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Ulrike Blank · Claudia Rückes · Wolfgang Clauss
Wolf-Michael Weber

Effects of nicotine on human nasal epithelium: evidence for nicotinic receptors in non-excitable cells

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Abstract We investigated the effects of nicotine and its derivate nicotine di-d-tartrate on primary cultured human nasal epithelial cells. Both substances evoked an increase in the intracellular free calcium concentration. In the presence of extracellular Ca^{2+} the cytosolic Ca^{2+} ($[Ca^{2+}]_i$) increase was long lasting, whereas in the absence of external Ca^{2+} there was a transient increase of $[Ca^{2+}]_i$ indicating that nicotine has an influence on Ca^{2+} conductances across the membranes and on intracellular Ca^{2+} stores. Both effects could be blocked by the nicotinic receptor antagonist methyllycaconitine (MLA). Apical or basolateral application of nicotine during transepithelial transport measurements with confluent monolayers of cultured human nasal cells resulted in a significant, reversible decrease of amiloride-sensitive sodium absorption with an apparent half-maximal blocker concentration of about 950 μM. To exclude the possibility that remnant neuronal components were responsible for the observed effects we used tetrodotoxin and verapamil to block putative neuronal channels and 4-(4-diethylamino)styryl-*N*-methylpyridinium iodide (4-di-2-Asp) to stain neuronal tissue. Both experimental approaches demonstrated that there were no neuronal-mediated effects. These results indicate the direct effects of nicotine on human nasal epithelium, giving the first evidence of the existence of nicotinic receptors in non-excitable cells.

Key words Human nasal epithelium · Nicotine · Nicotinic receptor · Epithelial Na^+ channel · Intracellular Ca^{2+}

Introduction

In excitable cells nicotine is a dominant neurotransmitter. In general, neurotransmitter receptors are grouped into two major populations: they comprise an integral ion pore or they are coupled to G proteins [10]. In excitable cells cholinergic stimulation of nicotinic acetylcholine receptors (nAChR) leads to a depolarization of the membrane and, in turn, activates voltage-dependent Ca^{2+} channels which allows Ca^{2+} to enter the cell [2]. As well, there is some evidence that nAChR activation is followed by a release of Ca^{2+} from intracellular stores [1].

The influences of nicotine on some non-excitable tissues have been investigated [19,29], but in these studies the nicotine effects were always mediated via the activity of sympathetic nerve endings connected to the investigated tissues. It was demonstrated that nicotine, a major component of tobacco smoke which is thought to be responsible for addiction, is able to penetrate epithelia [5] and that it interferes with cell energy metabolism [18]. Nicotine is also able to influence ion transport processes across epithelia [7]. However, direct effects of nicotine on non-excitable, nerve-free tissues have never been described.

We used a combination of electrophysiological methods and intracellular Ca^{2+} measurements to assess the effects of nicotine on human airway epithelium. We demonstrate that externally applied nicotine elevates the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) which could be blocked by the nicotinic antagonist methyllycaconitine (MLA). In Ussing chamber experiments apical or basolateral application of nicotine was followed by changes in transepithelial ion transport. Apical nicotine reduced the amiloride-sensitive portion of short-circuit current with an apparent half-maximal blocker concentration of about 950 μM. Higher concentrations of nicotine (5 mM) damaged the cells of the monolayer irreversibly. Possible influences of remnant neuronal components could be excluded by the use of specific blockers of neuronal ion channels and staining with a dye specific for neuronal tissue.

To the best of our knowledge, this is the first report of the direct effects of nicotine on non-excitable cells and the first hint of the existence of nicotinic receptors on epithelial cells.

U. Blank · C. Rückes · W. Clauss · W.-M. Weber (✉)
Institute for Animal Physiology,
Justus-Liebig-University, Wartweg 95,
D-35392 Giessen, Germany

Materials and methods

Cell culture

Nasal specimens were obtained from patients undergoing surgery for nasal reconstruction. Typically they were nasal polyps of patients suffering from chronic sinusitis. The use of these tissues for research was approved by the committee for human studies of the Justus-Liebig-University Giessen and the patients gave informed consent. Our method of culturing human nasal epithelium was similar to that outlined recently [4,27].

