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A β_4 Isoform-specific Interaction Site in the Carboxyl-terminal Region of the Voltage-dependent Ca^{2+} Channel α_{1A} Subunit*

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The voltage-gated calcium channel β subunit is a cytoplasmic protein that stimulates activity of the channel-forming subunit, α_1, in several ways. Complementary binding sites on α_1 and β have been identified that are highly conserved among isoforms of the two subunits, but this interaction alone does not account for all of the functional effects of the β subunit. We describe here the characterization *in vitro* of a second interaction, involving the carboxyl-terminal cytoplasmic domain of α_{1A} and showing specificity for the β_4 (and to a lesser extent β_{2a}) isoform. A deletion and chimera approach showed that the carboxyl-terminal region of β_4, poorly conserved between β isoforms, contains the interaction site and plays a role in the regulation of channel inactivation kinetics. This is the first demonstration of a molecular basis for the specificity of functional effects seen for different combinations of these two channel components.

Voltage-dependent calcium channels have been classified into five groups, based on their electrophysiological and pharmacological properties. L-type channels are ubiquitous, present particularly in skeletal and cardiac muscle, where they play an essential role in excitation-contraction coupling. T-type channels are important for cardiac pacemaker activity and the oscillatory activity of several thalamic neurons, while N- and P/Q-type channels are important in the control of neurotransmitter release in the central and peripheral nervous systems, and the role of R-type channels remains unclear. Two of these channels have been purified to homogeneity, the skeletal muscle L-type channel and the brain N-type channel (1, 2). Although these channels differ dramatically in function, their subunit compositions are very similar, the core subunit composition of a high voltage-activated channel consisting of an α_1 subunit, the ionic pore of the channel, and two auxiliary subunits, β and $\alpha_2\delta$, that confer native biophysical and pharmacological properties to the channel. These subunits are encoded by at least six α_1, four β, and one $\alpha_2\delta$ gene, for which numerous splice variants have been identified (3).

The β subunit is a cytoplasmic protein of 52–78 kDa that, when coexpressed with the α_1 subunit, results in an increase (of up to 100-fold) in current amplitude, alteration of both the kinetics and voltage dependence of activation and inactivation, and an apparent increase in recognition sites for channel-specific toxins (*e.g.* see Refs. 4–8). The regulatory effects of β vary in importance, depending on the combination of channel subunits studied. Although β regulation seems to be highly conserved from β_1 to β_4 and on α_{1S} to α_{1E}, some important differences between these various isoforms have nevertheless been noted. The different β subunits produce consistently different channel inactivation behaviors, β_3 producing fast inactivation, β_2 slow channel inactivation, and β_1 and β_4 more intermediate behaviors (9–11). The β effect also appears to be α_1 isoform-dependent; the β-induced shift in voltage dependence of inactivation has been reported for non-L-type channels, A, B, and E (4, 12), whereas it is absent for L-type channels, S, C, and D (13). Since the interaction between calcium channel subunits is promiscuous, at least for α_1 and β subunits (11, 14), the heterogeneity of combinations observed so far in two native channel types (N-type (15) and P/Q-type (16)) must be of functional significance in cell biology.

Recent studies have identified complementary interaction domains on the α_1 and β subunits (17, 18). AID[1] ($\underline{\alpha}_1$ subunit $\underline{i}$nteraction $\underline{d}$omain), a highly conserved region in the cytoplasmic loop between transmembrane domains I and II, interacts with a stoichiometry of 1:1 (11) with BID ($\underline{\beta}$ subunit $\underline{i}$nteraction $\underline{d}$omain), a 30-residue region in the second conserved domain of the β subunit (domain IV in Fig. 5*A*). AID and BID appear to be essential for the subunit interaction and regulation by β subunits (17). Point mutations in AID and BID that disrupt this primary interaction also totally inhibit channel regulation by β, suggesting that it acts as an important anchoring site, due to its very high affinity (11). Several lines of evidence suggest, however, that, despite its importance in channel regulation, the AID-BID attachment site does not account for all of the regulatory potential of the β subunit. The deletion approach used to identify the BID site revealed that it may not carry all the current stimulatory function of β, the change in inactivation kinetics (17), nor the shift in voltage dependence of inactivation.[2] It is also interesting that BID represents only 30 residues in a region that shows 78% identity between β subunits over 200 residues and that, in addition, toward the amino-terminal of β there exists another highly conserved region (65% identity, over more than 100 residues) (17). The high level of sequence conservation is indicative of evolutionary constraint, suggesting that these regions are of functional importance. The remaining three less conserved domains (I, III, and V) undergo splicing and may also be functionally relevant to β-specific changes in inactivation as suggested by several studies (19, 20).

Viewed together, the inability of BID to account for all of the

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[1] The abbreviations used are: AID, α_1 subunit interaction domain; BID, β subunit interaction domain; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis.

[2] M. De Waard, unpublished data.

functional effects of the β subunit and the high level of conservation elsewhere in the sequence lead to the hypothesis that there may exist secondary sites of interaction between the α_1 and β subunits. Such sites would be dependent on the initial, highest affinity, essential interaction between AID and BID, and therefore need not be of high affinity themselves. The current work concerns the identification and characterization of interaction sites for the β subunit in the carboxyl-terminal domain of α_{1A}, which is the largest of the cytoplasmic regions and shows considerable degeneracy of sequence homology between α_1 subunit types and splice variants. We demonstrate that the carboxyl-terminal sequence of β_4 specifically interacts with this region of rabbit α_{1A} (BI-2) and is required for a proper regulation of channel inactivation.

