The $\alpha 2/\delta$ subunit of voltage sensitive Ca^{2+} channels is a single transmembrane extracellular protein which is involved in regulated secretion

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Abstract **The membrane topology of $\alpha 2/\delta$ subunit was investigated utilizing electrophysiological functional assay and specific anti-$\alpha 2$ antibodies. (a) cRNA encoding a deleted $\alpha 2/\delta$ subunit was coinjected with $\alpha 1C$ subunit of the L-type calcium channel into *Xenopus* oocytes. The truncated form, lacking the third putative TM domain ($\alpha 2/\delta\Delta$TMIII), failed to amplify the expressed inward currents, normally induced by α_{1C} coinjected with intact $\alpha 2/\delta$ subunit. Western blot analysis of $\alpha 2/\delta\Delta$TMIII shows the appearance of a degraded $\alpha 2$ protein and no expression of the full-size two-TM truncated-protein. The improper processing of $\alpha 2/\delta\Delta$TMIII suggests that the $\alpha 2/\delta$ is a single TM domain protein and the TM region is positioned at the δ subunit. (b) External application of anti-$\alpha 2$ antibodies, prepared for an epitope within the alternatively spliced and 'intracellular' region, inhibits depolarization induced secretion in PC12, further supporting an external location of the $\alpha 2$ subunit and establishing δ subunit as the only membrane anchor for the extracellular $\alpha 2$ subunit.**

Key words: Ca^{2+} channel; PC12 cell; $\alpha 2$ Subunit; δ Subunit; Regulated secretion; Exocytosis

1. Introduction

Diverse types of voltage-activated Ca^{2+} channels have been identified of which L- and N-type have been isolated and their subunits described. The L-type consists of four different subunits $\alpha 1$, $\alpha 2/\delta$, β and γ which have been characterized in detail for rabbit skeletal muscle (for reviews, see [1–6]). The N-type Ca^{2+} channel consists of $\alpha 1$, $\alpha 2/\delta$, and β subunits and an additional 95 kDa polypeptide [7] which is absent from the subunit composition of the L-type channel.

Most attention has been focused on the functional $\alpha 1$ subunit which introduces Ca^{2+} across the cell membrane independently of the other auxiliary subunits [8,9]. The $\alpha 2/\delta$-subunit is the largest integral component of purified voltage-sensitive Ca^{2+} channels and the cDNA encoding the $\alpha 2/\delta$ subunit was first cloned and sequenced from rabbit skeletal muscle [10] and later from rat brain [11] and human brain [12]. A single $\alpha 2/\delta$ precursor encodes two polypeptides, the $\alpha 2$ and δ [13]. A proteolytic cleavage site (Ala^{934}–Ala^{935}), distinguishes between the two polypeptides which are connected by a disulfide bond [14]. The hydropathy plot predicts three putative TM segments [10]. This model was challenged by a biochemical study, suggesting a single TM region in the δ-peptide, placing the $\alpha 2$ peptide entirely in the extracellular domain [13]. Co-expression of $\alpha 2/\delta$ subunit with $\alpha 1C$ (cardiac muscle) subunit in *Xenopus* oocytes increases inward current amplitude without affecting either the kinetics or the number of the channels expressed [15–18]. In addition, expression of $\alpha 1$ is required for proper targeting and distribution of $\alpha 2$ subunit [19], and $\alpha 2/\delta$ modulates the binding affinity of ω-CgTx to the N-type Ca^{2+} channel [20]. No specific function has yet been associated with the $\alpha 2/\delta$ subunit although the existence of five different spliced variants suggests a functional importance [20,21]. Recently, a single TM protein model for the $\alpha 2$ subunit was proposed by the use of site directed antibodies [22].

We performed a deletion analysis of $\alpha 2/\delta$ in order to study its interaction with the $\alpha 1$ subunit and to distinguish between the two proposed secondary structures. In addition, we have generated antibodies against an epitope within the extracellular region, according to the single TM model, and applied them to PC12 cells, monitoring their effect on the L-type Ca^{2+} channel by measuring depolarization mediated secretion.

2. Materials and methods

Expression vector pQE-30 and Ni/NTA agarose were obtained from Qiagen, and pGEX-4T and glutathione-S-Sepharose 4B were obtained from Pharmacia LKB Biotechnology. The ECL detection kit for immunoblotting was obtained from Amersham (UK). [^{3}H]Dopamine from New England Nuclear US. All other reagents were obtained from commercial sources.

2.1. Plasmid construction and cRNA synthesis in vitro

Rabbit skeletal muscle $\alpha 2/\delta$ subunit was obtained from Dr. A. Schwartz (Ohio, USA).

