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Neuronal-type nicotinic receptors in human neuroblastoma and small-cell lung carcinoma cell lines

Paola Tarroni[a], Francesca Rubboli[a], Bice Chini[a], Ruud Zwart[b], Marga Oortgiesen[b], Emanuele Sher[a] and Francesco Clementi[a]

[a]*CNR Center of Cytopharmacology, Department of Medical Pharmacology, University of Milan, Milan, Italy* and [b]*Research Institute of Toxicology, University of Utrecht, Utrecht, The Netherlands*

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A β subunit of the neuronal nicotinic receptor, sharing 88% homology with the rat β_4 subunit, has been cloned from a human neuroblastoma cell line. The gene encoding the human β_4 subunit is expressed in association with the α_3 gene in neuroblastoma and small-cell lung carcinoma cell lines. Patch-clamp experiments and radioligand binding assays confirm that these neuroendocrine tumor cell lines express functional neuronal nicotinic receptors. We suggest that these receptors might play a crucial role in the control of neurotransmitter and hormone secretion from neurosecretory human tumors.

Nicotine; Receptor; Cloning; Neuroblastoma; Small-cell lung carcinoma

1. INTRODUCTION

Neuronal nicotinic receptors are present in the central nervous system (CNS), the peripheral ganglia and the adrenal medulla. In these tissues the activation of nicotinic receptors causes a localized depolarization of the plasma membrane which may also lead to neurotransmitter release [1,2]. Different gene products (α_2–α_8; β_2–β_4), identified in the rodent and avian systems, participate in the formation of neuronal nicotinic receptors [3], which have distinct localization [3] and pharmacological profiles [4]. Very little information is available on the molecular properties of human neuronal nicotinic receptors. We took advantage of previously characterized human cell lines with neuronal properties. Neuroblastoma (NB) is a tumor of the peripheral nervous system which is known to release amines and peptide hormones into the circulation [5]. Over recent years, in vitro-stabilized NB cell lines have proved to be invaluable tools for the characterization of different types of neuronal receptors [6–8]. On the other hand, small-cell lung carcinoma (SCC) cell lines derived from epithelial lung tumors consist of secretory cells which also express a number of neuronal traits [9]. Binding studies have previously shown that SCC cell lines express muscarinic cholinergic receptors [10], but recent evidence suggests that they may also express cholinergic receptors of the nicotinic type [11,12].

We here report (i) the cloning of the human β_4 nicotinic subunit, (ii) the selective expression of α_3 and β_4 nicotinic subunits in human NB and SCC cell lines, and (iii) the presence in the same cells of functional nicotinic receptors.

Correspondence address: P. Tarroni, CNR Center of Cytopharmacology, via Vanvitelli 32, 20129 Milano, Italy. Fax: (39) (2) 749 0574.

The hβ_4 nucleotide sequence has been deposited in the EMBL data library under accession number x68275.

Abbreviations: SCC, small-cell lung carcinoma; NB, neuroblastoma; Ach, acetylcholine; Cch, carbachol; TPMP$^+$, triphenylmethylphosphonium; nt, nucleotides.

2. MATERIALS AND METHODS

2.1. *Library screening*

A λgt10 cDNA library from the IMR32 human NB cell line (kindly provided by H. Soreq) was screened under low stringency conditions (hybridization was performed in 43% v/v formamide, 5× SSC, 1× Denhardt's, 20 mM sodium phosphate buffer, pH 6.8, 0.1% w/v SDS, and 20 μg/ml denatured fish sperm DNA at 37°C. Filters were washed at a final stringency of 2× SSC, 0.1% SDS at 60°C) using probe PCX49 (kindly provided by S. Heinemann) which encodes the rat β_2 nicotinic subunit [13]. Two positive overlapping clones, λh21 (nt 221–1,372) and λh22 (nt 881–1,360), were isolated (numbers in parentheses indicate the nucleotide residues of the cDNA carried by the clone). DNA sequencing was perfomed in M13mp18 and M13mp19 using the Sequenase Version 2.0 kit (USB). The two clones were found to contain fragments of the human β_4 subunit cDNA. Further information (nt 1–346) was obtained by sequencing the PCR amplification products from IMR32 mRNA described below.

