Modulation of the smooth-muscle L-type Ca^{2+} channel $\alpha 1$ subunit ($\alpha 1\text{C-b}$) by the $\beta 2\text{a}$ subunit: a peptide which inhibits binding of β to the I–II linker of $\alpha 1$ induces functional uncoupling

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Modulation of the smooth-muscle Ca^{2+} channel $\alpha 1\text{C-b}$ subunit by the auxiliary $\beta 2\text{a}$ subunit was studied in the HEK 293 (cell line from human embryonic kidney cells) expression system. In addition, we tested whether the $\alpha 1$ – β interaction in functional channels is sensitive to an 18-amino-acid synthetic peptide that corresponds to the sequence of the defined major interaction domain in the cytoplasmic I–II linker of $\alpha 1\text{C}$ (AID-peptide). Ca^{2+} channels derived by co-expression of $\alpha 1\text{C-b}$ and $\beta 2\text{a}$ subunits exhibited an about 3-fold higher open probability (P_o) than $\alpha 1\text{C-b}$ channels. High- P_o gating of $\alpha 1\text{C-b} \cdot \beta 2\text{a}$ channels was associated with the occurrence of long-lasting channel openings [mean open time (τ) > 10 ms] which were rarely observed in $\alpha 1\text{C-b}$ channels. Modulation of fast gating by the $\beta 2\text{a}$ subunit persisted in the cell-free, inside-out recording configuration. Biochemical experiments showed that the AID-peptide binds with appreciable affinity to $\beta 2$ subunits of native Ca^{2+} channels. Binding of the $\beta 2$

protein to immobilized AID-peptide was specifically inhibited (K_i of 100 nM) by preincubation with free (uncoupled) AID-peptide, but not by a corresponding scrambled peptide. Administration of the AID-peptide (10 μM) to the cytoplasmic side of inside-out patches induced a substantial reduction of P_o of $\alpha 1\text{C-b} \cdot \beta 2\text{a}$ channels. The scrambled control peptide failed to affect $\alpha 1\text{C-b} \cdot \beta 2\text{a}$ channels, and the AID-peptide (10 μM) did not modify $\alpha 1\text{C-b}$ channel function in the absence of expressed $\beta 2\text{a}$ subunit. Our results demonstrate that the $\beta 2\text{a}$ subunit controls fast gating of $\alpha 1\text{C-b}$ channels, and suggest the $\alpha 1$ – β interaction domain in the cytoplasmic I–II linker of $\alpha 1\text{C}$ (AID) as a possible target of modulation of the channel. Moreover, our data are consistent with a model of $\alpha 1$ – β interaction that is based on multiple interaction sites, including AID as a determinant of the affinity of the $\alpha 1$ – β interaction.

INTRODUCTION

Gating as well as regulation of class C high-voltage-activated Ca^{2+} channels is for a large part determined by the interaction between $\alpha 1$ and β subunits [1–6].

In tissues of the cardiovascular system, L-type channels are formed by one out of two splice variants of $\alpha 1\text{C}$ (a and b [7,8]) that are preferentially associated with β subunits of the $\beta 2$ and $\beta 3$ [9–11] isoform. A significant fraction of the L-type channels in various smooth-muscle tissues appear to be comprised of $\alpha 1\text{C-b}$ and $\beta 2$, and the subunit composition is considered essential for fine tuning of channel function [11]. Divergent modulatory effects have been observed with different β subunits, with $\beta 2\text{a}$ being an effective modulator of channel inactivation and open probability (P_o) [6,9,12]. So far, the susceptibility of the two class-C $\alpha 1$ splice variants to modulation by $\beta 2$ species has not been compared in detail. A structure involved in the $\alpha 1$ – β interaction has been located in the cytoplasmic loop between domains I and II of the $\alpha 1$ subunit [13,14]. Recent data support the view that modulation of the gating behaviour of the $\alpha 1$ subunit by β proteins involves multiple interaction sites [12]. Moreover, Shistik et al. [5] suggested the N-terminal tail of $\alpha 1\text{C}$ as a crucial determinant of β subunit-dependent control of Ca^{2+} channel gating. Since the cardiac- and the smooth-muscle splice variants of $\alpha 1\text{C}$ differ substantially in the N-terminal sequence, divergent modulation of these splice variants by β subunits may

