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A New β Subtype-specific Interaction in α_{1A} Subunit Controls P/Q-type Ca^{2+} Channel Activation*

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The cytoplasmic β subunit of voltage-dependent calcium channels modulates channel properties in a subtype-specific manner and is important in channel targeting. A high affinity interaction site between the α_1 interaction domain (AID) in the I-II cytoplasmic loop of α_1 and the β interaction domain (BID) of the β subunit is highly conserved among subunit subtypes. We describe a new subtype-specific interaction (Ss1) between the amino-terminal cytoplasmic domain of α_{1A} (BI-2) and the carboxyl terminus of β_4. Like the interaction identified previously (21) between the carboxyl termini of α_{1A} and β_4 (Ss2), the affinity of this interaction is lower than AID-BID, suggesting that these are secondary interactions. Ss1 and Ss2 involve overlapping sites on β_4 and are competitive, but neither inhibits the interaction with AID. The interaction with the amino terminus of α_1 is isoform-dependent, suggesting a role in the specificity of α_1-β pairing. Coexpression of β_4 in *Xenopus* oocytes produces a reduced hyperpolarizing shift in the *I-V* curve of the α_{1A} channel compared with β_3 (not exhibiting this interaction). Replacing the amino terminus of α_{1A} with that of α_{1C} abolishes this difference. Our data contribute to our understanding of the molecular organization of calcium channels, providing a functional basis for variation in subunit composition of native P/Q-type channels.

Despite their functional diversity, high voltage-gated Ca^{2+} channels have three subunit types in common (1, 2). The α_1, pore-forming component of the channel is associated with a cytoplasmic β subunit of 52–78 kDa and a largely extracellular $\alpha_2\delta$ component, anchored by a single transmembrane domain. These subunits are encoded by at least 7 α_1, 4 β, and 1 $\alpha_2\delta$ genes, respectively, of which numerous splice variants exist (3).

The β subunit, when coexpressed with the α_1 subunit, results in an increase in current density, alteration of the voltage dependence and kinetics of both inactivation and activation, and an increase in the number of recognition sites for channel-specific ligands (for review, see Refs. 4 and 5). These effects reflect not only conformational modulation but also an increase in the number of channels properly addressed to the cell surface, suggesting multiple roles for the β subunit. Although the effects of β are highly conserved, significant differences are seen depending on the combination of α_1 and β subunits studied. For example, the kinetics of inactivation shows a general trend of variation with β subtype (6–9), whereas a shift in the voltage dependence of inactivation has been reported only for non-L-type, A, B, and E (10–12), and not L-type channels (13). β subunits also seem to differ in the mechanism by which they become localized to the plasma membrane (14, 15), perhaps suggesting that they are differentially targeted. Finally, α_1 and β subtypes differ in their potential (based on sequence predictions) to be phosphorylated by various protein kinases. These factors together point to a functional explanation for the growing evidence that the *in vitro* promiscuity of α_1-β interactions is reflected by a heterogeneity of combinations in native channels (N (16), P/Q (17), and L type (18)).

Preliminary studies (10, 19) have identified a high affinity interaction between a highly conserved region in the cytoplasmic loop linking transmembrane regions I and II of α_1 (AID,[1] or α_1 interaction domain) and a 30-residue region in the second conserved domain of β subunits (BID, or β interaction domain). This interaction occurs with a stoichiometry of 1:1 (20) and (at least *in vitro* and in expression systems) occurs between all combinations of α_1 and β subtypes tested so far. We have since reported (21) the existence of a subunit-specific interaction between the carboxyl-terminal domain of α_{1A} and the most carboxyl-terminal 109 residues of β_4, and a similar interaction has been reported (22, 23) between α_{1E} and β_{2a}. The comparative high affinity of the AID-BID interaction (20, 21), coupled with the abolition of all β modulatory effects by mutation of residues critical to the interaction between AID and BID (10, 19), suggests that this interaction represents a primary, anchoring interaction upon which further, secondary, interactions might depend. The specificity of such secondary interactions, or at least differences in affinity, represents a potential source for the variation seen for different α_1 and β combinations, in terms of both the electrophysiological properties of the channel and potential differences in control by other cellular factors, such as protein kinases and G proteins. We therefore set out to determine whether further secondary interaction sites exist. The present report describes the identification of an interaction between the amino-terminal cytoplasmic region of α_{1A} and the β_4 subunit of P/Q channels providing a refreshed

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[1] The abbreviations used are: AID, α_1 interaction domain; BID, β interaction domain; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; MBP, maltose-binding protein; ^{35}S-β_4, [^{35}S]methionine-labeled β_4 subunit; PAGE, polyacrylamide gel electrophoresis. Fusion proteins are referred to as, for example, GST-NT_A for that containing the entire amino-terminal region of α_{1A}, and GST-$NT_{A,2-52}$ for the truncated form of this which contains only residues 2–52.

understanding of the molecular organization of voltage-dependent calcium channels. The interaction plays a critical role in the precise positioning of the channel activation process on the voltage axis. It constitutes yet another molecular determinant underlying functional differences among various β subunits and, by extension, probably among various native P/Q channel subtypes.

EXPERIMENTAL PROCEDURES

GST Fusion Proteins—Regions of the rabbit brain α_{1A} cDNA (BI-2 (24)) corresponding to residues 2–98 (*i.e.* the entire amino-terminal region), 2–52, 43–77, 66–98, and 76–98 were amplified by PCR and, with the aid of *Bam*HI and *Eco*RI restriction sites included in the primers, were subcloned into pGEX2TK (Amersham Pharmacia Biotech). The resulting recombinant plasmids were expressed in *Escherichia coli* BL21, and the GST fusion proteins were purified as described previously (20). Fusion proteins expressing the entire amino-terminal regions (minus start codon) of α_{1B} (amino acids 2–95, GenBank M92905 (25)), α_{1C} (amino acids 2–151, M57974 (26)), and α_{1S} (amino acids 2–49, M23919 (27)) were constructed and purified similarly. The resulting fusion proteins are referred to as, for example, GST-NT_A for that containing the entire amino-terminal region of α_{1A} and GST-$NT_{A,2-52}$ for the truncated form of this which contains only residues 2–52.

In Vitro Translation of β Subunits—β_{1b}, β_{2a}, β_3, and β_4 cDNA clones were as described previously (21). Truncated derivatives of β_4 were constructed by PCR amplification of the corresponding regions of cDNA and subcloning into pcDNA3 (Invitrogen), using *Hin*dIII and *Bam*HI sites (added to the PCR primers) with the addition of a Kozak sequence (28) and initiation codon (ACCATGG) or termination codon (TGA) as necessary. The $\beta_{3/4}$ chimera construct ($\beta_{3\ 1-360}/\beta_{4\ 402-519}$ in pcDNA3) is as described previously (21). ^{35}S-Labeled β subunits were synthesized *in vitro* using the TNT™-coupled Transcription/Translation System (Promega). Non-incorporated [^{35}S]methionine was removed by purification on a PD10 column (Amersham Pharmacia Biotech).

