Identification of critical amino acids involved in α_1–β interaction in voltage-dependent Ca^{2+} channels

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Abstract In voltage-dependent Ca^{2+} channels, the α_1 and β subunits interact via two cytoplasmic regions defined as the Alpha Interaction Domain (AID) and Beta Interaction Domain (BID). Several novel amino acids for that interaction have now been mapped in both domains by point mutations. It was found that three of the nine amino acids in AID and four of the eight BID amino acids tested were essential for the interaction. Whereas the important AID amino acids were clustered around five residues, the important BID residues were more widely distributed within a larger 16 amino acid sequence. The affinity of the AID_A GST fusion protein for the four interacting β_{1b} BID mutants was not significantly altered compared with the wild-type β_{1b} despite the close localization of mutated residues to disruptive BID amino acids. Expression of these interactive β mutants with the full-length α_{1A} subunit only slightly modified the stimulation efficiency when compared with the wild-type β_{1b} subunit. Our data suggest that non-disruptive BID sequence alterations do not dramatically affect the β subunit-induced current stimulation.

Key words: Voltage-dependent calcium channel; Interaction site; α_1 Subunit; β Subunit; *Xenopus laevis* oocyte; Subunit coexpression

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1. Introduction

Voltage-dependent Ca^{2+} channels are composed of a minimum of three subunits: α_1, a pore-forming protein, β, a cytoplasmic protein and $\alpha_2\delta$, a transmembrane and glycosylated component of less well understood function [1,2]. Despite important molecular diversity in α_1 and β subunits [3], two well-conserved sites were recently identified in both subunits, the α_1 interaction domain or AID [4] containing nine conserved amino acids and a larger 30 amino acid β interaction domain or BID [5]. Both domains determine the attachment of the β subunit to the α_1 channel. This linkage is required for the observed β subunit regulation of the current properties of the α_1 subunit, that is the increase in current amplitude [6–10] and the modifications in kinetics and voltage dependence of the channel [11–14].

Although there have been several reports investigating the mechanism of β-induced current stimulation, the results have been controversial [15–16]. One report suggests that the increase in current amplitude was largely due to important conformational changes in the α_1 subunit that would ultimately lead to an increase in single-channel activity [17]. Since changes in conductance values have been ruled out [18], this change in channel activity would solely be the result of an increase in opening probability. However, such a mechanism contradicts several other reports. In particular, it appears that these same conformational changes are required to account for the observed increase in total number of drug/toxin-binding sites, but without altering binding affinity [19–20]. This result was surprising since conformational changes in α_1 subunit that are extensive enough to uncover additional drug-binding sites (i.e. dihydropyridines on α_{1C} and ω-CTx GVIA on α_{1B}) would also be expected to change drug-binding affinity, especially since both dihydropyridines and ω-CTx GVIA bind to spatially separated and different epitopes on their respective α_1 subunits. In an alternative mechanism, the role of β subunits may be to increase the number of functional α_1 subunits at the plasma membrane, although changes in kinetics and voltage-dependent parameters and some slight changes in opening probability as reported [18] would be the result of somewhat more subtle conformational changes. The present identification of critical residues in BID and the recent development of a biochemical assay to measure the affinity between α_1 and β subunits [21], provided a unique alternative to examine further either one of these hypotheses. Herein, we have investigated the role of several as yet uncharacterized AID and BID amino acids in the anchoring of the β subunits to α_1 channels. We have created four novel point mutations in BID that do not affect the α_1–β affinity despite the possibility that these mutations could induce subtle changes in the regulatory input of BID by their close localization to disruptive amino acids.