be anticipated. Modulation by $\beta 2\text{a}$ of the cardiac isoform of $\alpha 1\text{C-a}$ has recently been characterized at the single-channel level [6], but so far a study on functional modulation of the smooth-muscle $\alpha 1\text{C-b}$ isoform is lacking. The present study was designed to analyse the effects of $\beta 2\text{a}$ on the gating properties of $\alpha 1\text{C-b}$ channels in intact cells as well as in excised inside-out patches. Cell-free, inside-out recording of Ca^{2+} channel activity was used to test for a role of the interaction domain in the I–II linker of $\alpha 1\text{C-b}$ by disruption of the $\alpha 1$ – β interaction in this motif using an 18-amino-acid synthetic peptide (QQLEEDLKGYLDWI-TQAE) that corresponds to the sequence of the proposed $\alpha 1$ – β interaction domain in the I–II linker of class C $\alpha 1$ subunits. Our results demonstrate that fast gating of the smooth-muscle isoform of $\alpha 1\text{C}$ is modulated by the $\beta 2\text{a}$ in a manner similar to $\alpha 1\text{C-a}$. Moreover, we report for the first time that the $\alpha 1$ – β interaction within a functional Ca^{2+} channel is reversed by a synthetic peptide that interferes with binding of β to the I–II linker of the Ca^{2+} channel $\alpha 1\text{C}$ subunit.

EXPERIMENTAL

Materials

The $\alpha 1\text{C}$ – β interaction (AID) peptide as well as a corresponding control peptide of random amino acid sequence (scrambled peptide) was synthesized and purified by BioSyntan (Berlin,

Abbreviations used: AID, $\alpha 1\text{C}$ – β interaction domain in the cytoplasmic I–II linker of the Ca^{2+} channel $\alpha 1\text{C}$ subunit; HEK 293, cell line from human embryonic kidney cells; P_o , open probability; τ , mean open time.

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Germany). The 18-amino-acid AID-peptide (QQLEEDLKGY-LDWITQAE) comprised the sequence 477–494 of the smooth-muscle $\alpha 1C$ splice variant [7] designated as $\alpha 1C$ -b. This sequence is identical with amino acids 458–475 of the cardiac Ca^{2+} channel $\alpha 1$ sequence ([13]; designated as $\alpha 1C$ -a). The AID-peptide corresponds to the conserved sequence of the $\alpha 1$ - β interaction domain (QQ-E-L-GY-WI-E) within the I-II linker of $\alpha 1$ subunits reported by Pragnell et al. [14]. The sequence of the scrambled peptide was TDIQDEGALWEQYLQKEL. Tissue-culture medium was from Gibco BRL (Vienna, Austria); the dihydropyridine- Ca^{2+} channel activator S(-)-BayK 8644 was from Research Biochemicals Incorporated (Natick, MA, U.S.A.), and all other chemicals were from Sigma Chemical Co. (Vienna, Austria).

Cell culture and transfection

HEK 293 (human embryonic kidney cells) were stably transfected with the coding region of the $\alpha 1C$ -b subunit (HEK $\alpha 1C$ -b) as described in [16]. To study the function of channels comprised of $\alpha 1C$ -b [7] and $\beta 2a$ [9] subunits, HEK $\alpha 1C$ -b were transiently transfected with $\beta 2a$ subunit as described in [17]. Expression of CD8 antigen and binding of CD8-antibody-coated beads was used as a marker to identify cells which were successfully transfected. HEK293 cells were cultured in Dulbecco's modified Eagle's medium that was supplemented with 10% (v/v) foetal calf serum and contained 0.178 g/litre geneticin.

Measurement of single-channel currents

Ba^{2+} currents through single Ca^{2+} channels were recorded in the membrane of intact cells (cell-attached configuration) and in inside-out patches. Cell potentials were set to approximately zero by use of a high- K^+ low- Cl^- extracellular solution that contained (in mM): 110 potassium aspartate, 20 KCl, 2 MgCl₂, 20 Hepes, 2 EGTA; the pH was adjusted to 7.3 with *N*-methyl-D-glucamine, and pCa was adjusted to 7.5 using a Ca^{2+} -sensitive electrode. Patch pipettes were fabricated from borosilicate glass (Clark Electromedical Instruments, Pangbourne, Reading, Berks., U.K.), and had resistances of 5–10 M Ω . The pipette solution contained (in mM): 10 BaCl₂, 100 NaCl, 30 tetraethylammonium chloride and 15 Hepes, pH adjusted to 7.4. S(-)-BayK 8644 (1.0 μ M) was included in the pipette solution to facilitate stabilization of channel activity in inside-out patches as well as in intact cells. To prevent run-down of channel activity in inside-out experiments, 2 mM ATP/Na₂ and 1 unit/ml calpastatin were added to the bath solution before patch excision [18]. Exchange of bath solutions and administration of the $\alpha 1$ - β interaction peptide (10 μ M) or a scrambled control peptide was performed during constant-flow perfusion. All experiments were performed at room temperature.

