Nicotinic Receptors Mediate the Release of Amino Acid Neurotransmitters in Cultured Cortical Neurons

Nicotine stimulation of cortical neurons obtained from gestation day 19 rats provoked a dose-dependent release of aspartate, glutamate, glycine and GABA, indicating a functional role for the nicotinic receptor in this model. This release was exclusively Ca\(^{2+}\)-dependent (vesicular release) in the case of aspartate and dual (Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent) for glutamate, glycine and GABA. Nicotine also raised the membrane potential and the intracellular calcium concentration. These effects were specific, since they were reversed by hexamethonium, an antagonist of the nicotinic receptor. It was shown that L, N, and P/Q type Ca\(^{2+}\) channels are involved in nicotine-mediated Ca\(^{2+}\) entry into cortical neurons. Evaluation of the effects of nicotine on Ca\(^{2+}\) entry in isolated cells showed that 100% of the cells responded to nicotine, although the intensity of the response was variable: 53% of the neurons showed an increase in intracellular Ca\(^{2+}\) of 152 ± 5 grey levels, 25% of 88 ± 12 grey levels and 12% of 48 ± 1 grey levels. Tetrodotoxin, which blocks voltage-dependent Na\(^{+}\) channels, completely reversed nicotine-induced Ca\(^{2+}\) entry into single cells. This suggests that the Ca\(^{2+}\) increment is mediated by opening of Ca\(^{2+}\) channels and not by the nicotinic receptor.

Introduction

Although its physiological and psychological effects have long suggested that nicotine exerts specific actions in the brain, the identification of neuronal nicotinic receptors has only been possible in the last few years due to the development of molecular biology techniques [reviewed by McGehee and Rolen (McGehee and Rolen, 1995)]. A variety of nicotinic acetylcholine receptor (nAchR) complexes in the central nervous system (CNS) are formed by a diverse array of subunits which confer different pharmacological and physiological properties to the receptors (Sargent, 1993; Rust et al., 1994). Although there is little experimental evidence (Anano et al., 1991; Cooper et al., 1991), the neuronal nAchR channel is assumed to be a pentamer formed by α and β subunits, although many alternative α/β stoichiometries may exist, and this could contribute to the particular functionality of the nAchR channel. The expression of specific nAchR subunits is important during brain development since it influences neuronal excitability (Margiotta and Gurantz, 1989). Nicotinic AChRs are present on autonomic nerves and adrenal chromaffin cells of the peripheral nervous system and on many neurons in the CNS. Many of the properties of the nAchR channels, such as ion selectivity and gating properties, resemble those of muscle AChR. However, neuronal AChRs are clearly distinct from muscle and are themselves diverse. The most important role performed by cholinergic receptors in the CNS is their participation in neuronal excitability modulation (Aquilonius and Gillberg, 1990; Vernino et al., 1992) and in neurotoxicity events (Slotkin et al., 1997). Cumulative evidence from animal and human studies has indicated that nicotinic systems play a major role in higher cognitive functions and dysfunctions. Nakayama et al. were able to show the presence of nicotinic receptors in cerebral cortex through immunocytochemical techniques (Nakayama et al., 1995). The number of these receptors seems to be diminished in neurological disorders such as Parkinson’s and Alzheimer’s diseases (Araujo et al., 1988; Schroder et al., 1995; Chesselli, 1997; Zamanim et al., 1997) and schizophrenia (Miller et al., 1996). Pharmacological and electrophysiological studies appear to suggest the existence of different isoforms of the nicotinic receptor in the neocortex and hippocampus (Wada et al., 1989, 1990; Seguela et al., 1993; Lobron et al., 1995), although the exact location of these isoforms is not known. This diversity of nicotinic receptors in the CNS makes functional and pharmacological variations possible (Papke et al., 1989; Luetje and Patrick, 1991). In dopaminergic nigrostriatal axons nicotine induces dopamine release after previous stimulation of glutamatergic neurons, which release glutamate (García Muñoz et al., 1996). In hippocampal synaptosomes release of noradrenaline may be modulated by nicotinic receptors containing α\(_3\) and β\(_4\) subunits (Clarke and Reuben, 1996). Furthermore, nicotine may induce increases in glutamate, dopamine, serotonin and acetylcholine release in the hippocampus, cerebellum, nucleus accumbens and striatum (Thoth et al., 1992; Marshall et al., 1996, 1997; Wilkie et al., 1996; Ferger and Kuschinsky, 1997). Conversely, Izenwasser et al. found that in the striatum nicotine inhibits \(^{[3]H}\)dopamine uptake and induces its release (Izenwasser et al., 1991). Moreover, Waniweski and Martin reported that the stimulation of sympathetic ganglia with nicotinic or muscarinic agonists provoked the release of \(^{[3]H}\)taurine (Waniweski and Martin, 1994). Although there is much data on the properties and functional characteristics of the different cholinergic receptors (AchR) in the CNS, most of these may be considered presynaptic receptors since the action of nicotine has generally been evaluated in terminal neurons (synaptosomes). However, nicotinic receptors are distributed throughout the neurons. Few investigations have centred on the action of AchRs on neurotransmitter release in neuronal preparations, although the effects of nicotine in \textit{in vitro} systems have also been studied (Thoth et al., 1993). The aim of the present study was to investigate the nicotinic receptor-mediated modulation of synaptic transmission in cortical neurons. Our results suggest that in cortical neurons in culture nicotine induces aspartate release by an exocytotic pathway and glutamate, glycine and GABA release by a dual mechanism. It was also established that nicotine increased the intracellular calcium concentration in all of the neurons examined.