Binding Assays—These were carried out using fusion proteins coupled to glutathione-agarose in Tris-buffered saline as described previously (21). Binding reactions were incubated for 5 h unless otherwise stated.

Peptides—A 21-amino acid peptide containing the AID_A sequence QQQIERELNGYMEWISKAEEV and a 21-amino acid peptide containing residues 76–96 of the α_{1A} amino-terminus (RSLFLFSEDNVVRKYAKKITE) were synthesized by Genosys (United Kingdom).

Competition Experiments—For competition experiments, a maltose-binding protein (MBP) in fusion with the carboxyl-terminal binding site of α_{1A} (amino acids 2120–2275) was constructed using the *Bam*HI/*Sal*I sites of pMAL-c2 (MBP-$CT_{A,2120-2275}$). The effects of β-AID_A and β-CT_A associations on β-AID_A, β-CT_A, and β-NT_A interaction were analyzed by saturating each β site by preincubating the [^{35}S]methionine-labeled β_4 subunit (^{35}S-β_4) with 10 μM AID_A peptide (1 h) or 2 μM MBP-$CT_{A,2120-2275}$ (4 h). Binding of ^{35}S-β_4 to various α_{1A} binding sites was then tested by a 4-h incubation with 250 nM GST (control), 250 nM GST-AID_A (AID site), 2 μM GST-NT_A, and 2 μM GST-$CT_{A,2090-2424}$, precipitation of glutathione-agarose beads, gel electrophoresis, and autoradiography.

Chimera α_{1A} Subunit—Pairs of primers CSl-N1(+) 5′-GGGTCGACTAAAACGTAAAGTATTACTAAAACCTCAATTTGCAG-3′ and BIC-N1-(−) 5′-GTACTCAAAGGGTTTCCACTCGACGATGCT-3′, and primers BIC-N1(+) 5′-GTCGAGTGGAAACCCTTTGAGTACATGATT-3′ and BINt-N1(−) 5′-GAGCGGCCGCAGCACCCGCACTGC-3′ were combined with the templates pCARD3 (29) and pSPBI-2 (24), respectively, in PCR amplification using the Advantage PCR kit (CLONTECH). The resulting PCR products and the primers CSl-N1(+) and BINt-N1(−) were subjected to subsequent PCR amplification to yield a chimeric sequence that contains nucleotides −191 to 462 from the α_{1C} sequence (29) and 336–650 from the α_{1A} sequence (24). The chimeric fragment was digested with *Sal*I and *Not*I and ligated with the 11.8 kilobase *Not*I (partially digested)/*Sal*I fragment from pSPBI-2 to yield pSP72C(N)-BI-2 ($\alpha_{1A}(NT)_C$ subunit).

Electrophysiology—*Xenopus* oocytes were prepared as described previously (6). Stage V and VI oocytes were injected with α_{1A} (BI-2 (24)) or $\alpha_{1A}(NT)_C$-specific mRNA (0.3 μg/μl) either alone or in combination with β_3- or β_4-specific mRNA (0.15 μg/μl) and maintained for 3–4 days before recording in defined nutrient oocyte medium (6). Two-electrode voltage clamp recording was performed at room temperature (18–20 °C) using 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, 0.5; pH 7.4 with methanesulfonic acid. Electrodes filled with 3 M KCl had a resistance of 0.1 megohm. Current records were filtered at 1 kHz, leak-subtracted on-line by a P/6 protocol, and sampled at 5 kHz. Residual capacitative currents were blanked. Data were analyzed using pCLAMP version 6.03 (Axon Instruments). All values are mean ± S.D.

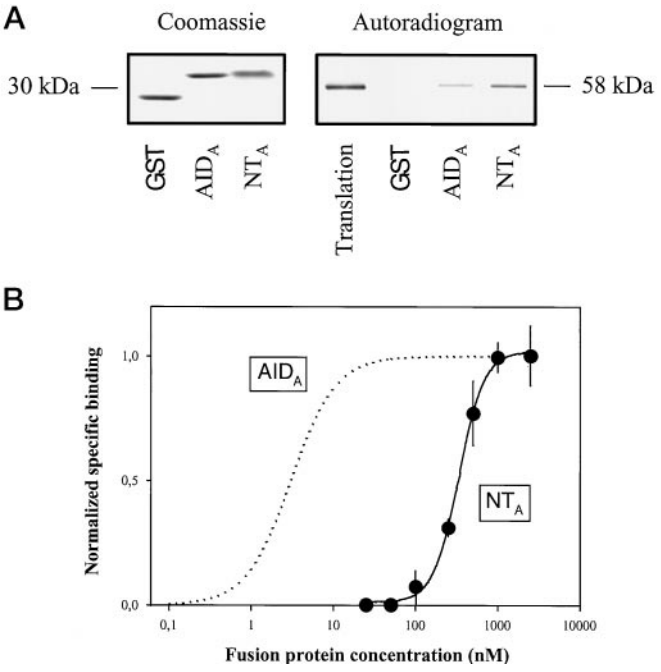

FIG. 1. ***In vitro* binding of ^{35}S-labeled β_4 to the amino-terminal region of α_{1A}.** *Panel A*. *Left*, Coomassie Blue-stained SDS-PAGE showing the GST fusion proteins used (5 μg). *Right*, autoradiogram of the binding assay. *In vitro* translated β_4 was assayed for binding to the fusion proteins indicated (5 μM). *GST*, glutathione *S*-transferase alone; *GST-AID_A*, GST fused to AID region (residues 369–418) of α_{1A}; *GST-NT_A*, GST fused to entire amino-terminal cytoplasmic domain (residues 2–98) of α_{1A}. After binding interactions as described under "Experimental Procedures," washed beads were analyzed by SDS-PAGE and autoradiography. *Panel B*, various concentrations of GST-NT_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 concentration) and normalized by expression as a proportion of maximal binding. *Error bars* indicate normalized S.D. Data are described by a logistic function $f = [(a - d)/(1 + (x/c)^b] + d$ where $a = 1.019$ and $d = 0.01022$ are the asymptotic maximum and minimum, respectively; x is the fusion protein concentration; $c = 336$ nM is the K_d; and $b = -2{,}816$ is the slope of the curve. For comparison purposes, the saturation curve for AID_A-GST (K_d = 3 nM, *dashed line*) is also shown (20).

