Mechanisms of mitogen-activated protein kinase activation by nicotine in small-cell lung carcinoma cells

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We have previously reported that nicotine stimulates cell proliferation of three small-cell lung carcinoma (SCLC) cell lines by activating nicotinic receptors of the neuronal type. Here we report that, in the GLC-8 SCLC cell line, nicotine stimulates mitogen-activated protein (MAP) kinase activity in a concentration- and time-dependent manner ($ED_{50} = 10$ nM). The nicotine effect was antagonized by mecamylamine, an antagonist specific for neuronal nicotinic receptors. The absence of extracellular Ca^{2+} , or pretreatment with pertussis toxin or the tyrosine kinase inhibitor genistein inhibited the action of nicotine on

MAP kinase. Moreover, supernatants from nicotine-stimulated cells transferred to cells pretreated with mecamylamine were still capable of activating MAP kinase. On the other hand, the same supernatants transferred to cells pretreated with mecamylamine and pertussis toxin or genistein failed to activate MAP kinase. These findings suggest that nicotine elicits its stimulatory effect on MAP kinase in SCLC cells indirectly by inducing the production and/or release of a factor which then acts via a pertussis toxin- and tyrosine kinase-sensitive route.

INTRODUCTION

Tobacco smoking is causally linked to a variety of serious diseases ranging from cardiovascular diseases to lung cancer [1]. Recently it has been shown that benzopyrene and its metabolites derived from cigarette smoke cause selective damage to the p53 gene, thus confirming the major role of these polycyclic aromatic hydrocarbons in human tumorigenesis [2]. However, it is not known to what extent nicotine, the major ingredient of tobacco, contributes to the adverse effects of chronic tobacco use, apart from its psychoactive actions and dependence properties [3].

Small-cell lung carcinoma (SCLC), one of the most aggressive human lung cancers, is highly associated with tobacco smoking [4]. Cell lines derived from this tumour possess some neuroendocrine properties (e.g. the presence of secretory granules containing hormones and neuropeptides that, once released, act as autocrine/paracrine growth factors [5]). They also contain enzymes such as L-dopa decarboxylase and have voltage-operated Ca^{2+} channels [6,7]. However, since SCLC is considered the 'smoker's tumour', the most remarkable feature displayed by these cells is the presence of nicotinic receptors of the neuronal type (nAChR). It has been found that the α_3 , α_7 , β_2 and β_4 neuronal subunits of the nicotinic receptor are expressed in these cells [8]. On the basis of molecular and electrophysiological evidence it has been suggested that different subunits can associate in various combinations leading to receptor channels with different properties and functions [9]. We have previously shown that the stimulation of the nAChR by nicotine induced a significant increase in [³H]thymidine incorporation into DNA and in cell number in three SCLC cell lines, designated GLC-8, N-592 and H-69 [10]. The mitogenic effect of nicotine was antagonized by mecamylamine and α -bungarotoxin. Considering the interest in the physiological role of nAChRs and the dearth of information still available about their functions, we investigated some cellular mechanisms by which the stimulation of these receptors could

elicit cell proliferation in the GLC-8 cell line. Since mitogen-activated protein (MAP) kinase signalling appears to be essential for activating the expression of genes that control the response to mitogens [11], we studied the capacity of nicotine to stimulate MAP kinase activity in GLC-8 cells. Subsequent to observing such an effect we attempted to establish the possible mechanism(s) by which nicotine stimulates this activity.

MATERIALS AND METHODS

Cell culture

The SCLC cell lines GLC-8 and N-592 were routinely grown in Roswell Park Memorial Institute 1640 medium (RPMI 1640) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, U.S.A.). The cell lines were kept at 37 °C in a humidified atmosphere of 5% CO_2 in air.

MAP kinase activity

In all experiments the cells were deprived of serum for 48 h in RPMI alone. The cells (1.5×10^6 – 2×10^6) were then incubated at 37 °C with the various substances for the times indicated. For the experiments described in Table 1, RPMI medium was supplemented with appropriate EGTA/ $CaCl_2$ mixtures to maintain the indicated extracellular free Ca^{2+} concentrations. After stimulation, the medium was removed, and the cells were washed with ice-cold PBS before the addition of 0.5 ml of homogenization buffer, containing 50 mM β -glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM sodium vanadate, 1 mM benzamide, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 2 μ g/ml pepstatin. After sonication (2×7 s) and centrifugation (15000 g for 10 min at 4 °C), the supernatants

Abbreviations used: SCLS, small-cell lung carcinoma; nAChR, nicotinic acetylcholine receptor; FBS, fetal bovine serum; MBP, myelin basic protein.