2.2. *PCR amplification*

First strand cDNAs were synthesized from poly(A) RNA using the first strand cDNA synthesis kit (Stratagene) and the supplied random hexamer primer. 1/10th of the first strand reaction was amplified as previously described [14]. The β_4 5′ sense primer, 5′rβ_4-S (CAA CAA CCT GAT CCG CCC AGC; nt 177–197), was drawn on the β_4 rat seqence [15]. The primers for human β_4 (this paper), human α_3 [16] and β-actin [17] are as follows: 3′hβ_4-AS (GAA GGG AAA GTA CTT

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CAC CTC; nt 326–346); 5′hα$_3$-S (CGA CAT CAA GTA CAA CT; nt 558–574); 3′hα$_3$-AS (TAG AGC TTC TCG TGA GG; nt 1,161–1,177); 5′β-act-S (CGT GGG GCG CCC CAG GCA CCA GGG; nt 143–167); 3′β-act-AS (CGG TTG GCC TTG GGG TTC AGG GGG G; nt 364–388).

The ampification products were analyzed by Southern blot using the full-length α_3 and the λh21 insert as probes, and human β-actin was detected by ethidium bromide staining. The identity of β_4 PCR products was confirmed by DNA sequencing.

2.3. *Electrophysiological recordings*

Electrophysiological experiments on SK-N-BE cells were carried out using the standard whole-cell patch-clamp technique [18]. Resting potential was from −50 to −65 mV. Patch electrode resistance was 3–4 MΩ, and 70–75% of the series resistance was compensated for. Membrane potential was held at −80 mV. Membrane currents were low-pass filtered (8 pole Bessel; −3 dB at 1 kHz), digitized (12 bits; 1,024 points/record) and stored on magnetic discs for off-line computer analysis.

The external solution contained (in mM): NaCl 135, KCl 5.5, $CaCl_2$ 1.8, $MgCl_2$ 0.8, HEPES 20, glucose 25, and sucrose 36.5 (pH 7.3, 340 mOsm). The pipette solution contained (in mM): KCl 100, KF 50, HEPES 10, sucrose 50, and EGTA 10 (pH 7.2, 340 mOsm). For whole-cell superfusion, a continuous flow (1.5 ml/min) of external solution was applied from a 1 mm diameter capillary positioned within 0.5 mm of the cell. The cells were superfused at room temperature (RT) with known concentrations of the agonist and/or antagonists. To improve recovery from desensitization, cells were washed with external solution for 4 min between each response.

2.4. *[^{3}H]TPMP$^+$ binding assay*

[^{3}H]TPMP$^+$ binding assays were performed as in [20], with some modifications. The different human cell lines were obtained and grown as described [19]. The binding assay was performed at RT on cell homogenates in a buffer (in mM: NaCl 160, KCl 5, $CaCl_2$ 2, phenylmethylsulfonyl fluoride 0.1, and HEPES 10, pH 7.4) containing 0.5 mg/ml BSA, [^{3}H]TPMP$^+$ 50 nM (46.1 Ci/mmol; DuPont-NEN) and different concentrations of carbachol (Cch) (Sigma). Non-specific binding was measured in the presence of 5 mM unlabeled TPMP$^+$. After incubation, the samples were resuspended in 3 ml of buffer containing BSA (1 mg/ml), and filtered on GF/B filters (Whatman). The filters were washed 3 times with 3 ml buffer and radioactivity counted.

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human β4                              ...TSSSQLISIKLQLSLAQLISVNER  24
                                      ||||||||| | ||| ||||||||
rat β4   RLANAEEKLMDDLLNKTRYNNLIRPATSSSQLISIRLELSLSQLISVNER  50
                                    °
human β4 DEIMTTNVWLKQEWTDYRLTWNSSRYEGVNILRIPAKRIWLPDIVLYNNA  74
         ||||  |||||||||||| |||| ||||||||||||| ||||||||||
rat β4   EQIMTTSIWLKQEWTDYRLAWNSSCYEGVNILRIPAKRVWLPDIVLYNNA 100
                              °          *          °*
human β4 DGTYEVSVYTNLIVRSNGSVLWLPPAIYKSACKIEVKYFPFDQQNCTLKF 124
         ||||||||||| |||||||| ||||||||||||||||| |||||||| ||
rat β4   DGTYEVSVYTNVIVRSNGSIQWLPPAIYKSACKIEVKHFPFDQQNCRVKF 150

human β4 RSWTYDHTEIDMVLMTPTASMDDFTPSGEWDIVALPGRRTVNPQDPSYVD 174
         |||| |||||||||  ||| ||||||||||||||||||||||||||||||
rat β4   RSWTSDHTEIDMVLKSPTAIMDDFTPSGEWDIVALPGRRTVNPQDPSYVD 200