RESULTS

A GST fusion protein, GST-NT_A, expressing the entire amino-terminal cytoplasmic region of α_{1A} (splice variant BI-2) was assayed for *in vitro* binding to ^{35}S-β_4. As Fig. 1*A* shows, the NT_A region exhibits a significant and specific interaction with β_4 which is comparable to the binding observed to a GST fusion protein carrying the AID_A sequence. The binding of GST-NT_A to ^{35}S-β_4 appears slightly stronger than the binding of GST-AID_A, but the relative efficiency of binding of these fusion proteins varied slightly depending on the β-translation reactions used. The affinity of this interaction was determined by carrying out similar binding assays using a range of concentrations of GST-NT_A fusion protein. Fig. 1*B* shows the resulting saturation curve, which is compared with that observed previously for the interaction of ^{35}S-β_4 with GST-AID_A. The affinity of interaction of GST-NT_A is 100-fold lower (k_D = 336 nM) than that for the AID interaction (close to 3 nM). These data are in favor of the idea that the AID-BID interaction represents a primary anchoring site of interaction between the two subunits which allows secondary interactions of lower affinity to occur. As already mentioned, it is interesting that in Fig. 1*A*, GST-NT_A

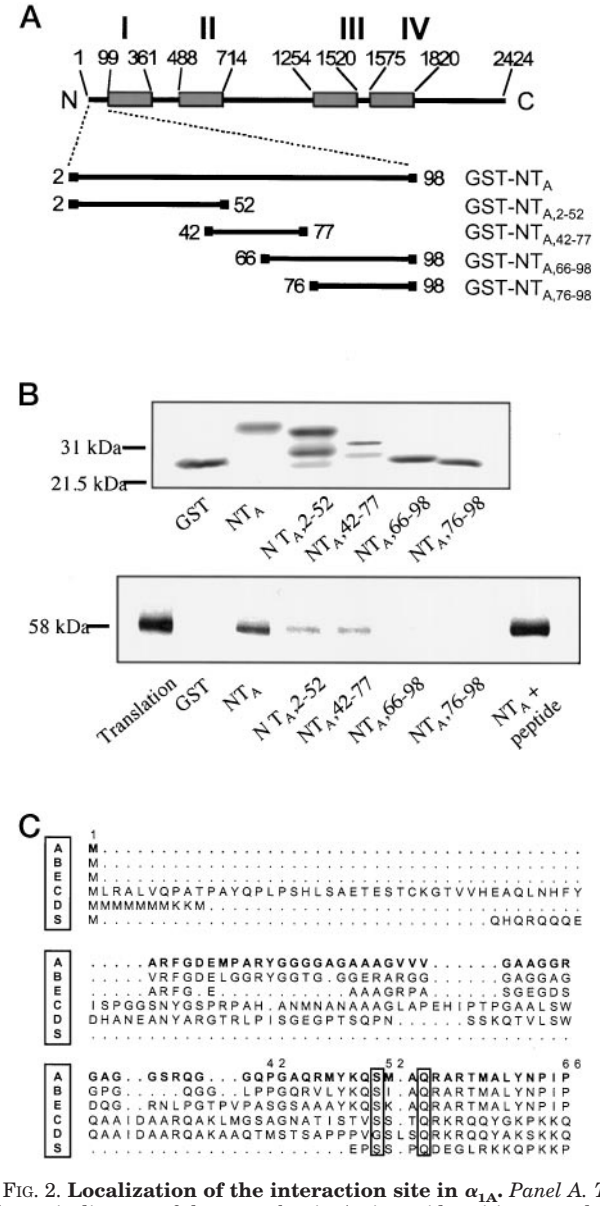

FIG. 2. **Localization of the interaction site in α_{1A}.** *Panel A. Top*, schematic diagram of the α_{1A} subunit. Amino acid positions are shown above, transmembrane domains (each composed of six membrane-spanning segments) are shown as *dark boxes* and *numbered* (I–IV) above. *Bottom*, enlargement of the amino-terminal domain, showing GST fusion proteins constructed with amino acid positions in α_{1A} marked at the extremities. *Panel B*. *Top*, Coomassie Blue-stained SDS-PAGE showing fusion proteins used (5 μg). *Bottom*, capacity of 5 μM purified fusion proteins to interact with ^{35}S-β_4. After the binding reactions, washed beads were analyzed by SDS-PAGE and autoradiography. *Panel C*, amino-terminal binding site of α_{1A} (BI-2, amino acids 1–66) and its alignment with sequences of other calcium channels (GenBank accession codes M92905, α_{1B}; X67855, α_{1E}; X15539, α_{1C}; M57682, α_{1D}; and M23919, α_{1S}).

demonstrates greater binding than GST-AID$_A$ to ^{35}S-β_4. Given that both fusion proteins are at concentrations giving maximal binding (Fig. 1*B*), this demonstrates a difference in maximal binding which appears to reflect a difference in conformational requirements, coupled with conformational heterogeneity in the ^{35}S-β_4 preparation (permissive and nonpermissive binding states; data not shown).

To characterize more precisely the region of α_{1A} responsible for interaction with β_4, a series of GST fusion proteins carrying truncations of the region concerned (depicted in Fig. 2*A*) was constructed, and the proteins were assayed for their capacity to interact with ^{35}S-β_4. As Fig. 2*B* shows, removal of the most carboxyl-terminal amino acids, or of the 42 most amino-terminal, does not abolish the capacity to interact with β_4. Concomitantly, fusion proteins corresponding to the most carboxyl-terminal region, which is most highly conserved among α_1 subtypes, are incapable of binding. In addition, the interaction between GST-NT$_A$ and ^{35}S-β_4 was not inhibited by addition to the binding reaction of a peptide (500 μM) corresponding to amino acids 76–98. These data suggest that the β_4 binding site concerns a region between residues 1 and 66 of α_{1A}, maybe comprising, but not necessarily limited to, residues 42–52. The reduced binding to NT$_A$,2–52 and NT$_A$,42–77 compared with full-length NT$_A$ probably reflects instability and/or sequence reduction of the interaction site. The reduction in binding of smaller deleted derivatives meant that we were unable to pursue this approach further. A sequence alignment of this α_{1A} binding domain with equivalent domains of other α_1 subunits (α_{1B}, α_{1E}, α_{1C}, α_{1D}, and α_{1S}), some used in this investigation, suggests a relatively low level of sequence conservation, although α_{1B} and α_{1A} show some similarity (Fig. 2*C*). This observation implies that the interaction may not be conserved, a prediction that we went on to test (see Fig. 5).

To identify the region of β_4 which interacts with the amino-terminal region of α_{1A}, we initially analyzed the binding capacity of several deleted derivatives of β_4, translated *in vitro* (Fig. 3, *A* and *B*). These derivatives lacked either the amino-terminal, carboxyl-terminal, or both regions, which shows a low level of conservation among β subunit subtypes. As Fig. 3*B* shows, removal of the amino-terminal region had no effect, whereas removal of the carboxyl-terminal abolished binding completely, illustrating the importance of this region in the interaction. We also found that although β_3 does not interact with GST-NT$_A$ (see Fig. 5), the opposite is true for a β_3-β_4 chimera, in which the nonconserved carboxyl terminus of β_3 is replaced by the equivalent domain of β_4 (Fig. 3*B*).