EXPERIMENTAL PROCEDURES

Preparation of Fusion Proteins—Regions of the rabbit brain α_{1A} cDNA (BI-2) (21) corresponding to residues 1889–2126, 2090–2424, 2275–2424, 2070–2275, 2070–2196, 2120–2196, and 2197–2275 were amplified by PCR, and, with the aid of *Bam*HI and *Eco*RI or *Xba*I restriction sites included in the primers, subcloned into these sites in pGEX2TK or pGEXKG (Pharmacia Biotech Inc.). The resulting recombinant plasmids were expressed in *Escherichia coli* BL21, and GST fusion proteins were purified as described previously (11). The newly purified fusion proteins are referred to as, for example, GST-1889–2126_A. GST alone and a GST fusion protein of the AID region of α_{1A} (11) were prepared in the same way.

In Vitro Translation of β Subunits—β subunit cDNA clones used were rat β_{1b} (L11453), rabbit heart β_{2a} (X64297), rabbit heart β_3 (M88751), and rat brain β_4 (A45982). Truncated derivatives of β_4 were constructed by PCR amplification of the corresponding regions of cDNA and subcloning into pCDNA3, using *Hin*dIII and *Bam*HI sites (added to the PCR primers), with the addition of a Kozak (22) sequence and initiation codon (ACCATGG) or termination codon (TGA) as necessary. For construction of a chimera between $\beta_{3\,(1–360)}$ and $\beta_{4\,(402–519)}$, a two-step PCR approach was used with the following primers: β_3, forward: 5′-ACGTAAGCTTACCATGGATGACGACTCGTAGGTGCCC-3′, reverse: 5′-GCTTGTGTGGGTGGCGCGCCAGTAAACCTCTAGGTA-3′; and β_4, forward: 5′-GAGGTTTACTGGCGCGCCACCCACACAAGCAGTAGC-3′, reverse: 5′-CGCGGATCCTCAAAGCCTATGTCGGGAGTCATGGCTGCATCC-3′. The reverse primer for β_3 and the forward primer for β_4 contain complementary sequences, allowing annealing of the two PCR products to give the template for the second round of amplification, using the β_3 forward and β_4 reverse primers. Restriction sites *Hin*dIII and *Bam*HI were included in the external primers, allowing subcloning into pCDNA3.

^{35}S-Labeled β subunits were synthesized *in vitro* using the TNT™ coupled transcription/translation system (Promega). Nonincorporated [^{35}S]methionine was removed by purification on a PD10 column (Pharmacia).

Binding Assays—Purified GST fusion proteins were coupled to glutathione-agarose beads (Sigma) by incubation for 30 min, before addition of the translation mixture (approximately 500 pM final concentration). Binding assays were carried out in a final volume of 200 μl, in Tris-buffered saline (0.1% Triton X-100, 25 mM Tris, 150 mM NaCl, pH 7.4), at 4 °C, for 6 h, unless otherwise stated. Beads were washed four times in binding buffer, and then analyzed either by SDS-PAGE and autoradiography or by scintillation counting.

A peptide corresponding to the AID site of α_{1A} (RQQIERELNGYMEWISKAE from Genosys) was dissolved in phosphate-buffered saline (154 mM NaCl, 40 mM Na_2HPO_4, 11.5 mM NaH_2PO_4, pH 7.4) at 500 μM and added to binding reactions at 100 μM final concentration. Since addition of phosphate-buffered saline to the binding reactions introduced slight changes in binding affinity, an equal volume of phosphate-buffered saline was added to control reactions.

Electrophysiological Recordings—Stage V and VI *Xenopus* oocytes were injected with BI-2-specific mRNA (400 ng/μl) in combination with β_4-specific mRNA (100 ng/μl) or truncated $\beta_4\Delta$C mRNA (100 ng/μl). Cells were incubated for 3 days in defined nutrient oocyte medium as described previously (11). Whole cell recordings were performed at room temperature (22–24 °C) using the two-microelectrode voltage clamp configuration of a GeneClamp amplifier (Axon Instruments, Foster City, CA). The extracellular recording solution was of the following composition (in mM): $Ba(OH)_2$ 40, NaOH 50, KCl 3, HEPES 5, niflumic acid 1, pH 7.4 with methane sulfonic acid. Electrodes filled with 500 mM cesium acetate, 10 mM EGTA, 3 mM KCl, and 10 mM HEPES, pH 7.2, had resistances comprised between 0.5 and 2 megohms. The bath solution was clamped to a reference potential of 0 mV. Current records were filtered at 1 kHz, leak-subtracted on-line by a P/6 protocol, and sampled at 2–4 kHz. Data were analyzed using pCLAMP version 6.02 (Axon Instruments). All values are mean ± S.D.

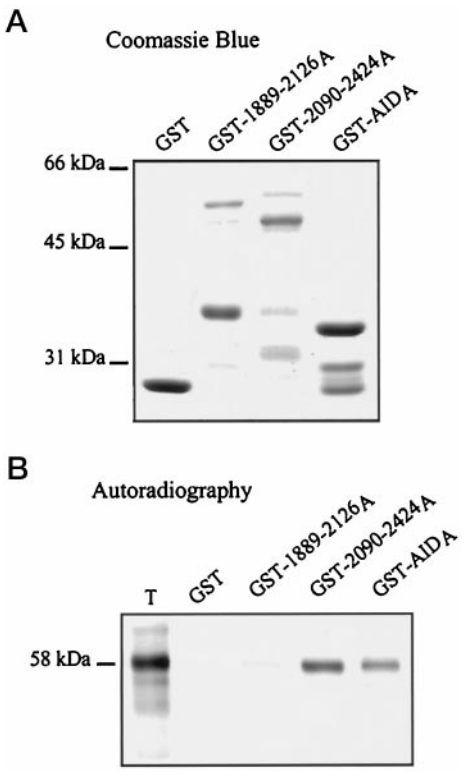

FIG. 1. ***In vitro* binding of ^{35}S-β_4 to the carboxyl-terminal region of α_{1A}.** *A*, Coomassie Blue-stained SDS-PAGE (9%), showing GST fusion proteins used (5 μg). *B*, autoradiogram of binding assay. *In vitro* translated β_4 (*T*) was assayed for binding to the fusion proteins indicated (5 μM). Following binding interactions (as described under "Experimental Procedures"), washed beads were resuspended in SDS-PAGE loading buffer.