For measurements of $[Ca^{2+}]_i$ nasal epithelial cells were cultured on coverslips with a diameter of 15 mm coated with collagen (type I, from calf skin). The coverslips were transferred to 4-well plates and 1 ml serum-free, hormone-supplemented Ham′s F12 medium was added to each well. The cells were seeded at 5×10^3 cells/cm^2 and medium was changed every day.

For transepithelial measurements nasal epithelial cells were grown on a permeable collagen membrane with a diameter of 14 mm (Cellagen disc CD-24, ICN Biomedicals, Costa Mesa, USA). These preparations were also cultured with serum-free, hormone-supplemented Ham′s F12 medium which was changed daily. The cell cultures were incubated at 37°C and 5% CO_2 in humidified atmosphere.

$[Ca^{2+}]_i$ measurements with Fura-2

When they were 3–5 days old, cultures were loaded with fura-2/AM (MoBiTec, Göttingen, Germany) at a final concentration of 2 μM at 37°C. After 1 h the cells were washed two times with NaCl Ringer solution to remove the unloaded dye. The coverslips with the cells attached were mounted into a chamber over the objective of an inverted fluorescence microscope (Axiovert 10, Zeiss, Oberkochen, Germany) and covered with NaCl Ringer solution. Measurements of $[Ca^{2+}]_i$ were obtained at 24°C, the observed effects were similar to those measured at 37°C as also reported recently [21]. The fluorescence from the cells was monitored alternately at 360 and 390 nm by a dual wavelength photometer (Luigs and Neumann, Rathingen, Germany). Fluorescence at >470 nm was recorded by a photomultiplier (Luigs and Neumann) and a computer with an interface (DAS801, Keithley, Frankfurt/Main, Germany).

All fluorescence measurements were corrected for background. The corrected ratio (360/390 nm) was converted to the intracellular calcium concentration using an external calibration standard and the Grynkiewicz equation [11].

Cell staining

Cells were incubated with 4-(4-diethylamino)styryl-*N*-methylpyridinium iodide (4-di-2-Asp, 10 μM, Molecular Probes, Eugene, Ore., USA) for 3 min. This dye concentration has been reported to be more than sufficient for the staining of living nerve cells [17]. To monitor the fluorescence cells were subsequently mounted in a perfusion chamber on an inverted microscope (Axiovert 135 M, Zeiss) connected to a laser scanning confocal imaging system (MRC-1000, Bio-Rad, Hempstead, UK) equipped with an argon ion laser.

Transepithelial measurements

After 7–10 days in culture the nasal epithelial cells reached confluency on permeable membranes of the culture discs. These preparations were mounted in a modified Ussing chamber with a 0.5 cm^2 aperture designed to hold the intact filter cup without damage to the edge of the cultured monolayer. The two compartments of the Ussing chamber were continuously perfused with 37°C NaCl Ringer solution. Transepithelial voltage and current electrodes were AgCl wires connected to the bath by KCl agar bridges. The transepithelial resistance (R_T) was calculated from superimposed 10 mV pulses of 500 ms duration according to Ohm′s law and corrected for filter blanks. The short-circuit current (I_{sc}) was continuously recorded by a stripchart recorder and a computer (Apple Mac IIcx) with a MacLab interface and a chart recorder program (Analog Digital Instruments, Castle Hill, Australia). All stated electrical parameters are normalized to an area of 1 cm^2.

Solutions

The composition of the NaCl Ringer solution for $[Ca^{2+}]_i$ and transepithelial measurements was (in mM): 130 NaCl, 5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 25 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) and adjusted to pH 7.3. In Ca^{2+}-free solutions $CaCl_2$ and $MgCl_2$ were substituted by tetramethylammonium chloride (TMACl) and 0.5 mM EDTA was added. In Na^+-free solutions NaCl was substituted completely by TMACl; nicotine [(–)-1-methyl-2-(3-pyridyl)pyrrolidine], (–)-nicotine di-d-tartrate, MLA and amiloride were applied either to the apical or the basolateral solution as indicated for the particular experiment. All these chemicals were purchased from Sigma, Deisenhofen, Germany.

