hall, L. M., and Glossmann, H. (1994) *J. Biol. Chem.* **269,** 23668–23674
23. Mori, Y., Friedrich, T., Kim, M.-S., Mikami, A., Nakai, J., Ruth, P., Bosse, E., Hofmann, F., Flockerzi, V., Furuichi, T., Mikoshiba, K., Imoto, K., Tanabe, T., and Numa, S. (1991) *Nature* **350,** 398–402

24. Eppig, J. J., and Dumont, J. N. (1976) *In Vitro* **12,** 418–427
25. Sakamoto, J., and Campbell, K. P. (1991) *J. Biol. Chem.* **266,** 18914–18919
26. Witcher, D. R., De Waard, M., and Campbell, K. P. (1993) *Neuropharmacology* **32,** 1127–1139
27. Hullin, R., Singer-Lahat, D., Freichel, M., Biel, M., Dascal, N., Hofmann, F., and Flockerzi, V. (1992) *EMBO J.* **11,** 885–890
28. Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kajima, M., Matsuo, H., Hirose, T., and Numa, S. (1987) *Nature* **328,** 313–318
29. Ruth, P., Rohrkasten, A., Biel, M., Bosse, E., Regulla, S., Meyer, H. E., Flockerzi, V., and Hofmann, F. (1989) *Science* **245,** 1115–1118
30. Castellano, A., Wei, X., Birnbaumer, L., and Perez-Reyes, E. (1993) *J. Biol. Chem.* **268,** 12359–12366
31. Starr, T. V., Prystay, W., and Snutch, T. P. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5621–5625
32. De Waard, M., and Campbell, K. P. (1995) *J. Physiol.*, in press
33. Love, D. R., Hill, D. F., Dickson, G., Spurr, N. K., Byth, B. C., Marsden, R. F., Walsh, F. S., Edwards, Y. H., and Davies, K. E. (1989) *Nature* **339,** 55–58