2.1.1. Construction of truncated $\alpha 2/\delta$ subunit. To create an in-frame deletion of the third putative transmembrane domain designated $\alpha 2/\delta$ ΔTMIII, the enzyme *Eco*RV was used to excise from the skeletal muscle $\alpha 2/\delta$ clone a fragment of 332 bp at *Eco*RV site upstream to TMIII (nt 3470–3473). At the 3′ end, *Spe*I was used to cut at nt 3802–3805, downstream to TMIII and the end of the coding region. The truncated $\alpha 2/\delta$ was ligated to give a 332 bp shorter cDNA that was verified by cutting the original and the mutated plasmid with *Xba*I to yield a 600 bp shorter fragment when compared with the original full length cDNA. $\alpha 2/\delta$ ΔTMIII was identical in all other respects (ATG site and poly(A)) to the original $\alpha 2/\delta$ plasmid. The deletion resulted in the removal of 110 amino acids Ilu^{997}–Leu^{1080} which included the third putative TM domain present in the δ subunit. During the ligation 7 amino acids were added at the carboxy-terminal end. The new deleted construct was transcribed to give the appropriate RNA size (Fig. 2A).

2.2. Synthesis of cRNA and expression in Xenopus oocytes

Cardiac α_{1C} subunit mutant (dN60-del1733; α*1C) (5 μg) kindly obtained from Dr. Birnbaumer, (Los Angeles CA), was linearized with *Hin*dIII; and rabbit skeletal $\alpha 2/\delta$ (5 μg) and $\alpha 2/\delta\Delta$TMIII (5 μg) were linearized with *Sca*I. In vitro transcription was carried out at 37°C for 120 min in a volume of 50 μl using an in vitro transcription kit (Stratagene), 5 μg linearized DNA and T7 RNA polymerase (2 units;

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Boehringer-Mannheim). Complementary RNAs were extracted with phenol/chloroform and recovered by ethanol precipitation. The cRNAs obtained were dissolved in water and RNA size and amounts were determined by RNA gel [23]. Frog oocytes were surgically removed from *Xenopus laevis* ovaries and treated with collagenase (Worthington) in calcium-free ND96 buffer for 120 min at 20°C with shaking to remove follicular cells. Stage V and VI oocytes were incubated overnight in ND 96: 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES pH 7.4, supplemented with 100 units/ml penicillin, 100 μg streptomycin and 2.5 mM Na^+-pyruvate at 20°C, before RNA injection. RNAs were transcribed in vitro using T7 polymerase. Oocytes were co-injected with cRNAs of cardiac α*1C (50 pg/μl) with either skeletal α2/δ subunit (50 pg/μl) or α2/δΔTMIII (50 ng/μl), in a final volume of 50 nl/oocyte using the Drummond microdispenser. Oocytes were maintained at 19°C in ND96 buffer, and tested 5–8 days after injection.

2.3. Electrophysiological recordings

Whole cell Ba^{2+} currents were recorded using the two microelectrodes voltage clamped (Dagan 8500) on line with IBM compatible PC using PCLAMP software version 5.5 (Axon Instruments). Voltage and current agar cushioned electrodes (0.3–0.6 MΩ tip resistance) filled with 3 M KCl were used. The oocytes were impaled in ND96 and inward current of 500 ms duration were monitored by voltage steps from −80 to +60 mV, with 30 s intervals, in Ba^{2+} buffer: 40 mM $Ba(OH)_2$, 50 mM NMDG (*N*-methyl-D-glucamine), 1 mM KOH, 0.5 mM niflumic acid, and 5 mM HEPES adjusted to pH 7.4 with methansulphonic acid. Leak and capacitance currents were subtracted on-line by P/4 protocol.

2.4. Generation of GST/α_2 subunit fusion protein and preparation of polyclonal antibodies

An Xba (2675) cDNA fragment of the α_2 subunit, was filled-in, cut with *Dra*I (2160), isolated and subcloned into the bacterial expression vector pGEX-KG [24]. The GST/α2 fusion protein, induced by IPTG (0.1 M) in TG1 cells (*Escherichia coli*), was affinity purified on GST-agarose according to protocols of the manufacturer. The sequential *E. coli* lysate extractions generated a fusion protein with minor impurities in a Coomassie staining. Two rabbits were repeatedly injected with the GST/α2 fusion protein (1 mg/injection in ABM adjuvant). A larger fragment, *Dra*I (2160) and *Nsi*I (2798), was subcloned into the bacterial expression vector pQE30 (Qiagen). The His_6-fusion protein was affinity purified on a Ni/NTA agarose according to manufacturer's protocols. The antibodies were affinity purified on His_6-α2 fusion protein bound to Ni/NTA agarose beads according to manufacturer instructions.

