A New β Subtype-specific Interaction in α1A 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 subunit interaction domain (AID) in the I-II cytoplasmic loop of α subunit 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 α1A (B1-2) and the carboxyl terminus of β. Like the interaction identified previously (21) between the carboxyl termini of α1A and β (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 subunit and are competitive, but neither inhibits the interaction with AID. The interaction with the amino terminus of α1 is isoform-dependent, suggesting a role in the specificity of α1-β pairing. Coexpression of β subunit in Xenopus oocytes produces a reduced hyperpolarizing shift in the I-V curve of the α1A channel compared with β subunit (not exhibiting this interaction). Replacing the amino terminus of α1A with that of α1C 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 subunits, pore-forming component of the channel is associated with a cytoplasmic β subunit of 52–78 kDa and a largely extracellular αδ component, anchored by a single transmembrane domain. These subunits are encoded by at least 7 α, 4 β, and 1 αδ genes, respectively, of which numerous splice variants exist (3).

The β subunit, when coexpressed with the α 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 subunits. 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, α1 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 α1-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 α1 (AID, or α subunit 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 subunit and β subtypes tested so far. We have since reported (21) the existence of a subunit-specific interaction between the carboxyl-terminal domain of α1A and the most carboxyl-terminal 109 residues of β2, and a similar interaction has been reported (22, 23) between α1C and β2A. 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 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 α1A and the β4 subunit of P/Q channels providing a refreshed

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§ The abbreviations used are: AID, α interaction domain; BID, β interaction domain; GST, glutathione S-transferase; PCR, polymerase chain reaction; MFB, maltose-binding protein; [35S]BSA, [35S]methionine-labeled β subunit; PAGE, polyacrylamide gel electrophoresis. Fusion proteins are referred to as, for example, GST-NTA1 for that containing the entire amino-terminal region of α1A, and GST-NTA1,2-52 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 α₁A cDNA (BI-2 (24) containing residues 2–36 (i.e. the entire amino-terminal region) were subjected to subsequent PCR amplification to yield a chimeric and subcloning into pcDNA3 (Invitrogen), using constructed by PCR amplification of the corresponding regions of cDNA (Promega). Non-incorporated [35S]methionine was removed by purification on a PD10 column (Amersham Pharmacia Biotech). The exenclamp recording was performed at room temperature (18–20 °C) using recording in defined nutrient oocyte medium (6). Two-electrode voltage clamp recordings used. The results, summarized in Table I, show that the α₂₃₄₅ sixty-nine channel subtypes.

**Peptides—**A 21-amino acid peptide containing the AID sequence QQQIERELNGYMEWISKAEEV and a 21-amino acid peptide containing the AID A sequence BINt-N1(9). Pairs of primers CSl-N1(9) and BINt-N1(9) were constructed by PCR amplification using the Advantage PCR kit (CLONTECH). The resulting recombinant plasmids were expressed in E. 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 α₁A (amino acids 2–95, GenBank M29905 (25), α₁C (amino acids 2–151, M57974 (26), and α₁B (amino acids 2–49, M23919 (27) were constructed and purified similarly. The resulting fusion proteins are referred to as, for example, GST-NTA, that containing the entire amino-terminal region of α₁A and GST-NTN25–25 for the truncated form of which this contains only residues 2–25.

**In vitro Translation of β Subunits—**β₁, β₂, and β₃ cDNA clones were as described previously (21). Truncated derivatives of β₁ were constructed by PCR amplification of the corresponding regions of cDNA and subcloning into pcDNA3 (Invitrogen), using HindIII and BamHI sites (added to the PCR primers) with the addition of a Kozak sequence (28) and initiation codon (ACCAATG) or termination codon (TGA) as necessary. The β₂₄ chimaera construct (β₂, 1-sal/Pr4, 402–519 in pCDNA3) is as described previously (21). [35S]-Labeled β subunits were synthesized in vitro using the TNT coupled Transcription/Translation System (Promega). Non-incorporated [35S]methionine was removed by purification on a PD10 column (Amersham Pharmacia Biotech). The exenclamp recording was performed at room temperature (18–20 °C) using recording in defined nutrient oocyte medium (6). Two-electrode voltage clamp recordings used. The results, summarized in Table I, show that the α₂₃₄₅ sixty-nine channel subtypes.