Materials and Methods

Materials

Fura 2AM and bis-[1,3-diethylthiobarbiturate]trimethine oxonol (bis-oxonol) were obtained from Molecular Probes (Eugene, OR). Eagle’s minimum essential medium (EMEM) was supplied by Bio-Whittaker and...
foetal calf serum (FCS) and horse serum (HS) by Sera-Lab (Sussex, UK). Nicotine, hexamethonium and muscimol came from Sigma (St Louis, MA). The remaining chemicals were reactive grade products from Merck (Darmstadt, Germany).

Cell Isolation and Culture

Brain neurons were obtained from foetal rat brains at gestation day 19 following the procedure described by Segal (Segal, 1983) with minor modifications. Isolated neurons were suspended in EMMEM containing 0.3 g/l glutamine, 0.6% glucose, 10% FCS, 1% HS, 100 U/ml penicillin, 100 µg/ml streptomycin, 40 µg/ml gentamycin and 5 µg/ml imipenem. Cells, at a density of 2 × 10^6 cells/well, were plated onto plastic Petri dishes treated with 10 µg/ml poly-lysine to aid attachment. The plates were incubated in a humidified incubator at an atmosphere of 5% CO2/95% air at 37°C. After 72 h the incubation medium was replaced with fresh medium to which 10 µM cytochrome arabinoside was added to prevent overgrowth of contaminating glial cells. Cells were used after 10–15 days culture. Cell viability was checked by the trypan blue exclusion method. Viability was routinely >95%. Cell purity was checked by both cell staining with cresyl violet to identify neurons and with a specific anti-GFAP antibody to identify glial cells.

Glutamic Acid Release by Neuronal Cultures

Cytosolic [Ca2+]

Changes in intracellular calcium concentration, [Ca^2+], were monitored by Fura 2 AM fluorescence. Six day cultured cells were detached from the culture plates with trypsin, as indicated below. Cells were the fixed (for 30 min) in 2% paraformaldehyde and washed in phosphate-buffered saline (PBS) followed by treatment (1 h) with anti-rabbit GFAP antibody (diluted 1/500). Cells were once again washed in PBS and treated with anti-rabbit FITC-conjugated IgG at a dilution of 1/100 for 30 min and identified by flow cytometry. Under these conditions the glial cells in the cultures were estimated at 9 ± 3% of the total cell population (neural + glial cells).

Cytosolic [Ca2+]

Changes in intracellular calcium concentration, [Ca^2+], were monitored by Fura 2 AM fluorescence. Six day cultured cells were detached from the culture plates with trypsin (0.25% trypsin and 0.02% EDTA in Dulbecco’s phosphate-buffered saline containing without calcium or magnesium) and washed twice using 1 ml of a Krebs HEPES solution (Locke medium) containing 140 mM NaCl, 4.4 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 4.0 mM NaHCO3, 5.5 mM glucose, 0.58 mM ascorbic acid and 10 mM HEPES, adjusted to pH 7.5 and incubated with 5 µM Fura 2 AM for 45 min at 37°C. Excessive dye was removed by washing the cells twice with fresh Locke medium followed by suspension in this medium at 1 × 10^5 cells/ml. After Fura 2 AM treatment, cell viability was checked as indicated previously. Fluorescence (excitation wavelength 340/380 nm, emission wavelength of 510 nm) was monitored at 37°C in a well stirred cuvette indicated previously. Fluorescence (excitation wavelength 340/380 nm, emission wavelength of 510 nm) was monitored at 37°C in a well stirred cuvette using a Perkin Elmer spectrofluorimeter. Drugs were added at the indicated concentrations. Controls were performed using Locke medium in place of the drug. Fluorescence intensity was reported in arbitrary units.

