β Subunit heterogeneity of L-type Ca^{2+} channels in smooth muscle tissues

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Abstract Various β subunit isoforms stabilize different gating properties of voltage-gated L-type Ca^{2+} channels. We therefore investigated the expression of Ca^{2+} channel β subunit isoforms in different smooth muscle types on the protein level by immunoblotting and immunoprecipitation employing β subunit-selective sequence-directed antibodies. From the four known β subunit isoforms only β2 and β3 were detected in porcine uterus, bovine trachea and bovine aorta membranes. Multiple immunoreactive β2 bands were detected in a tissue-selective manner indicating structural heterogeneity of β2. Immunoprecipitation of (+)-[^{3}H]isradipine-prelabeled channels revealed that β2 and β3 participate in Ca^{2+} channel formation in uterus and trachea, and β3 in aortic smooth muscle. We conclude that β2 and β3 subunits form L-type Ca^{2+} channels in smooth muscle tissues. This subunit heterogeneity may be important to fine-tune channel function.

Key words: Ca^{2+} channel; Subunit; Smooth muscle; 1,4-Dihydropyridine

1. Introduction

Voltage-gated Ca^{2+} channels mediate depolarization-induced Ca^{2+} influx into electrically excitable cells and thereby control important physiological processes, such as muscle contraction, neuronal plasticity, neurotransmitter and hormone secretion, and gene transcription. At least six different types of Ca^{2+} channels (high voltage-activated L-, P-, Q-, R- and N-type channels; low voltage-activated T-type channels) can be discriminated based on pharmacological and biophysical criteria [1]. Different α1 subunit isoforms (α1A to I and α1S) comprise the pore-forming region of the various channel types [1]. α1 subunits of high voltage-activated channels form hetero-oligomeric complexes with accessory α2-δ, β and γ subunits, which modulate channel function and expression [2]. Whereas all channel types are found in neurons, muscle cells only express L- and T-type Ca^{2+} channels.

Ca^{2+} channel function is fine-tuned by different mechanisms including phosphorylation/dephosphorylation [3], G-protein modulation [4,5] and subunit composition [6,7]. The physiological importance of channel fine-tuning by accessory subunits is evident from recently described genetic disorders leading to dysfunction of Ca^{2+} channels in neurons. Disruption of one of the four β subunit genes (β4) in the *lethargic* mouse mutant results in seizures and ataxia [8]. A similar phenotype is found in *stargazer* mutant mice characterized by a defect neuronal γ subunit ('stargazin', [9]). These examples illustrate that even minor changes in channel expression and/or gating cause alterations in depolarization-dependent Ca^{2+} influx incompatible with normal neuronal function. Consequently, our understanding of physiological channel function in neuronal and non-neuronal tissues requires the detailed biochemical analysis of their subunit composition.

Considerable plasticity of Ca^{2+} channel function is caused by the existence of four distinct β subunit isoforms (β1–β4), which differentially affect the voltage-dependent gating of the pore-forming α1 subunit [6,10–13]. Recent biochemical studies have shown that all four isoforms can associate to various extents with P/Q-, N- or L-type channels in mammalian brain [14–16], whereas only β2 subunits were found to form L-type channels in heart muscle [16]. In contrast, such information is lacking for smooth muscle L-type Ca^{2+} channels, whose functional fine-tuning is important to maintain normal muscular tone in tissues such as blood vessels, airway and uterus smooth muscle.

In the present study we therefore determined the β subunit expression in aorta, trachea and uterus smooth muscle on the protein level as well as their association with L-type Ca^{2+} channels.

2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources: (+)-[^{3}H]isradipine, (±)-[^{3}H]isradipine from New England Nuclear (Vienna, Austria); protease inhibitors, bovine serum albumin (essentially fatty acid free), protein A-Sepharose, lectin from triticum vulgaris, and CHAPS from Sigma (Vienna, Austria); digitonin from Fluka Biochemica (Basel, Switzerland); prestained molecular weight markers from Biorad; glutathione-Sepharose from Pharmacia (Vienna, Austria).