We have shown previously (21) that the carboxyl-terminal region of β_4 also interacts with the carboxyl-terminal cytoplasmic domain of α_{1A} (BI-2). We therefore wanted to map the two interaction sites more precisely, for which we constructed two additional derivatives of β_4, lacking a third (residues 483–519) and two-thirds (residues 447–519) of the carboxyl terminus (Fig. 3, *A* and *B*). As Fig. 3*C* shows, deletion of residues 483–519 of β_4 had no effect on its capacity to bind to GST-NT$_A$, whereas truncation of the carboxyl terminus of β_4 up to residue 446 resulted in a total loss of binding capacity. This indicates that the NT$_A$ binding region is located between residues 446 and 482 of β_4. Analysis of the capacity of these truncates to bind to a GST fusion protein of the carboxyl-terminal region (residues 2090–2424) of α_{1A} (GST-CT$_A$) resulted in binding capacity being gradually lost with each further deletion. This suggests that the binding site of CT$_A$ spans a wider region than the NT$_A$ binding site, is dependent on secondary or tertiary structures that are disrupted by the deletions, or consists of a series of dispersed sites. It is noteworthy that the previously characterized α_{1A} carboxyl-terminal binding site was also difficult to define, in that deleted derivatives over a long region retained binding capacity, giving support to the hypothesis that there are microdomains of interaction between these two sites (21). In contrast, the NT$_A$ site and corresponding domain on β_4 are shorter and seem more easily delineated. In any case, the different patterns of interaction capacities seen for GST-NT$_A$ and GST-CT$_A$ suggest that these two regions of α_{1A} occupy different but overlapping sites on β_4.

The involvement of overlapping regions of β_4 in interactions with the amino- and carboxyl-terminal domains of α_{1A} also raised the question as to whether these interactions could occur simultaneously or whether they were mutually exclusive. To

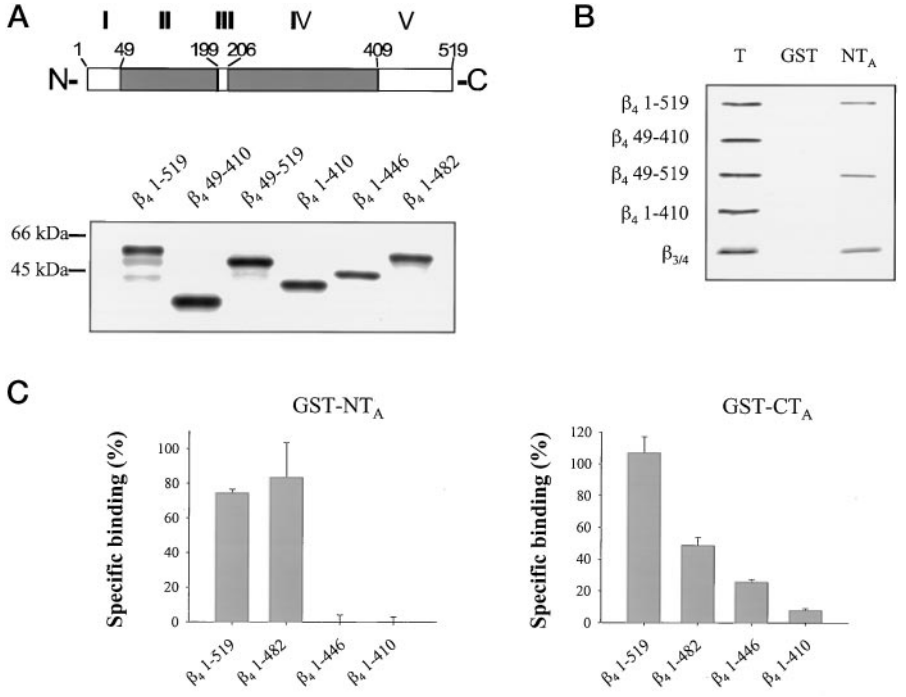

FIG. 3. **Localization of the interaction site in the β_4 subunit.** *Panel A. Top*, schematic map of β_4 subunit, dividing the protein into five domains. *Darker* domains (II and IV) represent sequences of highest conservation among β subtypes (10). *Bottom*, autoradiography of SDS-PAGE showing β_4 and deleted derivatives translated *in vitro* in the presence of [^{35}S]methionine. *Panel B*, *in vitro* translated full-length, truncated β_4 derivatives and a β_3-β_4 chimera (*T*) were assayed for their capacity to interact with GST and GST-NT_A fusion proteins (2.5 μM) as described under "Experimental Procedures," and washed beads were analyzed by SDS-PAGE and autoradiography. *Panel C*, ^{35}S-β_4 and carboxyl-terminal deleted derivatives were assayed for their capacity to interact with 2.5 μM GST-NT_A and a GST fusion protein containing residues 2090–2424 of α_{1A} (GST-CT_A (21)). Specific binding was calculated by subtraction of binding to GST (at the same concentration) and normalized by expression as a percentage of maximal binding to GST-AID_A. *Error bars* represent normalized S.D.

investigate this as well as their relationship with the AID-BID interaction, we tested whether the binding of AID_A (21-amino acid peptide) or GST-CT_A to ^{35}S-β_4 could prevent its interaction with GST-NT_A. The results, illustrated in Fig. 4, show that although the AID peptide was effective in preventing the interaction of β_4 to GST-AID_A, it did not prevent the concomitant interaction with either GST-NT_A or GST-$CT_{A,2070-2275}$ (Fig. 4*A*). On the other hand, the association of MBP-$CT_{A,2120-2275}$ with β_4 blocked the ability of β_4 to interact with GST-$CT_{A,2070-2275}$ and also significantly reduced the binding of β_4 to GST-NT_A (Fig. 4*B*), suggesting that β_4 is able to interact with AID and only one of the secondary interaction sites at a time.

We have shown previously (21) that β subtypes differ in their capacity to interact with the carboxyl-terminal region of α_{1A}, with β_4 interacting with greatest affinity, β_{2A} with a lesser affinity, and β_{1b} and β_3 showing no significant interaction. We therefore wished to determine whether the same was true for interaction with the amino-terminal domain. In addition, because the β interaction site in the amino-terminal region of α_{1A} shows a variable level of conservation among α_1 subtypes, we wished to investigate whether β interaction capacities were conserved among them. Both of these questions were addressed by constructing a series of GST fusion proteins carrying the amino-terminal cytoplasmic region of α_{1B}, α_{1C}, and α_{1S} (Fig. 5*A*). These fusion proteins, along with GST alone, GST-AID_A (for comparison purposes), and GST-NT_A, were assayed for their ability to interact with four different β subtypes, translated *in vitro* in presence of [^{35}S]methionine (Fig. 5*B*). Interestingly, interaction with GST-NT_A showed a pattern similar to that observed for the carboxyl-terminal region of α_{1A} (21) in that β_4 exhibited the most significant interaction, β_{2a} interacted to a lesser degree, and β_{1b} and β_3 showed no significant interaction. The amino-terminal domains of α_{1B} showed no significant interaction despite its closer sequence relatedness to α_{1A}. GST-NT_S, on the other hand, showed significant interaction with all four β subunits, whereas GST-NT_C, another L-type channel member, showed no interaction with any of the β subunits.