Amino Acid Secretion

High performance liquid chromatography (HPLC) of amino acids was performed according to the methods described by Márquez et al. (Márquez et al., 1986). Cells were washed twice at 10 min intervals with 1 ml of Locke medium. After removal of the medium cells were stimulated for 15 min periods at 37°C with 0.5 ml of fresh Locke medium containing the different secretagogues. The stimulating medium was then withdrawn and cells ruptured by the addition of 0.5 ml of distilled water. The concentration of amino acids was determined by reversed phase HPLC using pre-column derivation with dansyl chloride and UV detection at 254 nm. Integration of peaks was achieved using a Spectraphysis integrator. Peaks were quantified by comparison with those obtained using simultaneously prepared amino acid standards. Separation of dansyl derivatives was performed using a 5 µM Spherisorb-ODS-2 column (15 × 0.46 cm).

Proteins were identified according to Bradford (Bradford, 1976). Results were expressed as nmol neurotransmitter/mg protein/well or as the percentage of amino acids released into the incubation medium with respect to the total amino acid content (incubation medium + cells).

Data Presentation

Data are presented as the means of three or four separate experiments performed on different cell cultures. Each experiment was performed in duplicate using different batches of cells. Student’s t-test was used to statistically compare data.

Results

A dose-dependent release of the amino acids aspartate, glutamate, glycine and GABA was shown when cortical neurons were stimulated with nicotine. The effect of nicotine was first observed at a nicotine dose of 50 µM. Glycine was released in greatest quantity (Fig. 1). Measurement of secretion mediated by nicotine in a medium with and without external calcium revealed that aspartate release was exclusively Ca2+-dependent (vesicular release), while the release of the remaining amino acids occurred through Ca2+-dependent and Ca2+-independent processes. The order of Ca2+-dependent release of these amino acids at a nicotine concentration of 200 µM was: aspartate =
glycine > glutamate > GABA. The Ca²⁺-dependent release of all of these amino acids was higher than that observed in calcium-free medium, except in the case of GABA, which was preferentially released by a Ca²⁺-independent process (Fig. 2).

The nicotinic acetylcholine receptor antagonist hexamethonium completely blocked the release of these amino acid neurotransmitters evoked by nicotine, which implies that the effect may be attributed to activation of the nicotinic receptor (Fig. 3).

Nicotine also increased both the membrane potential, measured as arbitrary fluorescence units, and intracellular calcium levels in a dose-dependent manner. Both effects were inhibited by hexamethonium (Fig. 4A, B).

Release of the four amino acid neurotransmitters evoked by 200 μM nicotine was inhibited by verapamil, an antagonist of L type Ca²⁺ channels; ω-conotoxin GVIA, an antagonist of N type Ca²⁺ channels, inhibited the release of all the amino acid neurotransmitters with the exception of aspartate. Further, ω-agatoxin GIVA, which blocks P/Q type Ca²⁺ channels, inhibited the release of aspartate, glycine and GABA, but not of glutamate (Fig. 5).

When the nicotine-mediated increments in intracellular calcium levels were measured in single cells it was observed that all of the neurons responded to nicotine, although these responses varied depending on the cell. Increases in intracellular Ca²⁺ levels of 152 ± 5 grey levels were recorded in 63% of the cortical neurons, of 90 ± 10 in 25% and of 48 ± 1 in 12% (Fig. 6).

TTX, which blocks voltage-dependent Na⁺ channels, completely reversed the Ca²⁺ entry mediated by nicotine in 100% of the cells under study (Fig. 7).