2.2. Membrane preparation

Rabbit cerebral cortex membranes were prepared as described [16]. Porcine heart and uterus were rapidly excised in a local slaughter house and immediately placed in ice-cold homogenization buffer (20 mM $NaHCO_3$, containing a protease inhibitor mix consisting of 0.2 mM PMSF, 0.5 mM benzamidine, 2 mM iodacetamide, 1 μM pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin). The tissues were

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Abbreviations: AIDA, α1A-β subunit interaction domain; AO, aortic smooth muscle; GST, glutathione-*S*-transferase; HT, heart muscle; SDS, sodium dodecylsulfate; TR, tracheal smooth muscle; UT, uterus smooth muscle; WGA, wheat germ agglutinin

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then homogenized by a polytron (2×20 s) and by 10–15 strokes in a Dounce homogenizer. Microsomes were collected by centrifugation at 45 000×*g* (10 min; 4°C) and washed three times with buffer A (50 mM Tris–HCl, pH 7.4, containing the above protease inhibitor mix). Membranes were resuspended in the same buffer at a membrane protein concentration of about 5 mg/ml and stored at −80°C until use. Bovine trachea and aorta sarcolemmal membrane vesicles were prepared as described previously [17].

2.3. Affinity purification of β subunits

Glutathione-*S*-transferase (GST) and a GST-fusion protein (GST-AIDA) of the α1 subunit interaction domain of the α1A subunit (AIDA) were prepared as described previously [16]. All further steps were carried out on ice or at 4°C. 10–20 mg microsomal protein isolated from rabbit brain, porcine heart or smooth muscle tissues were solubilized in 9 ml of buffer A containing 1 M NaCl and 1% (w/v) CHAPS. 10 μg AIDA-GST or GST were coupled to 30 μl aliquots of glutathione-Sepharose equilibrated in buffer B (buffer A containing 0.1 M NaCl and 0.1% (w/v) CHAPS) and washed three times with the same buffer. Solubilized membranes were diluted 10-fold in buffer A and 4 ml were mixed with the coupled glutathione-Sepharose beads overnight. The beads were washed three times with 1.5 ml of buffer B, mixed with SDS–polyacrylamide gel electrophoresis sample buffer (3 min, 95°C) and the eluted proteins separated on 8% or 10% SDS–polyacrylamide gels.

Western blotting was carried out according to standard procedures [16].

2.4. Sequence-directed antibodies

Sequence-directed antibodies (raised as described previously in rabbits [16]) against the following peptide epitopes were employed (amino acid positions are given according to the sequences in [18]): β1b, 516–530; β2, 595–604; β3, 470–483; β4, 460–474. Anti-βcom was raised against residues 61–79 in β1a [19], and anti-α1C against α1C residues 818–835 [20].

2.5. Partial purification and immunoprecipitation of prelabeled Ca^{2+} channels

Typically 10–20 mg of membrane protein was prelabeled with 1–2 nM (+)-[^{3}H]isradipine for 60 min at 25°C in buffer A containing 0.1 mM $CaCl_2$. Prelabeled membranes were solubilized in 1% (w/v) of digitonin and purified by wheat germ agglutinin (WGA) affinity chromatography [16]. Purified fractions were immediately used in immunoprecipitation experiments or stored at −80°C. Protein concentrations were determined according to Bradford [21] using bovine serum albumin as a standard.

For immunoprecipitation of prelabeled Ca^{2+} channels saturating concentrations of affinity-purified antibodies were coupled to protein A-Sepharose. The antibody-protein A-Sepharose complex was then washed three times with ice-cold RIA buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5 mg/ml bovine serum albumin, 0.1% (w/v) digitonin) and incubated with 0.05–0.25 ml (3000–4000 dpm) of WGA-purified prelabeled Ca^{2+} channels. After four 1.5 ml washes in RIA buffer bound radioactivity was determined by liquid scintillation counting. Channel-associated (+)-[^{3}H]isradipine was determined by filtration over GF/C Whatman filters as described [16].