Because the amino-terminal sequences of α_{1A} and α_{1S} are very different, we checked whether binding of β_4 to NT_S involved the same interaction domain of β_4. Fig. 6 demonstrates

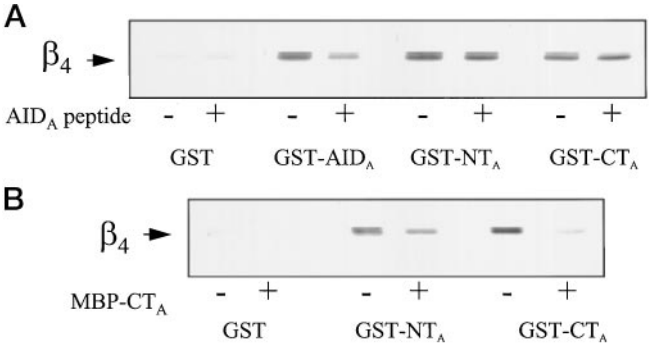

FIG. 4. **Multiple site occupancies on β_4 subunit.** *Panel A*, effect of 1-h AID_A peptide (10 μM) preincubation with ^{35}S-β_4 on control GST (250 nM), GST-AID_A (250 nM), GST-NT_A (2 μM), and GST-CT_A (2 μM) binding to ^{35}S-β_4. The 4-h binding reaction was conducted in the continued presence of AID_A peptide. *Panel B*, effect of 4-h MBP-CT_A (2 μM) preincubation with ^{35}S-β_4 on control GST (250 nM), GST-NT_A (2 μM), and GST-CT_A (2 μM) binding to ^{35}S-β_4. The 4-h binding reaction was conducted in the continued presence of MBP-CT_A. Complete inhibition of GST-NT_A binding to ^{35}S-β_4 by MBP-CT_A was difficult to achieve because MBP-CT_A bound only a subset of the available ^{35}S-β_4, which was presumably in a more favorable conformation.

that, as for NT_A, the carboxyl terminus of β_4 was required for binding to NT_S, and the use of deleted derivatives of the carboxyl terminus of β_4 also indicates an important role for residues 446–482 of β_4 in this interaction. These results suggest that the interaction site is defined more by the tertiary structure of the α_1 amino-terminal region than by its primary sequence, also explaining why the NT_A site could not be localized more precisely than to residues 1–66 (Fig. 2).

Finally, we questioned the relevance of the interaction between the amino terminus of α_{1A} and the carboxyl terminus of β_4 in terms of channel functioning. First, because β_3, in contrast to β_4, does not interact with the amino terminus of α_{1A}, we investigated whether there were significant differences in terms of channel regulation by these two subunits. We found that in addition to triggering different inactivation kinetic behaviors (6), the two subunits differed in terms of their ability to shift the activation curve toward hyperpolarized potentials (Fig. 7*A*). Although both β subunits shifted the activation curve

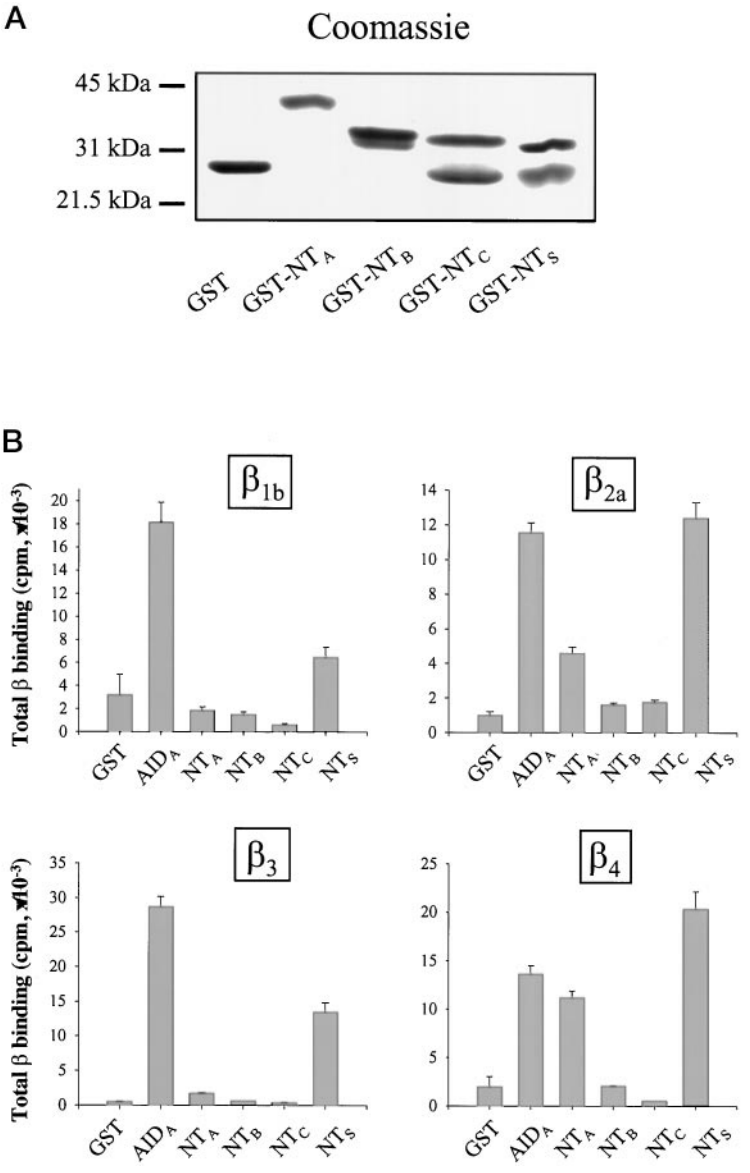

FIG. 5. **α_1 amino-terminal specificity of interaction with β subunits.** *Panel A*, Coomassie Blue-stained SDS-PAGE showing various GST fusion proteins used (5 μg). *Panel B*, *in vitro* translated β subunits were assayed for their capacity to interact with 5 μM GST fusion proteins, and the remaining radioactivity associated with washed beads was quantified by counting. *GST*, control; *AID_A*, GST-AID_A; *NT_A*, *NT_B*, *NT_C*, *NT_S*, GST fusion proteins containing amino-terminal cytoplasmic domains of α_{1A}, α_{1B}, α_{1C}, and α_{1S}, respectively. *Error bars* represent S.D.