RESULTS

Purified GST fusion proteins carrying amino acids 1889–2126 (GST-1889–2126_A) and 2090–2424 (GST-2090–2424_A) of the α_{1A} subunit carboxyl-terminal region were coupled to glutathione-agarose beads at a concentration of 5 μM and assayed for interaction with a ^{35}S-labeled *in vitro* translated rat β_4 subunit (Fig. 1). GST-1889–2126_A showed no significant binding, as seen for the control GST protein alone, while GST-2090–2424_A showed a significant level of interaction, comparable to that seen for a 500 nM, saturating (11) concentration of a GST fusion protein carrying the AID region of α_{1A} (GST-AID_A).

Analysis of the binding of various concentrations of GST-2090–2424_A to ^{35}S-β_4 (Fig. 2*A*) demonstrates that binding is saturable; specific binding appears at about 25 nM and saturates at 500 nM. Comparison of the saturation curve of GST-2090–2424_A binding to β_4 to the dose-response curve of GST-AID_A reveals a dissociation constant (K_d) of 93 nM for GST-2090–2424_A, which is an approximately 30-fold lower affinity compared with the GST-AID_A-β_4 interaction. Association kinetics (Fig. 2*B*) are relatively slow compared with those previously seen for the AID-BID interaction (11), with a half-time of association of approximately 120 min at 5 μM compared with 20 min at 500 nM GST-AID_A.

We next tested whether the AID-BID interaction had any effect on the interaction between GST-2090–2424_A and β_4. In the presence of a 21-amino acid synthetic peptide containing