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were fractionated on DEAE-cellulose minicolumns as described by Seger et al. [12]. Briefly, the supernatants were loaded on the minicolumns and washed twice with 0.75 ml of buffer A (50 mM β -glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol and 0.1 mM sodium vanadate) + 0.02 M NaCl. MAP kinase activity was eluted with 0.75 ml of buffer A + 0.22 M NaCl. Aliquots (12.5 μ l) of this fraction were incubated with myelin basic protein (MBP) in the presence of [γ - 32 P]ATP (2 μ Ci/sample; specific radioactivity 3000 Ci/mmol; Amersham) as described by Seger et al. [12]

Materials

All reagents were purchased from Sigma, St. Louis, MO, U.S.A. unless otherwise indicated. Nimodipine was from RBI, Natick, MA, U.S.A.

RESULTS

Activation of MAP kinase by nicotine

When we examined the ability of nicotine to stimulate MAP kinase activity in GLC-8 cells, using MBP as an exogenous substrate, we observed a concentration-dependent activation of the enzyme (Figure 1). The threshold concentration of nicotine was about 1 nM and EC_{50} was about 10 nM, with maximum stimulatory effects (3–4-fold stimulation over basal) measured at 100 nM. We observed that the degree of stimulation and the level of basal activity were sometimes variable, depending on the batch of cells used.

This kind of concentration–response curve is similar to, although not identical with, that of nicotine-induced DNA synthesis in the same cells [10]. To confirm that the effect of nicotine was specifically receptor-mediated, we used mecamylamine, an antagonist of the nAChR. Mecamylamine, at a concentration of 1 μ M, completely abolished nicotine-stimulated MAP kinase activity (inset of Figure 1).

Kinetic analysis of MAP kinase activity in response to nicotine (100 nM) showed that nicotine activation is detectable 1 min after its addition and the effects are maximal after 2 min, remaining elevated for up to 10 min (Figure 2A). This time

Figure 1 Concentration–response relationship of nicotine-induced MAP kinase activity

Serum-deprived GLC-8 cells were treated for 5 min with the indicated concentrations of nicotine. Inset: GLC-8 cells were pretreated for 10 min with medium alone (open bar) or 1 μ M mecamylamine (solid bar) before stimulation with nicotine (100 nM) for 5 min. MAP kinase activity was determined as described in the Materials and methods section. The data are expressed as fold increase in 32 P incorporated into MBP (pmol/min per mg of protein extract) under basal conditions. The data represent means $\pm$ S.E.M. for three experiments, each performed in duplicate.

Figure 2 Comparison of the short (A) and extended (B) time course of nicotine- and serum-induced MAP kinase activity

Serum-deprived GLC-8 cells were stimulated with nicotine (100 nM, ●) or 20% FBS (○). At the indicated times, cells were harvested and MAP kinase activity was determined as described in the Materials and methods section. The data are expressed as in Figure 1 and represent means $\pm$ S.D. for duplicate samples from the representative experiment which was repeated once with similar results.

course is delayed in comparison with that measured using serum as a stimulus, where the effect on enzyme activity is half-maximal within 30 s (Figure 2A). As can be seen in Figure 2(B), in which a more extended time course is presented, nicotine leads to a biphasic activation of MAP kinase. After an early peak observed between 5 and 10 min, a decrease to basal level is reached at 30 min and then kinase activity increased again after 60 min. Serum induced a similar biphasic response (figure 2B).

Ca²⁺-dependence of the nicotine effect

It is known that the nicotinic receptor is a channel permeable to Na⁺ and Ca²⁺ ions and that, in addition, the induced depolarization causes the opening of voltage-operated Ca²⁺ channels. To evaluate the contribution of Ca²⁺ signal-transduction pathways to nicotine-stimulated MAP kinase activity, the kinase activity was examined in cells treated with Ca²⁺ EGTA buffer so that extracellular [Ca²⁺] was held at 200 nM. Under these conditions nicotine failed to activate MAP kinase, indicating that Ca²⁺ influx is required for nicotine-induced MAP kinase activation. Under the same experimental conditions the stimulatory effect of serum was preserved (Table 1).