Voltage clamp and current amplification was performed with a List (Darmstadt, Germany) EPC/7 patch-clamp amplifier. Current records were filtered at 1 kHz (–3 dB) and digitized at a rate of 5 kHz. Experiments were controlled using pClamp software (Axon Instruments, Foster City, CA, U.S.A.). For idealization of current records, custom-made level-detection software was employed [19]. Ca^{2+} channel activity was calculated as the mean number of open channels (NP_o) during depolarizing pulses, and the gating properties of single channels were analysed in terms of channel availability (P_s), i.e. the probability that a channel will open upon depolarization, as well as the open probability (P_o) of available channels as described previously [20,21].

Events were compiled in open-time histograms constructed

with variable bins (logarithmic scale) and fitted with multiple exponential functions.

Ca^{2+} channel preparations

Cardiac homogenate and cytoplasmic proteins were prepared from adult Wistar rats (strain Schö:Wist) and used as a source of native Ca^{2+} channel $\beta 2$ subunits. Frozen cardiac tissue was homogenized as in [22] using a Polytron PT homogenizer and buffer A, consisting of 5 mM histidine/HCl, pH 7.4, 120 mM KCl, 50 mM Na₂P₂O₇, 25 mM NaF, 10 mM EDTA, 0.2 mM dithiothreitol, 0.1 mM PMSF, 1 μ M pepstatin A, 17 μ g/ml calpain I and 7 μ g/ml calpain II. The cytoplasmic fraction was prepared by high-speed centrifugation of the homogenate proteins at 100 000 g for 2 h. Homogenate proteins were solubilized with 1% digitonin as described in [23].

Affinity binding of $\beta 2$ subunits to immobilized $\alpha 1C$ - β interaction peptide

To achieve covalent binding of the $\alpha 1C$ - β interaction peptide (AID-peptide) to agarose beads, the AID-peptide was synthesized with a N-terminal monochloroacetylglucyl extension. The peptide was immobilized to ω -aminohexyl-agarose for 3 h at a peptide concentration of 6 mg/ml of packed gel via activation of the ω -amino groups as previously described [24]. *In vitro* binding assays were performed by incubation of solubilized rat heart homogenate proteins (0.5 mg) or cytosolic proteins (0.5 mg) with 10 μ l of beads in buffer A (supplemented with 0.1% digitonin) for 2 h at 4 °C on a rotating wheel. The beads were extensively washed with digitonin-containing buffer A, extracted with SDS-sample buffer, and the eluted proteins separated on 7% (w/v) polyacrylamide gels. Competition experiments were done by incubation of cardiac $\beta 2$ protein samples with either free AID-peptide or scrambled peptide overnight at 4 °C prior to binding on to AID-affinity beads.

SDS/PAGE and Western-blot analysis

Protein samples were subjected to SDS/PAGE and Western-blot analysis according to standard protocols. Briefly, the blots were incubated with the affinity-purified, $\beta 2$ isoform-specific antibody (directed against the C-terminal $\beta 2$ epitope EWNRDVYIRQ) at a concentration of 1 μ g IgG/ml for 90 min and with the secondary peroxidase-conjugated antibody (anti-rabbit IgG; BioGenex, Berlin, Germany; diluted 1:10 000) for 60 min at room temperature as described in [22]. Immunoreactive $\beta 2$ protein bands were revealed by enhanced chemiluminescence reaction kit (ECL[®], Amersham) using a X-ray film. The antibody recognized the 85-kDa $\beta 2$ subunit expressed in brain [25], cardiac [25,26] and smooth muscle [10]. For comparison, the $\beta 3$ -isoform-specific antibody was employed. It stained an approx.-60-kDa protein [22,25]. ECL[®] signals were evaluated by computer-assisted scanner densitometry (ScanPack; Biometra, Goettingen, Germany). Densitometric analysis of the bands was within the linear range of immunoreaction.