2. Materials and methods

Site-directed mutagenesis of the α_{1A} epitope were performed mostly on the pGEX2TK vector expressing the AID_A epitope as previously described [4] by using the Transformer Site-Directed mutagenesis system (Clontech). The following mutagenic primers were used with the underlined sequences denoting differences in codon sequences: 5′-CCTGAAGCTGCGGCGGCAGGCGCAGATTGAACGCGAGC-3′ ($AID_{A\ Q383A}$), 5′-GCTGCGGCGGCAGCAGGCGATTGAACGCGAGCTCAACG-3′ ($AID_{A\ Q384A}$), 5′-GCGGCAGCAGCAGATAGACCGCGAGCTCAACGGG-3′ ($AID_{A\ E386D}$), 5′-CAACGGGTACATGGAGGCGATCTCAAAAGCAGAAGAGG-3′ ($AID_{A\ W395A}$) and 5′-CAACGGGTACATGGAGTGGGCCTCAAAAGCAGAAGAGG-3′ ($AID_{A\ I396A}$). Mutants $AID_{A\ L389H}$, $AID_{A\ Y392F}$ and $AID_{A\ E400A}$ were described elsewhere [4,22]. The $AID_{A\ G391R}$ mutant was constructed by cassette mutagenesis using the polymerase chain reaction (PCR) with the mutagenic primer 5′-GAACGCGAGCTCAACCGGTACATGGAGTGGATC-3′. The final PCR product was cleaved with *Bam*H1 and *Eco*R1, two restriction sites conveniently introduced in the forward and reverse primers, respectively, and subcloned into pGEX2TK for the expression of GST fusion proteins. Point mutations in BID on the full-length β_{1b} sequence were performed as described elsewhere [5]. The following mutagenic primers were used: 5′-CCCTATGACGTGGTGAGATCTATGAGGCCCATCATC-3′ ($\beta_{1b\ P227R}$), 5′-CCCTATGACGTGGTGCCGGCCATGAGGCCCATCATC-3′ ($\beta_{1b\ S228A}$), 5′-

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GTGGTGCCTTCCATGCGCAGGATCATCCTGGTGGGA-3′ ($\beta_{1b\ P231R}$), 5′-ATCCTGGTGGGACCAGCGCTTAAGGGCTATGA-GGTA-3′ ($\beta_{1b\ S238A}$), 5′-GGACCATCGCTCAAGCGCTATGAGGT-AACTGACATG-3′ ($\beta_{1b\ G241R}$), 5′-GGACCATCGCTCAAGGGTCT-AGAGGTAACTGACATG-3′ ($\beta_{1b\ Y242L}$). Mutants $\beta_{1b\ P221R}$ and $\beta_{1b\ P237R}$ were described elsewhere [5]. Proteins were analysed by SDS-PAGE (3–12% gradient gels) using the buffer system of Laemmli [23]. The gels were stained with Coomassie blue and/or dried and exposed to X-ray film. The ^{35}S-labeled wild-type and mutant β_{1b} subunits were synthesized using the TNT-coupled reticulocyte lysate system (Promega). Purification of the Glutathione S-Transferase (GST) fusion proteins and binding of these fusion proteins to the wild-type or mutant β_{1b} subunits were performed as described previously [21]. *Xenopus laevis* oocyte preparation and maintenance, in vitro transcription, and cRNA injection were performed according to protocols described elsewhere [24]. Briefly, 50 nl of various transcribed cRNAs were injected into each oocyte at the following concentrations (μg/μl): α_{1A} (0.4) and wild-type or mutant β_{1b} (0.2). Two-electrode voltage clamp was performed 4–5 days after injection using the following extracellular solution (mM): $Ba(OH)_2$ (40), NaOH (50), KCl (2), niflumic acid (1), EGTA (0.1), HEPES (5) at pH 7.4 and 3 M KCl filled electrodes. Mature *X. laevis* female frogs were from Nasco (Wisconsin, USA). The α_{1A} subunit (pSPCBI-2) was from Mori et al. [6] and the β_{1b} clone from Pragnell et al. [25].

