Greater diversity than previously thought of chromaffin cell Ca^{2+} channels, derived from mRNA identification studies

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Abstract Using reverse transcription followed by PCR amplification (RT-PCR), we have identified multiple messenger RNAs encoding for the neuronal pore-forming Ca^{2+} channel subunits α_{1A} (P/Q channel), α_{1B} (N channel), α_{1D} (neuronal/endocrine L channel), α_{1E} (R channel), α_{1G-H} (T channel) and α_{1S} (skeletal muscle L channel) in bovine chromaffin cells. mRNAs for the auxiliary β_2, β_3, β_4, α_2/δ and γ_2 subunits were also identified. In agreement with these molecular data, perforated patch-clamp recordings of whole-cell Ca^{2+} currents reveal the existence of functional R-type Ca^{2+} channels in these cells that were previously undetected with other techniques. Our results provide a molecular frame for a much wider functional diversity of Ca^{2+} channels in chromaffin cells than that previously established using pharmacological and electrophysiological approaches. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Chromaffin cell; Calcium channel; R-type current; α_1 Subunit; β Subunit; γ_2 Subunit

1. Introduction

On the basis to their biophysical, pharmacological and molecular properties, two classes of voltage-dependent Ca^{2+} channels have been identified in neurons. The first class comprises the high-threshold voltage-activated (HVA) Ca^{2+} channels, including the L-, N-, P/Q- and R-subtypes [1–3]. The second class corresponds to the low-threshold voltage-activated (LVA) Ca^{2+} channels or T-type channels [4,5]. HVA Ca^{2+} channels are multimeric complexes formed by a pore-forming α_1 subunit and several auxiliary subunits (β, α_2/δ and γ). Attention has been mostly focused on the functional α_1 subunit, which introduces Ca^{2+} across the cell membrane, independently of the other auxiliary subunits. So far, the Ca^{2+} channel α_1 subunits isolated, sequenced and expressed encode functional L-type (α_{1C}, α_{1D}, α_{1F}, α_{1S}), N-type (α_{1B}), P/Q-type (α_{1A}), R-type (α_{1E}) and T-type (α_{1G}, α_{1H}, α_{1I}) Ca^{2+} channels [6,7].

Several types of HVA Ca^{2+} channels, including L-, N- and P/Q-types, have been well characterized in bovine chromaffin cells using pharmacological and electrophysiological approaches [8–12]. However, controversial results were obtained regarding the existence of R- and T-type Ca^{2+} channels in these cells [9–11,13–18]. To determine, in a more definitive way, the variety of Ca^{2+} channel subtypes expressed by chromaffin cells, we decided to recourse to the more powerful molecular biology approach. Hence, in this study we have investigated the expression of mRNAs for several pore-forming α_1 and auxiliary Ca^{2+} channel subunits in bovine chromaffin cells. Our results reveal that, along with predictable subunits (α_{1A}, α_{1B}, α_{1D}, β_2, β_3, β_4 and $\alpha_2\delta$), mRNAs for unexpected subunits (α_{1E}, α_{1G-H}, α_{1S} and γ_2) are also present in these cells. The identification of the α_{1E} mRNA forced us to reevaluate the presence of a functional R-type Ca^{2+} channel in these cells, using the perforated configuration of the patch-clamp technique; these results are also presented here.

2. Materials and methods

2.1. Isolation and culture of bovine chromaffin cells and RNA extraction

Chromaffin cells were isolated and cultured from adult bovine adrenal glands as previously described [19]. Total RNA was extracted from fresh acutely isolated cells using a Ultraspec™ RNA Isolation System (Biotecx Laboratories, Inc., TX, USA).