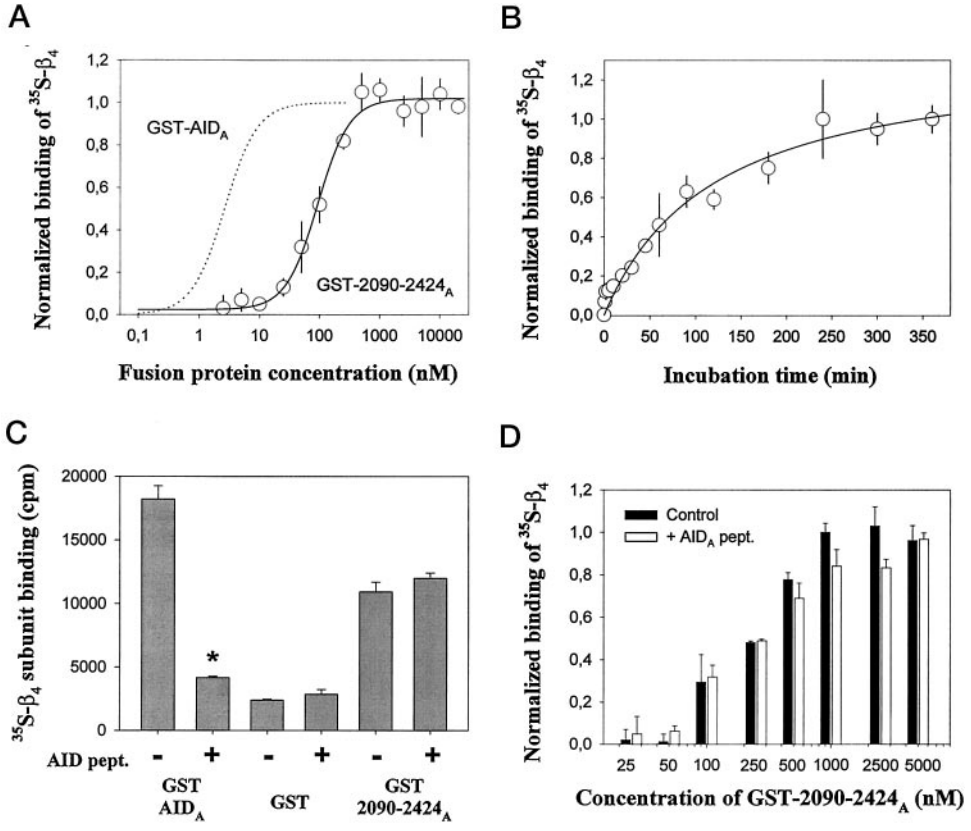

FIG. 2. ***In vitro* characterization of the α_{1A}-β_4 interaction.** *A*, various concentrations of GST-2090–2424$_A$ fusion protein were assayed for binding to ^{35}S-β_4, and binding was quantified by counting. Specific binding was calculated by subtraction of binding to GST (at the same concentrations) and normalized by expression as a proportion of maximal binding. *Error bars* represent normalized S.D. The *dotted line* represents binding of ^{35}S-β_4 to GST-AID$_A$ under the same conditions, with a K_d of 3 nM, shown for comparison. Data were fitted by a logistic function $y = (a - d)/(1 + (x/c)b) + d$ where $a = 1.019$ and $d = 0.02$ (the asymptotic maximum and minimum, respectively); $c = 93$ nM (the K_d); and $b = -1.6$ (the slope of the curve). *B*, kinetics of 5 μM GST-2090–2424$_A$ fusion protein binding to ^{35}S-β_4 subunit at 4 °C. The data were fitted by an hyperbolic function $y = (a \cdot t)/(b + t)$, where t is the time of association before washing, $a = 1.34$ (the theoretical maximum of binding), and $b = 120$ min (the time of half-maximum binding). *C*, fusion proteins (500 nM GST-AID$_A$, 2.5 μM GST, and 2.5 μM GST-2090–2424$_A$), were assayed for binding to ^{35}S-β_4 in the presence or absence of 100 μM AID$_A$ peptide and quantified by counting. Results are shown as counts/min; *error bars* represent S.D.; the *asterisk* represents a statistically significant effect of the AID$_A$ peptide. *D*, various concentrations of GST-2090–2424$_A$ fusion protein were assayed for binding to ^{35}S-β_4 in the presence or absence of 100 μM AID$_A$ peptide and quantified by counting. Results were normalized by subtraction of binding to GST under the same conditions and expression as a proportion of maximal binding in the absence of peptide. *Error bars* represent normalized S.D.

the AID sequence of α_{1A}, specific binding of β_4 to GST-AID$_A$ is diminished by over 90%, demonstrating the effectiveness of the peptide. The same peptide had no significant effect on the binding of GST-2090–2424$_A$ to β_4 (Fig. 2*C*), however, indicating that the BID region is not implicated in this interaction. The peptide did not modify the maximum binding of GST-2090–2424$_A$, indicating that, at least *in vitro*, binding of AID to the β subunit does not induce conformational changes capable of favoring (or indeed disfavoring) this interaction. This was further investigated by analyzing the effects of AID$_A$ peptide on the binding to β_4 at various concentrations of GST-2090–2424$_A$ (Fig. 2*D*). The data show that the peptide also had no significant effect on the affinity of β_4 for GST-2090–2424$_A$.

To identify the region of the carboxyl-terminal domain that interacts with β_4, we constructed a series of smaller GST fusion proteins encoding smaller fragments of this region (Fig. 3*A*) and compared their binding to ^{35}S-labeled β_4 (Fig. 3*B*). Within the region from residue 2120 to the carboxyl terminus of the molecule, a whole series of subcloned fragments maintained an ability to interact with ^{35}S-β_4. Further investigations suggested, however, that these interactions occur with a weaker affinity than GST-2090–2424$_A$. For example, we found a K_d of 225 nM for GST-2070–2196$_A$ binding to β_4 (data not shown), *i.e.* 2-fold lower. These data indicate that a series of "microsites" are responsible for the binding activity of the α_{1A} carboxyl terminus, perhaps together forming a binding pocket, although dependence on overall conformation of the binding domain appears to be limited. This further contrasts with the β interaction to AID, which relies on only three crucial AID residues (14).

All four β subunit isoforms show a fairly similar affinity for AID. However, the functional effects of coexpression of these isoforms vary considerably and also depend on the α_1 subunit tested. Functional differences among β subunits may be a reflection of the differing capacities of β isoforms to form secondary interactions with the α_1 subunit concerned. We therefore tested whether the interaction observed between β_4 and GST-2090–2424$_A$ also existed for other β subunits translated *in vitro*. Fig. 4 shows a comparison of binding of ^{35}S-β subunits to three different concentrations of GST-2090–2424$_A$, to GST alone, and to a GST fusion protein expressing AID$_A$, at a concentration expected to yield maximal binding. β_4 interacts with GST-2090–2424$_A$ with a high affinity, showing maximal binding at 1 μM fusion protein concentration. β_{2a} binds with a much lower affinity, showing only limited binding at 10 μM fusion protein concentration, while binding of β_3 and β_{1b} is insignificant even at this concentration of fusion protein.

The differences in interaction affinity observed for different β subunits and the fact that the main regions of sequence divergence among β subunits are the carboxyl- and amino-