On account of the extracellular [Ca²⁺]-dependence of the action of nicotine, we next examined the effect on MAP kinase activity of Ca²⁺ influx induced by either a Ca²⁺ ionophore or a depolarizing stimulus, which opens voltage-dependent Ca²⁺ chan-

Table 1 Effect of extracellular Ca^{2+} concentration on MAP kinase activation by nicotine or serum

GLC-8 cells were incubated for 5 min with medium alone, nicotine or FBS in the presence of the indicated extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_e$). Results are expressed as pmol of ^{32}P incorporated into MBP/min per mg of protein and represent means $\pm$ S.E.M. for three experiments, each performed in duplicate.

	MAP kinase activity (pmol/min per mg)	
	500 μM $[\text{Ca}^{2+}]_e$	200 nM $[\text{Ca}^{2+}]_e$
Basal	1.54 $\pm$ 0.64	1.55 $\pm$ 0.82
100 nM Nicotine	4.15 $\pm$ 0.88	1.3 $\pm$ 0.68
20% FBS	4.30 $\pm$ 0.81	3.98 $\pm$ 0.94

Table 2 Effect of various treatments on MAP kinase activation

GLC-8 cells were stimulated with nicotine, ionomycin or FBS at the indicated concentrations for 5 min or with KCl for 10 min. Where indicated, GLC-8 cells were pretreated with pertussis toxin (PTx; 100 ng/ml; 4 h) or genistein (50 μM ; 15 min) before stimulation. Results are expressed as in Table 1 and represent means $\pm$ S.E.M. for three experiments, each performed in duplicate.

	MAP kinase activity (pmol/min per mg)		
	Control	+ Genistein	+ PTx
Basal	0.22 $\pm$ 0.011	0.22 $\pm$ 0.009	0.28 $\pm$ 0.019
100 nM Nicotine	0.99 $\pm$ 0.241	0.27 $\pm$ 0.032	0.34 $\pm$ 0.022
60 mM KCl	0.59 $\pm$ 0.005	0.23 $\pm$ 0.007	0.27 $\pm$ 0.060
1 μM Ionomycin	0.61 $\pm$ 0.006	0.24 $\pm$ 0.007	0.38 $\pm$ 0.040
20% FBS	0.95 $\pm$ 0.311	0.26 $\pm$ 0.032	0.67 $\pm$ 0.082

nels. Table 2 shows that both the depolarization of GLC-8 cells with 60 mM KCl and the administration of ionomycin (1 μM) induced MAP kinase activation to an extent comparable with that induced by nicotine.

Effect of tyrosine kinase inhibitors on nicotine action

To determine whether a tyrosine kinase-sensitive step is involved in the action of nicotine on MAP kinase, GLC-8 cells were pretreated with the tyrosine kinase inhibitor genistein (50 μM ; 15 min pretreatment). As can be seen in Table 2, the drug completely suppressed MAP kinase activity stimulated not only by nicotine, but also by KCl, ionomycin and serum. The basal levels were not affected. Almost superimposable results were obtained by using a second tyrosine kinase inhibitor, the tyrphostin AG18 (not shown).

Effect of PTx treatment on nicotine action

In an attempt to collect more information about the cellular mechanism(s) by which nicotine activates MAP kinase, we evaluated the possible involvement of a G-protein. GLC-8 cells were treated with PTx (100 ng/ml) for 4 h before stimulation with nicotine or serum. As can be seen in Table 2, PTx pretreatment completely inhibited the effect of nicotine (100 nM, applied for 5 min) on MAP kinase activation, whereas the stimulatory response to serum was only partially affected. PTx pretreatment also caused inhibition of MAP kinase activity stimulated by ionomycin and high K^+ (Table 2).

The above results indicate that nicotine activation of MAP kinase is dependent on the presence of extracellular Ca^{2+} and on a tyrosine kinase activity, and that a PTx-sensitive G-protein is

Table 3 Activation of MAP kinase by supernatants obtained from GLC-8 and N-592 cells treated with nicotine

A set of cells were treated with or without nicotine (100 nM for GLC-8 cells, 1 μM for N-592 cells) for 2 min. The supernatants obtained from these cells were then transferred for 5 min to another set of serum-deprived cells (represented in the Table) pretreated, where indicated, with mecamylamine (1 μM for GLC-8 cells, 2 μM for N-592 cells; 10 min), PTx (100 ng/ml; 4 h) or genistein (50 μM ; 15 min). The data, expressed as pmol of ^{32}P incorporated into MBP/min per mg of protein, are means $\pm$ S.D. for duplicate samples from a representative experiment which was repeated once with similar results.