2.2. Reverse transcription-polymerase chain reaction (RT-PCR) and sequencing of amplified products

cDNA was synthesized from 5 μg of total RNA with 400 U Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) in a total volume of 20 μl. The PCR was carried out in a volume of 100 μl containing 4 μl of cDNA, 10 pmol of each oligonucleotide primer, 100 μM of dNTP and 5 U of *Taq* polymerase (Quiagen Inc., Germany). The PCR reaction was performed using 40 cycles (94°C for 30 s, 55°C for 30 s, 72°C for 35 s). The following sets of primers were designed to amplify the different Ca^{2+} channel subunits from 5′ to 3′; numbers in brackets indicate the accession number (before) and initial position of the primer (after) the sequence: rat brain α_{1A} (M64373) sense GAT GAA CAA GAA GAG GAA GAG G (2182), α_{1A} antisense CTT GTT GGT GTT GTT GTT ACG G (2514); rat brain α_{1B} (M92905) sense TGG AGG GCT GGA CTG ACA T (936), α_{1B} antisense GCG TTC TTG TCC TCC TCT GC (1218); rat brain α_{1C} (M67515) sense AAG ATG ACT CCA ACG CCA CC (712), α_{1C} antisense GAT GAT GAC GAA GAG CAC GAG G (1106); rat brain α_{1D} (M57682) sense TGA GAC ACA GAC GAA GCG AAG C (2560), α_{1D} antisense GTT GTC ACT GTT GGC TAT CTG G (2926); rat brain α_{1E} (L15453) sense ATC TTA CTG TGG ACC TTC GTG C (4833), α_{1E} antisense CTC AGT GTA ATG GAT GCG GC (5339); rat brain α_{1G}/human heart α_{1H} (AF027984 and AF073931) sense GTG GTC CTC GTC WTC ATC TTC C (α_{1G} 4260, α_{1H} 3969), α_{1G-H} antisense AAT GAT GAA GAA GGC ACA GC (α_{1G} 4694, α_{1H} 4403); rat skeletal muscle α_{1S} (L04684) sense CAG TAT CAG GTG TGG TAT GTC G (1183), α_{1S} antisense CTG GAA GGA CTT GAT GAA CG (1656); human brain β_1 (M92303) sense CGA GGA GAA GAC TAT GAG GAA (1799), β_1 antisense ATC CAT CCA CAA CAG GCA GG (2155); human

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brain β_{2a-c} (AF137377 and HSU95019) sense AAC AAT GAC TGG TGG ATA GGG (β_{2a} 304, β_{2c} 799), β_{2a-c} antisense CAT CAT ACG GAG GAG TGT GC (β_{2a} 670, β_{2c} 1165); rat brain β_3 (M88751) sense CTG AGT GAC ATT GGC AAC CG (523), β_3 antisense TCT TGC CAG GAT TGT TCA GC (796); human brain β_4 (AF038852) sense CGG ATT CCT ACA CAA GCA GG (133), β_4 antisense ATC CGT ATG TTC TCC AAT CTG A (463); rat brain α_2/δ (M86621) sense GAG GAC CTA TTC AGT TGG ATG GC (1338), α_2/δ antisense CTT CCA GAG ACA CAT CAA CGC C (1637); mouse brain γ_2 (AF077739) sense CTC CTG AGA ACC AGA AGT CG (344), γ_2 antisense CAC TCA GGA TGA TGT TGT GGC G (801).

After PCR amplification, 10 μl of each individual reaction sample was run on a 1.5% agarose gel stained with ethidium bromide, using a 100 bp DNA ladder as molecular weight marker (GIBCO/BRL). The predicted sizes of the PCR products were (in base pairs): α_{1A} (332), α_{1B} (282), α_{1C} (394), α_{1D} (366), α_{1E} (506), α_{1G-H} (434), α_{1S} (437), β_1 (356), β_{2a-c} (366), β_3 (273), β_4 (330), α_2/δ (299), γ_2 (457). Reaction samples containing the RT-PCR products with the predicted sizes were run in an agarose gel and the bands extracted using a commercial kit according to the manufacturer's instructions (Geneclean Kit from BIO101, CA, USA). Sequencing of these PCR products was performed using the Big Dye method and the automatic sequencer ABI PRISM 377 (Perkin Elmer).

2.3. Electrophysiological recordings of whole-cell Ca^{2+} channel currents using the perforated patch configuration

Ca^{2+} currents were recorded in 2–5 days old cells with borosilicate glass electrodes of 2–4 MΩ resistance, mounted on the headstage of a DAGAN PC-ONE patch-clamp amplifier. The pipette solution contained (in mM): Cs–glutamate 145, NaCl 8, $MgCl_2$ 1, HEPES 10, as well as 0.5 mg/ml amphotericin B freshly prepared (pH 7.2 with CsOH). The bath solution had the following composition (in mM): NaCl 145, KCl 5.5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 10 (pH 7.2 with NaOH). Currents were filtered at 3 kHz and sampled at 12.5 kHz. Only cells with resting currents smaller than 5 pA were used. Cadmium subtraction was performed off line for analysis of Ca^{2+} currents [20]. An Instrutech ITC-16 controlled by a Macinstosh Power PC 8200/120 running IGOR (Wavemetrics, Lake Oswego, OR, USA), and the Pulse Control XOP's (J. Herrington and R.J. Bookman, University of Miami) were used as acquisition system. The analysis of the data was conducted on a Macintosh computer using IGOR Pro (Wavemetrics, Lake Oswego, OR, USA).