**Electrophysiology—**Xenopus oocytes were prepared as described previously (6). Stage V and VI oocytes were injected with α₁A (BI-2 (24) or α₁A(NTA)–specific mRNA (0.5 μg/μl) either alone or in combination with β₁D or β₂D (0.5 μg/μl) mRNA injected 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): BaOH₂, 40; NaOH, 50; KCl, 3; HEPES, 5; niflumic acid, 0.5; pH 7.4 with methanesulfonic acid. Electrodes filled with 3 mM KCl had a resist ance of 0.1 meq/m. 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.

**RESULTS**

A GST fusion protein, GST-NTA, expressing the entire amino-terminal cytoplasmic region of α₁A (splice variant BI-2) was assayed for binding to [35S]-β₄. As Fig. 1A shows, the NTA region exhibits a significant and specific interaction with β₄ which is comparable to the binding observed to a GST fusion protein carrying the AIDA sequence. The binding of GST-NTA to [35S]-β₄ appears slightly stronger than the binding of GST-AID₄, but the relative efficiency of binding of these fusion proteins varied slightly depending on the β-interaction reactions used. Therefore, the interaction was determined by carrying out similar binding assays using a range of concentrations of GST-NTA fusion protein. Fig. 1B shows the resulting saturation curve, which is compared with that observed previously for the interaction of [35S]-β₄ with GST-AID₄. The affinity of interaction of GST-NTA is 100-fold lower (kᵣ = 336 nm) than that for the AID-A protein carrying the AID A sequence. The binding of GST-AID₄ to GST-NTA, which allows secondary interactions of lower affinity to occur. As already mentioned, it is interesting that in Fig. 1A, GST-NTA.
Figure 2. Localization of the interaction site in α1A
Panel A, Top, schematic diagram of the α1A 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.

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 35S-β4. After the binding reactions, washed beads were analyzed by SDS-PAGE and autoradiography.

Panel C, amino-terminal binding site of α1A (BI-2, amino acids 1–66) and its alignment with sequences of other calcium channels (GenBank accession codes [M92905, X67855, X15539, M57682, and M23919, α1A]).

Based on the data presented, it is evident that GST fusion proteins constructed with amino acid positions in the extremities interact with 35S-β4, whereas fusion proteins encompassing the amino-terminal domain (I–IV) above do not interact, as shown in Figure 2A (top) and Figure 2B (bottom). This suggests a low level of sequence conservation among α subunits. In contrast, the NTA site and corresponding domain retained binding capacity, giving support to the hypothesis that the binding site of CTA spans a wider region than the amino-terminal domain of α1A, as depicted in Figure 2A (top). This implies that the interaction may not be conserved, suggesting a relatively low level of sequence conservation, although α1B and α1A show some similarity (Figure 2C). This observation indicates that the interaction of GST-NTA and 35S-β4 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 β4 binding site concerns a region between residues 1 and 66 of α1A, comprising, but not necessarily limited to, residues 42–52. The reduced binding to NTA4–27 and NTA4–22 compared with full-length NTA 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 α1A binding domain with equivalent domains of other α subunits (α1B, α1E, α1C, α1D, and α1E), some used in this investigation, suggests a relatively low level of sequence conservation, although α1B and α1A show some similarity (Figure 2C). This observation indicates that the interaction may not be conserved, a prediction that we went on to test (see Figure 5).