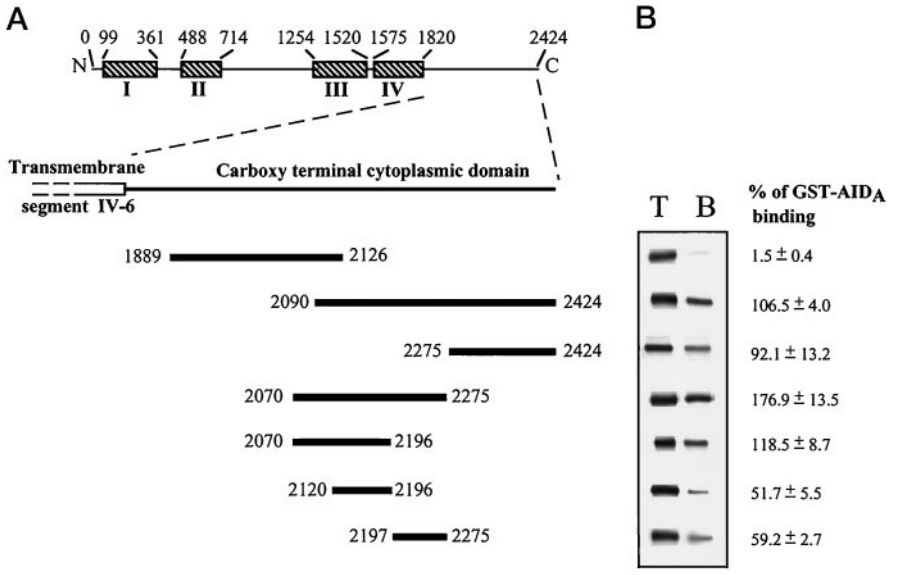

FIG. 3. **Localization of the interaction site in α_{1A}.** *A*, *above*, schematic diagram of α_{1A}. Amino acid positions are shown above, transmembrane domains (each composed of six membrane-spanning segments) are shown as *hatched boxes*. *Below*, enlargement of the carboxyl-terminal domain, showing GST fusion proteins constructed, with amino acid positions marked at the extremities. *B*, capacity of fusion proteins (5 μM) to interact with ^{35}S-β_4 and binding was quantified both by SDS-PAGE and autoradiography and by β counting. *T*, equivalent volume of *in vitro* translation of ^{35}S-β_4 used in binding assays. *B*, ^{35}S-β_4 that remained bound following washing of beads. Percentage values indicate specific binding (under the same experimental conditions, quantified by counting) as a percentage of binding to GST-AID$_A$ (500 nM, *i.e.* at saturation).

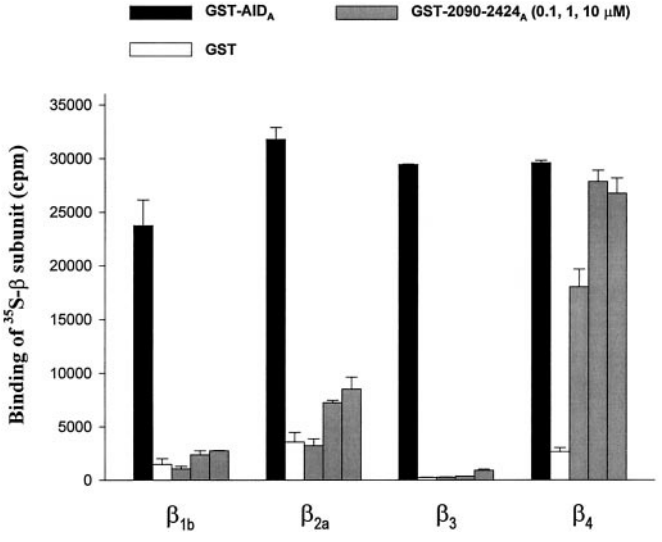

FIG. 4. **β subunit specificity.** Fusion proteins (500 nM GST-AID$_A$, 10 μM GST, and 100 nm GST-2090–2424$_A$, 1 and 10 μM final concentrations, shown from *left* to *right*) were assayed for binding to ^{35}S-labeled β_{1b}, β_{2a}, β_3, and β_4. Results are shown as counts/min, with *error bars* representing S.D.

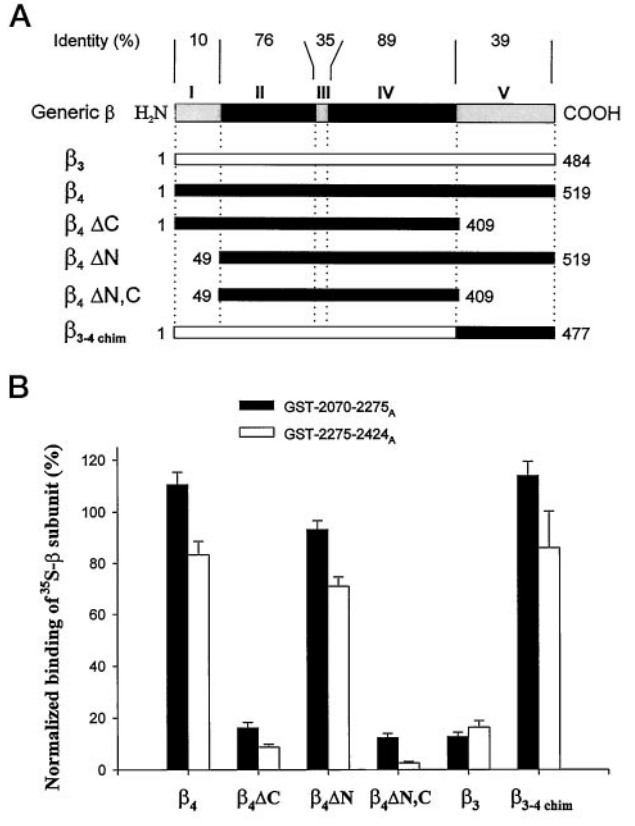

FIG. 5. **Localization of the interaction site in β_4.** *A*, map of a β subunit, dividing the protein into five domains according to the degree of sequence conservation between isoforms. Percentage identity between β_3 and β_4 is shown *above*, for each domain. *Below*, map of β subunits and derivatives constructed (β_4, full-length; $\beta_4\Delta$C, β_4 residues 1–409; $\beta_4\Delta$N, β_4 residues 49–519; β_4 ΔN,C, β_4 residues 49–409; β_3, full-length; and $\beta_{3-4chim}$, chimera between β_3 residues 1–360 and β_4 residues 402–519). *B*, fusion proteins (GST-2070–2275$_A$ and GST-2275–2424A, 5 μM) were assayed for binding to *in vitro* translated β subunits and derivatives.