2.5. Preparation of antibodies against the α2 subunit peptide

Antiserum was raised in two rabbits against a 19 amino acid peptide (Lys^{508}–Ile^{526}) of the α2 subunit conjugated to hemocyanin from keyhole limpet hemocyanin (KLH). The peptide is within the alternative spliced region and conserved in rat brain, PC12 cells and skeletal muscle [21]. The antibodies were tested for specificity by enzyme linked immunoabsorbant assay (ELISA) and were shown to interact specifically with the α2 subunit in PC12 cells membranes in a Western analysis.

2.6. Gel electrophoresis and immunoblotting

Oocytes (20–30), preinjected with full-length α2/δ or the truncated form α2/δΔTMIII, were homogenized in 150 μl boiling lysis buffer (5% mercaptoethanol, 2% sodium dodecyl sulfate, 10% glycerol and 50 mM Tris-HCl buffer, pH 6.8) at 100°C for 15 min then spun down at 100,000 × *g* for 30 min at 4°C to pellet the yolk platelets and melanin pigment, and protein was determined according to Peterson method [25]. PC12 cells were solubilized in lysis buffer (see above), centrifuged for 10 min at 10000 × *g* and the supernatant protein content was determined (see above). Proteins were separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using mini gel protein apparatus (Bio-Rad, Hercules, CA) and than transferred to nitrocellulose using a semi-dry electrotransfer apparatus (Pharmacia-LKB-Novablot). The blots were incubated with blocking buffer containing 0.02% Tween-20 and 5% non-fat dry milk in Tris-buffered saline (TBST) overnight, followed by affinity purified anti GST/α2 antibodies (see above) in blocking buffer for 1 h. After five washes with 0.02% Tween-20 in TBS the blot was exposed to peroxidase-conjugated affinity-pure goat anti-rabbit IgG (1:10,000) for 1 hr, washed (× 5) and detected by enhanced chemiluminescence detection system (ECL).

2.7. Cell growth

PC 12 Cells were kindly provided by B. Cohen (Berkeley, CA). Growth medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (Hyclone), supplemented with 10% horse serum, 5% fetal calf serum, 130 units/ml penicillin and 0.1 mg/ml streptomycin. For assays, cells were removed using 1 mM EDTA, replated on collagen coated 12-well plates, and assayed 24 h later.

2.8. [^{3}H]DA release assay

Transmitter release was determined essentially as previously described [26]. Briefly, cells were incubated for 1.5 h at 37°C with 0.5 ml growth medium 0.85 μl [^{3}H]DA (41 Ci/mmol) and 10 μg/ml pargyline, followed by extensive washings with medium (3 × 0.5 ml) and release buffer consisting of (mM): 130 NaCl, 5 KCl, 25 $NaHCO_3$, 1 NaH_4, 10 glucose, and 1.8 $CaCl_2$. In a typical experiment, cells were incubated with 0.5 ml buffer for five consecutive incubation periods of 3 min each at 37°C. Spontaneous [^{3}H]DA release was measured by collecting the medium released by the cells during the two initial 3 min periods. Stimulation (60 mM KCl) of release was monitored during the third period. The remaining [^{3}H]DA was extracted from the cells by overnight incubation with 0.5 ml of 0.1 N HCl. [^{3}H]DA release during each 3 min period was expressed as a percentage of the total ^{3}H content of the cells. Net evoked release was calculated from [^{3}H]DA released during the stimulation period after subtracting basal [^{3}H]DA release in the preceding baseline period.

3. Results

*3.1. Current amplification of α1C and α*1C coexpressed with α2/δ subunit*

To characterize current and kinetic alterations of α1 subunit of the cardiac L-type channel (α1C) by the α2/δ subunit, *Xenopus* oocytes were preinjected with equimolar cRNAs of α1C and α2/δ of rabbit skeletal muscle. An amplified (5-fold) inward current (−150 ± 30 nA) was observed as compared with current mediated by α1C alone (Table 1). The mutated α1C subunit (dN60-del1733; Qin and Birnbaumer, personal communication; [27]); designated α*1C) expresses a large inward current (−270 ± 60 nA) when expressed alone, and an amplified (~13 fold) current (−3499 ± 244 nA; Table 1) when coexpressed with α2/δ subunit of rabbit skeletal muscle. The observed current amplification suggests a strong interaction between α1C and α2/δ.

Hydropathy plot analysis infers a 3TM orientation for α2/δ [9,10], while a biochemical study predicts a single TM region at the δ subunit with α2 flanking outside the cell [13]. To distinguish between these two models and to better define the

Table 1
Amplitude of inward Ca^{2+} currents in oocytes preinjected with cRNA of α1 subunit or its mutated form α*1C alone, or combined with cRNA of the α2/δ subunit of rabbit skeletal muscle

Channel subunit	Current nA	Fold amplification
α1C	−30 ± 30 (n = 6)	
α1C/α2/δ	−150 ± 38 (n = 12)	5
α*1C	−270 ± 60 (n = 4)	
α*1C/α2/δ	−3499 ± 243 (n = 9)	13
α*1C/α2ΔTMIII	−180 ± 34 (n = 9)	none

α*1C is a deleted carboxyl- and amino-terminal cardiac class L-type Ca^{2+} channel (dN60-del1733, 27); α2/δ subunit is the rabbit skeletal muscle [10].
The data correspond to the mean ± S.E.M.; the number of oocytes is shown in brackets. Two-sample Student's t-test were carried out assuming unequal variance. Values of $P < 0.001$.

