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ANTI-β2 SUBUNIT ANTISENSE OLIGONUCLEOTIDES MODULATE THE SURFACE EXPRESSION OF THE α1 SUBUNIT OF N-TYPE ω-CTX SENSITIVE Ca^{2+} CHANNELS IN IMR 32 HUMAN NEUROBLASTOMA CELLS

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Summary - High voltage activated Ca^{2+} channels are heteropolymeric complexes in which the α_1 subunit forms the channel, while the α_2-δ and β subunits are important for the assembly and regulation of the biophysical properties of the channel. We have tested the role of the β_2 subunit on the expression and electrophysiological properties of the ω-conotoxin GVIA-sensitive Ca^{2+} channel expressed in the IMR 32 human neuroblastoma cell line. Anti-β_2 subunit antisense oligonucleotides supplied to the cells in culture induced a time-dependent increase in the number of [^{125}I]-ω-conotoxin binding sites on the cell surface, which was not paralleled by an increase in current amplitude. We suggest that a reduction in the expression of β_2 stimulates the transport to the plasma membrane of non-functioning Ca^{2+} channels and, in particular, of the α_1 ω-conotoxin binding subunit.

Several subtypes of high voltage activated (HVA) Ca^{2+} channels are expressed in neurons and neuroendocrine cells (1, 2) and seem to share a common structure with the well-characterised DHP-sensitive Ca^{2+} channels of skeletal muscle (3): an α_1 dihydropyridine (DHP) binding subunit forming the ion channel (175 kd), and three auxiliary proteins, α_2-δ (160 kd), γ (32 kd) and β (52 kd) (4). Five classes of cDNA (A to E) coding for Ca^{2+} channel α_1 subunits have been cloned from rat brain (5, 6). Several cDNAs encoding β subunits and one gene encoding the neuronal α_2-δ subunit have also been cloned from neuronal tissues (7-10). Human B and D α_1 subunits, respectively blocked by ω-conotoxin GVIA (ω-ctx) and DHPs, as well as β_2 and α_2-δ clones, have been characterized in the IMR 32 neuroblastoma cell line (7, 11). These human tumoral cells have been previously shown to express [^{125}I]-ω-conotoxin binding sites (12) and HVA Ca^{2+} currents with a predominant ω-ctx component (13). The growing number

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of identified Ca^{2+} channel subunits opens the question of their roles in channel physiology and of their specific associations. Although the contribution of the various α_1 subunits to the pharmacological diversity of HVA Ca^{2+} channels has been partially defined, the role of α_2-δ and β subunits is still poorly understood. Studies in reconstituted systems like *Xenopus* oocytes and transfected mammalian cells come to the conclusion that the coexpression of β and/or α_2-δ subunits with α_1 enhances the amplitude of the expressed currents, increases the activation and inactivation rates of the channels, and also changes some of their pharmacological properties (7,11, 14-18). In these systems all of the β subunits tested can influence the expression of different types of α_1 subunit in reconstituted system, albeit with different potency (5). Therefore one of the questions not solved by reconstituted systems obviously concerns the "in vivo" combination of the different β subunits with the different α_1 subunits. The depletion of a single specific protein in a polymeric complex could give valuable information on its function in a system not far from its natural environment (19). Using anti-β_2 antisense oligonucleotides, we have studied the possible interaction of the β_2 subunit (the expression of which seems to be specifically restricted to neuronal and neuroendocrine cells) with the α_1 subunit of ω-ctx sensitive channel, finding that supplementation of the medium with anti-β2 oligonucleotides increases the number of surface [^{125}I]-ω-ctx binding sites without causing a comparable increase in current amplitude. These data suggest that "in vivo" β2 subunits interact with ω-ctx sensitive α_1 subunits, and that a reduction in β2 expression stimulates the transport to the membrane of a ω-ctx binding α_1 subunit, which fails to give functional channels.