2.4. Materials and solutions

The products not specified were purchased from Sigma (Madrid, Spain). Amphotericin B and tetrodotoxin were obtained from Calbiochem-Novabiochem (La Jolla, CA, USA); CsOH was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). ω-Conotoxin GVIA and ω-conotoxin MVIIC were purchased from Bachem (Bubendorf, Switzerland); ω-agatoxin IVA was from Peptide Institute (Osaka, Japan). Toxins were prepared in distilled water at 10^{-4} M. Nifedipine was dissolved in dimethylsulfoxide (DMSO) and kept at 10^{-2} M as stock solution protected from light. Concentrate stock solutions were aliquoted and stored at −20°C until use. Final concentrations of drugs were prepared in the HEPES extracellular solution.

3. Results and discussion

3.1. mRNA expression of multiple α_1 subunits encoding for Ca^{2+} channels in bovine chromaffin cells

RT-PCR was performed on total RNA isolated from bovine chromaffin cells using a selective and powerful set of primers designed against the different α_1, β, α_2/δ and γ subunits identified in other tissues and species. From the seven pairs of primers for the α_1 subunits tested, six of them amplified products corresponding to the predicted sizes of α_{1A}, α_{1B}, α_{1D}, α_{1E}, α_{1G-H} and α_{1S} subunits (Fig. 1); the identity of these products was further confirmed by subsequent sequencing (see GenBank accession numbers at the end of this section). However, since PCR can amplify even a single transcript, and it is virtually impossible to purify chromaffin cells to 100% homogeneity, we cannot exclude that some of the above transcripts might come from cells other than chromaffin cells. Nevertheless, the identification of α_{1A}, α_{1B} and α_{1D} mRNAs in the present study agrees well with previous pharmacological and electrophysiological data indicating the presence of functional P/Q (α_{1A}), N (α_{1B}) and L channels (α_{1D}) in bovine chromaffin cells; in fact, the α_{1B} subunit from bovine chromaffin cells has been recently cloned [21]. Our study shows, for the first time, that the dihydropyridine-sensitive Ca^{2+} channel subtype present in bovine chromaffin cells corresponds to the L-type channel encoded by one α_{1D} gene, highly homologous to that identified in brain and endocrine cells [22,23]. However, since α_{1D} and α_{1C} subunits were expressed together in many peripheral tissues [24], it is likely that this last subunit was also present in chromaffin cells. Thus, the fail to detect α_{1C} transcripts in these cells could reside in the low homology between bovine chromaffin cells and rat brain in this region of the α_{1C} gene and/or the low expression level of the α_{1C} subunit mRNA in bovine chromaffin cells. In addition, we have identified the mRNA encoding for the α_{1S} subunit in chromaffin cells, although we do not know whether this subunit is translated into a functional skeletal-like L-type Ca^{2+} channel.

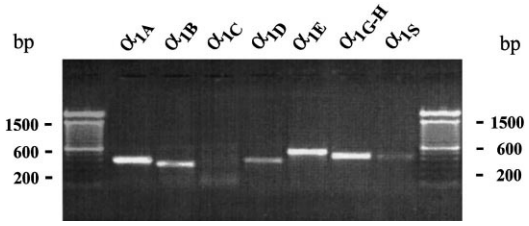

Fig. 1. mRNA expression of different Ca^{2+} channel α_1 subunits in chromaffin cells. Agarose gel separation of the RT-PCR products obtained from total RNA of bovine chromaffin cells; selective primers for α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, α_{1G-H} and α_{1S} subunits were designed as described in Section 2. Molecular weights of the standards are shown on the left and right sides of the figure. Three different and independent RNA extractions from batches of cells isolated in different days, provided similar results to those shown in this figure.