Results are expressed as means ± standard error of the mean (SEM) and *n* is the number of preparations.

Results

$[Ca^{2+}]_i$ measurements were made from single cells or two to three neighbouring cells. Basal $[Ca^{2+}]_i$ was 110 ± 3 nM (n = 33). $[Ca^{2+}]_i$ did not change significantly with the calcium content of the surrounding solution. Figure 1A depicts a typical experiment with fura-2-loaded nasal epithelial cells. Addition of nicotine (3 mM) resulted in a rapid increase in the fluorescence, caused by a rise in $[Ca^{2+}]_i$ which stabilized at this elevated level and persisted as long as nicotine was present. While the extent of the intracellular Ca^{2+} increase was quite varied and reached values of up to 5000 nM in some experiments, the mean nicotine-induced $[Ca^{2+}]_i$ was 800 ±141 nM (n = 15). With nicotine di-d-tartrate, a nicotinic agonist, an application of 200 μM was sufficient to evoke a similar, albeit lower, increase in $[Ca^{2+}]_i$ (450 ± 41 nM, n = 5, Fig. 1B).

Application of nicotine (3 mM) to the preparation in Ca^{2+}-free buffer resulted in a rise in $[Ca^{2+}]_i$ to a transient peak value of 536 ± 85 nM (n = 7, Fig. 1C). $[Ca^{2+}]_i$ then rapidly returned to the basal value, even in the continued presence of nicotine (n = 7). This lower and transient response to nicotine under external Ca^{2+}-free conditions must be due to the emptying of internal Ca^{2+} stores. The higher and long-lasting increase of $[Ca^{2+}]_i$ in the presence of external Ca^{2+} indicates the participation of Ca^{2+} conductances.

We investigated the effect of MLA, a potent and specific nicotinic antagonist that binds to nicotinergic binding sites [26]. Application of MLA alone was without effect on $[Ca^{2+}]_i$ in nasal epithelial cells. Addition 5 μM MLA together with nicotine produced a very delayed increase in $[Ca^{2+}]_i$ in Ca^{2+}-containing Ringer solution that could be completely abolished by further application of the antagonist (Fig. 1D). Initial addition of higher concentrations of MLA totally prevented the rise in $[Ca^{2+}]_i$ (n = 5).