MATERIALS AND METHODS

Cell culture and oligonucleotide treatment - IMR32 human neuroblastoma cells (20), were grown as previously described in the presence of 1 mM dibutyrryl-cAMP and 2.5 μM 5-bromodeoxyuridine to achieve a more differentiated phenotype (21). The cells were plated at a concentration of $10^4/cm^2$ in 24-well plates for [^{125}I]-ω-ctx binding assay, and on glass coverslips coated with 0.01% gelatine for electrophysiological recordings. After 6 days of differentiation, the cells were divided into three groups and further cultured with medium containing 5 μM sense, antisense or no oligonucleotide. The medium was replaced every 48 hr. The phosphorotioate oligonucleotide sequences targeted to the ATG codon were as follows: AS β_2-CAT GGT GGT GAA TCT; S β_2-AGA TTC ACC ACC ATG.

RT-PCR analysis of messenger RNA - Reverse transcription of RNA was performed using a first strand cDNA synthesis kit (Stratagene). Polymerase chain reaction was performed according to Promega instructions during 24 cycles (94°C 1 min, 56°C 1 min, 72°C 1 min). PCR primers and internal oligonucleotides for hybridisation, drawn on β_2 published sequence (9), were as follows: 5'pr-CTC GGC TCC AGC AAA TCA GG; 3'pr-TGT CAG CAT CCA GAG CGA CCA; I.ol-CTG AAG GGC TAC GAG GTC ACA G. The amplification products were analyzed by means of Southern blot, using the internal oligo (I.ol) as ^{32}P radiolabelled probe.

Western blot - A polyclonal antibody (pAb.β_2#2)was raised against the C-terminal sequence of the human β_2 (7) and affinity purified against the same synthetic peptide (GGLESSQRGSVVPQEQENAM). The specificity of the antibody was tested by ELISA against the specific peptide and peptides drawn on other human β subunits. For Western blot analysis, cells harvested after 6 days of oligonucleotide treatment were homogenized in IB buffer (250 mM sucrose, 5 mM Hepes, 2 μM leupeptin, 1 μM pepstatin, 0.5 mM PMSF, pH 7.4) and centrifuged at 1000 g for 10 min. The supernatant was centrifuged at 36500 g for 20 min. The pellet thus obtained was dissolved in IB buffer. 50 μg samples separated on SDS-PAGE were transferred to nitrocellulose membranes that were probed with 0.8 μg/ml affinity-purified

antibody. Developing was performed using the ECL chemiluminescent method (Amersham). The quantitation of signal intensity by image analysis was performed using Macintosh IMAGE 1.47 software.

[^{125}I]-ω-ctx binding assay - A modification of a previously described binding assay protocol (12) was used. Intact adherent cells. were washed twice with Dulbecco's modified PBS containing 0.1% BSA. 50 pM [^{125}I]-ω-ctx was then added to the cells for 2h at room temperature. After washing the cells were extracted in 0.5N NaOH and bound radioactivity determined. Each point was evaluated in triplicate. Non-specific [^{125}I]-ω-ctx binding in the cells was evaluated in parallel in the presence of 200 nM unlabelled toxin. Under these conditions, non-specific binding was about 15% of total binding.

Electrophysiological recordings - Electrophysiological experiments on IMR 32 cells were carried out using the standard whole patch clamp technique (22) as previously described (13). After establishing the whole-cell clamp conditions, the cells were held at -90 mV. Step depolarizations from the holding potential lasted 100-200 ms, and were applied at intervals of 5-10 s to allow recovery from inactivation. The external bath contained (in mM): 125 NaCl, 10 $BaCl_2$, 1 $MgCl_2$, 10 Na Hepes (pH 7.3) and 1 μM tetrodotoxin. The solution filling the pipette contained (in mM): 110 CsCl, 30 tetraethylammonium chloride, 2 $MgCl_2$, 10 EGTA, 8 glucose, 4 Mg ATP and 10 CsHEPES (pH 7.3).