To identify the region of β4 which interacts with the amino-terminal region of α1A, we analyzed the binding capacity of several deleted derivatives of β4. Translated in vitro (Figure 3A and B), these derivatives lacked either the amino-terminal, carboxyl-terminal, or both regions, which shows a low level of conservation among α subunit subtypes. As Figure 3B shows, removal of the amino-terminal domain had no effect, whereas removal of the carboxyl-terminal domain abolished binding completely, illustrating the importance of this region in the interaction. We also found that although β4 does not interact with GST-NTA (see Figure 5), the opposite is true for a β2-β4 chimera, in which the nonconserved carboxyl terminus of β4 is replaced by the equivalent domain of β2 (Figure 3B).

We have shown previously (21) that the carboxyl-terminal region of β4 also interacts with the carboxyl-terminal cytoplasmic domain of α1A (BI-2). We therefore wanted to map the two interaction sites more precisely, for which we constructed two additional derivatives of β4, lacking a third (residues 483–519) and two-thirds (residues 447–519) of the carboxyl terminus (Figure 3A and B). As Figure 3C shows, deletion of residues 483–519 of β4 had no effect on its capacity to bind to GST-NTA, whereas truncation of the carboxyl terminus of β4 up to residue 446 resulted in a total loss of binding capacity. This indicates that the NTA binding region is located between residues 446 and 482 of β4. Analysis of the capacity of these truncates to bind to a GST fusion protein of the carboxyl-terminal domain (residues 2090–2424) of α1A (GST-CTA) resulted in binding capacity being gradually lost with each further deletion. This suggests that the binding site of CTA spans a wider region than the NTA 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 α1A 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 NTA site and corresponding domain on β4 are shorter and seem more easily delineated. In any case, the different patterns of interaction capacities seen for GST-NTA4 and GST-CTA suggest that these two regions of α1A occupy different but overlapping sites on β4.

The involvement of overlapping regions of β4 in interactions with the amino- and carboxyl-terminal domains of α1A also raised the question as to whether these interactions could occur simultaneously or whether they were mutually exclusive. To...
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-$\beta_4$ 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 $\beta_4$ to GST-AIDA$_A$, it did not prevent the concomitant interaction with either GST-NT$_A$ or GST-CT$_A$. The 4-h binding reaction was conducted in the continued presence of AID$_A$ peptide. Panel B, effect of 4-h MBP-CT$_A$ (250 nM) preincubation with $^{35}$S-$\beta_4$ on control GST (250 nM), GST-NT$_A$ (2 M), and GST-CT$_A$ (2 M), and binding to $^{35}$S-$\beta_4$. 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-$\beta_4$ by MBP-CT$_A$ was difficult to achieve because MBP-CT$_A$ bound only a subset of the available $^{35}$S-$\beta_4$, which was presumably in a more favorable conformation.

that, as for NT$_A$, the carboxyl terminus of $\beta_4$ was required for binding to NT$_B$, and the use of deleted derivatives of the carboxyl terminus of $\beta_4$ also indicates an important role for residues 446–482 of $\beta_4$ in this interaction. These results suggest that the interaction site is defined more by the tertiary structure of the $\alpha_1$ 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 $\alpha_1$ and the carboxyl terminus of $\beta_4$ in terms of channel functioning. First, because $\beta_4$, in contrast to $\beta_3$, does not interact with the amino terminus of $\alpha_1$, 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. 7A). Although both $\beta$ subunits shifted the activation curve
along the voltage axis, the shift induced by $\beta_3$ was significantly more pronounced than the one produced by $\beta_4$. The estimated half-activation potential shifted from 17 mV ($\alpha_{1A}$-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 $\beta_3$ and $\beta_4$ subunits. In addition, we found that depending on the $\beta$ subunit being expressed, the channels differed in their voltage dependence of inactivation with $-50$ 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 $\beta$ subunits may be the result of differences in interaction levels between $\alpha_{1A}$ and the two $\beta$ subunits, we determined the role of the NT A site in $\beta$-induced channel regulation. We took advantage of the observation that essential differences were found in $\beta$ subunit association with amino-terminal sequences of various $\alpha_1$ subtypes. We constructed a chimera $\alpha_{1A}$ subunit ($\alpha_{1A NT}C$), in which we replaced the amino terminus of $\alpha_{1A}$ (interacts with $\beta_4$ but not $\beta_3$) with the amino terminus of $\alpha_{1C}$ (does not interact with either $\beta_4$ or $\beta_3$). Coexpression of this chimeric channel with $\beta_3$ or $\beta_4$ triggers high voltage-activated currents in Xenopus oocytes (Fig. 7B). The amplitude of the currents elicited by membrane depolarization are reduced slightly compared with those obtained for the wild-type $\alpha_{1A}$ channel. Cells expressing $\alpha_{1A}\beta_3$, for instance, have a peak current amplitude of $1,001 \pm 651$ nA ($n = 7$, S.D.), whereas cells expressing $\alpha_{1A}(NT)\beta_4$ peak at $423 \pm 655$ nA ($n = 12$), which corresponds to a 2.37-fold