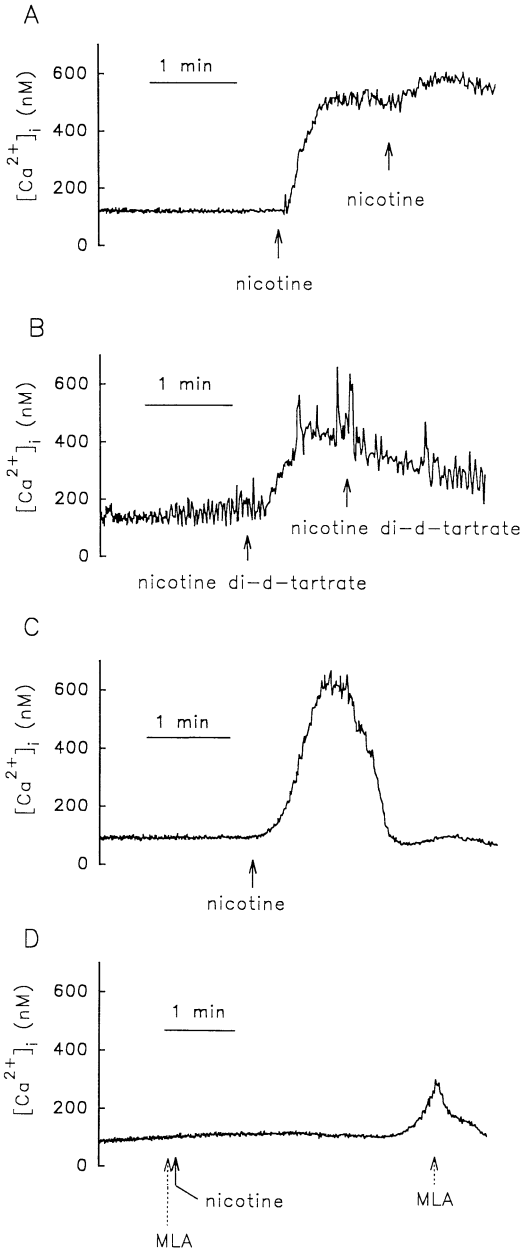

Fig. 1A–D Changes in $[Ca^{2+}]_i$ levels of fura-2-loaded primary cultured human nasal epithelial cells. **A** Apical addition of 3 mM nicotine (*arrows*) to cells bathed in Ca^{2+}-containing Ringer solution drastically raised $[Ca^{2+}]_i$. **B** Nicotine di-d-tartrate (200 μM) was followed (*arrows*) by a similar but lower increase in $[Ca^{2+}]_i$. **C** Addition of 3 mM (–)nicotine (*arrow*) to Ca^{2+}- and Mg^{2+}-free Ringer solution superfusing nasal epithelial cells evoked a transient peak in $[Ca^{2+}]_i$. **D** Application of 5 μM methyllycaconitine (*MLA, dotted arrow*) and subsequent addition of 3 mM nicotine (*solid arrow*) in the presence of MLA led to a markedly delayed small increase in $[Ca^{2+}]_i$ after nicotine administration. This increase could be reversed entirely by additional application of 5 μM MLA (*second dotted arrow*)

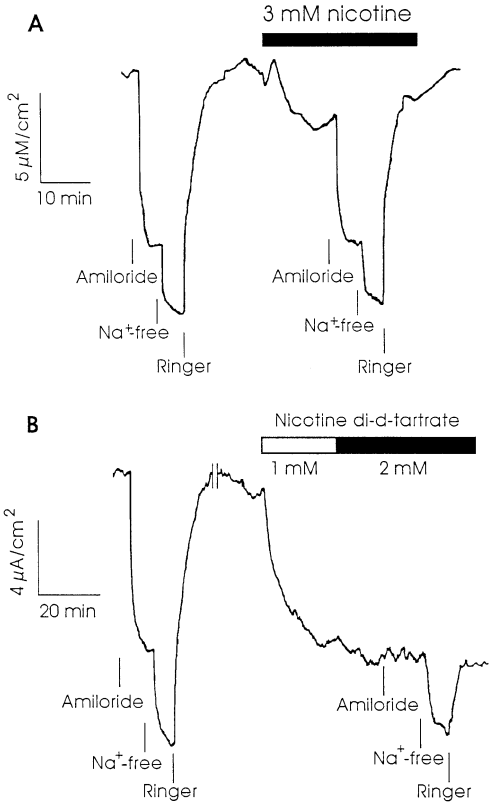

Fig. 2A, B Time course of typical experiments showing dependence of short-circuit current (I_{SC}) on apical nicotine. **A** Application of 3 mM nicotine reduced the total I_{SC} and the amiloride-sensitive I_{SC}, whereas the amiloride-insensitive Na^+ absorption was not affected by nicotine. The effects of nicotine were reversible. **B** Addition of 1 mM nicotine di-d-tartrate to the apical superfusate drastically reduced I_{SC}. Application of higher concentrations of nicotine di-d-tartrate or addition of amiloride (100 μM) did not further lower I_{SC}. Again, amiloride-insensitive I_{SC} was not affected. The effects of nicotine di-d-tartrate were not reversible