RESULTS

β2 antisense oligonucleotides up-regulate ω-ctx binding sites on IMR 32 plasma membrane. The effects of AS oligonucleotides on β_2 synthesis in IMR 32 cells was checked by means of Western blot analysis. In Western blots, the pAb.β_2#2 antibody recognised a protein with an apparent molecular weight of about 60KD. This protein was not present in a non-neuronal human adenocarcinoma cell line (A549) (Fig.1A), which also did not express β_2 subunit mRNA (Fig.1B). The level of expression of this protein in AS-treated IMR 32 cells (mean value 130±34 in arbitrary units) was reduced by more than 50% (n=4) in comparison with control cells (mean value 267±57 in arbitrary units) (Fig.2). The surface expression of ω-ctx α_1 calcium channel subunit was monitored by means of [^{125}I]-ω-ctx binding every 2nd day from the beginning of the oligonucleotide treatment. A reduction in β_2 expression was accompanied by a time-dependent increase in surface [^{125}I]-ω-ctx binding (Fig. 3), which reached its maximum (437%±22.2 of control values; n=3) on day 6 and returned to baseline levels on day 8. [^{125}I]-ω-ctx binding in S-treated IMR 32 cells remained constant (Fig. 3). The three differentially-treated groups of cells did not differ in terms of their morphologic aspect, level of differentiation or in cells number. It can therefore be concluded that treatment with antisense oligonucleotides targeting the β2 Ca^{2+} channel subunit specifically and dramatically increases the number of ω-ctx binding sites expressed on the plasma membrane of IMR 32 cell in a time-dependent manner.

β2 antisense oligonucleotide effect on HVA macroscopic Ca currents expressed by IMR 32 cells. The whole cell patch clamp technique was used to measure the inward Ba^{2+} currents carried by voltage dependent Ca^{2+} channels in the same differentiated IMR 32 cells treated with S and AS oligonucleotides. The cells from all three groups expressed HVA Ca^{2+} channels. Peak current amplitudes were measured holding the cells at a Vh of -90 mV and using pulses to 10 mV. Control cell had currents ranging from 20 to 1100 pA, with mean of 255 (n=63); AS

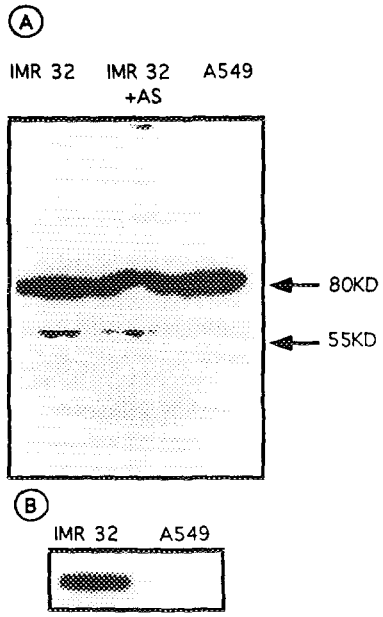
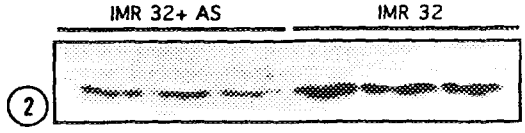


Fig. 1. **(A)** Western blot analyses with pAb.β_2#2 antibody of samples enriched with membrane proteins. From left to right, 50μg samples from: IMR 32; IMR 32 treated with AS oligonucleotides; and A549 adenocarcinoma cells. Two bands were recognized by pAb.β_2#2 antibody (MW=60 and 80 kd). β_2 was expected to be the one of 60KD for its size; indeed the 60 kd protein was specifically expressed, in concomitance with β_2 mRNA, in IMR 32 cells and not in A549 cells, while the 80KD protein was expressed also in A549 cells.
(B) Southern blot of RT-PCR amplification products with specific ^{32}P-probes for the β_2 subunit showing that, while β_2 mRNA is present in IMR 32 cells, it is not expressed in A549 cells.

treated cells had currents ranging from 0 to 1070 pA, with mean of 220 (n=88); S treated cells had currents ranging from 0 to 1100 pA, with mean of 260 (n=36). Furthermore pharmacology of the observed currents did not reveal any significant differences between the three groups.