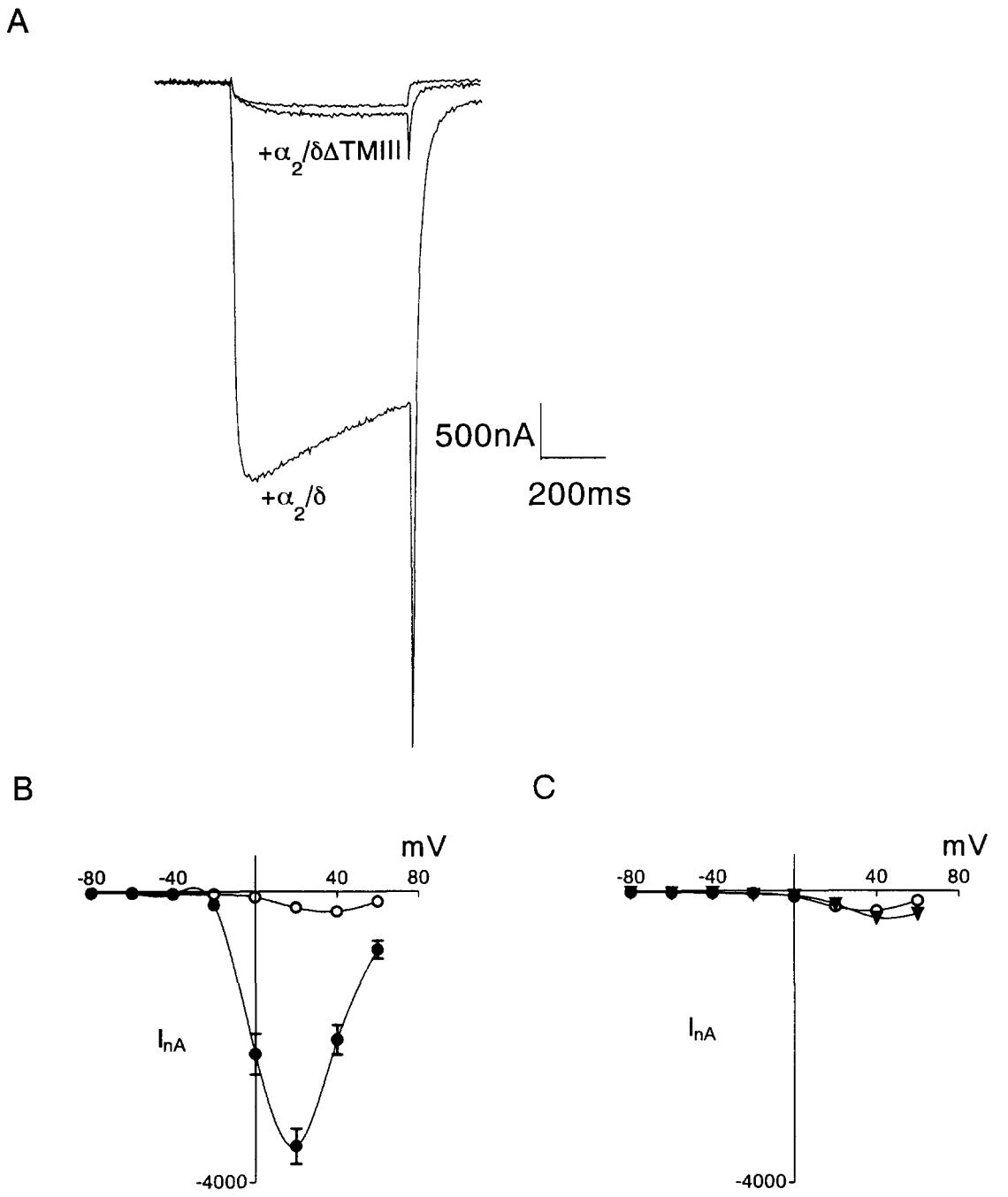

Fig. 1. Amplification of α*1C inward currents by α2/δ subunit and by α2/δDTMIII, a deleted α2/δ subunit. (A) Superposition of macroscopic whole-cell Ba^{2+} currents mediated by α*1C, α*1C/α2/δ and α*1C/α2/δΔTMIII. Currents are evoked in response to a 500 ms test pulse from a holding potential of −80 mV to +20 mV. (B) Leak subtracted current–voltage relationship of α*1C injected alone (○) and in the presence of α2/δ (●). (C) Leak subtracted current–voltage relationship of α*1C expressed alone (○) and in the presence of α2/δΔTMIII (●). Currents were evoked in response to a 500 ms pulse from a holding potential of −80 mV to various test potentials at 10 mV increments (as indicated). The data points correspond to the mean ± S.E.M. ($n = 8$).

topology of a functional interaction between the two channel subunits, we utilized two different approaches.