3. Results

To examine the specific interaction between the α_1 and β subunits, we have developed an in vitro binding assay [21]. In this affinity assay, the AID_A is expressed as a 50 amino acid GST fusion protein and coupled at various concentrations to glutathione-Sepharose beads to form a ligand defined herein as AID_A-Sepharose beads. The binding of this ligand to in vitro translated ^{35}S-labeled β subunits ([^{35}S]β_{1b}) can then be measured by the formation of a radioactive complex. To characterize the role of various amino acids in the α_1–β interaction, we mutated all conserved AID amino acids normally present in each class of α_1 subunit cloned so far. The purity of the wild-type and various mutant AID_A GST fusion proteins was demonstrated by SDS-PAGE and Coomassie blue staining (Fig. 1a). The ability of 100 nM purified mutant AID_A GST fusion proteins to interact with wild-type [^{35}S]β_{1b} subunit was examined and compared with that of the wild-type AID_A GST fusion protein at the same concentration (Fig. 1b). At this saturating concentration (100 nM), as previously determined [21], the wild-type AID_A-Sepharose beads bound approximately 57% of the total in vitro translated [^{35}S]β_{1b} protein. This fraction was comparable to the amount of [^{35}S]β_{1b} (41.4 ± 4.5%) that could be immunoprecipitated by $VD2_1$ a monoclonal antibody that recognizes a conserved sequence in β subunits (data not shown). This suggests that a proportion of the translated [^{35}S]β_{1b} may undergo some misfolding during in vitro synthesis. The binding of wild-type AID_A GST fusion protein to [^{35}S]β_{1b} was normalized to 100% and compared with the binding of each of the AID_A mutants. Noticeably, there were only small reductions in the total binding to [^{35}S]β_{1b} for $AID_{A\ Q383A}$ (67 ± 3%), $AID_{A\ Q384A}$ (79 ± 5%), $AID_{A\ L389H}$ (76 ± 5%), $AID_{A\ G391R}$ (71 ± 3%) and $AID_{A\ E400A}$ (59 ± 2%) (Fig. 1b). It is, therefore, possible that these mutants induced small reductions in binding affinity although these changes were not sufficient to prevent native subunit association as previously demonstrated for $\alpha_{1A\ E400A}$, $\alpha_{1A\ L389H}$ and $\alpha_{1A\ E386S}$ in expression systems [4]. A small increase in binding was even observed for $AID_{A\ E386D}$ (111 ± 3%), suggesting that this mutant was able to bind a slightly larger fraction of the in vitro translated [^{35}S]β_{1b} subunit than the wild-type AID_A GST fusion protein perhaps by compensating for a small fraction of the β misfolding. In contrast, the $AID_{A\ Y392F}$, $AID_{A\ W395A}$ and $AID_{A\ I396A}$ GST fusion proteins bound to [^{35}S]β_{1b} with significantly reduced efficiencies at 22 ± 2%, 0.2 ± 0.5% and 6 ± 2% of the control value, respectively. We conclude, therefore, that, of the nine conserved amino acids that compose each AID sequence, only three of them are absolutely required for the binding to the β subunits.

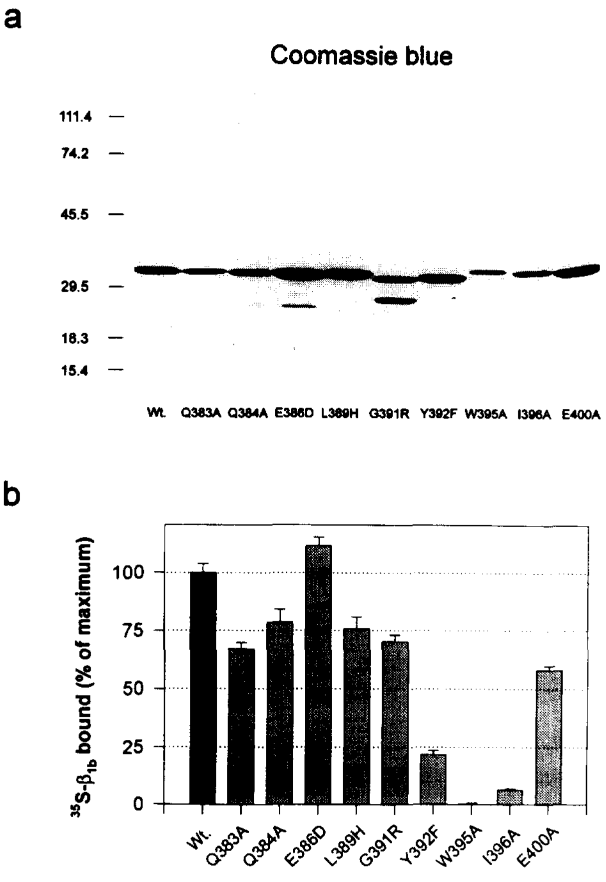

Fig. 1. Identification of all critical AID amino acids involved in the α_1–β interaction. (a) Coomassie blue-stained SDS-PAGE gel showing the purity of wild-type and mutant AID_A fusion proteins. Approximately 50–200 pM of the fusion proteins were loaded in each lane. Note the shifts in mobility introduced by some point mutations. Molecular weight standards (×10^{-3}) are indicated on the left. (b) [^{35}S]β_{1b} bound to 100 nM mutant AID_A-Sepharose beads expressed as a percentage of [^{35}S]β_{1b} bound to 100 nM wild-type AID_A-Sepharose beads (6 h reaction time).