Primary cultured monolayers of nasal epithelial cells grown on permeable collagen membranes had a transepithelial potential (V_T) of –3.5 ± 0.3 mV and an R_T, corrected for filter blanks, of 314 ± 20 Ω · cm² (n = 47). I_{SC} averaged 15.6 ± 1.4 μA/cm² and was mainly generated by transepithelial Na^+ absorption without any remarkable contribution of other ion transport mechanisms, as also reported previously [4]. Figure 2A shows the trace of I_{SC} in a typical experiment. Addition of 100 μM amiloride to the apical solution reduced total I_{SC} by about 80%. This blocker concentration was reported to block entirely all amiloride-sensitive Na^+ conductances of nasal epithelial cells in primary culture [3]. Subsequent removal of Na^+ from the apical solution by substitution with the impermeant cation TMA^+ further decreased I_{SC} until it was nearly completely abolished. After readdition of Na^+ I_{SC} reached its former value again. Addition of nicotine (3 mM) to the solution superfusing the apical side of the epithelium produced a small transient increase in I_{SC}, which was followed by a larger reduction of I_{SC}. Total I_{SC} was decreased by nicotine to 75 ± 5% (n = 4) of its former value, while R_T did not change significantly indicating that the observed current decrease also reflects transepithelial depolarizations. The unaltered R_T is also proof that no leakages occurred in the epithelial preparations after nicotine

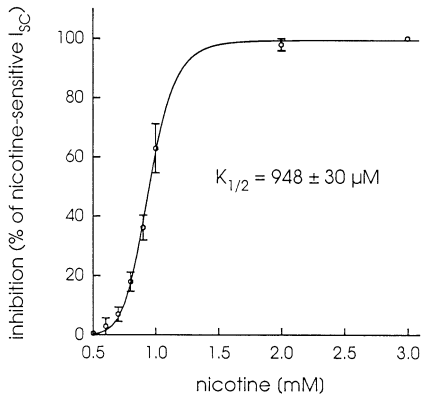

Fig. 3 Nicotine dose/response relationship. Increasing apical nicotine concentrations led to successively decreasing I_{SC}. The *solid line* represents a fit of the Hill equation to the data points; $K_{1/2}$ is indicated on the figure

administration. Additional application of 100 µM amiloride decreased I_{SC} again down to the same value as it did without nicotine. The reduction of I_{SC} caused by amiloride in the presence of nicotine was 21 ± 4% (n = 4) smaller compared to the reduction in the absence of nicotine, demonstrating an effect of nicotine on amiloride-sensitive Na^+ conductances. The amiloride-insensitive, Na^+-mediated I_{SC} was not affected by nicotine. Similar results were obtained when the same experimental protocol was used in combination with basolaterally applied nicotine (3 mM, n = 5). In this case, 33.3 ± 4.72% of total I_{SC} was blocked reversibly while the amiloride-insensitive portion of I_{SC} again remained uninfluenced.

Application of nicotine at concentrations higher than 5 mM led to a rapid and drastic decrease of R_T, indicating irreversible damage of the tissue. Subsequent optical investigations revealed a structural impairment of the monolayer by nicotine in such high doses (n = 6).

Figure 2B shows the effects of nicotine di-d-tartrate on I_{SC}, which is decreased drastically. Additional application of 100 µM amiloride did not further reduce I_{SC}. Again, replacement of Na^+ lowered the current. Readdition of Na^+ and withdrawal of nicotine di-d-tartrate were followed by a small increase in I_{SC}, but the effect was not completely reversible. Contrary to nicotine, its analogue nicotine di-d-tartrate completely and irreversibly inhibited all amiloride-sensitive Na^+ conductances in human nasal epithelium.

Transepithelial short-circuit measurements were also used to determine the concentration of nicotine which induced a half-maximal inhibition of Na^+ transport. Figure 3 presents the dose/response relationship of apical nicotine on I_{SC}. Half-maximal current appeared at nicotine concentrations of 948 ± 30 µM (n = 6). Similar results were obtained with basolateral nicotine and measurements of $[Ca^{2+}]_i$ after application of different nicotine concentrations yielded comparable half-maximal blocker concentrations. Dose/response measurements with acetylcholine (ACh) yielded a half-maximal inhibition of Na^+ transport at an ACh concentration of 21 ± 6 µM.