terminal regions (Fig. 5*A*) suggested that one of these regions was responsible for the interaction. We investigated this possibility (Fig. 5*B*) by deleting either or both regions from the β_4 cDNA. We assayed the capacity of the resulting *in vitro* translated proteins to bind to two fusion proteins, GST-2070–2275$_A$ and GST-2275–2424$_A$, which represent approximately the two halves of the region of α_{1A} under investigation (Fig. 2*B*). Deletion of the amino-terminal 48 amino acids of β_4 had no effect on the binding of GST-2070–2275$_A$ and GST-2275–2424$_A$. In contrast, deletion of the carboxyl-terminal 109 amino acids of β_4 drastically interferes with its capacity to interact with either fusion proteins. Residual weak binding of both fusion proteins seemed to be present. To check whether this residual binding was due to the amino terminus, we tested the binding of these fusion proteins to the double mutant $\beta_4\Delta$N,C. The results show that there was no difference between $\beta_4\Delta$C and $\beta_4\Delta$N,C, confirming the absence of a binding function for the amino terminus. The importance of the carboxyl-terminal region was confirmed by constructing a chimera in which the carboxyl-terminal region of β_3 was replaced by the corresponding region of β_4 (Fig. 5*B*). The inability of β_3 to interact with either α_{1A} fusion protein was successfully "rescued" by replacement of this region, resulting in a binding capacity approaching that of the full-length β_4 subunit.

Interestingly, the results obtained with the full-length, truncated, and chimera β_4 were very similar for the two fusion proteins assayed. This further suggests that the carboxyl ter-