In a similar attempt to characterize the BID amino acids required for the α_1–β interaction, we mutated several amino acids in the BID sequence of the full-length β_{1b} subunit. Fig. 2a shows the results of the binding of 100 nM AID_A-Sepharose beads to the wild-type or to several [^{35}S]β_{1b} mutants. The fraction of wild-type or mutant β subunit bound to the AID_A-Sepharose beads (lane 2) was compared with the amount of subunit initially present in the lysate (lane 1). As discussed previously, 100 nM AID_A-Sepharose beads bound only 56.1% of the [^{35}S]β_{1b} initially present in the lysate. By comparison, the AID_A GST fusion protein bound similar fractions with 68.8% of $\beta_{1b\ P221R}$, 60.5% of $\beta_{1b\ S228A}$, 63.6% of $\beta_{1b\ S238A}$ and 53.3% of $\beta_{1b\ G241R}$, suggesting that these amino acids were not critical for the α_1–β interaction. In contrast, equal amounts of AID$_A$-Sepharose beads bound 15.4% of $\beta_{1b\ P227R}$, 6.8% of $\beta_{1b\ P231R}$, 11.8% of $\beta_{1b\ P237R}$ and 30% of $\beta_{1b\ Y242L}$ indicating that these BID amino acids are probably required to maintain a stable and high-affinity interaction between both subunits. These four mutations in BID, therefore, reduce the affinity of the α_1–β interaction to values lower than 100 nM. This represents a 20-fold decrease in the binding affinity as compared with that of the wild-type AID$_A$ GST fusion protein to the β_{1b} subunit [21]. Fig. 2b summarizes the results of the AID and BID mutations and localizes the critical residues in both domains. This schematic representation of the α_1–β interaction demonstrates that in addition to Y392 of AID$_A$ and P237 of BID$_{1b}$, two and three more critical AID and BID residues have now been identified, respectively. The results reveal the conformational importance of certain proline residues within the BID sequence.

Particularly interesting was the presence of a cluster of amino acids **LXGY** present in both AID and BID. In all BID sequences, the X amino acid, located between the L and G, is a lysine that is conserved in some AID sequences (AID$_C$ and AID$_D$), but replaced by an arginine in the skeletal muscle α_1 subunit (AID$_S$) or an asparagine in the non-L-type α_1 subunits (AID$_A$, AID$_B$ and AID$_E$). Effects of amino acid mutations in these clusters followed the same pattern for both AID$_A$ and BID$_{1b}$. Whereas the mutations of G391 in AID$_A$ and G241 in BID$_{1b}$ to an arginine were without effect on the subunit interaction in both cases, changing a tyrosine residue in either AID$_A$ (Y392) or BID$_{1b}$ (Y242) reduced the binding of AID to the β subunit to a similar extent.

Although there are drastic reductions in the binding of AID$_A$ Sepharose beads to several β mutants, the assay described in Fig. 2a does not test for small variations in binding affinity to the four interacting mutants: $\beta_{1b\ P221R}$, $\beta_{1b\ S228A}$, $\beta_{1b\ S238A}$ and $\beta_{1b\ G241R}$. A binding assay was performed as described elsewhere [21] to test for possible differences in affinity. Analysis of the binding of various concentrations of AID$_A$ Sepharose beads to these mutant β_{1b} subunits demonstrates that the binding is saturable and of high affinity in all four cases (Fig. 3). For $\beta_{1b\ P221R}$ and $\beta_{1b\ S238A}$ a second site of much lower affinity (400–900 nM) appears that represents somewhere between 37 and 50% of the total binding. This lower-affinity component was previously shown to represent the binding of AID$_A$ Sepharose beads to proteolyzed fractions of these mutant β subunits [21]. Increased misfolding in the β subunit that would be introduced by these mutations can probably be excluded since the AID$_A$ Sepharose beads bound the same maximum fraction of in vitro translated β subunit (Fig. 2a). Interestingly, mutations of these BID amino acids did not significantly alter the high-affinity component of AID$_A$ interaction with the β_{1b} subunit despite the proximity of the mutated residues to essential BID amino acids. The high-affinity K_d values were 5.6 nM ($\beta_{1b\ P221R}$), 7.2 nM ($\beta_{1b\ S228A}$), 5.8 nM ($\beta_{1b\ S238A}$) and 7.9 nM ($\beta_{1b\ G241R}$) which in all cases were not significantly different from the 5.8 nM K_d value of the wild-type β_{1b} subunit [21].