human β4 VTYDFIIKRKPLFYTINLIIPCVLITTLLAILVFYLPSDCGEKMTLCISVL 224
         |||||||||||||| |||||||| | ||||||||||||||||||||||||
rat β4   VTYDFIIKRKPLFYIINLIIPCVLITSLAILVFYLPSDCGEKMTLCISVL 250

human β4 LALTFFLLLISKIVPPTSLDVPLIGKYIMFTMVLVTFSIVTSVCVLNVHH 274
         |||||||||||||||||||| ||||||| |||||||||||| ||||||||
rat β4   LALTFFLLLISKIVPPTSLDIPLIGKYLLFTMVLVTFSIVTTVCVLNVMH 300

human β4 RSPSTHTMAPWVKRCFLHKLPTFLFMKRPGPDSSPARAFPPSKSCVTKPE 324
         ||||||||| ||| |||||||||||||||||   | |  ||
rat β4   RSPSTHTMASWVKECFLHKLPTFLFMKRPGLEVSLVRVPMPSQLHLATAD 350

human β4 ATATS----TSPSNFYGNSMYFVNPASAASKSPAGSTPVAIPRDFWLRSS 370
           |||    ||||| || ||||||| || ||  |    |||  ||||
rat β4   TAATSALGPTSPSNLYGSSMYFVNPVPAAPKSAVSSHTAGLPRDARLRSS 400

human β4 GRFRQDVQEALEGVSFIAQHMKNDDEDQSVVEDWKYVAMVVDRLFLWVFM 420
         |||| | |||||||||||||||  || |||| |||| ||||||||||||
rat β4   GRFREDLQEALEGVSFIAQHLESDDRDQSVIEDWKFVAMVVDRLFLWVFV 450

human β4 FVCVLGTVGLFLPPLFQTHAASEGPYAAQRD stop                451
         ||| ||| |||||||||| || |
rat β4   FVCILGTWGLFLPPLFQIHAPSKDS stop                     475
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Fig. 1. Alignment of human and rat β_4 subunit amino acid sequences. The one-letter amino acid notation is used. Putative transmembrane regions are underlined. Asterisks (*) indicate the cysteines conserved in all ligand-gated ion channels. Circles (°) indicate glycosylation sites.

3. RESULTS AND DISCUSSION

3.1. *Human β_4 nicotinic receptor subunit*

Fig. 1 shows an alignment of the amino acid sequences of the human and rat β_4 subunits. The deduced protein sequence of the human β_4 subunit (451 amino acids) was incomplete at its N-terminal but revealed the canonic primary strucutre of a nicotinic receptor subunit, which was characterized by four hydrophobic putative membrane-spanning regions and a large cytoplasmic loop. At the amino acid level, the homology between human and rat subunits was 88%. The most divergent region was represented by the cytoplasmic domain (77% homology), where the human subunit also had a deletion of four amino acids.

3.2. *Expression of hα$_3$ and hβ$_4$ in neuronal and non-neuronal cell lines*

It is known that rat and chick α_3 and β_4 subunits are highly expressed in the peripheral nervous system and that , when expressed in *Xenopus* oocytes, they yield functional nicotinic receptors with a ganglionic pharmacology [21]. IMR32 and SK-N-BE human NB cells have already been shown to express α_3 [16] and α_5 nicotinic subunits, but not α_1 [12]. By means of Northern blot analysis (Fig. 2), we now show that both IMR32 and SK-N-BE express a single β_4 transcript of about 3.1 kb. Given that NB originates from ganglionic peripheral neurons, this findings in human cells is in line with previous reports on rat.

Furthermore, PCR amplification (Fig. 3) showed that the α_3 and β_4 nicotinic subunits were expressed in two different SCC cell lines (NCI-H-69; NCI-N-592 not shown) although they were not expressed in either a lung non-SCC (A549) or a rhabdomyosarcoma (TE671) cell line. Thus the expression of the α_3 and β_4 nicotinic subunits is not confined to the nervous system but also appears in cells of the neuroendocrine type.

Fig. 2. Northern blot analysis of RNA from human neuroblastoma cells. Analysis of poly(A) RNA from SK-N-BE and IMR32 cells (10 mg each) was performed under high stringency conditions (final wash stringency: 0.1× SCC, 0.1% SDS, 50°C) using the λh21 clone insert as probe. Autoradiography was at −70°C for 2 days. 3.1 indicates the length of the transcript in kb.