One unexpected finding was the detection of two PCR products corresponding to the α_{1E} and α_{1G-H} subunit mRNAs. The sequenced region of the α_{1E} subunit amplified by RT-PCR from bovine chromaffin cells shows the highest homology (92%) with nucleotides 4787–5286 from human neuronal α_{1E} subunit (accession no. L277745). Also, the sequenced region of α_{1G-H} subunit in bovine chromaffin cells shows the highest homology with its counterpart in humans: 91 and 86% homology respectively, with nucleotides 3964–4403 from human heart α_{1H} subunit (accession no. AF073931), and nucleotides 3947–4381 from human neuronal α_{1G} subunit (accession no. AF126966). Thus, it is likely that both α_{1E} and α_{1G-H} mRNAs encode for functional R- and T-type Ca^{2+} channels in bovine chromaffin cells. Concerning the α_{1G-H} subunit, most of the studies performed until now have failed to detect the presence of a functional T-type Ca^{2+} channel in chromaffin cells. As far as we know, only two groups have reported a tetrodotoxin-resistant and low-threshold transient component of the Ca^{2+} current in a subset of bovine (91 of 130 cells tested) and rat (68 of 290 cells tested) chromaffin cells [13,18]. We do not know why these currents were not detected by other authors, including our own laboratory; possible explanations could be the enzymatic digestion used for the isolation of the cells, which might modify the set

of proteins expressed at the membrane. However, we have to consider that the identification of α_{1G-H} mRNA in chromaffin cells does not mean that the amount of protein synthesized and incorporated into the cell membrane is high enough to be easily detected as a functional T-type current.

3.2. Identification of a functional R-type Ca^{2+} current in bovine chromaffin cells

A great controversy exists in the literature regarding the presence or absence of R-type Ca^{2+} currents in chromaffin cells. Thus, using the whole-cell configuration of the patch-clamp technique [25], we and others have been unable to detect a functional R-type current in chromaffin cells of different species [14–16,20,26,27]. In contrast, Hollins and Ikeda [18] and Currie and Fox [17], using the same patch-clamp configuration, found a small resistant current after the perfusion of rat or bovine chromaffin cells with different Ca^{2+} channel blockers. However, these authors have used ω-conotoxin MVIIC as blocker of P/Q channels, and 10 mM Ca^{2+} as charge carrier. We have described previously that the binding of toxins (particularly ω-conotoxin MVIIC) is extremely affected by the concentration of the extracellular divalent cation [14,28], and hence, the resistant current observed could be due to an artifact. Our present finding of one mRNA for the α_{1E} subunit in bovine chromaffin cells forced us to reconsider the existence of a functional R-type Ca^{2+} current in these cells, and to perform new patch-clamp experiments.

This time, in order to detect more accurately the presence of the R current, we decided to use the perforated patch-clamp technique, to prevent the run-down of the current components, which might explain previous failures in detecting the current. In addition, to avoid excess Ca^{2+} which might reduce the toxin binding to its channel receptor, 2 mM Ca^{2+}, and not 10 mM Ca^{2+}, was used. Ca^{2+} currents (I_{Ca}) were elicited by 50 ms depolarizing pulses of 0 mV, applied to bovine chromaffin cells held at a membrane potential (V_m) of −70 mV. Fig. 2A shows the time course of the I_{Ca} blockade produced by a cocktail containing supramaximal concentrations of three Ca^{2+} channel blockers (3 μM nifedipine for the L-type, 1 μM ω-conotoxin GVIA for the N-type, and 5 μM ω-conotoxin MVIIC for the P/Q-type). The initial current of 180 pA was reduced to 25 pA after superfusion with the cocktail (14% of the initial I_{Ca}). After the remaining current reached a steady-state, two different concentrations of ω-agatoxin IVA (300 nM or 2 μM), sequentially added to the cocktail to ensure the complete blockade of P/Q-type channels [3], did not produce any additional inhibition of the current. These results indicate that, under our experimental conditions, 5 μM ω-conotoxin MVIIC was able to fully block P/Q-type channels. Finally, the resistant current was completely inhibited by perfusion of 200 μM $CdCl_2$. Original traces of I_{Ca}, upon the addition of the different blockers, are shown in Fig. 2B; results reveal that the resistant current (traces b, c and d) did not exhibit an increased inactivation with respect to the control current (trace a). In 12 cells tested as above, the resistant I_{Ca} contributed 14.5 ± 1.5 and 10.8 ± 1% to the control Ca^{2+} current, when currents were measured at the peak or at the end of the pulse, respectively (Fig. 2C).