To investigate the possible presence of nerve endings in our primary culture preparation, which could have influenced our experiments, we used two different strategies: application of neuronal ion channel blockers and staining of neuronal components with a specific sensitive dye. However, both methods clearly showed that our primary culture of human nasal epithelium was entirely free of any detectable neuronal components. Apical or basolateral application of tetrodotoxin (TTX, 1 µM), a specific blocker of Na^+ channels in excitable tissues [24], had no influence on I_{SC}. Verapamil, known to block Ca^{2+} channels effectively in nerve tissues [15], also had no effect on the electrical parameters of human nasal epithelium, indicating the absence of these neuronal channels in our preparations.

In another attempt to exclude potential neural-mediated effects we used a styryl pyridinium dye to stain putative nerve cells in our preparation of human airway epithelium. 4-di-2-Asp has been shown to stain nerve fibres and terminals effectively in a variety of tissues derived from different species [17]. However, in our preparations of primary cultured human airway epithelium, even at high 4-di-2-Asp concentrations (1 mM), no staining could be observed, more strong evidence for the absence of any remnant neuronal components.

Discussion

Until now nicotine was thought to have direct effects only in excitable cells or tissues still connected to sympathetic nerve endings. However, in the present study we used primary cell cultures of human nasal epithelium recently introduced as a model system for non-excitable cells free of any nerve endings [3]. The lack of any neuronal components was proved by application of specific blockers of neuronal ion channels, TTX and verapamil: neither showed any effect on the electrical parameters or $[Ca^{2+}]_i$. Staining with a dye specific for nerve fibres and terminals was also without effect, giving additional strong evidence for the absence of nerve endings which may have unwanted influences on our results.

The present report is the first study, to the best of our knowledge, that describes direct effects of nicotine on human nasal epithelial cells. Cigarette smoke contains various substances, including nicotine; therefore, the airways of smokers and passive smokers are in contact with nicotine. The consequences of cigarette smoke for the airway epithelium are complex. Cigarette smoke is responsible for impaired mucociliary clearance, increased recruitment of inflammatory cells and nasal respiratory epithelial hyperplasia. It is also known to decrease the tracheal potential difference and transepithelial ion transport [8]. As to which of the components of cigarette smoke are responsible for these various effects is poorly understood. In our study we investigated the impact of nicotine on monolayer cultures of human nasal epithelium. Nicotine was applied to the apical side of the epithelium, the appropriate side for investigating the action for

environmental cigarette smoke, as well as to the basolateral side. In both experimental approaches nicotine had similar effects on the epithelium. However, the concentrations of nicotine that were required to induce the effects on $[Ca^{2+}]_i$ and I_{SC} were quite high compared to data already published on the neuronal nAChR, at which nicotine acts in micromolar doses. It was also found that the nicotine blockage is highly co-operative (Hill coefficient of nearly 4) which is in good accordance with findings from studies of neuronal nAChR.

We found a large increase of $[Ca^{2+}]_i$ after application of nicotine or its analogue nicotine di-d-tartrate in nasal epithelial cells. In the presence of extracellular Ca^{2+} this intracellular Ca^{2+} elevation is extensive and persistent. In the absence of extracellular Ca^{2+}, nicotine application evokes only a small, transient increase in $[Ca^{2+}]_i$. We conclude that nicotine evokes a biphasic increase in $[Ca^{2+}]_i$ that seems to be composed of mobilization of calcium from intracellular stores and influx of Ca^{2+} from the extracellular space through Ca^{2+} conductances in the apical membrane. In airway epithelial cells comparable biphasic Ca^{2+} increases were observed after application of histamine [14], menthol [23] or ATP [6,12]. Moreover, previous studies of mouse myotubes [10] and bovine adrenal medullary cells [1] revealed similar effects of nicotine on $[Ca^{2+}]_i$. In these cells nicotine application was followed by an independent acquisition of Ca^{2+} from inositol trisphosphate-sensitive stores and Ca^{2+} influx through voltage-gated Ca^{2+} channels. In rat hippocampal neurons nicotinic receptor activation leads to an increase of $[Ca^{2+}]_i$ by promoting the influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels that can be abolished in the presence of MLA, which acts as a powerful antagonist of the nAChR [2,26]. In our experiments MLA, which is extracted from the seeds of *Delphinium brownii*, suppresses the action of nicotine. MLA is reported to bind specifically to nAChR thereby inducing complete block of the receptors in excitable cells. The sensitivity of nasal epithelial cells to MLA confirms the assumption that the nicotinic effects are also mediated by nAChR in these non-excitable cells the presence of which we proved further by dose/response measurements with ACh.