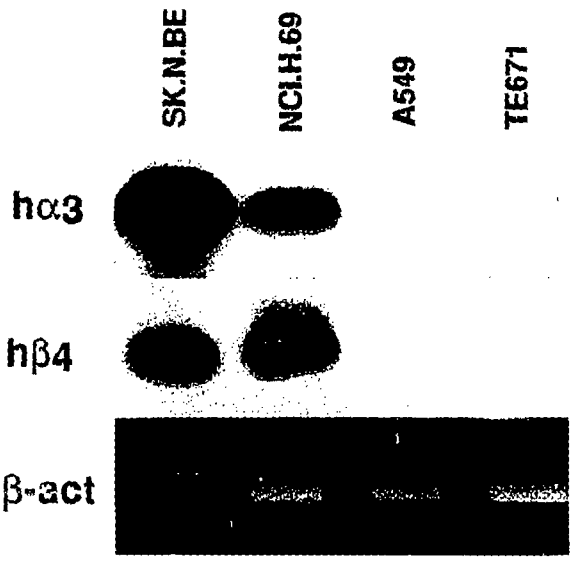

Fig. 3. PCR analysis of α_3 and β_4 RNA in different human cell lines. Human α_3 and β_4 amplification products from SK-N-BE, NCI-H-69, A 549 and TE 671 cells were analyzed by Southern blot with specific cDNA probes. The PCR products of hα_3 and the hβ_4 were only present in SK-N-BE and NCI-H-69 cells. The same results were obtained in NCI-N-592 cells (not shown). The β-actin control (β-act) was detectable by ethidium bromide staining in all of the lanes.

3.3. *Nicotinic receptor-mediated ion currents in human NB cells*

Superfusion with 1 mM acetylcholine (Ach) resulted in transient inward currents in most of the SK-N-BE (n = 13 cells tested) (Fig. 4) with peak amplitudes ranging from 0.25–3.50 nA. The Ach-induced inward current was completely and reversibly blocked by both 10 μM d-tubocurarine (Fig. 4a) and 1 μM $TPMP^+$ (Fig. 4b), thus confirming the nicotinic nature of the Ach-induced currents. SK-N-BE and IMR32 are the first human neuronal cell lines of peripheral origin found to express the α_3 and β_4 nicotinic subunits which might be involved in the formation of the functional nicotinic receptors here described. However, we cannot exclude the possibility that other α and β nicotinic subunits may be expressed in these cells and contribute towards the formation of different functional nicotinic channels. Several attempts were made to patch-clamp SCC cells, but the recordings were insufficiently stable to permit the characterization of the acetylcholine-induced currents. We therefore used the following approach to obtain indirect proof of the presence of functional nicotinic receptors.

3.4. *[^{3}H]TPMP$^+$ binding to human NB and SCC cell lines.*

$TPMP^+$ binding to the nicotinic receptor is allosterically modulated by cholinergic effectors [20]. This channel blocker binds to residues located inside the pore of the channel, which become available after receptor activation. The blocking effects of $TPMP^+$ on the muscular nicotinic receptor have been previously characterized [22], and we have shown here that it also blocks neuronal nicotinic currents in SK-N-BE cells (see above). As expected [^{3}H]$TPMP^+$ binding in SK-N-BE cells was positively modulated, in a dose-dependent manner, by Cch (Fig. 5B). The specific binding of [^{3}H]$TPMP^+$ was maximally stimulated by 10^{-6} M Cch. Cch also stimulated the specific binding of [^{3}H]$TPMP^+$ in NCI-H-69 (Fig. 5C) and NCI-N-592 (not shown) SCC cell lines which, like NB, express the α_3 and β_4 nicotinic receptor subunits. On the other hand, in the lung non-SCC cell line (A549), which does not express either α_3 of β_4 subunits, the binding of [^{3}H]$TPMP^+$ was not stimulated by Cch (Fig. 5D). As a control, an allosteric modulation of [^{3}H]$TPMP^+$ binding was also shown in TE671 cells, which are known to express muscle-type nicotinic receptors and the α_1 nicotinic subunit (Fig. 5A).