2.6. Radioligand binding studies

Modulation of (+)-[^{3}H]isradipine binding to cardiac and uterus microsomal membranes by unlabeled Ca^{2+} antagonists and divalent cations was performed as described [22] employing 0.2–0.3 mg/ml of membrane protein and 0.2 nM of radioligand. Incubation was for 45 min at 37°C. Non-specific binding was measured in the presence of 1 μM (±)-isradipine. The concentration of bound radioactivity was determined by rapid filtration over GF/C Whatman filters, pretreated for 45 min with 0.1% (v/v) polyethylenimine.

2.7. Statistics

Data are given as means ± S.E.M. for the indicated number of experiments. Binding parameters were calculated by computer fitting the data to the general dose-response equation [23].

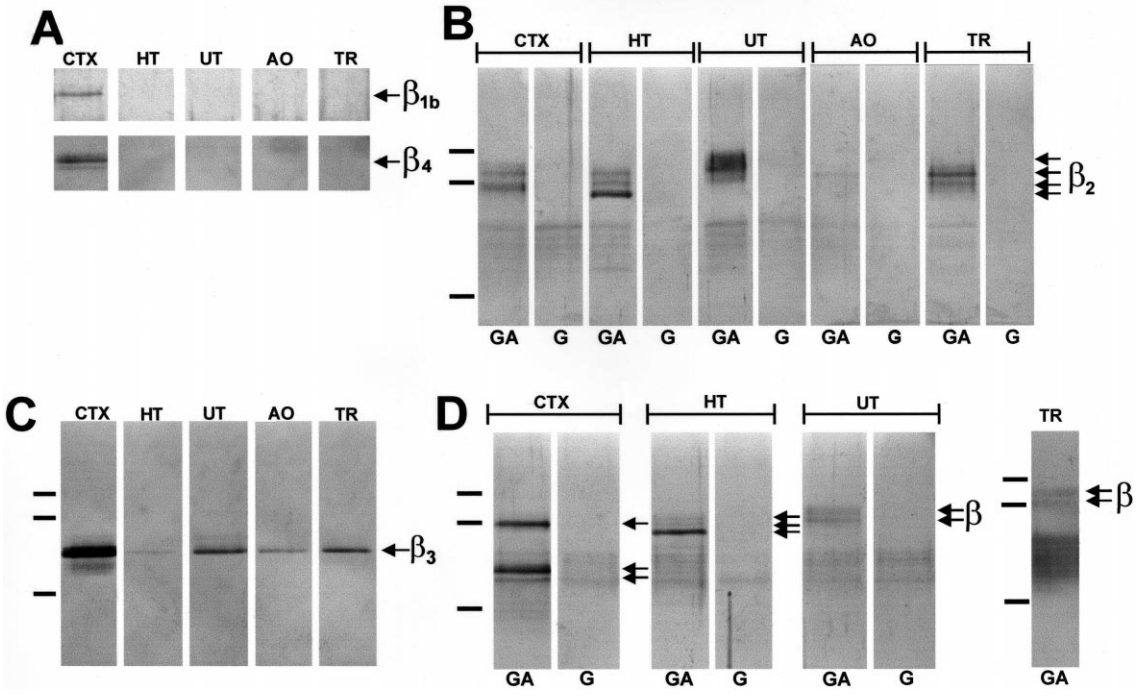

Fig. 1. Expression of β subunits in cerebral cortex, heart microsomes and different smooth muscle tissues. The results of Western blot analysis are shown. β subunits were purified from membrane proteins isolated from cerebral cortex (CTX), heart (HT) and different types of smooth muscle (UT, AO, TR) by GST-AIDA affinity chromatography, separated by SDS–polyacrylamide gel electrophoresis together with prestained molecular weight markers and subjected to immunoblot analysis with β subunit isoform-selective antibodies. For all tissues the same amounts of membrane protein were used for solubilization and subsequent affinity purification (4 ml of diluted extracts, 35–58 μg of protein). The migration of specific immunoreactivity as discussed in the text is indicated by arrows for β1b (A), β2 (B), β3 (C), β4 (A) and βcom (D). The specificity of the affinity purification step is shown in panels B and D where either GST-AIDA (GA) or GST alone (G) was used for affinity purification. The migration of prestained marker proteins (112 in panel A; 112, 78 and 49 kDa in panels B–D) is indicated on the left. The apparent molecular masses of β1b and the β4 doublet were 79 and 59/62 kDa, respectively. One of at least four independent experiments yielding similar results is shown.

3. Results

3.1. β Subunit expression in smooth muscle

As no systematic analysis of β subunit isoform expression has been reported on the protein level we investigated the expression of β subunits in Western blots employing subunit-selective sequence-directed antibodies in smooth muscle from bovine aorta (AO), trachea (TR) and porcine uterus (UT) membranes. To achieve maximal sensitivity and specificity β subunits were enriched from CHAPS extracts of bovine AO, bovine TR and porcine UT by