In airway epithelial cells different consequences of intracellular Ca^{2+} increases have been reported. Intracellular Ca^{2+} elevation could be responsible for enforced ciliary beat frequency [20] or changes in cellular transport processes, such as increased secretion of Cl^- and HCO_3^- across the apical membrane [25] and increased K^+ permeability of the basolateral membrane [6]. From studies of toad bladder epithelium it is known that increases in $[Ca^{2+}]_i$ induce inhibition of apical amiloride-sensitive Na^+ channels [9].

To examine possible influences of the nicotine-produced increase in $[Ca^{2+}]_i$ on ion conductances in nasal epithelial cells we measured transepithelial ion transport of monolayer cultures in modified Ussing chambers. Under physiological conditions Na^+ absorption is the dominant transport process in primary cultured nasal epithelial cells [3] and other ion transport processes such as Cl^- secretion are similarly small [4]. The majority of Na^+ transport is sensitive to the classic Na^+ channel blocker amiloride or its analogues. We found that the amiloride-sensitive portion of Na^+ absorption is affected by apical or basolateral application of nicotine or its derivate nicotine di-d-tartrate. Application of nicotine produces a transient increase of I_{SC} which is followed by a sustained decrease of I_{SC} due to reduced amiloride-sensitive Na^+ absorption. This reduction is reversible after withdrawal of nicotine. With regard to the nearly unchanged R_T and the reversibility of the effect we conclude that nicotine, at these concentrations, does not damage the epithelial cells. Similar traces of I_{SC} after application of nicotine were obtained in studies of rat colon, but they were mediated indirectly by stimulating the release of an intermediary neurotransmitter [30]. Nevertheless, also in rat colon, nicotine was found to be responsible for a transient increase of Na^+ absorption followed by a decline.

Inhalation of pure nicotine affects the respiratory system by producing a concentration-dependent cough and airway obstruction, but these effects may be due to stimulation of afferent nerve endings and mediated through parasympathetic cholinergic pathways [13]. In rat trachea nicotine induces the release of calcitonin gene-related peptide from peripheral nerve terminals [16]. Nicotine is well known to have toxic effects in many tissues, such as the pancreas [22] and liver [28]. In rat neonatal lung nicotine was reported to damage type I pneumocytes, rupturing the blood–air barrier [18]. In our freshly cultured nasal epithelial cells we also observed destruction of the tissue after application of high doses of nicotine, which was obvious because of the rapid decrease of R_T. Short-lasting applications of concentrations of up to 5 mM nicotine or application of its derivate nicotine di-d-tartrate had no noticeable cytotoxic effects on human nasal epithelium.

All these data give evidence that apical and basolateral membranes of human nasal epithelial cells possess receptors for nicotine. Binding of nicotine to these receptors could lead to the activation of Ca^{2+} conductances, thereby increasing $[Ca^{2+}]_i$. On the other hand, via as yet unidentified second messenger processes intracellular Ca^{2+} stores are emptied independently of an extracellular Ca^{2+} supply. Both of these mechanisms leading to increased $[Ca^{2+}]_i$ may be the cause of the inhibition of amiloride-sensitive Na^+ absorption by nicotine. However, from our experimental data we can only speculate about this proposed mechanism.

In conclusion, our experiments demonstrate a direct regulation of physiological processes in human nasal epithelium by nicotine. That nicotine has an effect on both sides of the epithelium may indicate the presence of a specific receptor in this membrane. While the effect of nicotine on epithelial amiloride-sensitive Na^+ conductances is obvious, possible influences of nicotine on other transport systems of the cell remain to be elucidated. However, with these findings we add another aspect to the long list of unwanted nicotine-induced effects.

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