The expression of functional neuronal nicotinic receptors in SCC cell lines is a novel and intriguing finding. We suggest that these receptors should be considered as new members of the growing list of neuronal markers selectively expressed by this type of lung tumor. Neuronal nicotinic receptors are known to mediate the secretagogue effects of endogenous acetylcholine

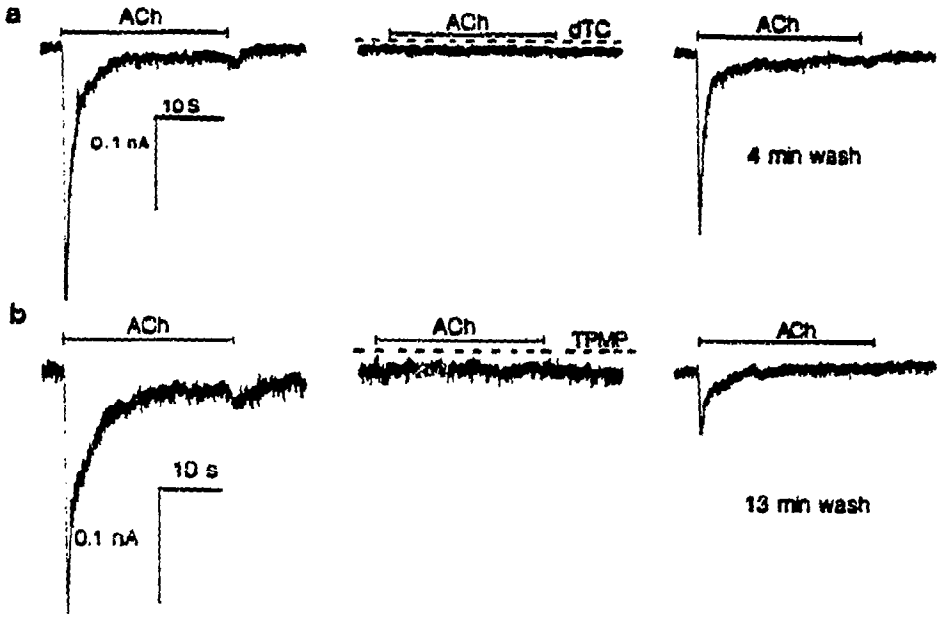

Fig. 4. Electrophysiological recordings in SK-N-BE cells. (a) Inward control currents induced by 1 mM Ach (left) are completely blocked after 3 min of superfusion with 10 μM d-tubocurarine (center). Recovery from the block is observed after 4 min of washing with control external medium (right). (b) Inward control currents induced by 1 mM Ach (left) are completely blocked after 7 min of superfusion with 1 μM TPMP$^+$ (center). Recovery from the block is observed after 13 min of washing with control external medium (right). Superfusion periods are indicated by bars.

and exogenous nicotine in a number of tissues, and we have also demonstrated the secretagogue effects of nicotine on SCC cell lines (E. Sher, manuscript in preparation). The effects of nicotine on cell proliferation have previously been described [11,23,24], but no information is available on the molecular nature of nicotine targets. The present characterization of neuronal nicotinic receptors in SSC, a subtype of human lung cancer, the association of which cigarette smoking is well demonstrated [25], offers new insights into the possible mechanisms by which nicotine might affect tumor cell physiology and proliferation.

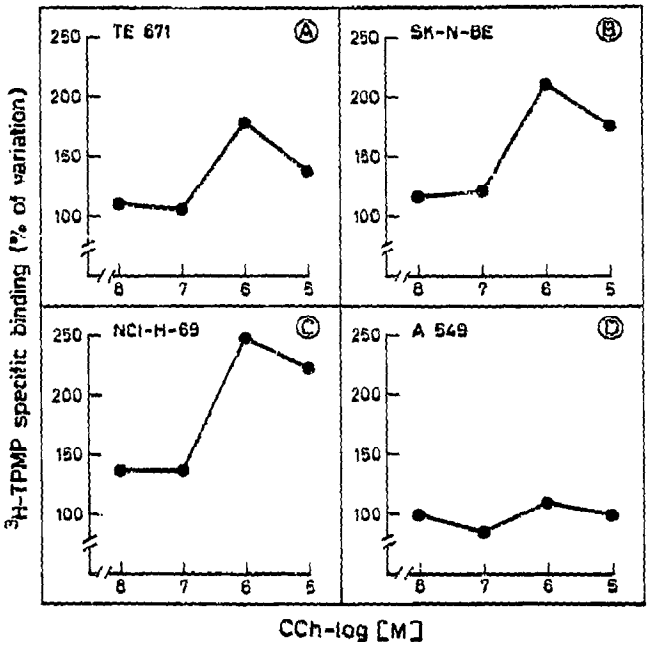

Fig. 5. Dose-response curves of the effects of Cch on [^{3}H]TPMP$^+$ binding. Cch-induced increases in [^{3}H]TPMP$^+$ binding were evaluated in TE 671 (A), SK-N-BE (B), NCI-H-69 (C) and A549 (D) cells. Specific [^{3}H]TPMP$^+$ binding in the absence of carbachol was considered as 100%. Values represent the average of 3–8 experiments, each performed in triplicate.

