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FEBS Letters 342 (1994) 286–290

FEBS 13887

FEBS LETTERS

Serotonin release and cell proliferation are under the control of α-bungarotoxin-sensitive nicotinic receptors in small-cell lung carcinoma cell lines

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Received 27 January 1994; revised version received 10 March 1994

Abstract

Neuronal type nicotinic acetylcholine receptors (nAchRs) have recently been identified in small-cell lung carcinoma. We here show that both nicotine and cytisine stimulate [^{3}H]serotonin release in a dose-dependent manner; this effect is antagonized by α-bungarotoxin (αBgtx) and α-conotoxin MI (αCtx). Nicotine and cytisine stimulate in vitro SCLC proliferation and this effect is completely antagonized by both αBgtx and αCtx. By PCR analysis, we demonstrate the presence in SCLC of both the α_7 and the β_2 nAchR subunits mRNA. These data show that nAchRs play an important role in the biology of SCLC, and that αBgtx-sensitive receptors of the α_7 subtype are crucially involved in both the secretagogue and mitogenic effects of nicotinic agonists.

Key words: Human small cell lung carcinoma; Nicotinic acetylcholine receptor; Serotonin; Secretion; Proliferation

1. Introduction

Until recently, small cell lung carcinoma (SCLC) cells have been though to express only cholinergic receptors of the muscarinic type [1]. In the last few years, however, cholinergic nicotinic receptors have also been described in these cells [2–5].

Most interestingly, SCLC nicotinic receptors have been shown to be of neuronal type [3,4]: in particular, SCLC cells express α_3 and β_4 neuronal nicotinic receptor subunits, charbacol-sensitive [^{3}H]TPMP binding sites [4] and αBgtx binding sites with neuronal characteristics. These αBgtx binding sites are not recognized by a monoclonal antibody (mAb35) recognizing muscle type nicotinic receptors, and, furthermore, SCLC cells do not express the α_1 muscle type αBgtx-binding subunit [3] .

Nicotine is known to be mitogenic for several types of lung tumors [5–8], including SCLC [2,8]. We have recently shown that nicotine is also a potent secretagogue for SCLC cells, stimulating the release of substances, such as serotonin, that, in turn, act as potent mitogens [8]. Therefore, determining the mechanisms and the subtypes of receptors involved in the secretagogue effects of nicotine is important for understanding its mitogenic effects.

We now report that SCLC cells express the α_7 nicotinic subunit mRNA, which codes for the major αBgtx-binding nicotinic subunit in the nervous system, and that both αBgtx and αCtx block the secretagogue and mitogenic effects of nicotine.

These results represent the first demonstration of a physiological role of αBgtx-sensitive, neuronal type nicotinic receptors in lung cancer, and might help in elucidating its role in the nervous system as well.

2. Materials and methods

2.1. Cell lines

Human SCLC cell lines (GLC8, NCI-N592 and NCI-H-69) were kindly provided by Prof. G. Gaudino (Dept. of Biomedical Sciences and Oncology, Turin, Italy). They were grown in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 mg/ml streptomycin in 10-cm diameter Falcon tissue culture dishes. Cells were incubated at 37°C in a humidified atmosphere of 10% CO_2 in air.

2.2. [^{3}H]Serotonin release

SCLC cells were recovered from the dishes and passed three times through a sterile syringe in order to dissociate the cell clusters. After centrifugation (5 min, 800 rpm), the cells were resuspended in a Krebs–Ringer–HEPES (KRH) solution containing (in mM) 150 NaCl, 5 KCl, 1.2 $MgSO_4$ and KH_2PO_4, 2 $CaCl_2$, 6 glucose and 25 HEPES-NaOH (pH 7.4). The medium also contained equal amounts (0.1 mg/ml) of ascorbic acid (Merk) and of the monoamine oxidase inhibitor pargyline (Sigma), plus [^{3}H]5HT (specific activity 11.6 Ci/mmol; final concentration 500 nM, Amersham). After 30 min at 37°C, the cells were centrifuged

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Abbreviations: SCLC, small-cell lung carcinoma; nAchR, nicotinic acetylcholine receptor; 5HT, 5-hydroxy-tryptamine; α and κBgtx, α- and κ-bungarotoxin; αCtx, α-conotoxin MI