**Fig. 5.** $\alpha_1$ amino-terminal specificity of interaction with $\beta$ subunits. Panel A, Coomassie Blue-stained SDS-PAGE showing various GST fusion proteins used (5 µg). Panel B, in vitro translated $\beta$ 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; AIDA, GST-AIDA; NTN, GST NTN, GST-NTN, GST-NTS, GST fusion proteins containing amino-terminal cytoplasmic domains of $\alpha_{1A}, \alpha_{1T}, \alpha_{1C}$, and $\alpha_{1S}$, respectively. Error bars represent S.D.

**Fig. 6.** The carboxyl terminus of $\beta_4$ is also involved in NT S binding. $^{35}$S- $\beta_4$ 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-AIDA (500 nM). Error bars represent normalized S.D.
Role of a New \( \alpha_{1A}^{{\text{NT}}_A} \beta_4 \) Interaction Site in Activation

A. \( \beta_3 \) and \( \beta_4 \) differ in their ability to shift the voltage dependence of activation of \( \alpha_{1A} \). 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 \( \alpha_{1A} \) channel alone is shown for comparison purpose (\( n = 8 \)). The experimental data were fitted with a modified Boltzmann equation \( I_{\alpha_{1A}} = \frac{g(V - E)}{1 + \exp(-(V - \frac{1/2}{\sqrt{2}})/k)} \), where \( g \) is the normalized conductance \( g = 0.032, 0.018, + \beta_4 \), and 0.026, + \( \beta_3 \); \( V_{1/2} \) is the half-activation potential \( (V_{1/2} = 17 \text{ mV, } 0 \beta; 13 \text{ mV, } + \beta_4 \); and \( 1.5 \text{ mV, } + \beta_3 \)) is the reversal potential \( (E = 67 \text{ mV, } 0 \beta; 63 \text{ mV, } + \beta_4 \); and 58 mV, + \( \beta_3 \)) and \( k \) is the range of potential for an \( e \)-fold change around \( V_{1/2} \) \((k = 7.9 \text{ mV, } 0 \beta; 4.2 \text{ mV, } + \beta_4 \); and 5.8 mV, + \( \beta_3 \)) (52).

B. Change in difference in the \( \beta \)-induced I-V shift by \( \alpha_{1A} \) 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 \( \alpha_{1A}(\text{NT})_B \beta_3 \) and \( \alpha_{1A}(\text{NT})_B \beta_4 \) channels. Right, corresponding average I-V curves for \( \alpha_{1A}(\text{NT})_B \beta_3 \) (\( n = 13 \)) and \( \alpha_{1A}(\text{NT})_B \beta_4 \) channels (\( n = 12 \)). The fit of the experimental data yields \( V_{1/2} = -13 (+ \beta_4) \) and \(-9 \text{ mV } (+ \beta_3) \); \( k = 4.2 (+ \beta_4) \) and \( 4.4 \text{ mV } (+ \beta_3) \); \( g = 0.017 (+ \beta_4) \) and 0.018 (+ \( \beta_4 \)); and \( E = 60 (+ \beta_4) \) and 63 mV (+ \( \beta_3 \)).