Statistics

Averaged results are given as means $\pm$ S.E.M. for the indicated number of experiments. Two-tailed *t* tests were used for statistical comparisons, using either the unpaired or paired format. Differences were considered statistically significant at $P < 0.05$.

RESULTS

The $\beta 2a$ subunit promotes high P_o gating and induces ultralong openings of $\alpha 1C-b$

Figure 1 shows representative single-channel recordings obtained with $\alpha 1C-b$ and $\alpha 1C-b \cdot \beta 2a$ channels in the cell-attached re-

cording configuration. Multiple channels were detected in the majority of experiments in cells co-expressing $\alpha 1C-b$ and $\beta 2a$ subunits. The observed functional channels/patch were in the range of 2–6 in cells co-expressing $\alpha 1C-b$ and $\beta 2a$, but 1–3 in cells expressing $\alpha 1C-b$ only. Gating properties were analysed with a recently developed method [20] for the analysis of multichannel

Figure 1 $\beta 2a$ subunit increases the fraction of long-lasting openings of $\alpha 1C-b$ channels in cell-attached patches

Top: individual current responses recorded during depolarizing voltage pulses. Bottom: open dwell-time histograms calculated from the experiments shown above. Dwell-time histograms were fitted with exponential functions (continuous lines) using Marquardt-least-squares or maximum-likelihood method. Time constants (τ_1 , τ_2) and percentual fractions of dwell times (p_1 , p_2) are given.

Table 1 Effects of $\beta 2a$ on gating of $\alpha 1C$ -b channels

Values are means $\pm$ S.E.M. for the indicated number of experiments. *Denotes statistically significant difference versus channels derived by expression of $\alpha 1C$ -b only. n.d., not detectable.

	P_o (%)	P_s (%)	Mean open time (ms)	Proportion of long openings ($\tau > 10$ ms) (%)
Cell-attached				
$\alpha 1C$ -b ($n = 5-7$)	3.3 ± 0.7	42.2 ± 8.2	2.3 ± 0.3	n.d.
$\alpha 1C$ -b $\cdot$ $\beta 2a$ ($n = 10$)	$11.1 \pm 1.9^*$	48.3 ± 2.2	$9.3 \pm 2.2^*$	35.0 ± 11.4
Inside-out				
$\alpha 1C$ -b ($n = 8$)	3.6 ± 1.0	50.1 ± 2.2	3.5 ± 0.4	6.0 ± 4.55
$\alpha 1C$ -b $\cdot$ $\beta 2a$ ($n = 8-10$)	$7.3 \pm 0.7^*$	56.9 ± 2.5	$6.2 \pm 1.0^*$	15.1 ± 5.16

patches that allows for calculation P_o as a parameter of fast gating, and availability (P_s) as a parameter that characterizes the slow gating between available and unavailable states of the Ca^{2+} channels. This analysis revealed that co-expression of the $\beta 2a$ subunit increased not only the mean number of functional channels in the patches of membrane, but, in addition, clearly increased P_o of individual channels (Table 1). In contrast, availability (P_s) of Ca^{2+} channels was not significantly affected by expression of the $\beta 2a$ subunit. Channels comprising both $\alpha 1C$ -b and $\beta 2a$ subunits exhibited a fast gating pattern strikingly different from that of channels derived by expression of the $\alpha 1C$ -b subunit alone. In experiments with single-channel patches, or when mostly the opening of a single channel was recorded, open time distributions were analysed. As illustrated in Figure 1, the elevated level of P_o in $\alpha 1C$ -b $\cdot$ $\beta 2a$ channels was associated with a significantly increased mean open time and promotion of long-lasting channel openings. Open time distributions obtained with $\alpha 1C$ -b $\cdot$ $\beta 2a$ channels were best fitted by two, or in most cases, by three exponentials with an ultra-long-lived open state exhibiting a mean open time (τ) > 10 ms. The mean proportion of such ultra-long-lasting openings amounted to $35 \pm 11.2\%$ (Table 1). In cells expressing only the $\alpha 1C$ -b subunit, open time distributions were best fitted with two exponential components. Openings with $\tau > 10$ ms were not statistically resolved in the open-time histograms.