3.2. Current amplification of α*1C coexpressed with α2/δΔTMIII subunit

We deleted a 330 bp fragment at the C-terminal of α2/δ, excising about one-third of the δ subunit (see section 2), and eliminating the third putative transmembrane domain. The mutated α2/δ subunit, α2/δΔTMIII, was otherwise identical to intact α2/δ, including its initiation site and the poly(A) tail (see section 2).

Xenopus oocytes were injected with α*1C, intact or truncated α2/δ subunit, and their current properties tested. A superposition of α*1C current traces expressed alone and in the presence of intact or truncated α2/δ, clearly demonstrated a large inward current amplification by intact α2/δ and no change in current

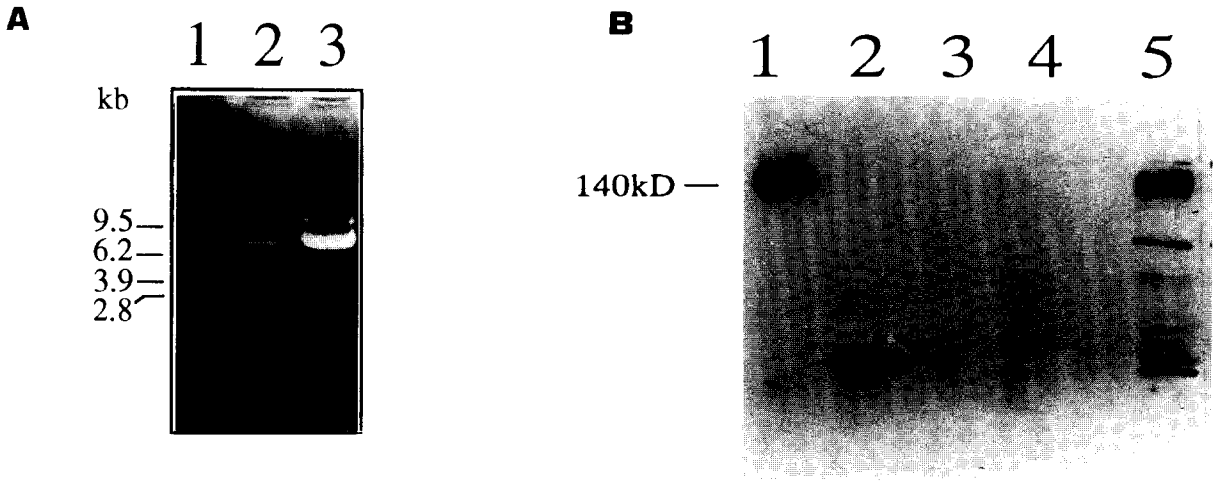

Fig. 2. RNA gel and Western analysis of intact and truncated $\alpha 2/\delta$ subunit ($\alpha 2/\delta\Delta$TMIII) of voltage-sensitive Ca^{2+} channels expressed in *Xenopus* oocytes. cRNAs of full-length and a truncated form of the $\alpha 2/\delta$ subunit (1–3470 bp) and intact $\alpha 2/\delta$ were injected into defolliculated oocytes and after 7 days a crude membrane fraction was prepared and analyzed. (A) RNA gel: RNA markers (lane 1), intact $\alpha 2/\delta$ (lane 2); and $\alpha 2/\delta\Delta$TMIII (lane 3). (B) Western blot analysis: intact $\alpha 2/\delta$ expressed in *Xenopus* oocytes (lane 1), truncated $\alpha 2/\delta\Delta$TMIII subunit expressed in *Xenopus* oocytes (lane 2), molecular weight markers (lane 3), uninjected oocytes (lane 4) and PC12 cell-extract (25 μg protein; lane 5) expressed in *Xenopus* oocytes. Antibodies against GST-fusion protein with $\alpha 2$ (aa 645–856). Protein of 7 oocytes could be detected as a distinctive band in 30 s.

amplitude in oocytes expressing the deleted subunit (Fig. 1A). Leak subtracted currents at increasing test potentials of α*1C expressed alone or with intact $\alpha 2/\delta$ (Fig. 1B) or $\alpha 2/\delta\Delta$TMIII (Fig. 1C) are presented as current–voltage relationships. Unlike intact $\alpha 2/\delta$, the truncated form of $\alpha 2/\delta$ does not amplify α*1C currents and their amplitudes remain similar to those expressed by α*1C alone (Fig. 1C).