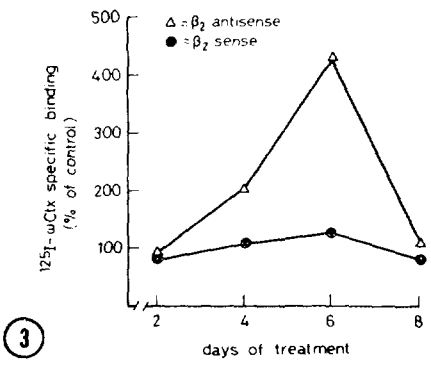


Fig. 2. Western blot analyses of samples from IMR 32 cells treated with AS oligonucleotides (left) and untreated control IMR 32 (right).

Fig. 3. [^{125}I]-ω-ctx specific binding to IMR 32 cells on different days of AS treatment (Δ). Control values were not significantly different from those of the S-treated cells (●). Each value in this representative experiment is the mean of triplicate samples. Non-specific binding was measured as described in the text.

5μM ω-ctx irreversibly blocked a major component of the HVA currents in all of the groups, leaving a minor nitrendipine-sensitive component (data not shown). There is therefore a large discrepancy between the 4-fold increase in the number of surface ω-ctx binding sites and the unchanged amplitude of currents.

DISCUSSION

In *Xenopus* oocytes and transfected human embryonic kidney cells, the human β_2 subunit can combine with either the DHP-sensitive α_{1D} subunit, or the ω-ctx sensitive α_{1B} subunit to give functional Ca^{2+} channels (7,11). In IMR 32 cells, that represent a good model of human peripheral neurons, we found that inhibition of the expression of the β_2 subunit cells alters the number of ω-ctx binding sites expressed in the plasma membrane. This finding strongly suggest that, in "in vivo" IMR 32 cells , the β_2 subunit interacts with the ω-ctx binding α_{1B} subunit. It has been recently demonstrated by Campbell and collaborators that, in rabbit brain, the ω-ctx binding α_1 subunit is associated with the β_3 subunit (3). His and our data indicate that there might be heterogeneity in subunit associations in different tissues from different species.
β coexpression with α_1 and α_2-δ subunits has been shown to affect both the amplitude of the currents and the number of expressed binding sites in a positive direction. Paradoxically, in our system, the effect of the decreased synthesis of β_2 is a sharp increase in the number of ω-ctx binding sites, indicating that, in its natural environment, the presence of β_2 subunit is not necessary for the transport of α_{1B} subunit to the plasma membrane. We also found that the increase in the number of surface α_{1B} subunit is not paralleled by an increase in current amplitude, suggesting that most of the α_{1B} subunits delivered to the membrane during β_2 synthesis inhibition are not functional channels. This finding is in line with previous reports indicating that some amounts of α_{1B} can be expressed on the surface of cells transfected with α_{1B} alone with no β coexpressed, but that HVA currents are almost absent under these circumstances. It remains unclear why the inhibition of β_2 stimulates the appearance of α_{1B} to the cell surface and the mechanism used. We have previously shown (23) that calcium channel blockers, such as cadmium and ω-ctx, can increase the numbers of ω-ctx binding sites at the cell surface and that increase is obtained through a recruitment of Ca^{2+} channels from a cytoplasmic pool. We may speculate that a similar mechanism operates in the case of a reduced β2 synthesis due to AS treatment. In this case cells could be induced to up regulate ω-ctx sensitive channels expression on plasma membrane in order to balance their decreased functionality. The return of basal levels of ω-ctx binding sites after 8 days from the treatment is probably due to a removal of the Ca channels from the cell surface through a normal turnover (23).

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