Thus, the present electrophysiological results support the existence of a functional R-type Ca^{2+} current in bovine chromaffin cells; this current was most likely due to the expression of the α_{1E} subunit identified in these cells. However, in contrast to the fast inactivation of the R-type current in cerebellar granule cells [3,29], or the inactivating α_{1E} and doe-1 currents expressed in *Xenopus* oocytes [30,31], the R-type Ca^{2+} current identified in bovine chromaffin cells inactivates relatively slowly; this current is, therefore, similar to other R-type currents (G2 and G3) identified in rat cerebellar granule cells [32]. This functional diversity of native R-type channels is consistent with the different biophysical properties reported for the α_{1E} subunit coexpressed with different β subunits [30,33,34].

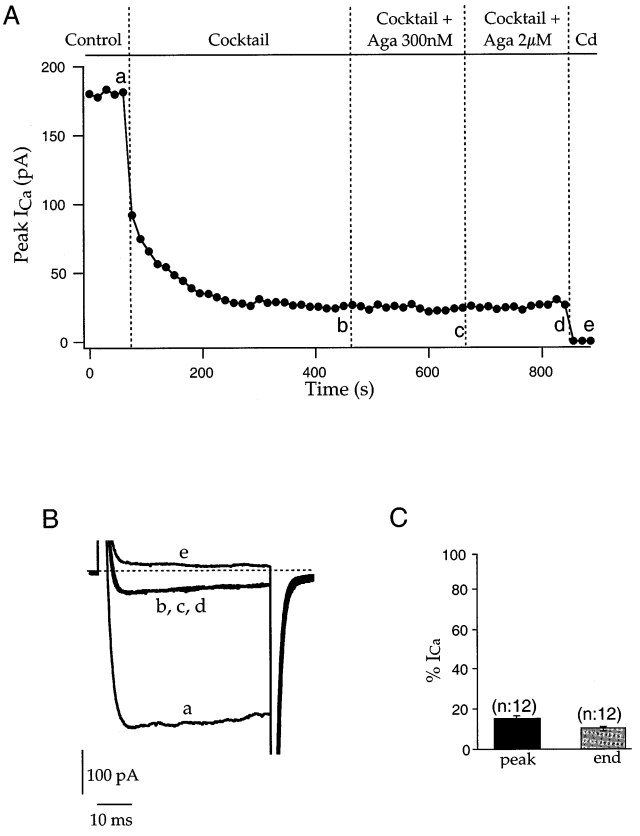

Fig. 2. A: Time course of Ca^{2+} current (I_{Ca}) blockade induced by various Ca^{2+} channel blockers in bovine chromaffin cells, under the perforated patch configuration. Cells were voltage-clamped at a holding potential of −70 mV; inward currents through Ca^{2+} channels were elicited by test depolarizing pulses to 0 mV, applied at 15 s intervals (abscissa). The cocktail of Ca^{2+} channel blockers containing 3 μM nifedipine, 1 μM ω-conotoxin GVIA and 5 μM ω-conotoxin MVIIC was superfused for 6.75 min; subsequently, 300 nM ω-agatoxin IVA (Aga, 3.75 min) or 2 μM ω-agatoxin IVA (3.25 min) were superfused along with the cocktail. At the end of the experiment, 200 μM $CdCl_2$ was superfused to block the resistant Ca^{2+} current. B: An example of original traces obtained in the absence of blockers (control I_{Ca}, trace a), in the presence of the cocktail (trace b), in the presence of the cocktail plus the lowest concentration of Aga (trace c), or in the presence of the cocktail plus the highest concentration of Aga (trace d); trace e represents the current upon the superfusion of $CdCl_2$. C: Bars represent the means ± S.E.M. of the resistant I_{Ca} measured at the peak and at the end of the 50 ms depolarizing pulse, upon the addition of the cocktail in the number of cells shown in parentheses; currents were expressed as percentage of the control I_{Ca}.