SSDI 0014-5793(94)00272-W

(5 min, 800 rpm), resuspended in KRH containing the 5HT reuptake inhibitor Cl-Imipramine (1 μM, RBI), and aliquoted in Eppendorf tubes containing the desidered drug concentration. To assay the block potency of nAchR antagonists on [^{3}H]5HT release, the cells were preincubated with drugs and toxins 30 min before, and during the stimulation period at 37°C. At the end of stimulation with agonists, carried out for 5 min (or different periods of time, in the case of the time course acquisition) at 37°C, the cells were washed three time with ice-cold buffer. Finally the pellets were counted to determine the amount of [^{3}H]5HT left over. Samples, dissolved in 300 μl of NaOH 1 mM and then in 5 ml of Atomlight (Du Pont), were counted in a β counter (Beckman Instruments, model LS7500) with an efficency of 40%. The amount of release is expressed as percent increase over basal.

All solution salts were purchased from Merk, nicotine and cytisine were obtained from Sigma, α- and κBgtx from Biotoxins, αCtx MI from RBI.

2.3. [^{3}H]Thymidine incorporation

SCLC cells were plated in RPMI 1640 in 96-well microtiter plates at a density of 2–5 × 10^3 cells/well. Cells were preincubated for 15′ before the addition of nicotine, cytisine or serotonin, and then incubated for 48 hrs at 37°C in a humified atmosphere of 10% CO_2 in air. Methyl[^{3}H]Thymidine (1 μCi/well; specific activity 2 Ci/mmol, Amersham) was added during the last six hours of incubation. At the end of the incubation period, cells were washed and lysed with distilled water and collected on filters with an automatic cell harvester (Titertek, Flow Laboratories). The filters were placed in Filter Count Scintillation fluid (Packard), and counted using standard procedures.

2.4. PCR analysis

First-strand cDNA was synthesized from 200 ng of Poly(A)$^+$ RNA using the first-strand cDNA synthesis kit (Stratagene) and the supplied random examer primers. 5% of the cDNA synthesized was amplified using *Taq* polimerase (Promega). 25 amplification cycles (95°C for 1′; 56°C for 1′; 72°C for 1′) were performed in the presence of specific primers following Promega procedure. RT-PCR amplification products were analysed by Southern blot with specific ^{32}P-labelled oligonucleotides as previously indicated [9]. Primers and oligonucleotidic probes, drawn on the published sequences, were as follows: 5′-α3S: CGA CAT CAA GTA CAA CT; C-α_3: AAG ACT GTA TTC TTA A; 3′-α_3AS: TAG AGC TTC TCG TGA GG; 5′-β_2S: GCC TGC GCC TGC GGC GAC GCC; C-β_2: GCC CCA GGG GCC GAC TCC TGC ACG TGC TTC GTC AAC CG; 3′-β_2-AS: CCT CAC TCA CGC TCT GGT CAT; 5′-α_7S: CCA CCA ACA TTT GGC TAC AAA TG; C-α_7: CAA GAG TTC CTG CTA CAT CGA TG; 3′α_7-AS: ATG GTC ACT GTG AAG GTG ACA TC; 5′-β_4S: CAA CAA CCT GAT CCG CCC AGC: C-β_4: GTA GAC AGA CAC CTC ATA GGT; 3′-β_4AS: GAA GGG AAA GTA CTT CAC CTC.

3. Results and discussion

3.1. Nicotinic agonists induce [^{3}H]serotonin release from SCLC cells

One of the effects of nicotinic receptor stimulation in the nervous system is the induction of neurotransmitter release [10]. We have recently shown that nicotine stimulates [^{3}H]5HT release from SCLC cells, a process strictly related to cell proliferation [8]. This effect was found to be dependent on extracellular Ca^{2+} and blocked by the ganglionic nicotinic antagonist mecamylamine [8].

We have now extended this functional analysis of SCLC nAchRs. Maximal nicotine-induced [^{3}H]5HT release from GLC8 SCLC cells is achieved after 5 min at 37°C (Fig. 1, inset) and for all further experiments we adopted this protocol of stimulation. Both nicotine and cytisine, a selective neuronal nicotinic receptor agonist, were found to be effective secretagogues for SCLC cells (Fig. 1). Both nicotine and cytisine effects were dose-dependent (Fig. 1) with nicotine being two orders of magnitude more potent than cytisine (EC_{50} of 27 pM and 1 nM for nicotine and cytisine, respectively).