It was previously shown on the basis of coexpression experiments that AID or BID point mutations which significantly modify the α_1–β interaction in vitro also prevented their native functional association in *Xenopus* oocytes [4–5]. Herein, we attempted to determine whether BID point mutations that would not alter the association of β subunit to the α_{1A} channel could significantly modify the regulatory input of the β_{1b} sub-

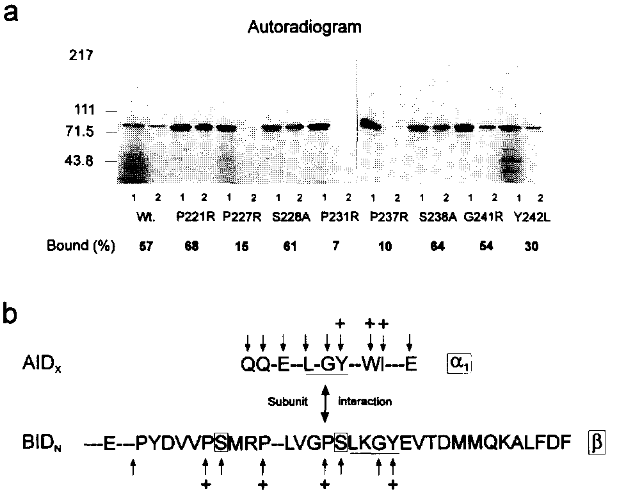

Fig. 2. Critical amino acid in BID involved in the α_1–β interaction. (a) Autoradiogram showing the fraction of wild-type (Wt.) and mutant β_{1b} subunits bound to 100 nM wild-type AID$_A$ GST fusion protein. The amount of wild-type or mutant β_{1b} subunit bound (lane 2) was compared with the amount originally present in the in vitro translate (lane 1). Molecular weight standards ($\times 10^{-3}$) are shown on the left. The % band denotes the fraction of in vitro translated subunit bound to the AID$_A$-Sepharose beads by cutting the major radioactive band out of the dried Coomassie blue gel and measuring the radioactivity present by scintillation counting. (b) Schematic illustrating AID and BID amino acids required to the α_1–β interaction. The arrows denote all the amino acids mutated and positive signs indicate the important residues. Underlined amino acids are present as a cluster in both AID and BID. Boxed amino acid are potential protein kinase C phosphorylation sites.