3.3. Identification of multiple auxiliary subunits encoding for Ca^{2+} channels in bovine chromaffin cells

Using specific primers for the mouse neuronal γ_2 subunit gene [35] we have identified, by the predicted size and sequence (see accession numbers at the end of this section),

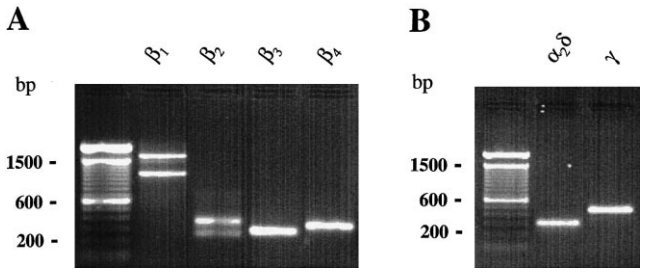

Fig. 3. Expression of the mRNAs encoding for different auxiliary Ca^{2+} channel subunits in chromaffin cells. A: Agarose gel electrophoresis of the products amplified by RT-PCR using total RNA obtained from bovine chromaffin cells; human brain (β_1, β_2 and β_4) and rat brain (β_3) primers were used. Two RT-PCR products for the β_2 subunit were identified; bands in the β_1 lane most likely correspond to non-specific amplifications. B: The same, but with rat brain $\alpha_2\delta$ and mouse brain γ_2 primers. On the left side of each panel, molecular weights of standards are shown.

the expression of one mRNA for this subunit in chromaffin cells (Fig. 3B). This finding, along with the recent cloning of two isoforms of the γ subunit gene in human brain [36], suggests that this subunit could be an additional component of different Ca^{2+} channel subtypes in neuronal cells, and not a specific subunit of the skeletal muscle L-type Ca^{2+} channels [37].

In addition, from the five pairs of primers specifically designed to detect other Ca^{2+} channel auxiliary subunits, four of them amplified chromaffin cell cDNA products corresponding to the predicted sizes and sequences for the β_2, β_3, β_4 and α_2/δ subunits (Fig. 3A and B; accession numbers at the end of this section); the two bands in the β_1 lane correspond to non-specific amplifications given their sizes and sequences. During the preparation of this manuscript, we have been aware of the paper of Cahill et al. [21] cloning the β_{1b}, β_{1c}, β_{2a}, β_{2b} and β_{3a} Ca^{2+} channel subunits of bovine chromaffin cells. Thus, the failure to detect β_1 subunits in our study was due to the low homology between the β_1 primers designed from human brain and the corresponding region of the β_1 gene in bovine chromaffin cells. The two RT-PCR products obtained for the β_2 subunit in the present study (Fig. 3A) most likely correspond with different isoforms of this subunit. The pair of primers used in our study recognized a region of the β_2 subunit gene shared by the isoforms β_{2a}-β_{2c} of human brain and the isoforms β_{2a}-β_{2b} of bovine chromaffin cells; hence, it is likely that one of the two RT-PCR products obtained in our study corresponds with the β_{2a} and the β_{2b} isoforms cloned in chromaffin cells (whose size was 366 bp), whereas the other amplified product (whose size was around 250 bp) could correspond to the β_{2c} isoform of chromaffin cells, which remains to be cloned. Furthermore, our results show, for the first time, the expression of a β_4 subunit mRNA in chromaffin cells.

It is known that α_1 subunits determine the major biophysical properties of the Ca^{2+} channel current subtypes, whereas different β subunits modify the properties of the channel. Our results reveal the presence of multiple types of pore-forming α_1 and auxiliary subunits (α_{1A}, α_{1B}, α_{1D}, α_{1E}, α_{1G-H}, α_{1S}, β_2, β_3, β_4, α_2/δ and γ_2), that offer multiple possibilities for combinations among them. We believe that the results of our study extend the actual view about the diversity of Ca^{2+} channel subtypes in chromaffin cells, and provide new basis for their biophysical characterization. The GenBank accession numbers for the newly identified subunits in chromaffin cells are: AF273327 (α_{1A}), AF273328 (α_{1D}), AF244126 (α_{1E}), AF245161 (α_{1G-H}), AF273329 (α_{1S}), AF273332 (β_4), AF273331 (α_2/δ) and AF273330 (γ_2).

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