Treatment of recipient cells	MAP kinase activity (pmol/min per mg)			
	GLC-8 cells		N-592 cells	
	Untreated	Nicotine-treated	Untreated	Nicotine-treated
None	0.21 $\pm$ 0.011	2.26 $\pm$ 0.006	1.28 $\pm$ 0.036	3.73 $\pm$ 0.037
Mecamylamine	0.22 $\pm$ 0.015	1.46 $\pm$ 0.015	1.25 $\pm$ 0.024	3.08 $\pm$ 0.054
PTx	0.20 $\pm$ 0.021	0.22 $\pm$ 0.011	1.34 $\pm$ 0.045	1.00 $\pm$ 0.029
Genistein	0.22 $\pm$ 0.004	0.23 $\pm$ 0.007	1.23 $\pm$ 0.012	1.19 $\pm$ 0.008
Mecamylamine + PTx	0.23 $\pm$ 0.008	0.22 $\pm$ 0.009	1.21 $\pm$ 0.048	0.92 $\pm$ 0.017
Mecamylamine + genistein	0.21 $\pm$ 0.009	0.20 $\pm$ 0.031	1.18 $\pm$ 0.003	1.07 $\pm$ 0.086

also critical for its action. It has recently been shown that, in neuronal cells, the Ras signalling pathway to MAP kinase can be stimulated by two different mechanisms, both set in motion by Ca^{2+} influx, i.e. the activation of a novel protein tyrosine kinase termed PYK2 [13] and an exchange factor named Ras-GRF/CDC25^{Mm} [14]. However, no sensitivity to PTx was reported in either case and immunoprecipitation studies with PYK2 antibodies (I. Dikic and J. Schlessinger, personal communication) and with Ras-GRF antibodies (R. Zippel, personal communication) failed to detect the two proteins in GLC-8 cells.

Thus in GLC-8 cells nicotine does not appear to activate MAP kinase via PYK2 or CDC25^{Mm} but rather through some other Ca^{2+} -, tyrosine kinase- and PTx-dependent mechanism.

One possible interpretation of these data is that nicotine (which acts as a secretagogue in neuronal cells) as well as ionomycin and high K^+ stimulate, in a Ca^{2+} -dependent manner, the production and/or release of a substance which then activates MAP kinase in a tyrosine kinase- and PTx-dependent manner, as many G_i-protein-coupled receptor agonists do [15]. To test this hypothesis we performed the experiments presented in Table 3. A set of GLC-8 cells were treated with or without nicotine for 2 min. The supernatants from treated and untreated cells were then transferred to a second set of serum-deprived GLC-8 cells, some of which had been treated with mecamylamine to block nicotinic receptors. As can be seen, the supernatant from the nicotine-stimulated cells, when added to the cells in which the nicotinic receptors were blocked by mecamylamine, was still capable of inducing a large increase in MAP kinase activity, indicating that a substance responsible for this stimulatory action had been released by nicotine. In addition, the supernatant from the nicotine-stimulated cells, when added to cells in the absence of mecamylamine, induced an even greater response, presumably due to the combined effect of nicotine and the still unidentified released substance.

Table 3 also shows results of the same experiment performed in parallel in which all the recipient cells were pretreated with PTx (4 h; 100 ng/ml) or genistein (15 min; 50 μM). The PTx and genistein pretreatment completely abolished the MAP kinase-

Figure 3 Activation of MAP kinase by supernatants obtained from GLC-8 cells treated with KCl

(A) GLC-8 cells were stimulated with KCl (60 mM; 10 min) in the absence (hatched bar) or presence (solid bar) of nimodipine (5 μ M). One set of cells (open bar) was treated with medium alone. (B) The supernatants obtained from GLC-8 cells treated with KCl (60 mM; 10 min) were transferred to another set of serum-deprived GLC-8 cells in the absence (hatched bar) or presence (solid bar) of nimodipine (5 μ M). The open bar received the supernatants from control cells incubated with medium alone. Data are expressed as pmol of 32 P incorporated into MBP/min per mg of protein and are means $\pm$ S.D. for duplicate samples from a representative experiment which was repeated once with similar results.

stimulatory effect of the transferred supernatant in the recipient cells.

The same type of experiment was carried out in a different SCLC cell line, N-592, with similar results (Table 3), indicating that this kind of behaviour is not restricted to a single SCLC cell line.