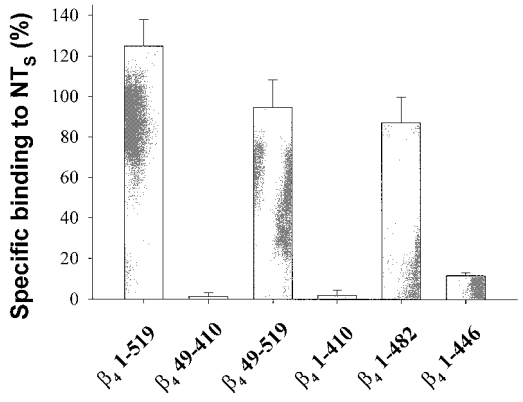

FIG. 6. **The carboxyl terminus of β_4 is also involved in NT_S binding.** ^{35}S-β_4 and deleted derivatives were assayed for their capacity to interact with GST-NT_S (5 μM). Specific binding was calculated by subtraction of binding to GST (at the same concentration) and normalized by expression as a percentage of maximal binding to GST-AID_A (500 nM). *Error bars* represent normalized S.D.

along the voltage axis, the shift induced by β_3 was significantly more pronounced than the one produced by β_4. The estimated half-activation potential shifted from 17 mV (α_{1A}-expressing oocytes) toward −13 mV ($\alpha_{1A}\beta_3$ oocytes) and 1.5 mV ($\alpha_{1A}\beta_4$ oocytes). There is thus an approximately 14–15 mV difference in the shift induced by the β_3 and β_4 subunits. In addition, we found that depending on the β subunit being expressed, the channels differed in their voltage dependence of inactivation with half-inactivation at −50 and −37 mV for $\alpha_{1A}\beta_3$ and $\alpha_{1A}\beta_4$ channels, respectively (data not shown). Because these differences in functional regulation by the various β subunits may be the result of differences in interaction levels between α_{1A} and the two β subunits, we determined the role of the NT_A site in β-induced channel regulation. We took advantage of the observation that essential differences were found in β subunit association with amino-terminal sequences of various α_1 subtypes. We constructed a chimera α_{1A} subunit ($\alpha_{1A}(NT)_C$), in which we replaced the amino terminus of α_{1A} (interacts with β_4 but not β_3) with the amino terminus of α_{1C} (does not interact with either β_4 or β_3). Coexpression of this chimeric channel with β_3 or β_4 triggers high voltage-activated currents in *Xenopus* oocytes (Fig. 7*B*). The amplitude of the currents elicited by membrane depolarization are reduced slightly compared with those obtained for the wild-type α_{1A} channel. Cells expressing $\alpha_{1A}\beta_3$, for instance, have a peak current amplitude of 1,001 ± 651 nA (n = 7, S.D.), whereas cells expressing $\alpha_{1A}(NT)_C\beta_3$ peak at 423 ± 655 nA (n = 12), which corresponds to a 2.37-fold