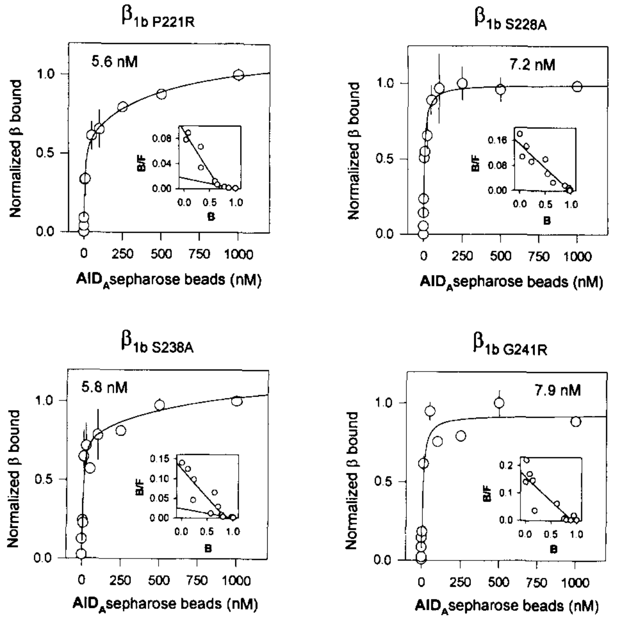

Fig. 3. Binding analyses of AID$_A$ GST fusion protein binding to interacting [^{35}S]β_{1b} mutants. The AID$_A$ GST fusion protein was coupled at various concentrations to glutathione-Sepharose beads and incubated overnight at 4°C with about 1–2 pM mutant [^{35}S]β_{1b} subunits. Saturation curves are shown for $\beta_{1b\ P221R}$, $\beta_{1b\ S228A}$, $\beta_{1b\ S238A}$ and $\beta_{1b\ G241R}$. Fitting of saturation data using the Grafit program yielded K_d values of 5.6 nM (50%) and 480 nM (50%) ($\beta_{1b\ P221R}$), 7.2 nM ($\beta_{1b\ S228A}$), 5.8 nM (63%) and 861 nM (37%) ($\beta_{1b\ S238A}$) and 7.9 nM ($\beta_{1b\ G241R}$). Scatchard representations are in inserts.

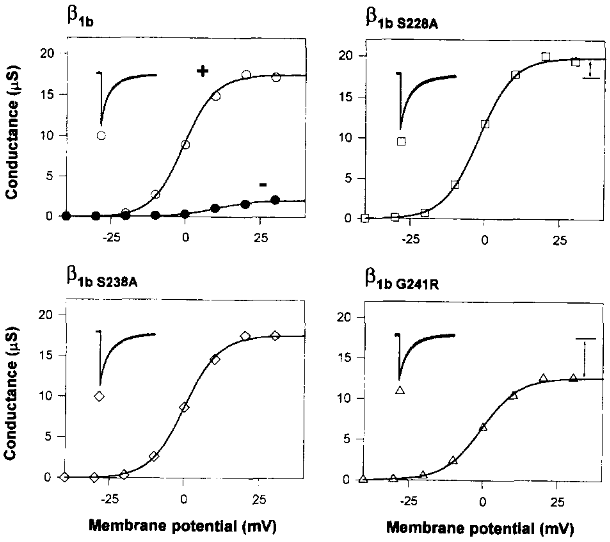

Fig. 4. Unaltered current stimulation efficiencies of non-disruptive point mutations. (a) Average voltage–conductance relationship of various Ca^{2+} channel complexes in *Xenopus* oocytes: $\alpha_{1A}\beta_{1b\,S228A}$, $\alpha_{1A}\beta_{1b\,S238A}$ and $\alpha_{1A}\beta_{1b\,G241R}$. The data were described by a Boltzmann curve and yielded the following parameters: half-conductance value (mV) 9.7 ($-\beta_{1b}$), −0.8 ($+\beta_{1b}$), −2.2 (S228A), 0.1 (S238A) and −0.6 (G241R), and slopes (mV) 6.6 ($-\beta_{1b}$), 5.3 ($+\beta_{1b}$), 5.8 (S228A), 5.7 (S238A) and 6.2 (G241R). Inset shows representative Ba^{2+} currents of these Ca^{2+} channels induced by depolarizing the cells for 2 s to 10 mV from a holding potential of −90 mV.

unit. We selected three BID mutants ($\beta_{1b\,S228A}$, $\beta_{1b\,S238A}$ and $\beta_{1b\,G241R}$) for their ability not to modify the α_1–β interaction in vitro and for their close proximity to critical BID amino acids (P227R, P237R and Y242L) and assessed their stimulation efficiencies. As previously demonstrated, coexpression of the β_{1b} subunit with α_{1A} subunit both enhances the conductance values of the α_{1A} channel and shifts the potential of half-activation by −12 mV (Fig. 4). The observed maximum conductance values of α_{1A} and $\alpha_{1A}\beta_{1b}$ were G_{max} = 2.3 µS and 17.3 µS and were reached at +30 mV and +20 mV, respectively. There is, thus, a 7.5-fold increase in the maximum conductance value induced by the β_{1b} subunit. In close agreement to the stimulation by the wild-type β_{1b} subunit, the mutant $\beta_{1b\,S228A}$, $\beta_{1b\,S238A}$ and $\beta_{1b\,G241R}$ subunits increased the maximum conductance of the channel by factors of 8.5, 7.6 and 5.5, respectively. These mutants also shifted the potential of half-activation by −14 (S228A), −11.3 (S238A) and −12 mV (G241R). It is, therefore, concluded that non-disruptive point mutations in BID produce only slight modifications in stimulation efficiencies of the β subunit.