Nicotine (100 nM) stimulated a similar amount of [^{3}H]5HT release from the three different SCLC cell lines tested, all of them known to express neuronal-type nicotinic receptors [3,4], the maximal release detectable with 100 nM nicotine (22.5 ± 1, 20 ± 2 and 23.5 ± 0.7% in NCI-N-592, NCI-H-69 and GLC8 cells, respectively) being comparable, in each cell line, to that obtained with a maximal effective concentration of 60 mM KCl (24 ± 3, 21 ± 2 and 28 ± 1.5% in NCI-N-592, NCI-H-69 and GLC8 cells, respectively).

3.2. Block of [^{3}H]serotonin release by αBungarotoxin and related toxins

Nicotine-induced [^{3}H]5HT release was inhibited in a dose-dependent manner by the two related nicotinic antagonists, αBgtx and αCtx (Fig. 2), with IC_{50}s of 1 nM and 10 pM, respectively. Also cytisine-induced (1 μM) [^{3}H]5HT release was efficiently antagonized by the two different α toxins (61 ± 2% and 60 ± 9% of inhibition at 625 nM αBgtx and 863 αCtx, respectively, $n = 3$).

αBgtx binding sites have been previously described in SCLC [1–3], but their functional role was not investigated. We here show, for the first time, that αBgtx-sensi-

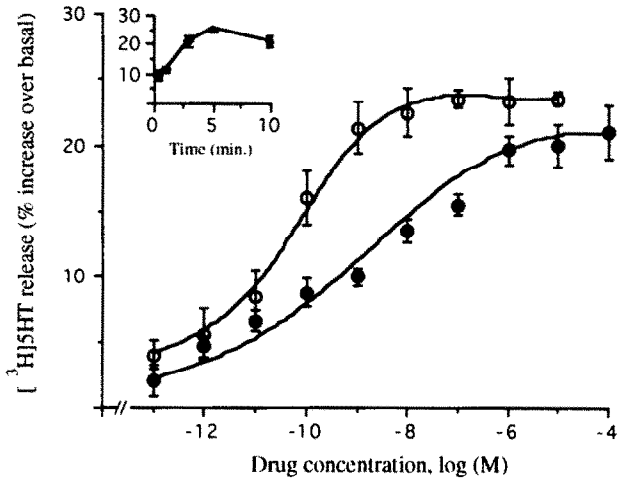

Fig. 1. Dose–response curve of nicotine- and cytisine-induced [^{3}H]5HT release from GLC8 SCLC cells. Nicotine (○) and cytisine (●) both stimulate [^{3}H]5HT release in a dose-dependent manner, but their efficacy is different, nicotine being more than one order of magnitude more potent than cytisine. Their EC_{50} were 27 pM and 1 nM for nicotine and cytisine, respectively. Furthermore, nicotine has the same effect in NCI-H69 and NCI-N592 cell lines (see text). Data are expressed as percentage increases over basal release. Each point represents the average ± S.E. of 5–8 experiments performed in quadruplicate as described. *Inset*: Time-course of nicotine-induced [^{3}H]-5HT release in GLC8 cells. We performed [^{3}H]-5HT release experiments with different periods of stimulation: 30″, 1′, 3′, 5′, 10′, and found that maximal secretion is achieved after 5′. We adopted this period for all subsequent experiments. Data are expressed as described.

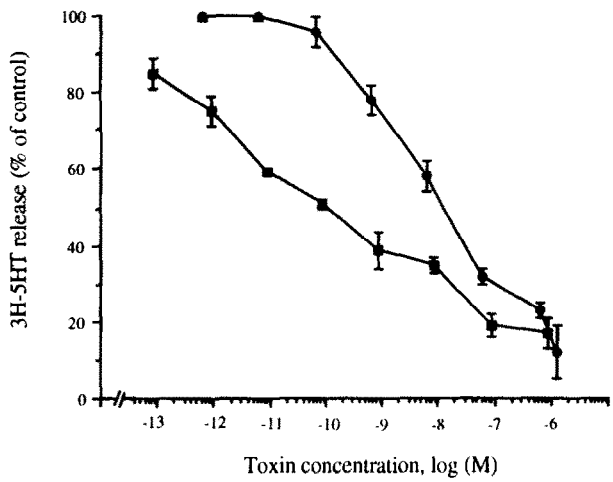

Fig. 2. Effect of nAchR toxins on nicotine-stimulated [^{3}H]5HT release. αBgtx (●) and αCtx MI (■) both inhibit nicotine-stimulated [^{3}H]5HT release in a dose-dependent manner with IC_{50} values of 1 nM and 10 pM, respectively. The maximal inhibition obtained was similar, varying from 88% with αBgtx to 83% with αCtx MI. Each point represents the average ± S.E. of 4–6 experiments performed in quadruplicate as described.