Discussion

Despite abundant data on AchR diversity in the CNS, there is still little evidence for classical nicotinic synaptic transmission. The results presented here clearly demonstrate that when cortical neurons are stimulated with concentrations of nicotine from 50 to 200 μM there is a dose-dependent release of aspartate, glutamate, glycine and GABA, which is exclusively Ca²⁺-dependent (exocytotic release) or dual (Ca²⁺-dependent and Ca²⁺-independent), depending on the amino acid. The nicotine concentration needed to induce amino acid release appears high compared with the findings of electrophysiological studies (McGehee et al., 1995). However, it is much lower than the concentrations used (1 mM or higher) in studies in which amino acid release was measured directly (Thoth et al., 1993). Besides its effects on amino acid neurotransmitter release, nicotine also induced membrane depolarization and intracellular Ca²⁺ increases. All these effects were blocked by hexamethonium, an antagonist of the nicotinic receptor, indicating their specificity. Intracellular calcium increases mediated by this cholinergic receptor were also shown by Mulle et al. in rat CNS neurons (Mulle et al., 1992). Further, Letz et al. observed that the stimulation of cholinergic receptors on pinealocytes may cause membrane depolarization and activation of L type Ca²⁺ channels (Letz et al., 1997).

Figure 1. Effect of different nicotine concentrations on amino acid neurotransmitter secretion in cortical neurons in culture. Basal is spontaneous release in the absence of nicotine, results without stimulation. Results are given as means ± SEM of two separate experiments, from cells of different cultures, each one performed in triplicate. Statistical significance is with respect to basal amino acids release. NS, not significant; ***, P < 0.001.

Figure 2. Effect of 200 μM nicotine on amino acid neurotransmitter release measured in a medium with (total release) and without external Ca²⁺ (Ca²⁺-independent release). Ca²⁺-dependent amino acid release is the difference between total release and release found in the absence of calcium in the medium. All results are given after subtracting the correspondent basal values. *, statistical significance with respect to the corresponding basal values (with and without external calcium). •, statistical significance between release measured in the presence and absence of external calcium. ** or ***, P < 0.01; ***, P < 0.001.
The data presented not only demonstrate the presence of nicotinic receptors in cultured cortical neurons, but also indicate their specificity and functionality. These findings are in accord with those obtained by Vidal and Changeus, who reported an increase in the negative wave of field potentials reflecting increased excitability of cortical neurons when neocortical slices were treated with acetylcholine or dimethylphenyl piperazinium (Vidal and Changeus, 1989).

In the present study nicotine was also able to induce the release of amino acid neurotransmitters in a calcium-free medium (Ca^{2+}-independent release). This Ca^{2+}-independent amino acid release may be attributable to the reverse action of amino acid transporters, given that the nicotinic receptor is a Na^{+}-permeable channel and, when open, the resulting increased intracellular Na^{+} level is a condition required to activate amino acid transporters.

Our data seem to indicate that different Ca^{2+} channels might be involved in release of the different amino acid neurotransmitters evoked by nicotine, since blockers of L, N and P/Q type Ca^{2+} channels inhibited release of the inhibitory amino acid neurotransmitters (glycine and GABA). However, N type Ca^{2+} channel blockers did not affect aspartate release and P/Q type Ca^{2+} channel blockers did not affect release of glutamate. These data would appear to suggest that opening of one Ca^{2+} channel or another is important in secretion of the different amino acid neurotransmitters mediated by nicotine.

The fact that nicotine-stimulated cortical neurons release
Ca²⁺ channels and not by the nicotinic receptor. In this case the evoked by nicotine appeared to be mediated by the opening of receptors, since, according to some authors, neuronal AchRs to activate Ca²⁺-dependent cellular processes. However, in the membrane depolarization, and/or Ca²⁺ entry through the nicotinic receptors, although the possibility that Ca²⁺ entry could be due to blockade of the nAChR. However, our results indicate that the effect of the toxins is mediated by Ca²⁺ channel blockade given that (i) the toxins show different sensitivity in inhibiting release of the different amino acid neurotransmitters and (ii) TTX, a voltage dependent Na⁺ channel blocker, completely abolished the Ca²⁺ entry mediated by 10 µM nicotine.

It may be concluded that: (i) cortical neurons contain functional nicotinic receptors since when stimulated with nicotine these neurons release aspartate, glutamate, glycine and GABA; (ii) the mechanism by which nicotine induces amino acid release is exocytotic or dual, depending on the amino acid; (iii) the effect of nicotine is specific since it is blocked by hexamethonium; (iv) L, N and P/Q type Ca²⁺ channels are involved in the nicotine effect.

Notes

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