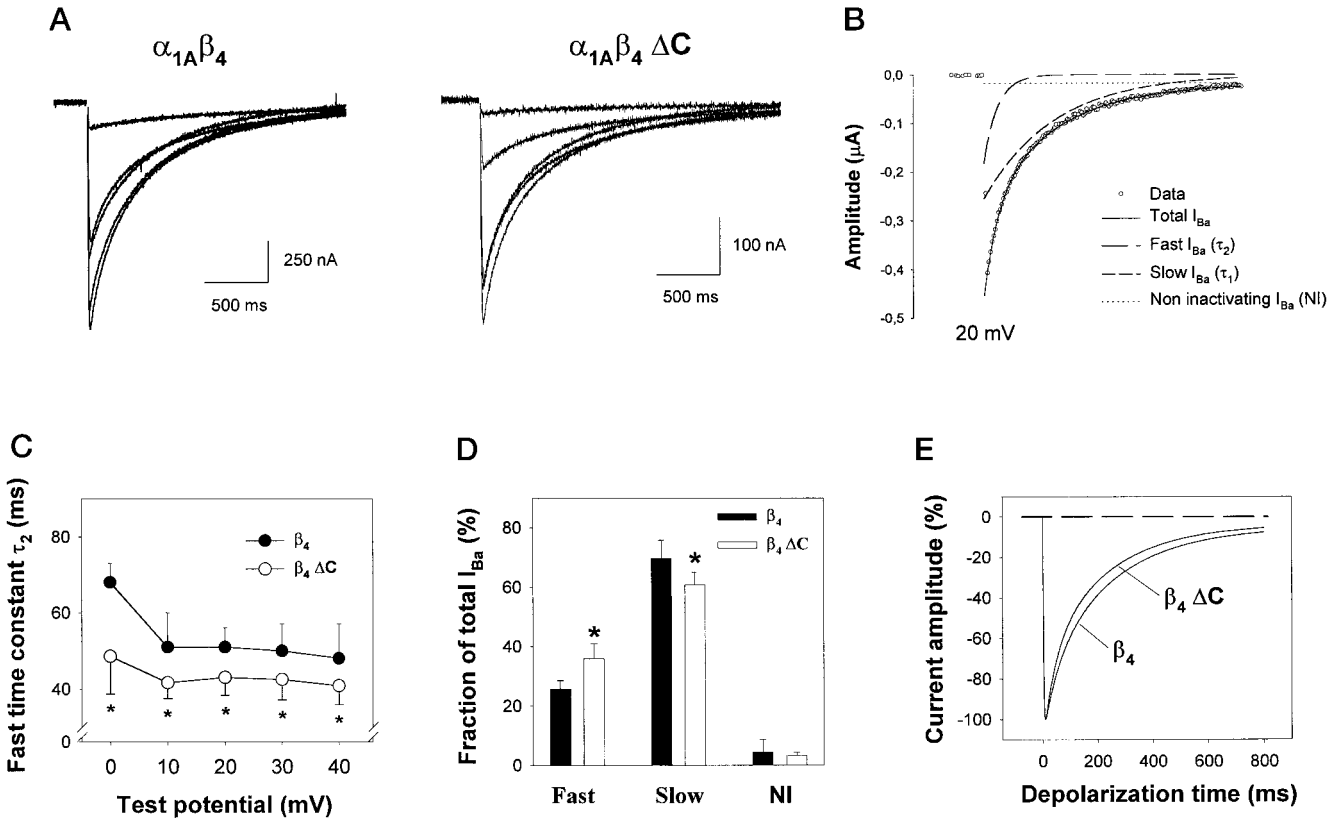

FIG. 6. **Electrophysiological analysis of the effects of carboxyl-terminal truncation of β_4.** Modification in channel inactivation induced by carboxyl-terminal truncation of the β_4 subunit. *A*, representative current traces of $\alpha_{1A}\beta_4$ and $\alpha_{1A}\beta_4\Delta$C injected oocytes depolarized to −10, 0, 10, 20, and 30 mV. *B*, inactivating components of a current trace obtained by depolarizing an $\alpha_{1A}\beta_4\Delta$C cell to 20 mV. Data were fitted by the Chebyshev method according to an exponential equation $I_{Ba} = -I2 \cdot \exp(-t/\tau_2) - I_1 \cdot \exp(-t/\tau_1) - NI$ where I_{Ba} is the total current, I_2 the fast inactivating component (I_2 = 0.137 μA, 34.6% of I_{Ba}), I_1 the slow inactivating component (I_1 = 0.241 μA, 60.8% of I_{Ba}), and *NI* the noninactivating component (*NI* = 0.018 μA, 4.5% of I_{Ba}). Time constants for current inactivation were τ_2 = 42.5 ms and τ_1 = 219.1 ms in this example. Data were fitted 12 ms after the start of depolarization. Data (*open symbols*) were shown after filtering 1/10. *C*, average τ_2 time constant as a function of depolarization value for $\alpha_{1A}\beta_4$ and $\alpha_{1A}\beta_4\Delta$C-injected oocytes. *Asterisks* represent data statistically different from control (*t* test; $p \leq 0.1$). *D*, average percentage of each inactivating component present in total current for $\alpha_{1A}\beta_4$ and $\alpha_{1A}\beta_4\Delta$C-injected oocytes. Statistically significant results are shown by the *asterisk* (*t* test; $p \leq 0.1$). *E*, modeled current inactivation based on average τ_2, τ_1, percent of I_2, percent of I_1, and percent of *NI* at 20 mV for both $\alpha_{1A}\beta_4$ and $\alpha_{1A}\beta_4\Delta$C.

minus of α_{1A} forms a single binding site and not several sites that would interact independently with diverse regions of β_4.

Since deletion of carboxyl-terminal sequences of the α_{1C} channel induces important modifications in channel gating and opening probability, we determined the functional importance of this α_{1A}-β_4 interaction by expression in *Xenopus* oocytes. Comparison of the effects of the full-length β_4 and $\beta_4\Delta$C revealed that the carboxyl terminus of the β subunit had little influence on the biophysical properties of the α_{1A} channel with the exception of a role in the control of inactivation kinetics (Fig. 6*A*). There were no noticeable differences in current amplitude between $\alpha_{1A}\beta_4$ and $\alpha_{1A}\beta_4\Delta$C-injected cells with average peak amplitudes of 898 ± 686 nA (n = 5) and 626 ± 413 nA (n = 6), respectively. In addition, no differences were detected in activation parameters with half-activation potentials of 1.5 ± 5.5 and 3.2 ± 3.5 mV, and slope values of 4.9 ± 1 and 5.2 ± 0.9 mV for $\alpha_{1A}\beta_4$ and $\alpha_{1A}\beta_4\Delta$C-injected cells, respectively. We also found no statistical difference in the voltage dependence of inactivation with half-inactivation potentials of −24.6 ± 5.8 mV (n = 5, $\alpha_{1A}\beta_4$) and −28.1 ± 4.7 mV (n = 6, $\alpha_{1A}\beta_4\Delta$C). Interestingly, there was a small but significant change in the rate of inactivation kinetics produced by the β_4 carboxyl-terminal deletion. Cells injected with $\alpha_{1A}\beta_4$ cRNAs inactivated rapidly after depolarization. The decay in current represents the sum of three components at all voltages (Fig. 6*B*), two of which are exponential, a fast decaying current with an average time constant of 51 ± 5 ms (25.7 ± 2.9% of total current at 20 mV), and a slow inactivating component with a time constant of 241 ± 30 ms (69.7 ± 6% at 20 mV) and a noninactivating current (4.5 ± 4.3%). The truncated $\beta_4\Delta$C increased the overall rate of inactivation by two essential modifications: (i) a decrease in the fast time constant to 43 ± 4.7 ms at 20 mV instead of 51 ms (Fig. 6*C*) and (ii) a change in the ratio between fast and slow inactivating components from 25.7 to 36 ± 5% (fast) and from 69.7 to 60.7 ± 4.2% (slow) (Fig. 6*D*). Although these effects were small, they were statistically significant and contributed to the overall average increase in channel inactivation as described in Fig. 6*E*. These results suggest that the carboxyl terminus of β_4 may actually contribute to a slowing in inactivation kinetics upon association to the carboxyl terminus of α_{1A}.

DISCUSSION

We describe here the identification of a new interaction site between the α_{1A} calcium channel subunit and the β_4 subunit. The interaction is of low affinity compared with that between the AID site on the I-II cytoplasmic loop of α_1 subunits and the BID site of β subunits. This and the fact that mutations in AID or BID disrupt the interaction between the two subunits in expression experiments (17, 18) suggest that such an interaction would be of a secondary nature, dependent on formation of the initial AID-BID interaction to bring the interaction sites into close proximity, or to introduce conformational constraints that favor the interaction. The alternative possibility, that two

β subunits would be associated to a single α_1 (*e.g.* as proposed by Tareilus *et al.* (20)), seems unlikely for several reasons. First, the molar ratio between α_1 and β subunits is 1:1 in purified channels (2, 23). Second, studies of the AID-BID interaction revealed that β subunits with affinities lower than 100 nM for AID do not associate with α_1 upon coexpression in oocytes (14), although, of course, it cannot be ruled out that in native conformation the affinity between the two carboxyl-terminal sequences is higher than that predicted from *in vitro* experience. Third, and most important, expression of β subunits containing disruptive BID mutations fail to modify channel properties, ruling out additional interactions in these conditions.

The carboxyl-terminal tails of α_1 subunits play various roles in channel function. In the α_{1C} subunit, deletion of the distal regions of the carboxyl terminus results in increased channel opening probability (24), and the more proximal EF-hand domain plays a role in Ca^{2+}-induced inactivation (25). The carboxyl-terminal tail is also known to undergo post-translational modifications in the form of phosphorylation and proteolysis, in some cases essential to channel function (26). Interestingly, there are many phoshorylation sites present in both the α_{1A} carboxyl-terminal and β_4 carboxyl-terminal sequence that may be of functional relevance. In β_4 several of these phosphorylation sites are unique to this subunit. These data point to the functional importance of this region in channel regulation and may also provide the key to the main function of the subunit interaction site we describe here.