4. Discussion

We have separated the AID residues into two groups: interacting and non-interacting AID amino acids. This segregation was based on the relative capacity of 100 nM of the mutant AID_A GST fusion proteins to bind to the β subunits. Amino acids that had affinities lower than 100 nM after mutating (less than 50% of the maximum wild-type AID_A-binding at 100 nM) were considered essential residues to the interaction, whereas, conversely, amino acids that induced affinities higher than 100 nM (greater than 50% maximum wild-type binding) were not considered as critical. Two observations support this separation into two groups of amino acids. On one hand, the binding of AID_A Sepharose beads to β_3 occurs with an affinity of 50 nM [21] which, although low, is not low enough to prevent the association of both α_{1A} and β_3 in expression systems [24]. On the other hand, the binding of 100 nM $AID_{A\,Y392F}$ represents only 22% of the wild-type fusion protein-binding and mutation of this amino acid has previously been shown to prevent native subunit association in an expression system [4]. These data suggest, therefore, that a K_d close to 100 nM in vitro reflects the 'cut-off' affinity that determines the ability of β subunits to anchor to the α_1 subunit in vivo.

Only three amino acids, located at the C-terminal portion of AID, were essential to the α_1–β interaction. The relative clustering of AID amino acids critical for the interaction is in contrast with the more disperse localization of important BID residues. These results strongly suggest that the functional conformation of the AID site is less dependent upon the presence and nature of flanking sequences than the BID site. These data also explain why synthetic peptides of the AID sequence but not of the BID sequence are able to compete for the α_1–β interaction both in vitro and in expression systems [21]. However, the importance of flanking AID sequences are probably not negligible in the α_1–β interaction. Sequences of the I-II cytoplasmic loop located downstream of AID are frequently the loci for alternative splicing [26–27] that regulate the affinity of interaction between α_1 and β subunits (unpubl. obs.).

The nature and role of non-conserved residues interspersed among the conserved AID residues also appear determinant for the α_1–β interaction. These amino acids may also be responsible for some of the differences in affinity already observed between α_{1A} and several β subunits [21]. Sequence modifications within and in close proximity of AID may, therefore, be determinant in defining the subunit composition of native calcium channels and their functional properties. The role of the N-terminal half of the conserved AID sequence (**QQXEXXLXG**) which does not seem to be essential to the binding of β subunits is intriguing. The conservation of this sequence is challenging and its proximity to essential AID amino acids suggest that it may be involved in some important regulatory function. Noticeably, it was recently demonstrated that $\beta\gamma$ subunits of G proteins could bind to a **QXXER** motif present on several effectors such as adenylyl cyclase 2 and muscarinic potassium channels [28]. This motif is also present in the AID sequence of three neuronal α_1 subunits (classes A, B and E) where the arginine residue represents a non-conserved AID amino acid which is absent in the remaining α_1 subunits (classes S, C and D). The functional importance of this sequence in the G protein regulation of several neuronal voltage-dependent Ca^{2+} channels is now under investigation.

Our data demonstrate that the expression of interacting β mutants results in only small changes in β stimulation efficiencies despite changes in sequence that may have induced conformational alterations in BID and also eventually in AID as a result of the α_1–β interaction. β-induced conformational changes in α_1 subunit are expected to account for the reported changes in voltage dependence and kinetics of the channels, and also for several other regulations. For instance, it is known that the interaction between α_1 and β is required to observe a functional regulation of calcium current by $\alpha_2\delta$ [24], suggesting that

α_1-$\alpha_2\delta$ interactions are conditioned by conformational changes induced by the β subunit at a site remote from the AID-BID interaction. Also, we found that the binding of $\mathrm{AID_A}$ GST fusion protein to $[^{35}\mathrm{S}]\beta_{1b}$ increases by 31% the maximum immunoprecipitation of β_{1b} by a monoclonal antibody $\mathrm{VD2_1}$ (data not shown), suggesting that multiple epitopes may be affected by the AID-BID interaction. The functional modifications in channel-gating induced by the β subunit do not necessarily have to be the result of conformational changes at the AID site itself but could occur from structural changes at sites different from AID or BID; structural changes that would, however, be contingent on the primary α_1–β interaction. With respect to the mechanism of β-induced current stimulation, it cannot be ruled out that β structural determinants other than BID itself might play essential roles in this process either by secondary interaction with the α_1 subunit or by an increased cell trafficking and plasma membrane insertion of the preformed $\mathrm{Ca^{2+}}$ channel.

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