affinity purification of β subunits on GST-AIDA-Sepharose beads as described in Section 2. Cerebral cortex and heart extracts were used as controls. Fig. 1 illustrates that, in accordance to previous work [16], all four β subunits were specifically enriched from cerebral cortex extracts. In all three types of smooth muscle only β2 and β3 isoforms were detected (Figs. 1B, C and 2), whereas β1b and β4 immunoreactivity were below the detection limit (Fig. 1A). Several bands ranging from apparent molecular masses of 65 to 107 kDa were specifically enriched and stained with our β2-specific antibody in a tissue-specific manner (see below). The density of β2 immunoreactivity in UT and TR was similar to cortex and heart, but was clearly lower in AO (only faint bands visible Figs. 1B and 2).

β3 subunits, migrating as 63 ± 1 ($n = 3$) kDa bands, were less abundant in all three types of smooth muscle as compared to cerebral cortex and again lowest in AO (Fig. 2B). In porcine heart membranes a very faint band of β3 immunoreactivity was detectable only in two out of eight experiments employing three different membrane preparations. This suggests that β3 subunits are expressed at significantly higher levels in TR, UT and even AO than in heart muscle.

To determine the relative abundance of β2 vs. β3 subunits, Western blots were stained with a generic β subunit antibody directed against an epitope conserved in all β subunit isoforms (anti-βcom). In cerebral cortex anti-βcom stained a heterogeneous 79 kDa band (which has recently been shown to comprise comigrating β1b and β2 immunoreactivities [16]) as well as a 63/59 kDa doublet (corresponding to β3 and β4 immunoreactivity [16]). In contrast, only bands corresponding to β2 immunoreactivity were stained by anti-βcom in heart, UT, and TR (arrows in Fig. 1D). Weak β2 staining was also present in AO (not shown). No bands corresponding to β3 immunoreactivity (Fig. 1C) could be demonstrated in these tissues due to the limited reactivity of this antibody. β2 must therefore represent the major β subunit isoform.

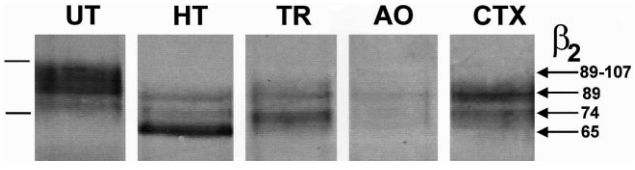

Fig. 2. β2 Heterogeneity in cerebral cortex, heart and smooth muscle tissues. Western blot analysis of β subunits affinity purified from cerebral cortex (CTX), heart (HT), uterus (UT), trachea (TR), and aorta (AO) membranes was carried out as in Fig. 1 with anti-β2 antibodies. Molecular weight markers are as in Fig. 1. Separation was on an 8% SDS–polyacrylamide gel. The arrows indicate the molecular masses (in kDa) of the individual β2 subunit bands.