We show that, at least *in vitro*, β_4 shows a much greater affinity for the carboxyl-terminal region of α_{1A} than does β_{2a}, while no interaction is detected for β_{1b} or β_3. These differences in affinity suggest a functional significance that may help to explain the differences in functional effects seen for different combinations of α_1 and β subunits. In light of this, it is interesting that β_4 is coexpressed in the same brain regions as is α_{1A}, particularly in the cerebellum (21, 27), and is the major β subunit associated with the α_{1A} subunit in the P/Q channel-type (16). A similar interaction has recently been reported between an α_{1E} splice variant and β_{2a} (20). It will be interesting to see whether this interaction also displays a specificity for a particular subset of β subunits.

The advantage of a form of subunit specificity in the α_1-β association remains largely to be investigated. Our data suggest that a secondary interaction site that favors β_4 association to BI-2 rather than β_3, the other predominant β in brain (28), could be determinant in underlying subtle kinetic differences induced by the various β subunits. Besides obvious functional differences, specific α_1-β associations may be determinant in various aspects of channel biosynthesis such as channel targeting. Brice *et al*. (29) and Chien *et al*. (30) have indeed demonstrated that β subunits are crucial to cell surface localization of α_1.

Alternatively, it is possible that the carboxyl-terminal sequences of both subunits contribute to the process of channel clustering known to occur in voltage-dependent calcium channels, with the carboxyl-terminal sequence of the β subunit interacting with the carboxyl terminus of an α_1 subunit other than the one that it is attached to via its BID site. Channel clustering is known to occur in various ion channels and has best been characterized for the shaker K^+ channel for which clustering is produced by the PSD-95 proteins (31). Calcium channel clustering is probably induced by a third party protein because the carboxyl-terminal interaction described herein may be of insufficient affinity to be the primary cause of such a clustering behavior.

Besides the existence of separate genes encoding Ca^{2+} channel subunits, alternative splicing is another process by which diversity can be introduced. The functional significance of alternative splicing in Ca^{2+} channel subunits is still largely unknown. Splicing can occur in several regions of the α_1 protein, including the amino terminus (32), the IS6 transmembrane sequence, the cytoplasmic linkers between domain I and II and between domain II and III (32), transmembrane segment IVS3, and the carboxyl-terminal sequences (21, 33). Particularly pertinent to the data presented here is the existence of two splice alternatives in α_{1A} described by Mori *et al.* (21) that result in an almost total divergence of sequence from residue 2230 onward, *i.e.* concerning the majority of the sequence responsible for the interaction studied here. It is therefore likely that the carboxyl terminus of the other α_{1A} splice variant, BI-1, may not interact with β_4. Generally, it remains to be seen whether the splicing occurring in the carboxyl-terminal tail of the α_1 subunit plays a role in the specificity of the secondary α_1-β interaction. Two case scenarios can be discussed. The first is that any deletion or insertion may modify the regulatory input of the associated β subunit at that location without modifying the type of β subunit associated. The second possibility is that splicing modifies the α_1-β interaction specificity and that it favors the association of another type of β subunit, presumably to specify a different membrane targeting of the channel. In the case of α_{1A}, it would be interesting to see whether the carboxyl terminus of BI-1 interacts with β_3, the other predominant β subunit known to interact with α_{1A} in the brain (16). Our data also shed new light on data obtained by other groups that report a lack of impact of α_1 carboxyl-terminal alternative splicing on β subunit regulation (for instance in α_{1C} (34), but see Soldatov *et al*. (35)) and suggest that negative data may well be due to the use of an inappropriate combination of α_1 and β subunits.

It is becoming increasingly obvious that a wide range of neurological and motor diseases result from mutations in the α_1 or β subunits, and a number of these are particularly relevant to the data we have presented here. In mice, the leaner phenotype, similar to absence epilepsy, has been attributed to a mutation in a splice donor consensus sequence of α_{1A}, resulting in aberrant splicing and therefore degeneration of the coding sequence corresponding to either residue 2026 or 2072 onward of the protein we have used (36), *i.e.* corresponding well to the region identified as interacting with β_4. In humans, a severe form of ataxia has been shown to be associated with a 5-base pair insertion close to the stop codon that extends the translated sequence to include a glutamine repeat of variable size (37), which would presumably entail conformational changes to this region. Concerning the β_4 subunit, its overall functional importance has been shown by the assignment of a lethargic phenotype in mice to a deletion of about 60% of the protein (38), although such a drastic alteration is likely to completely inactivate the β_4 subunit and at least leads to the loss of the BID in addition to the carboxyl-terminal site.

Finally, the data obtained here contribute to our understanding of the general organization of high voltage-activated calcium channels. The existence of several sites of interaction between the two channel components highlights the utility of an *in vitro* approach using fusion proteins, since it enables us to study such interactions individually and therefore to assess their functional impact and to gradually dissect the conformational basis of the relationship between the two subunits. It is not always possible, however, to extrapolate directly between the situation *in vitro* and that *in vivo*. For example, our inability to demonstrate an effect of AID-β association on the affinity of the interaction between the carboxyl termini of α_{1A} and β_4 probably reflects the absence of the remainder of the α_{1A} mol-

ecule and therefore a loss of integrity of the conformational constraints existing in native channels which may determine the overall manner in which the two subunits interact.

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