**Fig. 7.** Role of the \( \text{NT}_A \beta_4 \) interaction in the control of voltage dependence of activation. A. \( \beta_3 \) and \( \beta_4 \) differ in their ability to shift the voltage dependence of activation of \( \alpha_{1A} \). 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 \( \alpha_{1A} \) channel alone is shown for comparison purpose (\( n = 8 \)). The experimental data were fitted with a modified Boltzmann equation \( I_{\alpha_{1A}} = \frac{g(V - E)}{1 + \exp(-(V - \frac{1}{2}))/k} \), where \( g \) is the normalized conductance \( g = 0.032, 0.018, + \beta_4 \), and 0.026, + \( \beta_3 \); \( V_{1/2} \) is the half-activation potential \( (V_{1/2} = 17 \text{ mV, } 0 \beta; 13 \text{ mV, } + \beta_4 \); and \( 1.5 \text{ mV, } + \beta_3 \)) is the reversal potential \( (E = 67 \text{ mV, } 0 \beta; 63 \text{ mV, } + \beta_4 \); and 58 mV, + \( \beta_3 \)) and \( k \) is the range of potential for an \( e \)-fold change around \( V_{1/2} \) \((k = 7.9 \text{ mV, } 0 \beta; 4.2 \text{ mV, } + \beta_4 \); and 5.8 mV, + \( \beta_3 \)). B. Change in difference in the \( \beta \)-induced I-V shift by \( \alpha_{1A} \) 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 \( \alpha_{1A}(\text{NT})_B \beta_3 \) and \( \alpha_{1A}(\text{NT})_B \beta_4 \) channels. Right, corresponding average I-V curves for \( \alpha_{1A}(\text{NT})_B \beta_3 \) (\( n = 13 \)) and \( \alpha_{1A}(\text{NT})_B \beta_4 \) channels (\( n = 12 \)). The fit of the experimental data yields \( V_{1/2} = -13 (+ \beta_4) \) and \(-9 \text{ mV } (+ \beta_3) \); \( k = 4.2 (+ \beta_4) \) and \( 4.4 \text{ mV } (+ \beta_3) \); \( g = 0.017 (+ \beta_4) \) and 0.018 (+ \( \beta_4 \)); and \( E = 60 (+ \beta_4) \) and 63 mV (+ \( \beta_3 \)).

**DISCUSSION**

We describe the identification of a specific interaction site between the amino-terminal cytoplasmic region of the calcium channel \( \alpha_{1A} \) subunit and the \( \beta_4 \) subunit. The \( \beta_3 \) subunit is widely expressed in the brain, especially in the cerebellum (30).
On the basis of their colocalization in many tissue types, \( \beta_4 \) appears largely to be associated with the \( \alpha_{1A} \) subunit in native channels (17, 31). However, coimmunoprecipitation studies demonstrate that \( \alpha_{1A} \) is also found associated with \( \beta_{1B}, \beta_2, \) and \( \beta_4 \) (17) and that \( \beta_4 \) is also found associated with \( \alpha_{1B} \) (16). The importance of the \( \beta_4 \) subunit is illustrated by the recent demonstration that a lethargic phenotype in mice results from a deletion of approximately 60% of the \( \beta_4 \) coding sequence (32). This truncated \( \beta_4 \) subunit would lack all three of the interactions described with \( \alpha_{1A} \) (AID\( \alpha \) (19) and NT\( \alpha \) and CT\( \alpha \) (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 \( \beta \) subunits are expressed in parallel or that their expression is switched on to compensate for this deficiency (33). In fact, \( \beta_4 \) is known to be a normal constituent of about one-third of P/Q-type channels (17). Because \( \beta_4 \) expression is high in brain and parallels that of \( \beta_4 \) (34), it would be the most likely candidate for \( \beta_4 \) substitution in the lethal mice. Since \( \beta_4 \) lacks both secondary interaction sites described so far in \( \beta_4 \), such a substitution would not be functionally equivalent, perhaps explaining some of the neurological defects encountered in these mice.