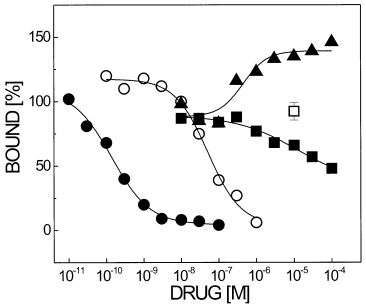

Fig. 3. Modulation of (+)-[^{3}H]isradipine binding to porcine uterus membranes: binding of (+)-[^{3}H]isradipine (0.2 nM) to uterus membranes (0.16–0.18 mg/ml of protein) was determined in the absence and presence of different unlabeled Ca^{2+} antagonists or Ca^{2+}. One representative experiment is shown. The following binding parameters (IC_{50} or EC_{50}, slope, binding inhibition/stimulation to % of control) were calculated from at least three independent experiments: (+)-isradipine (●): 0.3 ± 0.4 nM, 0.8 ± 0.1, 100%; (−)-isradipine (○): 53 ± 1 nM, 0.9 ± 0.1, 100%; (+)-verapamil (■): 8.6 ± 6.3 μM, 0.4 ± 0.1, 52 ± 2%; (−)-verapamil (□): no inhibition up to 100 μM; (+)-cis-diltiazem (▲): 0.91 ± 0.15 nM, 1.1 ± 0.1, 135 ± 6%. As a comparison the binding parameters obtained for cardiac muscle Ca^{2+} channels are also given ($n \geq 3$): (+)-isradipine: 0.4 ± 0.8 nM, 1 ± 0.1, 100%; (−)-isradipine: 63 ± 1 nM, 0.8 ± 0.1, 100%; (+)-verapamil: 1.7 ± 0.3 μM, 0.6 ± 0.3, 32 ± 3%; (−)-verapamil: no inhibition up to 100 μM; (+)-cis-diltiazem (▲): 7.3 ± 1.4 μM, 0.5 ± 0.1, 156 ± 5%.

3.2. β2 subunit heterogeneity

In contrast to β3 (Fig. 1C), β2 immunoreactivity was associated with multiple bands. In heart muscle the majority of immunostaining was associated with a 65 ± 1 kDa ($n = 4$) band, whereas two larger bands (89 ± 1 and 74 ± 2 kDa, $n = 3$) were less abundant (Figs. 1B and 2). The 89 and 74 kDa bands were detected as the major forms in cerebral cortex, TR and AO (Figs. 1 and 2). In contrast, in UT a diffuse 89–107 kDa band was most abundant. This staining pattern was tissue specific and reproducibly obtained in at least five independent purification experiments.

3.3. β subunit association with L-type Ca^{2+} channels

To investigate if β2, β3 subunits or both are associated with L-type Ca^{2+} channels in smooth muscle tissues, solubilized L-type channel complexes specifically prelabeled with (+)-[^{3}H]isradipine were partially purified by wheat germ lectin affinity chromatography and subjected to immunoprecipitation with saturable concentrations of βcom and isoform-selective antibodies. High affinity (+)-[^{3}H]isradipine binding has previously been shown to be associated with L-type Ca^{2+} channels in cerebral cortex, heart, TR and AO [24,25]. (+)-[^{3}H]isradipine binding to porcine UT membranes was also stereospecific and modulated by different classes of Ca^{2+} antagonists (stimulation by (+)-cis-diltiazem, partial inhibition by (+)-verapamil) (Fig. 3) similar to heart muscle (see legend to Fig. 3). In heart and uterus binding was stimulated by Ca^{2+} (maximal stimulation at 1 mM, $n = 3$). These properties demonstrate that specific (+)-[^{3}H]isradipine binding also occurs to L-type Ca^{2+} channels in porcine uterus. Saturating concentrations of anti-α1C immunoprecipitated 71 ± 5% of prelabeled Ca^{2+} channels in UT ($n = 3$), 57 ± 3% in TR ($n = 3$) and 63 ± 11% in AO ($n = 3$). Therefore the majority of L-type channels in these tissues contain α1C as a pore-forming subunit. Immunoprecipitation with anti-βcom revealed that most L-type Ca^{2+} channel complexes are also associated with β