When the expressed Ca^{2+} channels were studied in the excised inside-out configuration, differences in the gating pattern of channels comprised of $\alpha 1C$ -b plus $\beta 2a$ versus $\alpha 1C$ -b alone were again detectable (Figure 2). The most striking discrepancy concerned P_o , which was significantly (about 2-fold) higher in $\alpha 1C$ -b $\cdot$ $\beta 2a$ channels (Table 1). In inside-out recordings, ultra-long-lasting openings ($\tau > 10$ ms) were detectable in the open-time distributions of both $\alpha 1C$ -b $\cdot$ $\beta 2a$ and, albeit less frequently, also in $\alpha 1C$ -b channels (Table 1). Single-channel amplitude, P_o and P_s of channels did not change significantly with time in inside-out recordings (results not shown). Thus the functional $\alpha 1$ - β interaction was fairly stable in excised inside-out patches, as expected from the reported high-affinity interaction between $\alpha 1$ and β subunits [15]. To further investigate the properties of the $\alpha 1$ - β interaction, we performed experiments with a synthetic peptide that corresponds to the sequence of the conserved $\alpha 1$ - β interaction domain within the I-II linker of $\alpha 1C$. This peptide is expected to bind to Ca^{2+} -channel β subunits and to inhibit the interaction of β with AID under equilibrium binding conditions in a competitive manner. Where the stable interaction between $\alpha 1$ and β is based exclusively on a single high-affinity interaction site within AID, this peptide is not expected to promote

dissociation of β from AID in inside-out experiments. By contrast, where the $\alpha 1$ - β interaction involves multiple interaction sites, the AID peptide may promote dissociation by a step-by-step binding mechanism involving the formation of an unstable ternary complex.

A synthetic peptide corresponding to a sequence of the I-II linker of $\alpha 1C$ binds to the $\beta 2$ subunit and is able to disrupt $\alpha 1$ - β interactions

As an initial step we characterized the 18-amino-acid synthetic AID-peptide in terms of its ability to bind to $\beta 2$ subunits by an *in vitro* affinity-bead assay. The AID-peptide was covalently coupled to an agarose-gel matrix and incubated with the Ca^{2+} -channel $\beta 2$ subunit solubilized from rat cardiac tissue homogenates. Western-blot analysis with the $\beta 2$ -isoform-specific antibody showed that the $\beta 2$ subunit was recovered by affinity beads, but not by the agarose-gel matrix (Figure 3; + / - coupled peptide). Moreover, $\beta 2$ protein binding to affinity beads was entirely prevented by preincubation with 10 μ M AID-peptide (Figure 3; free peptide). By contrast, inclusion of the scrambled peptide (up to 20 μ M) did not interfere with $\beta 2$ binding under the same conditions (Figure 3). In addition to the 85 kDa $\beta 2$ subunit, a minor protein migrating at approx. 60 kDa was specifically recovered by AID-affinity beads too. Because of similarities in apparent molecular mass with the $\beta 3$ subunit [22], we tested the possibility of whether AID beads precipitate the $\beta 3$ subunit from heart homogenates. However, no $\beta 3$ subunit was detected by the $\beta 3$ -isoform-specific antibody (results not shown). Thus the 60 kDa protein is considered to be a $\beta 2$ subunit isoform which was also observed following protein kinase A-mediated phosphorylation [27,28].

Binding affinity of the major 85 kDa $\beta 2$ subunit was further assessed by competition experiments. Cytosolic proteins from rat heart were incubated with increasing concentrations of the AID-peptide (1 nM to 10 μ M) prior to binding on affinity beads. Densitometric evaluation of $\beta 2$ protein binding demonstrated that preincubation with the 18-amino-acid synthetic peptide prevented the association of native $\beta 2$ subunits with affinity beads with a K_i of 100 nM (Figure 4).

Administration of the AID-peptide to the cytoplasmic face of $\alpha 1C$ -b $\cdot$ $\beta 2a$ channels results in rapid removal of β -induced channel modulation

To investigate the functional role of the $\alpha 1$ - β interaction that takes place within the I-II linker of $\alpha 1C$, we studied the effect of the AID-peptide on gating of $\alpha 1C$ -b $\cdot$ $\beta 2a$ channels in the inside-out recording configuration. The peptide was administered at a concentration (10 μ M) that was sufficient to completely inhibit $\alpha 1$ - β interaction in *in vitro* affinity-bead assay. In five out of six experiments a substantial change in the gating properties was obtained during exposure of the cytoplasmic face of inside-out patches to AID-peptide. A typical experiment is illustrated in Figure 5(A). Channel activity ($N \times P_o$) declined rapidly (within 2-5 min) in the presence of the AID-peptide, whereas $N \times P_o$ of $\alpha 1C$ -b $\cdot$ $\beta 2a$ channels remained fairly constant within 20-30 min in control experiments (Figure 5, insert). The mean P_o of $\alpha 1C$ -b $\cdot$ $\beta 2a$ channels was decreased significantly from $9.3 \pm 1.5\%$ to $6.3 \pm 1.4\%$ ($N = 6$) by the AID-peptide. The reduction in channel activity was associated with a decrease of the mean open time and a reduction of the proportion of ultra-long-lasting channel opening (Figure 5B). A peptide-induced decrease in