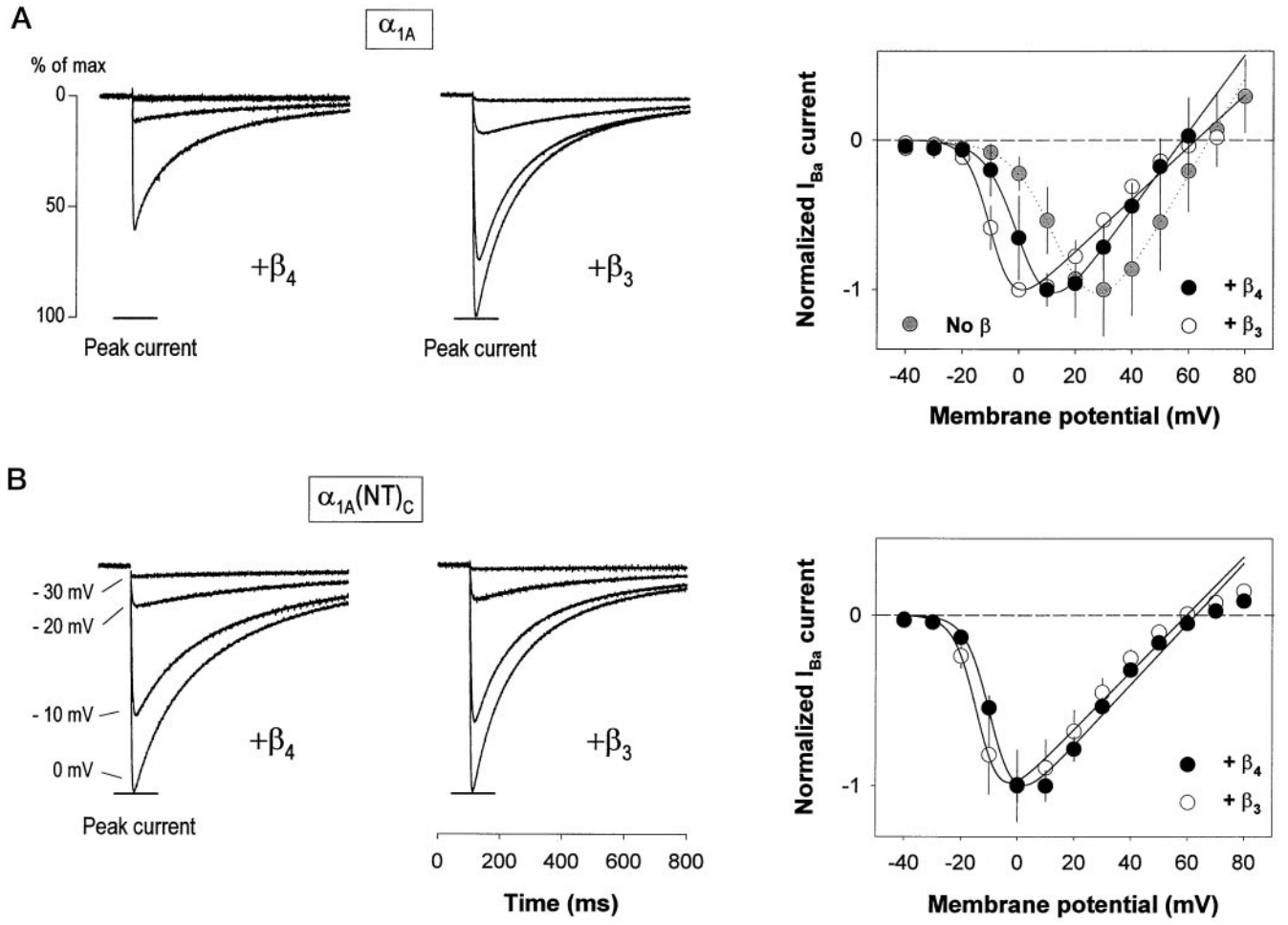

FIG. 7. **Role of the NT_A-β_4 interaction in the control of voltage dependence of activation.** *A*, β_3 and β_4 differ in their ability to shift the voltage dependence of activation of α_{1A}. *Left* and *center*, currents elicited by various membrane depolarizations (−30, −20, −10, and 0 mV) illustrating differences in threshold, intermediate, and peak activation of $\alpha_{1A}\beta_3$ and $\alpha_{1A}\beta_4$ channels expressed in *Xenopus* oocytes. *Right*, corresponding average current-voltage (*I-V*) relationship for $\alpha_{1A}\beta_3$ ($n = 7$) and $\alpha_{1A}\beta_4$ ($n = 6$) expressing cells. The *I-V* curve for cells expressing α_{1A} channel alone is shown for comparison purpose ($n = 8$). The experimental data were fitted with a modified Boltzmann equation $I_{Ba} = (g \cdot (V - E))/(1 + \exp(-(V - V_{1/2})/k))$, where g is the normalized conductance ($g = 0.032$, no β; 0.018, $+\beta_3$; and 0.026, $+\beta_4$); $V_{1/2}$ is the half-activation potential ($V_{1/2} = 17$ mV, no β; −13 mV, $+\beta_3$; and 1. 5 mV, $+\beta_4$); E is the reversal potential ($E = 67$ mV, no β; 63 mV, $+\beta_3$; and 58 mV, $+\beta_4$); and k is the range of potential for an e-fold change around $V_{1/2}$ ($k = 7.9$ mV, no β; 4. 2 mV, $+\beta_3$; and 5. 8 mV, $+\beta_4$). *B*, change in difference in the β-induced *I-V* shift by α_{1A} amino-terminal sequence substitution. *Left* and *center*, currents elicited by various membrane depolarizations (−30, −20, −10, and 0 mV) showing the absence of a difference in channel activation for $\alpha_{1A}(NT)_C\beta_3$ and $\alpha_{1A}(NT)_C\beta_4$ channels. *Right*, corresponding average *I-V* curves for $\alpha_{1A}(NT)_C\beta_3$ ($n = 13$) and $\alpha_{1A}(NT)_C\beta_4$ channels ($n = 12$). The fit of the experimental data yields $V_{1/2} = -13$ ($+\beta_3$) and −9 mV ($+\beta_4$); $k = 4.2$ ($+\beta_3$) and 4. 4 mV ($+\beta_4$); $g = 0.017$ ($+\beta_3$) and 0. 018 ($+\beta_4$); and $E = 60$ ($+\beta_3$) and 63 mV ($+\beta_4$).

reduction. A similar 2.06-fold reduction in current amplitude is seen when β_4 is coexpressed with $\alpha_{1A}(NT)_C$ (peak current 542 ± 240 nA, $n = 12$) rather than α_{1A} (peak current 1,118 ± 871 nA, $n = 6$). These results suggest that the amino terminus plays a role in channel expression levels at the plasma membrane but that β subunits and the NT_A interaction site have little influence on this process. Also, the amino-terminal substitution induced an important shift in the voltage dependence of inactivation with half-inactivation occurring at −52 mV for $\alpha_{1A}(NT)_C\beta_4$ channels compared with −37 mV for $\alpha_{1A}\beta_4$ channels (data not shown). Because a similar shift is seen with β_3 (not shown), this supports the idea that β subunit interaction with the amino terminus plays a minor role in this modification. In contrast, we found that the difference in the shift of voltage dependence of activation of $\alpha_{1A}(NT)_C\beta_3$ and $\alpha_{1A}(NT)_C\beta_4$ channels was reduced significantly (Fig. 7*B*). The average half-activation potential of $\alpha_{1A}(NT)_C\beta_3$ channels was −13 mV and thus remained identical to that of the $\alpha_{1A}\beta_3$ channels, whereas the $V_{1/2}$ of $\alpha_{1A}(NT)_C\beta_4$ channels was −9 mV, a significant hyperpolarizing shift compared with the $\alpha_{1A}\beta_4$ channels. These data suggest that in the absence of an NT_A/β_4 interaction, the *I-V* shift induced by the β_4 subunit resembles the shift induced by the β_3 subunit. Finally, the substitution of the α_{1A} NT_A sequence by NT_C produced a slowing of channel inactivation with β_4 but not with β_3. The decay of $\alpha_{1A}(NT)_C\beta_4$ currents occurred along two components with time constants of $\tau_1 = 80 \pm 4$ ms and $\tau_2 = 368 \pm 39$ ms (at 10 mV, $n = 7$) compared with $\tau_1 = 51 \pm 9$ ms and $\tau_2 = 246 \pm 27$ ms ($n = 6$) for $\alpha_{1A}\beta_4$ currents. In contrast, no significant differences were seen in inactivation kinetics of $\alpha_{1A}\beta_3$ or $\alpha_{1A}(NT)_C\beta_3$ channels with time constants at 10 mV of $\tau_1 = 65 \pm 15$ ms and $\tau_2 = 243 \pm 47$ ms ($n = 10$) for $\alpha_{1A}(NT)_C\beta_3$ currents and $\tau_1 = 62 \pm 14$ ms and $\tau_2 = 222 \pm 13$ ms ($n = 7$) for $\alpha_{1A}\beta_3$ currents. These data further confirm a functional role in inactivation kinetics of the carboxyl terminus of β_4 by its interaction with the carboxyl terminus (21) and amino terminus of the α_{1A} subunit.

DISCUSSION

We describe the identification of a specific interaction site between the amino-terminal cytoplasmic region of the calcium channel α_{1A} subunit and the β_4 subunit. The β_4 subunit is widely expressed in the brain, especially in the cerebellum (30).

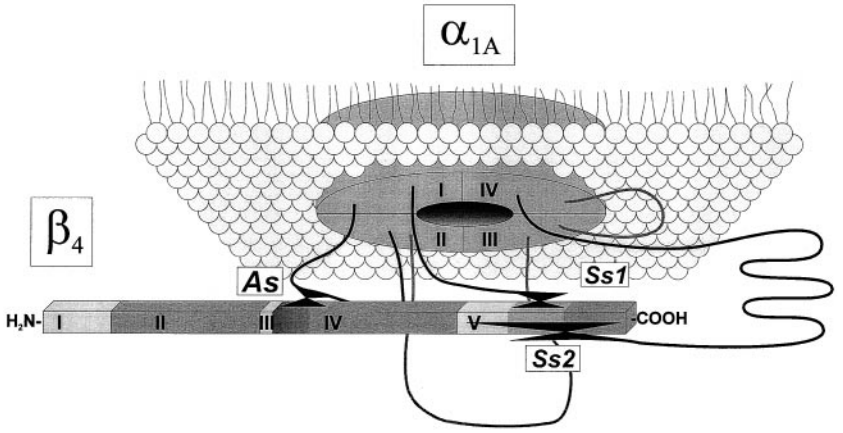

FIG. 8. **Schematic α_{1A}-β_4 interactions.** Shown are the AID-BID anchoring site (*As*) implicating the I-II loop of α_{1A} and the first 30 residues of domain IV of β_4; and the two secondary interactions sites, the first comprising NT_A (*Ss1*) and the second CT_A (*Ss2*), involving overlapping regions of domain V of β_4.