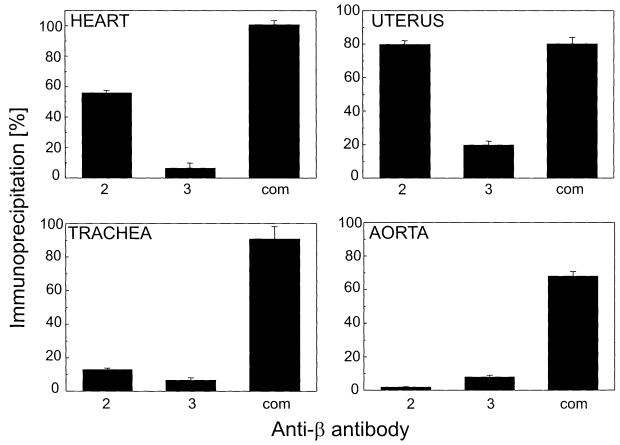

Fig. 4. Specific immunoprecipitation of solubilized (+)-[^{3}H]isradipine-labeled L-type Ca^{2+} channels from porcine heart and different smooth muscle tissues. Ca^{2+} channel complexes were labeled with 0.2 nM (+)-[^{3}H]isradipine, solubilized in 1% (w/v) digitonin and partially purified by affinity chromatography on WGA-Sepharose. Immunoprecipitation experiments were performed as described in Section 2. The radioactivity immunoprecipitated by saturating concentrations of anti-βcom or the isoform-selective antibodies were normalized with respect to the total channel-associated radioactivity employed in the assay (>1900 dpm HT, >2200 dpm UT, >2000 dpm TR, >1900 dpm AO). Numbers denote the β isoform to which antibodies were generated; com, anti-βcom. Immunoprecipitation by equal concentrations of non-immune rabbit immunoglobulins was less than 10% of channel-associated radioactivity, and was subtracted from the radioactivity immunoprecipitated by β subunit antibodies to yield specific immunoprecipitation.

subunits in smooth muscle tissues (Fig. 4). In UT the majority of channel complexes ($80 \pm 2\%$, $n = 5$) was associated with β2 and to a smaller extent with β3 ($20 \pm 2\%$, $n = 4$; Fig. 4). Anti-β2 and -β3 antibodies also immunoprecipitated (+)-[^{3}H]isradipine binding activity (13 ± 1, $n = 6$, and $7 \pm 1\%$, $n = 5$, respectively; Fig. 4) from solubilized TR membranes but together only accounted for about 20% of the total β immunoreactivity associated with L-type channels in this tissue (Fig. 4). In solubilized AO membranes a small fraction of channels was recognized by anti-β3 ($8 \pm 1\%$, $n = 5$), whereas no clear association with β2 ($2 \pm 1\%$, $n = 6$) could be demonstrated. In all cases immunoprecipitation was specific because it was not observed with equivalent concentrations of control rabbit immunoglobulins (see legend to Fig. 4).

4. Discussion

Our study provides the first analysis of the β subunit composition of L-type Ca^{2+} channels in different types of smooth muscle. Only significant expression of β2 and β3 subunits occurs in the smooth muscle tissues investigated whereas β1b and β4 polypeptides could not be detected. In UT, HT and TR β2 and β3 participate in the formation of L-type Ca^{2+} channel complexes. No evidence was obtained for association with β2 in AO, although it cannot be ruled out by our data. Detection of β3 subunit expression in both Western blot analysis and immunoprecipitation experiments clearly argues against the conclusions from a recent study in which no β3 subunit protein could be detected in aortic smooth muscle [26].