tive nicotinic receptors play a crucial role in SCLC cells, inhibiting nicotine-induced [^{3}H]5HT release. It is known that neuronal type αBgtx receptors represent functional ion channels, and that they are highly permeable to Ca^{2+} [11–13]; however their role in cellular processes is still largely unknown. We here show that neuronal-type αBgtx receptors mediate the secretagogue effects of nicotine in SCLC. It is tempting to speculate that they might play the same role in other neurosecretory cells and in the nervous system as well.

αCtx was as efficaceous as, and even more potent than αBgtx in inhibiting [^{3}H]5HT release (Fig. 2). This toxin is a potent antagonist of the αBgtx-sensitive muscular nAchRs [14], but did not block the neuronal nAchRs studied so far [15].

To our knowledge this is the first evidence that αCtx blocks a neuronal-type nAchR subtype, i.e. the αBgtx-sensitive one, suggesting a conserved binding site structure for both toxins in muscle and neuronal αBgtx-sensitive receptors. Therefore αCtx might become an important tool, together with αBgtx, for studying both SCLC and neuronal nicotinic receptor subtypes.

κBungarotoxin, a less selective polypeptide nicotinic antagonist [16], also inhibited potently and dose-dependently nicotinic-induced [^{3}H]5HT release (IC_{50} 1 pM). At 700 nM, κBgtx was as efficaceous as αBgtx and αCtx in inhibiting either 100 nM nicotine-(80 ± 4% of inhibition or 1 μM cytisine-(65 ± 3 of inhibition) [^{3}H]5HT release.

[^{125}I]κBgtx binds with high affinity and specificity to both NCI-H-69, NCI-N-592 and GLC8 SCLC cell lines (E. Sher et al., unpublished results). As in neurons, ^{125}I-κBgtx probably binds to heterogeneous nAchR subtypes in SCLC cells, since its binding is mostly, although not completely inhibited by either αBgtx, or αCtx. Therefore, while most binding sites are shared by the α and the κ toxins in SCLC cells, a small amount of αBgtx and αCtx insensitive nicotinic receptors are probably present. Further work will elucidate if they play any specific role in hormone secretion.

3.3. Nicotinic receptors and SCLC proliferation

We have recently shown that serotonin is a potent mitogen for SCLC cells and that nicotine-induced release of serotonin from SCLC is probably one of the main mechanisms mediating nicotine stimulation of SCLC growth [5]. Indeed, both nicotine-stimulated serotonin release and nicotine-stimulated cell proliferation were blocked by the ganglionic nicotinic antagonist mecamylamine.

The finding that also αBgtx and αCtx are potent antagonists of nicotine-induced serotonin release, prompted us to evaluate if these toxins were also able to inhibit nicotine-induced SCLC proliferation.

We have found that both αBgtx and αCtx (both at 1 μM) completely antagonized nicotine and cytisine stimulation of SCLC proliferation (Fig. 3) without affecting

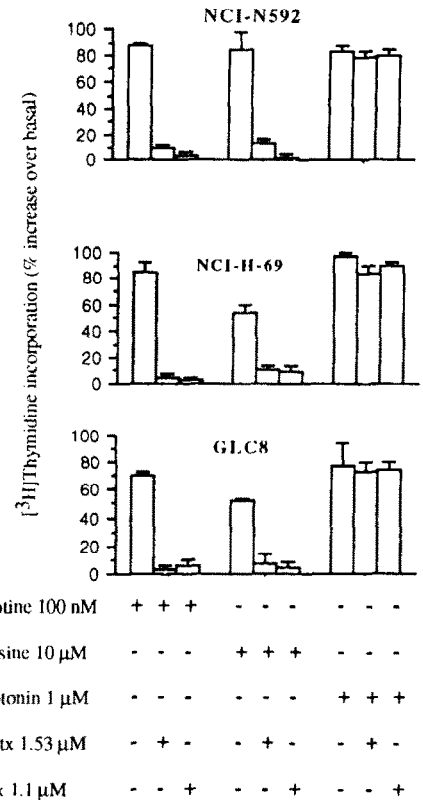

Fig. 3. Effect of αBgtx and αCtx on SCLC proliferation. Both nicotine- and cytisine-induced increases of cell proliferation were blocked by the two nicotinic α-toxins αBgtx and αCtx at maximal doses, while no effect is detectable with the serotonin-simulated cell proliferation. Similar results were obtained in three different cell lines: GLC8, NCI-N592 and NCI-H-69. Values are expressed as percentage increases over basal. Bars represent the average ± S.E. of 3 experiments, each performed in quadruplicate