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REFERENCES

[1] Wonnacott, S., Irons, J., Lunt, G.G., Rapier, C.M. and Albuquerque, E.X. (1988) in: Nicotinic Acetylcholine Receptors in the Nervous System (Clementi, F., Gotti, C. and Sher, E. eds.) NATO ASI Series H: Cell Biology vol. 25, pp. 41–60, Springer-Verlag, Berlin.
[2] Roll, L.W. and Perlman, R.L. (1983) Neuroscience 10, 979–985.
[3] Deneris, E.S., Connolly, J., Rogers, S.W. and Duvoisin, R. (1991) Trends Pharmacol. Sci. 12, 34–40.
[4] Luetje, C.W. and Patrick, J. (1991) J. Neurosci. 11, 837–845.
[5] Hsiao, R.J., Seeger, R.C., Yu, A.L. and O'Connor, D.T. (1990) J. Clin. Invest. 85, 1555–1559.
[6] Clementi, F., Cabrini, D., Gotti, C. and Sher, E. (1986) J. Neurochem. 47, 291–297.
[7] Gotti, C., Esparis Ogando, A. and Clementi, F. (1989) Neuroscience 32, 759–767.

[8] Abbracchio, M.P., Cattabeni, F., Clementi, F. and Sher, A. (1989) Neuroscience 30, 819–825.
[9] Teeling, M.E. and Carney, D.N. (1988) in: Biology of Lung Cancer: Diagnosis and Treatment Biochemical Markers of Lung Cancer (Rosen, S.T., Mulshine, J., Cutitta, F. and Abrams, P.C. eds.) pp. 43–58, Marcel Dekker, New York.
[10] Cunningham, J., Lennon, V., Lambert, E. and Scheithauer, B. (1985) J. Neurochem. 45, 159–167.
[11] Maneckjee, R. and Minna, J.D. (1990) Proc. Natl. Acad. Sci. USA 87, 3294–3298.
[12] Chini, B., Clementi, F., Hukovic, N. and Sher, E. (1992) Proc. Natl. Acad. Sci. USA 89, 1572–1576.
[13] Deneris, E.S., Connolly, J., Boulter, J., Wada, E., Wada, K., Swanson, L.W., Patrick, J. and Heinemann, S. (1988) Neuron 1, 45–54.
[14] Frohman, M.A., Dush, M.K. and Martin, G.R. (1988) Proc. Natl. Acad. Sci. USA 85, 8998–9002.
[15] Duvoisin, R.M., Deneris, E.S., Patrick, J. and Heinemann, S. (1989) Neuron 3, 487–496.
[16] Fornasari, D., Chini, B., Tarroni, P. and Clementi, F. (1990) Neurosci. Lett. 111, 351–356.
[17] Ponte, P., Ng, S.-Y., Engel, J., Gunning, P. and Kedes, L. (1984) Nucleic Acid Res. 12, 1687–1696.
[18] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflügers Arch. 391, 85–100.
[19] Sher, E., Pandiella, A. and Clementi, F. (1990) Cancer Res. 5, 3892–3896.
[20] Lauffer, L. and Hucho, F. (1982) Proc. Natl. Acad. Sci. USA 79, 2406–2409.
[21] Couturier, S., Erkman, L., Valera, S., Rungger, D., Bertrand, S., Boulter, J., Ballivet, M. and Bertrand, D. (1990) J. Biol. Chem. 265, 17560–17567.
[22] Spivak, C.E. and Albuquerque, E.X. (1984) Mol. Pharmacol. 27, 246–255.
[23] Shuller, H.M. (1989) Biochem. Pharmacol. 38, 3439–3442.
[24] Tominaga, K., Kinoshita, Y., Hato, F., Masuda, A. and Matsuyama, M. (1989) Cell. Mol. Biol. 35, 679–686.
[25] Iannuzzi, M.C. and Scoggin, C.H. (1986) Am. Rev. Resp. Dis. 134, 593–608.