On the basis of their colocalization in many tissue types, β_4 appears largely to be associated with the α_{1A} subunit in native channels (17, 31). However, coimmunoprecipitation studies demonstrate that α_{1A} is also found associated with β_{1b}, β_2, and β_3 (17) and that β_4 is also found associated with α_{1B} (16). The importance of the β_4 subunit is illustrated by the recent demonstration that a lethargic phenotype in mice results from a deletion of approximately 60% of the β_4 coding sequence (32). This truncated β subunit would lack all three of the interactions described with α_{1A} (AID_A (19) and NT_A and CT_A (21)), although such a deletion is also likely to result in severe conformational perturbation and probably degradation of the protein. This mutation is not entirely lethal, however, which is reminiscent of a growing number of experiments in which knockout of proteins of central importance does not turn out to be lethal. This is probably explained by a partial compensation by a related protein, in this case suggesting that other β subunits are expressed in parallel or that their expression is switched on to compensate for this deficiency (33). In fact, β_3 is known to be a normal constituent of about one-third of P/Q-type channels (17). Because β_3 expression is high in brain and parallels that of β_4 (34), it would be the most likely candidate for β_4 substitution in the lethargic mice. Since β_3 lacks both secondary interaction sites described so far in β_4, such a substitution would not be functionally equivalent, perhaps explaining some of the neurological defects encountered in these mice.

The NT_A interaction identified is of relatively low affinity, supporting the idea that this is one of several secondary interactions between the two subunits that rely on the initial, high affinity interaction between the AID and BID sites identified previously. This idea is supported by the observation that mutagenesis of AID or BID to disrupt interaction between the two sites also disrupts the ability of the β subunit to modify channel properties (10). It also stems from the fact that this is the third interaction site mapped between α_{1A} and β_4 and that binding of multiple β subunits to α_1 does not seem very plausible. The new interaction site that we describe involves the amino terminus of α_{1A} (residues 1–66) and carboxyl terminus (residues 446–482) of β_4. This is particularly interesting given the rather low level of sequence conservation in the two regions identified. With regard to α_{1A} splice variants, the sequence of the amino-terminal cytoplasmic region is identical in BI-1 and BI-2 subtypes, indicating conservation of this interaction (24). This is in contrast to the β_4 interaction site that we have identified previously in the carboxyl-terminal region of the BI-2 splice variant.

The low degree of sequence conservation observed for the respective interaction sites identified in the amino-terminal region of α_{1A} and the carboxyl-terminal of β_4 is reflected by the high degree of subtype specificity exhibited by this interaction with respect to both α_1 and β isoforms. Our results indicate that the equivalent amino-terminal regions of α_{1B} and α_{1C} did not interact with any of the β subunits tested. Because other β subtypes exist, we cannot rule out that this may reflect the use of an inappropriate α_1-β combination. Interestingly, we found that a fusion protein expressing the entire amino terminus of α_{1S} could interact with all four different β subunits tested. This is in contrast to the NT_A binding, which occurs only on β_4 and to a lesser extent on β_{2a}. These results are indicative of a potential interaction of α_{1S} with β subunits other than β_{1a}, the major β subunit of skeletal muscle, and parallel recent findings that β_3 (7, 32) and a β_1 splice variant other than β_{1a} (2) are also expressed, albeit at low levels, in skeletal muscle. Overall, our results are indicative of evolution to provide for α_1-β interaction specificity both within the α_1 amino-terminal and the β carboxyl-terminal sequences.

Fig. 8 summarizes what is now known about α_{1A}-β_4 interactions in terms of structure. One interesting aspect is that the β_4 subunit can interact simultaneously with AID and, via its carboxyl-terminal region, with either the amino- or carboxyl-terminal regions of α_{1A}, thereby defining two patterns of interactions. These interactions probably impose conformational constraints on the molecule which appear to affect channel function. It is also tempting to speculate that the conformational constraints are different depending on the patterns of interaction in use by the channel. The importance of the amino and carboxyl termini of α_{1A} are underlined by the observation that truncations of equivalent domains in α_{1C} result in enhanced current levels of the channel (35, 36). These enhanced current levels occur either by a greater membrane incorporation (amino terminus) or enhanced open probability (carboxyl terminus). Because β subunits also increase channel expression, and this effect varies in amplitude depending on the α_1 and β subtype studied, it is tempting to speculate that the secondary interaction sites described so far also intervene in α_{1A} channel expression by one of the two mechanisms described for α_{1C}. We did indeed find that substitution of the amino terminus of α_{1A} by the equivalent sequence of α_{1C} resulted in an important reduction in current density. This effect was, however, β subtype-independent, and it is therefore unlikely that the NT_A interaction site described here plays a role in β-induced enhancement of current amplitude. Despite this, secondary interactions appear to play other roles in several aspects of control of channel activity. We have shown previously (21) the importance of the carboxyl-terminal region of α_{1A} in the control of channel inactivation kinetics. Here, we demonstrate that the amino-terminal interaction site of α_{1A} is required for fine tuning the voltage dependence of activation. The NT_A interaction with β_4 appears to limit the amplitude of the hyperpolarizing β-induced shift of channel activation. By

this unique mechanism, it can be predicted that the β_3-containing P/Q channel subtype is activated more easily than the β_4-containing P/Q channel subtype. In addition, secondary interaction sites may serve to protect or uncover phosphorylation sites in the α_{1A} subunit, thereby altering the regulatory input of these. Another obvious possibility is that they play a role in the antagonistic relationship between the β subunit and G$\beta\gamma$ complex. In this respect, it is interesting that Qin *et al.* (23) have recently shown that, in addition to interacting with a region overlapping with the AID site (37, 38), G$\beta\gamma$ also interacts with the carboxyl-terminal domain of α_{1A}, α_{1B}, and α_{1E} and that the amino terminus has recently been recognized as another determinant for G$\beta\gamma$ regulation in α_{1E} subunits (39). Finally, the existence of secondary interactions in addition to the AID-BID interaction could serve to favor certain combinations of subunits in cells where several subtypes are expressed. Given that β subunits also play a role in the surface targeting of α_1 and $\alpha_2\delta$ (14), an interesting possibility is that specific β subunits serve to target α_1 subunits to specific regions of the cell surface.

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