We have recently also described β subunit heterogeneity of L-type Ca^{2+} channels in mammalian brain [16] where association with all isoforms could be demonstrated. β subunit isoforms differentially affect L-type Ca^{2+} channel function. This has been shown by their coexpression with L-type α1C subunits in heterologous systems. For example, in contrast to β3, β2 subunits slow voltage-dependent inactivation [11–13] and do not support prepulse facilitation [11,27]. Therefore subunit composition could play an important role to fine-tune channel function in mammalian brain as well as smooth muscle. In smooth muscle tissues L-type Ca^{2+} channel activity participates in the regulation of basal muscular tone by regulating steady-state intracellular free Ca^{2+} concentrations at resting membrane potentials. At these potentials macroscopic Ca^{2+} inward currents are absent but a constant Ca^{2+} channel-mediated Ca^{2+} influx occurs through non-inactivating channels ('window currents') [28–30]. Different extents of non-inactivating current components stabilized by β2 and β3 are therefore expected to affect steady-state intracellular free Ca^{2+} concentrations and thus muscular tone. Therefore the ratio of channels associated with β2 or β3 subunits may play an important role to fine-tune contractile smooth muscle activity and thereby affect physiological processes such as uterus contraction, airway and vascular resistance. Whether changes of the β subunit composition of L-type Ca^{2+} channels occurs under certain physiological (e.g. during pregnancy) or pathophysiological (e.g. hypertension or asthma) conditions and serves as a modulatory mechanism of voltage-gated Ca^{2+} influx must be determined in further studies. Evidence for changes in Ca^{2+} channel expression have already been obtained on the level of α1 subunits. For example, α1C expression and L-type Ca^{2+} current are upregulated in spontaneously hypertensive rats (SHR) [31] and changes in the relative expression of α1C splice variants are observed during pregnancy [32].

In UT the L-type Ca^{2+} channel activity immunoprecipitated by the β2- and β3-selective antibodies quantitatively accounted for all channel-bound β subunits as determined by immunoprecipitation with anti-βcom. Therefore we could quantify the β2/β3 ratio in this tissue. In contrast, only about 20% of the β subunit-associated channels were recognized by these isoform-selective antibodies in TR and AO. We reported a similar observation in mammalian brain, where isoform-selective antibodies together accounted for all β subunit-associated L-type channels solubilized from cerebral cortex but not from cerebellum (66%) and hippocampus (70%) [16]. This finding can be explained by different extents of C-terminal proteolysis of β subunits, which would remove the C-terminal epitopes of the isoform-selective antibodies but not the N-terminal epitope of anti-βcom. This proteolytic activity would have to occur despite the presence of protease inhibitors in our buffers and its extent must vary considerably between tissues. Alternatively, one or more so far not identified β subunit isoforms could exist, which could escape immunoprecipitation by isoform-selective antibodies but may contain the highly conserved epitope recognized by anti-βcom.

Another unexpected observation was the heterogeneity found for the β2 subunit immunoreactivity in immunoblots. The 42 kDa difference in molecular mass between the smallest (65 kDa, most abundant in cardiac muscle) and the largest band (80–107 kDa, most abundant in UT) cannot be explained by proteolysis because both bands are specifically recognized by anti-β2 as well as anti-βcom and must therefore contain the respective epitopes. C-terminal proteolysis can be ruled out because it would remove the anti-β2 epitope. Pro-

teolysis could only remove the portion N-terminal to the anti-βcom epitope. However, complete removal of this region would only account for a difference in molecular mass of 2–3 kDa (molecular mass of peptide from N-terminus to start of antibody epitope). Although we cannot rule out that proteolysis contributes to the observed β subunit heterogeneity to some extent, we propose that other factors such as post-translational modification or the existence of yet unknown splice variants must explain our findings.

Our finding should prompt further studies in e.g. β3 subunit-deficient mice which should reveal the physiological significance of β subunit heterogeneity predicted from our biochemical analysis.

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