Figure 2 $\beta 2a$ subunit increases the fraction of long-lasting openings of $\alpha 1C-b$ channels in excised patches

Top: individual current responses recorded during depolarizing voltage pulses. Bottom: open dwell-time histograms calculated from the experiments shown above. Dwell-time histograms were fitted with exponential functions (solid lines) using Marquardt-least-squares or maximum-likelihood method. Time constants (τ_1, τ_2) and percentual fractions (p_1, p_2) of dwell times are shown.

mean open times was clearly observed in the experiments which allowed for analysis of open time distributions ($N = 2$). The peptide decreased P_o , but did not affect availability of either $\alpha 1C-b \cdot \beta 2a$ or $\alpha 1C-b$ channels. Thus peptide-induced changes in gating behaviour were consistent with removal of β -induced

channel modulation. To further test the hypothesis that the observed effect of the AID-peptide was related to β -induced channel modulation, we performed control experiments with a scrambled control peptide and tested whether the AID-peptide would inhibit channels derived by expression of $\alpha 1C-b$ only.

Figure 3 Native $\beta 2$ subunit binds to the 18-amino-acid synthetic peptide resembling the $\alpha 1C$ interaction site within the I-II linker (AID)

Affinity beads were produced by covalent coupling of the AID-peptide to ω -aminoethyl agarose (above gel photograph, —/+ coupled peptide). The AID-affinity beads were incubated with rat heart homogenate proteins (0.5 mg) in the absence or presence of either the AID-peptide (free peptide) or the control peptide (scrambled peptide) as indicated (below gel photograph). Binding of the $\beta 2$ subunit was revealed by subsequent SDS/PAGE and immunoreaction with the $\beta 2$ -isoform-specific antibody.

Figure 4 Displacement of the $\beta 2$ subunit from AID-affinity beads by the $\alpha 1C$ - β interaction peptide

Rat heart proteins (0.5 mg) of the cytoplasmic fraction were incubated with increasing concentrations (1 nM–10 μ M) of the synthetic 18-amino-acid $\alpha 1C$ - β interaction peptide (AID-peptide) prior to binding on to AID-affinity beads. The amount of the $\beta 2$ subunit bound to the beads was estimated by densitometric evaluation of immunoblots.

Administration of a control (scrambled) peptide (10 μ M) did not affect channel activity of $\alpha 1C$ -b/ $\beta 2a$ channels in inside-out patches ($N = 2$), and the AID-peptide failed to modulate the function of $\alpha 1C$ -b in the absence of expressed $\beta 2a$ proteins ($N = 3$).

DISCUSSION

The present study demonstrates that the smooth-muscle isoform of the Ca^{2+} -channel $\alpha 1$ subunit is susceptible to modulation by

the $\beta 2a$ subunit. Moreover, we provide, for the first time, evidence for the principle reversibility of β modulation in a functional Ca^{2+} channel. We report on uncoupling of Ca^{2+} channel $\alpha 1$ and β subunits in cell-free patches by a peptide that interferes with binding of β to the conserved domain in the I-II linker of $\alpha 1$.

Control of fast gating of the smooth-muscle splice variant of $\alpha 1C$ by the $\beta 2$ subunit