3.3. *Expression of $\alpha 2/\delta$ and $\alpha 2/\delta\Delta$TMIII in* Xenopus *oocytes*

cRNAs of $\alpha 2/\delta$ and $\alpha 2/\delta\Delta$TMIII were prepared in vitro (see section 2) and analyzed on RNA gel presented in Fig. 2A. Both cDNAs and their corresponding cRNAs are detected and their expected sizes confirmed.

Protein expression of intact and truncated $\alpha 2/\delta$ subunits was tested 6 days after cRNA injection by Western analysis. Two types of antibodies were used: one type was generated against a GST-$\alpha 2$-fusion protein (Leu^{645}–Tyr^{856}; see section 2) and the other against a 19 amino acid peptide (Lys^{508}–Ile^{526}) within the alternatively spliced region [20–21]. As shown in Fig. 2B, the reduced $\alpha 2/\delta$ is expressed as a 140 kDa protein, identical in size to the reduced form of the $\alpha 2$ subunit present in PC12 cells (Fig. 2, lane 1 and 5, respectively). However, the expected 2TM protein, product of the truncated subunit $\alpha 2/\delta\Delta$TMIII, is not expressed in oocytes (Fig. 2, lane 2). Instead, a small size band recognized by anti $\alpha 2$ antibodies is detected (Fig. 2, lane 2), suggesting that the protein is not properly processed during maturation at the ER and/or onward. This result strongly supports a single TM protein which upon deletion of its membrane anchorage becomes either stacked at the ER with no destination, or a secreted protein. We failed to detect a secreted protein encoded by $\alpha 2/\delta\Delta$TMIII in the extracellular medium, most likely due to its degradtion in the ER. Assuming that the δ subunit is the only membrane-anchor for $\alpha 2$, a reduction of the S–S bond which connects the two parts of intact $\alpha 2/\delta$ subunit, should release $\alpha 2$ free to the extracellular milieu. Indeed, $\alpha 2/\delta$- expressing oocytes, treated with 10 mM DTT and 3 M urea, release free $\alpha 2$ (140 kDa protein) into the external solution (data not shown, see section 4).

3.4. *The effect of anti $\alpha 2$ peptide antibodies applied extracellularly to PC12 cells*

The second approach to establish the topology of $\alpha 2/\delta$ subunit and distinguish between the two existing models is by utilizing specific antibodies against the $\alpha 2$ subunit. Antibodies were prepared against a GST/$\alpha 2$ fusion-protein and a 19 amino acid peptide (section 2). Both epitopes, display 100% sequence homology among rat brain, PC12 cells and rabbit skeletal muscle [21] (epitope location is shown in Fig. 4). According to a 3TM model they are located intracellularly, separating two putative transmembrane domains residues 422–445 and 895–919 [10]. Alternatively, according to a single TM model, the

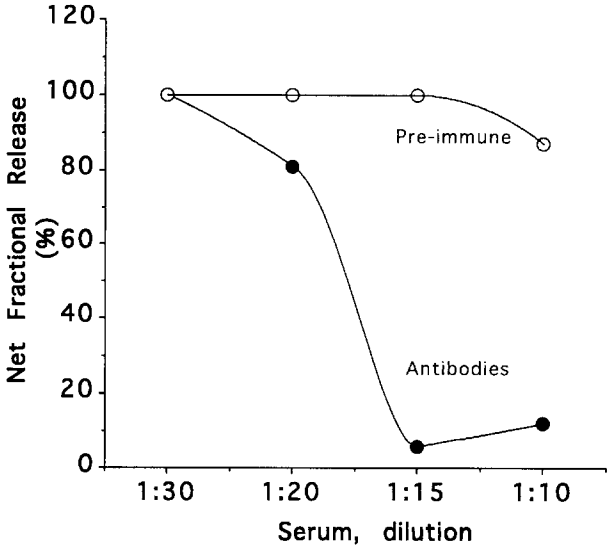

Fig. 3. Inhibition of depolarization induced [^{3}H]dopamine release by $\alpha 2$-antibodies in PC12 cells. Anti $\alpha 2$ antibodies were externally applied to a monolayer of PC12 cells at the dilution, as indicated, for 90 min prior to a 3 min period of KCl induction (60 mM). Inhibition of KCl induced [^{3}H]dopamine release by anti $\alpha 2$ antibodies and preimmune serum is presented as percentage of net fractional release (see section 2). Data is taken from 4 independent experiments using different batches of cells.