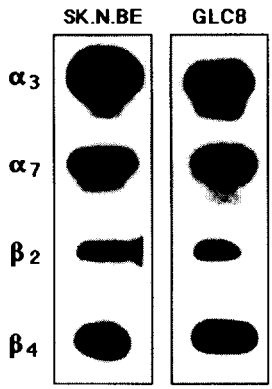

Fig. 4. RT-PCR analysis for transcripts encoding nicotinic receptor subunits in human neuroblastoma (SH-SKNBE) and small cell lung carcinoma (GLC8) cell lines. α_3, α_7, β_2 and β_4 amplification products from SH-SKNBE and GLC8 cells were analysed by Southern blot with specific oligonucleotidic probes. RT-PCR products of α_3, α_7, β_2 and β_4 are present in both cell lines. Control reactions, without reverse transcriptase, remained negative.

serotonin-stimulated cell proliferation, extending our previous findings indicating that neuronal nicotinic receptors mediate the biological effects of nicotine on SCLC cells. We suggest that nicotinic control of both hormone release and cell proliferation is carried out by the neuronal-type αBgtx receptor present in SCLC cells.

3.4. *Different α and β nicotinic subunit mRNA are expressed in SCLC cells*

We previously demonstrated the presence of both α_3 [4] and α_5 [3] nicotinic subunits in SCLC cells and the absence of α_1 [3]; the pharmacological and functional data here reported confirm that SCLC nAchRs are heterogeneous molecular entities.

However, besides some suggestions that α_5 does bind αBgtx under particular conditions [3,17], no clearcut evidence for αBgtx binding subunits in SCLC was yet avalaible. We here show that mRNA for α_7, the dominant and most diffuse αBgtx binding subunit of the nervous system, is expressed in SCLC cells (Fig. 4), suggesting that also in these cells it might represent the most important αBgtx binding subunit. We also found that SCLC express both β_2 and β_4 subunits (Fig. 4): interestingly β_4 is supposed to confer cytisine sensitivity to neuronal nAchRs, while β_2 is supposed to confer κBgtx sensitivity [15–18].

The functional and molecular findings that we have here reported suggest that the αBgtx-sensitive nicotinic receptor mediating nicotinic agonist-induced hormone secretion and cell proliferation is of the α_7 subtype. This α_7 nAchR is highly permeable to Ca^{2+} [11–13] and calcium influx is necessary for both hormone secretion and cell proliferation. Ca^{2+} ions might indeed enter the cells both through the nicotinic channel itself and through the voltage-dependent Ca^{2+} channels which will open in response to the nicotinic-induced cell depolarization. Which of the two pathways is more relevant to the control of secretion still has to be investigated. We have also shown that κBgtx specific receptors, although present, play a minor role in nicotinic agonist-induced [^{3}H]5HT secretion. Furthermore, mRNA for both β_2 and β_4 subunits are expressed in SCLC cells. These data, together with previous reports [2–4], confirm the heterogeneity of nAchRs in SCLC cells, and suggest that besides the α_7 subtype, other neuronal type nAchRs might have a specific, still undefined, role in the biology of SCLC.

Finally, our findings further confirm that, although of epithelial origin, SCLC cells have much in common with neurons. Voltage-operated calcium channel subtypes previously believed to be expressed only in neurons (N and P types) have recently been shown to be present, and involved in the control of hormone release from SCLC [19,20]. The neuronal type nicotinic receptors represent another family of 'neuronal' ion channels expressed by SCLC cells.

All these different membrane ion channels, both voltage and agonist-operated, so important in the control of hormone release from SCLC cells, might well become selective targets for new pharmacological approaches to the treatment of this very aggressive human cancer.

Acknowledgments: The paper was supported by a grant from the CNR, oriented project 'Clinical Application of Oncology Research' to L.M.Vicentini; M.G. Cattaneo is a recipient of a fellowship from A.I.R.C. (Associazione Italiana per la Ricerca sul Cancro).

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