Ca^{2+} -channel β subunits have been shown to potentiate Ca^{2+} currents in various expression systems [1,4,6,12,29]. This potentiation of the Ca^{2+} current is in part due to an increase in the number of channel complexes in the plasma membrane. In addition to this effect of β subunits on subcellular localization and targeting of the channel complex, evidence for an allosteric, β -induced modification of channels have been presented [1,4,6,12]. Here we demonstrate a substantial modification of the fast gating of the b- splice variant of the class C Ca^{2+} channel $\alpha 1$ subunit by a $\beta 2$ subunit ($\beta 2a$). These Ca^{2+} -channel subunits are expressed in cardiac and smooth muscle [8,10,11,25], and recent experimental evidence supports the view that complexes consisting of $\alpha 1C$ -b and $\beta 2a$ contribute to the formation of L-type channels in various smooth-muscle tissues [11]. Co-expression of the $\beta 2a$ subunit increased P_o of $\alpha 1C$ -b channels approx. 2-fold, due to promotion of long-lasting channel openings. This observation is consistent with the results of a previous study on the modulation by $\beta 2$ of the myocardial (a-) splice variant of $\alpha 1C$ [6]. Despite a high degree of homology, a- and b- splice variants of $\alpha 1C$ were reported to differ in their pharmacological properties [30,31]. Since the structural difference between a- and b- splice variants of $\alpha 1C$ resides for a large part in the N-terminal sequence, which has recently been suggested as a critical determinant of $\alpha 1$ - β interaction [5], a differential modulation by β subunits of the cardiac- and smooth-muscle splice variant of $\alpha 1C$ may be anticipated. Nonetheless, we observed a $\beta 2$ -induced modulation of fast gating behaviour of the smooth-muscle splice variant $\alpha 1C$ -b that is reminiscent of the modulation reported for $\alpha 1C$ -a channels [6]. The functional effect of $\beta 2a$ on both splice variants appears similar, in that frequent occupation of a long-lived open state was observed. Both the results reported by Costantin et al. [6], as well as our present results, were obtained in the presence of the Ca^{2+} channel agonist BayK 8644, which may to some extent interfere with the β -induced modulation of channel function. Nonetheless, β -induced prolongation of mean open time has been reported recently for $\alpha 1C$ -a channels to occur also in the absence of BayK 8644 [12]. It has been suggested that a long-lived open state exists also in the absence of exogenously expressed $\beta 2$ subunit, and that the auxiliary subunit promotes the occupation of this existing open state [6]. Consistently, we observed occasional ultra-long openings in $\alpha 1C$ -b channels. Our results demonstrate that the $\beta 2a$ subunit is able to modify fast gating of the smooth muscle (b-) splice variant of $\alpha 1C$ in a manner similar to that of the cardiac a-form, despite the structural difference in the N-terminus. Thus our results demonstrate that modulation by $\beta 2a$ of both class C splice variants are similar and argue against a crucial role of the N-terminal tail in fast-gating modulation by the $\beta 2$ subunit.

Role of the I-II linker of $\alpha 1$ in functional coupling between $\alpha 1$ and β subunits

The interaction between $\alpha 1$ and β proteins involves a conserved motif in the intracellular I-II connecting loop of $\alpha 1$ [14]. A recent

Figure 5 Synthetic $\alpha 1\text{C}-\beta$ interaction peptide decreases channel activity and the proportion of long-lasting channel openings in excised patches

(A) The upper panel shows the time course of Ca^{2+} -channel activity in an excised patch. Administration of $10 \mu\text{M}$ $\alpha 1\text{C}-\beta$ interaction peptide is indicated. The insert illustrates the stability of inside-out patches during control perfusion of the cytoplasmic side. The lower panel shows individual current responses during depolarizing voltage pulses before and after administration of $\alpha 1\text{C}-\beta$ interaction peptide. (B) Dwell-time histograms derived from the experiment shown in (A). Dwell-time histograms were fitted to exponential functions (continuous lines) using Marquardt-least-squares or maximum-likelihood method. Time constants (τ_1 , τ_2) and percentual fractions (p_1 , p_2) of dwell times are given.