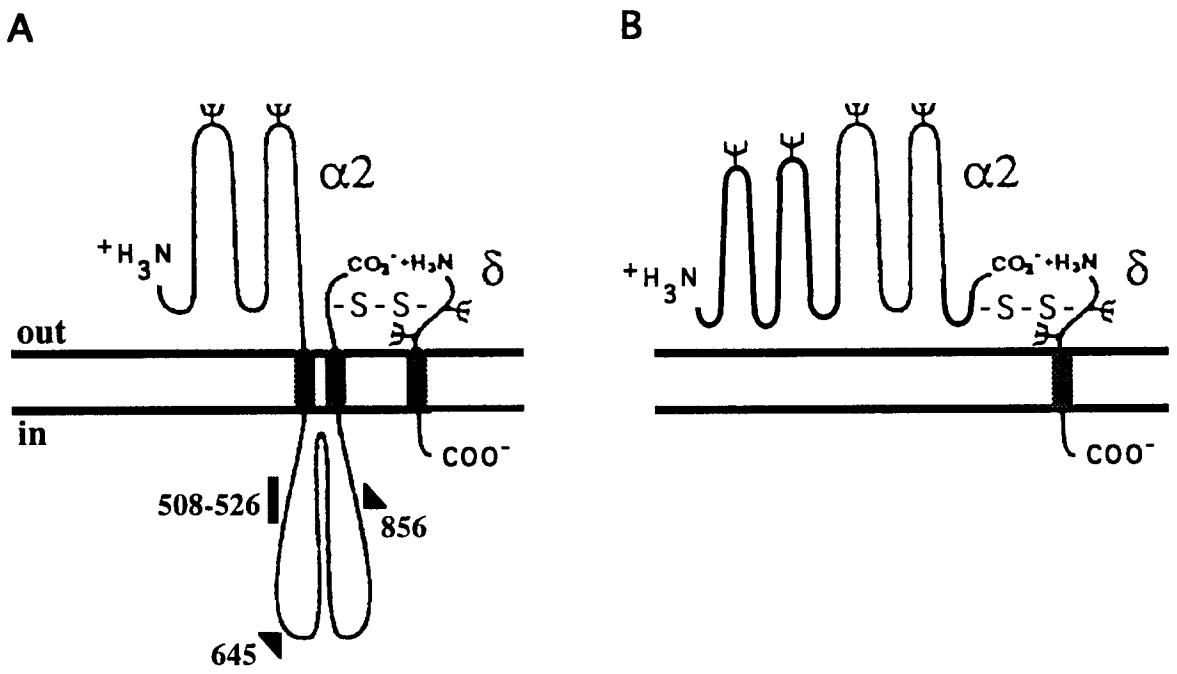

Fig. 4. Alignment of the $\alpha 2/\delta$ polypeptide of voltage-sensitive calcium channel according to current two models. The three TM model is based according to the calculated hydropathy plot [10] and the proposed model of one putative membrane spanning helices is based on results of a biochemical experiment [13]. The sequence location of the two epitopes used for generating antibodies, a 19 amino acid peptide within the alternative spliced region and a GST-fusion protein, are indicated.

epitopes are part of a large extracellular amino-terminal region [13]. The 19 amino acid peptide, used as an epitope, is located next to two deleted segments within the alternative spliced region, at a likely functionally diversified domain of the protein [20–21].

The antiserum raised against the synthetic peptide showed a high titer of anti peptide antibodies when assayed in ELISA using microtiter plate-bound immunizing peptide as antigen (data not shown). They recognized the intact denatured $\alpha 2/\delta$ subunit in a Western blot analysis (Fig. 2). These antibodies were externally applied to PC12 cells, and calcium-dependent [^{3}H]dopamine secretion was induced by high KCl (60 mM; see section 2). A strong reduction in transmitter release was observed at 1:15 and 1:10 dilution's as opposed to a significantly smaller inhibitory effect observed with pre-immune serum (Fig. 3). In addition, bradykinin induced [^{3}H]dopamine release in PC12 cells was not affected by anti-$\alpha 2$ antibodies, indicating the specific interaction of the antiserum with voltage-dependent secretion and excluding non-specific effects of the serum (data not shown).

Antibodies against the GST/$\alpha 2$ fusion protein were not effective at inhibiting KCl-evoked transmitter release, in spite of their high titer determined in ELISA and similar potency to anti-$\alpha 2$ peptide antibodies in a Western blot analysis (see section 4).

4. Discussion

*4.1. Modulation of α*1C by coexpression with intact and truncated α2 subunit*

The pore-forming α*1C subunit was coexpressed in *Xenopus* oocytes with intact $\alpha 2/\delta$ or truncated subunit lacking its third putative TM domain. Alterations of α*1C-mediated currents by $\alpha 2/\delta$ and $\alpha 2/\delta\Delta$TMIII were compared. The truncated form of the $\alpha 2/\delta$ subunit failed to amplify α*1C-mediated inward current. The deleted fragment (970–1080) consists of the putative TMIII and four amino acids placed intracellularly at the carboxy-terminal end. Therefore, lack of current amplification suggests either that $\alpha 2$ is no longer present in the membrane or a loss of $\alpha 2$ interaction site with the α1C subunit.