If the hypothesis of a substance released by nicotine into the medium being responsible for the effects on MAP kinase is correct, then the same kind of result should be obtained with KCl as secretagogue. We have previously shown [16] that the L-type Ca^{2+} -channel blocker nimodipine inhibits about 50% of the KCl-induced Ca^{2+} rise in GLC-8. Here in Figure 3 it is shown that nimodipine (5 μ M; 5 min pretreatment) partially inhibits MAP kinase activation by high K^{+} . However, the conditioned medium taken from KCl-depolarized GLC-8 cells and transferred to cells pretreated with nimodipine was still capable of stimulating MAP kinase activity to a similar extent to that in the control (compare solid bars in Figures 3A and 3B), which is consistent with the hypothesis that the conditioned medium contains a secreted active factor.

DISCUSSION

Nicotine acts as a mitogen in three SCLC cell lines tested in our laboratory by stimulating a nAChR [8,17]. In an attempt to understand the cellular mechanism(s) responsible for its mitogenic action we investigated the effect of nicotine on MAP kinase activation in the GLC-8 line because MAP kinase activation is considered to be a key event for growth factor signalling transmission to the nucleus [11].

Nicotine very potently stimulated MAP kinase activity with a biphasic response, evidenced by an early peak and a second increase in activity 1 h after stimulation. As suggested by Kahan et al. [18], it is this second phase of activity that is most probably related to the proliferative response. A previous paper has reported a stimulatory effect of nicotine on MAP kinase in bovine chromaffin cells, but in this case MAP kinase activity was suggested to be required for the nicotine-induced catecholamine secretion in these cells [19].

The Ca^{2+} -dependence of the nicotinic effect and the stimulation induced by the Ca^{2+} ionophore and KCl prompted us to

hypothesize that a Ca^{2+} -activated mechanism is responsible for the action of nicotine on MAP kinase. It has recently been shown that, in PC12 cells, a Ca^{2+} influx induced by carbachol via nicotinic receptors, activates PYK2 tyrosine kinase, which in turn is responsible for setting in motion the Ras/MAP kinase signalling cascade [13]. However, PYK2 tyrosine kinase involvement in our experiments must be excluded since this kinase is not expressed in the GLC-8 cell line. Also the Ca^{2+} -activated Ras-GRF/CDC25^{Mm} exchange factor is not expressed in the same cells. Of course, the existence of a Ca^{2+} -dependent Ras-GRF not yet cloned or other still unknown Ca^{2+} -activated pathways to Ras cannot be excluded.

However, SCLC cells are known to possess secretion granules, which contain peptides and other substances that, once released, might act as autocrine growth factors [5]. It is therefore reasonable to hypothesize that nicotine acts as a secretagogue, inducing Ca^{2+} -dependent release of a substance that then activates MAP kinase. This hypothesis is supported by three observations presented here. The delay in the onset of the action of nicotine in comparison with that of serum might indicate that some additional process occurs before MAP kinase activation. The PTx sensitivity of the nicotinic effect also suggests an indirect mechanism of action, since G_i and G_o -proteins are not reported to be involved in the functioning of nAChR. In addition, the PTx and genistein sensitivity not only of nicotine- but also of the ionomycin- and KCl-stimulated MAP kinase activity further strengthens the hypothesis of Ca^{2+} -dependent release of a factor that acts via a G-protein mechanism involving tyrosine kinase activity. Recent evidence suggests the existence of a G_i -Ras/MAP kinase connection where the $G_i\beta\gamma$ dimer is responsible for Ras/MAP kinase activation which involves the sequential interaction of one or more protein tyrosine kinases with the signalling proteins Shc, Grb-2 and SOS (for a review see [15]).

The final and strongest evidence that a substance is released by nicotine comes from the experiment in which supernatant from nicotine-treated cells was transferred to cells in which the nicotinic receptors had been blocked, and still MAP kinase activity was stimulated. Furthermore the transferred supernatant lost its stimulatory effect when the recipient cells were pretreated with PTx or genistein in addition to mecamylamine. The other secretagogue used, KCl, behaved in exactly the same way.

In conclusion, we provide evidence that, in GLC-8 cells, nicotine-stimulated MAP kinase activity is probably elicited by an indirect mechanism, i.e. the release, in a Ca^{2+} -dependent manner, of a substance that then activates MAP kinase in a tyrosine kinase- and PTx-dependent manner. Attempts to identify this substance(s) are under way.

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