study demonstrates that binding of β subunits to this motif is essential for membrane targeting of the $\alpha 1$ - β complex [32]. The role of this $\alpha 1$ - β interaction domain and of other hypothetical interaction sites in functional modulation of $\alpha 1$ by β subunits is still an open question. In the present study we employed a synthetic peptide corresponding to a partial sequence of the I-II linker of $\alpha 1$ (AID-peptide) as a tool to disrupt the $\alpha 1$ - β interaction. *In vitro* binding experiments confirmed that this peptide exhibits a reasonable affinity to cardiac $\beta 2$ subunits to serve this purpose. Administration of the AID-peptide to the cytoplasmic side of inside-out patches resulted in a significant change in fast gating of $\alpha 1C$ -b $\cdot$ $\beta 2a$ channels in that the P_o of the channels declined rapidly. This decrease in P_o was associated with prevention of entry into the ultra-long open state, i.e. a modulation consistent with elimination of the functional modulation of $\alpha 1C$ -b by the $\beta 2a$ subunit. The peptide did not exert any effect on the function of $\alpha 1C$ -b when expressed alone, excluding a direct inhibitory effect of the peptide on the $\alpha 1$ subunit. It appears thus reasonable to conclude that the employed AID-peptide inhibits Ca^{2+} -channel activity due to functional uncoupling of $\alpha 1C$ -b and $\beta 2a$ proteins. In the absence of AID-peptide, gating of $\alpha 1C$ -b $\cdot$ $\beta 2a$ channels was fairly stable in inside-out recordings up to 30 min, despite complete wash-out of any free cytosolic β proteins, indicating a tight high-affinity binding of the β subunit to $\alpha 1$. Such a high affinity $\alpha 1$ - β interaction has previously been demonstrated in *in vitro* binding experiments [15]. The observed rapid elimination of the typical β -induced gating behaviour upon addition of excess of the AID-peptide was interpreted in terms of an increased dissociation rate constant of the $\alpha 1$ - β complex and thus in terms of a decrease in $\alpha 1$ - β binding affinity by the peptide. The basis of this conclusion is that it appears reasonable to assume that free β subunit is rapidly removed by perfusion of the cytoplasmic face of inside-out patches, as the $\beta 2a$ subunit employed in the present study lacks structures for membrane anchoring [9]. Consistently, removal of the AID-peptide after elimination of β modulation did not result in recovery of high P_o gating of the channels (A. Hohaus and K. Groschner, unpublished work), indicating that reassociation of $\alpha 1$ and β is effectively prevented in the inside-out configuration. The observed AID-peptide-induced functional uncoupling of the subunits is therefore likely to reflect an increased dissociation rate of the $\alpha 1$ - β complex. Such a change in dissociation rate cannot be explained by a classical competitive interaction at a single interaction site according to the principal concepts of receptor-ligand interactions [33–35]. Hence the AID-peptide-induced reduction of the stability of $\alpha 1$ - β complexes is consistent with the hypothesis of multiple $\alpha 1$ - β interaction sites. Formation of a ternary complex comprised of $\alpha 1$, $\beta 2a$ and the AID-peptide is required to explain the observed uncoupling of $\alpha 1$ - β interaction in inside-out patches. Such a ternary complex involving multiple $\alpha 1$ - β attachment sites is likely to exhibit low stability, resulting in rapid dissociation and washout of AID-peptide-bound β subunit. This concept implies that, despite the high affinity of $\alpha 1$ - β interactions observed *in vitro*, an exchange of β subunits via a step-by-step binding mechanism is in principle possible in functional channels *in vivo*. On the basis of our present experiments we cannot distinguish whether suggested multiple attachment points are located within the same channel domain, i.e. the I-II linker, or whether attachment sites are located outside of this classical AID. Interaction domains in $\alpha 1C$ outside of the classical AID have so far not been detected, and recent experimental evidence argues against an interaction of β subunits with the cytoplasmic N- and C-termini of $\alpha 1C$ [5]. Nonetheless, a recent study shows that mutation of a single amino acid residue in AID of $\alpha 1C$ -a (Y467S, i.e. Tyr⁴⁶⁷ $\rightarrow$ Ser) is sufficient to disturb

the effect of $\beta 2$ on membrane targeting of the channels, but not to affect the allosteric modulation of channel function [12]. These findings were also interpreted in terms of multiple interaction sites between $\alpha 1$ and β subunits and in terms of the existence of one low-affinity site that contributes to functional coupling. A functionally relevant low-affinity interaction may well take place in $\alpha 1Y467S$ channels which exhibit none or weak AID interaction, as long as a sufficient concentration of cytosolic β proteins is maintained. Such low-affinity interaction may well explain the β -modulation of $\alpha 1Y467S$ channels observed in intact HEK 293 cells [12]. In the present inside-out experiments, disruption of the AID interaction resulted in rapid loss of β -modulation. These data suggest that the $\alpha 1$ - β interaction in AID is essential for stabilization of the subunit complex. It remains to be clarified to what extent the AID interaction contributes to functional coupling between α and β subunits.

Our results provide, for the first time, evidence for principal reversibility of Ca^{2+} -channel modulation by β subunits and suggest disruption of $\alpha 1$ - β interaction or exchange of β subunits as a possible mechanism of channel regulation.

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