The NT\( \alpha \) 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 \( \beta \) subunit to modify channel properties (10). It also stems from the fact that this is the third interaction site mapped between \( \alpha_{1A} \) and \( \beta_4 \) and that binding of multiple \( \beta \) subunits to \( \alpha_1 \) does not seem very plausible. The new interaction site that we describe involves the amino terminus of \( \alpha_{1A} \) (residues 1–66) and carboxyl terminus (residues 446–482) of \( \beta_4 \). This is particularly interesting given the rather low level of sequence conservation in the two regions identified. With regard to \( \alpha_{1A} \) 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 \( \beta_4 \) 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 \( \alpha_{1A} \) and the carboxyl-terminal of \( \beta_4 \) is reflected by the high degree of subtype specificity exhibited by this interaction with respect to both \( \alpha_1 \) and \( \beta_4 \) isoforms. Our results indicate that the equivalent amino-terminal regions of \( \alpha_{1B} \) and \( \alpha_{1C} \) did not interact with any of the \( \beta \) subunits tested. Because other \( \beta_4 \) subtypes exist, we cannot rule out that this may reflect the use of an inappropriate \( \alpha_1-\beta_4 \) combination. Interestingly, we found that a fusion protein expressing the entire amino terminus of \( \alpha_{1B} \) could interact with all four different \( \beta_4 \) subunits tested. This is in contrast to the NT\( \alpha \) binding, which occurs only on \( \beta_4 \) and to a lesser extent on \( \beta_2 \).

Fig. 8 summarizes what is now known about \( \alpha_{1A}-\beta_4 \) interactions in terms of structure. One interesting aspect is that the \( \beta_4 \) subunit can interact simultaneously with AID and, via its carboxyl-terminal region, with either the amino- or carboxyl-terminal regions of \( \alpha_{1A} \), 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 \( \alpha_{1A} \) are underlined by the observation that truncations of equivalent domains in \( \alpha_{1C} \) 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 \( \beta_4 \) subunits also increase channel expression, and this effect varies in amplitude depending on the \( \alpha_1 \) and \( \beta_4 \) subtype studied, it is tempting to speculate that the secondary interaction sites described so far also intervene in \( \alpha_{1A} \) channel expression by one of the two mechanisms described for \( \alpha_{1C} \). We did indeed find that substitution of the amino terminus of \( \alpha_{1A} \) by the equivalent sequence of \( \alpha_{1C} \) resulted in an important reduction in current density. This effect was, however, \( \beta_4 \) subtype-independent, and it is therefore unlikely that the NT\( \alpha \) interaction site described here plays a role in \( \beta_4 \)-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 \( \alpha_{1A} \) in the control of channel inactivation kinetics. Here, we demonstrate that the amino-terminal interaction site of \( \alpha_{1A} \) is required for fine tuning the voltage dependence of activation. The NT\( \alpha \) interaction with \( \beta_4 \) appears to limit the amplitude of the hyperpolarizing \( \beta_4 \)-induced shift of channel activation.
this unique mechanism, it can be predicted that the $\beta_2$-containing P/Q channel subtype is activated more easily than the $\beta_1$-containing P/Q channel subtype. In addition, secondary interaction sites may serve to protect or uncover phosphorylation sites in the $\alpha_1$ subunit, thereby altering the regulatory input of these. Another obvious possibility is that they play a role in the antagonistic relationship between the $\beta$ 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), $G\beta_4$ also interacts with the carboxyl-terminal domain of $\alpha_{1A}, \alpha_{1B},$ and $\alpha_{1E}$ and that the amino terminus has recently been recognized as another determinant for $G\beta\gamma$ regulation in $\alpha_{1B}$ 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 $\beta$ subunits also play a role in the surface targeting of $\alpha_1$ and $\alpha_2\delta$ (14), an interesting possibility is that specific $\beta$ subunits serve to target $\alpha_1$ subunits to specific regions of the cell surface.

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