4.2. Structural characterization of α2/δ subunit

If indeed, the δ-peptide solely anchors $\alpha 2$ subunit at the plasma membrane, $\alpha 2/\delta\Delta$TMIII protein should be processed as a soluble protein and be either stacked in the ER, or be excreted from the cell. In all cases the amplified inward currents should be lost. Alternatively, if the 3TM domain model is correct, a 110 amino acid shorter protein is expected to be expressed in the membrane. A degraded, small size <40 kDa protein is detected in a Western blot analysis of oocytes preinjected with $\alpha 2/\delta\Delta$TMIII (Fig. 2) but not in the extracellular medium. Most likely, an incorrect folded protein is expressed, stacked in the ER without a membrane anchor and therefore failing to be incorporated into the membrane.

These results (a) place the $\alpha 2$ entirely outside the cell and (b) outline the topology of a single transmembrane region to the δ subunit schematically presented in Fig. 4 supporting previous biochemical study [13]. Furthermore, a secondary structure of a single TM protein can better account for the 18 putative glycosylation sites of $\alpha 2/\delta$ placed outside the cell [10]. Unlike the β subunit, that displays pronounced effects on current kinetics [28–30], assigned to the intracellular α1 loop LI-II [30], the $\alpha 2/\delta$ extracellular location restricts its interactions to the extracellular domains of the α1 subunit.

4.3. Analogy to insulin receptor and common features with calcium/polyvalent cation sensing receptor

The structure of insulin receptor is analogous to $\alpha 2/\delta$ subunit of voltage-sensitive Ca^{2+} channels. The glycosylated single polypeptide precursor of the insulin receptor is cleaved into two mature polypeptides during its transport through the ER to Golgi and subsequently to the plasma membrane [31]. The mature insulin receptor is composed of two α and two β subunits, where the extracellular α subunit is linked to the intracellular β subunit by disulfide bonds [31,32]. Under reducing conditions and in the presence of urea, oocytes expressing intact $\alpha 2/\delta$ subunit released $\alpha 2$ subunit into the medium (data not shown). This experiment, similar to that carried out for the insulin receptor [32] further confirms that the S–S bond connects $\alpha 2$ and δ subunits, and the δ is the cell membrane anchor of the $\alpha 1$ subunit.

Recently a calcium/polyvalent cation sensing-receptor has been cloned and functionally expressed in oocytes and HEK cells [33,34]. A typical repeated motif of two adjacent negatively charged amino acids was noticed at the large extracellular region of the receptor and proposed to be the binding site for divalent cations [33,34]. Interestingly, a similar frequency of acidic amino acid pairs is apparent at the $\alpha 2$ sequence, which may serve as a divalent cation-binding site at the $\alpha 2/\delta$ subunit.

While the insulin receptor is activated in response to an exogenous signal, additional experiments will be required to determine whether $\alpha 2$ might interact with an exogenous ligand, and divalent cations, which activate calcium/polyvalent cation sensing-receptor, would be the potential candidates.

4.4. α_2 antibodies

Interaction of anti-$\alpha 2$ antibodies with $\alpha 1C$ was analyzed indirectly by monitoring depolarization-induced [^{3}H]dopamine release. In these cells, depolarization induced secretion is mediated by Ca^{2+} entry through the L-type Ca^{2+} channels (rbC-I transcript; [26]). Depolarization induced [^{3}H]dopamine release was strongly inhibited in cells pre incubated with anti-$\alpha 2$ peptide-serum. On the other hand, bradykinin-induced [^{3}H]dopamine release in PC12 cells was not affected under identical experimental conditions, inferring a specific effect of anti-$\alpha 2$ antibodies on $\alpha 1C$ mediated secretion. The strong inhibition by externally applied antibodies determine extracellular topology of the $\alpha 2$ region recognized by anti-$\alpha 2$-antibodies. Absence of glycosylation sites in bacteria-processed GST/$\alpha 2$ fusion-protein could account for intact $\alpha 2/\delta$ subunit-failing to be recognized by anti GST/$\alpha 2$ antibodies.

Finally, these results suggest that possible interaction(s) between the pore forming subunit $\alpha 1$, and its largest auxiliary subunit $\alpha 2/\delta$, would be restricted to their extracellular domains. In addition, furthere experimentation will be required to evaluate whether the $\alpha 2/\delta$ subunit may play an additionally independent role in regulating depolarization-mediated secretion. In addition, further experimentation will be required to evaluate whether the $\alpha 2/\delta$ subunit may play an additionally independent role in regulating depolarization-mediated secretion.

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