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Short Communication

EFFECT OF NICOTINE ON DOPAMINE UPTAKE IN COS CELLS POSSESSING THE RAT DOPAMINE TRANSPORTER AND IN PC12 CELLS

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Abstract—The effect of nicotine on the uptake of dopamine (DA) is not completely understood. We studied its effect on PC12 cells and on COS cells transfected with the rat DA transporter cDNA (pcDNADAT1). DA uptake by PC12 cells was inhibited by nicotine in a concentration-related fashion. Treatment of PC12 cells with nerve growth factor (NGF) increased such inhibition. This inhibitory effect was abolished by hexamethonium and mecamylamine, indicating that nicotine acted via the nicotinic acetylcholine (nACh) receptors in PC12 cells. This view is also supported by evidence that acetylcholine (ACh) reduced the uptake of DA in a hexamethonium-, but not atropine-, sensitive fashion. However, nicotine failed to inhibit DA uptake by COS cells possessing the DA transporter. These results suggest that the inhibitory effect of nicotine on DA uptake, when coupled with an nACh receptor leading to an indirect action on the transporter, may play a role in regulating extracellular concentrations of DA.

Key words: nicotine; dopamine transporter; nicotinic acetylcholine receptor; COS cell; PC12 cell; nerve growth factor

Several epidemiological studies indicate a negative relationship between the incidence of Parkinson's disease and smoking, independent of other factors [1]. Evidence of a possible functional interaction between nicotine and the nigrostriatal dopaminergic system suggests new approaches to the treatment or prevention of various neurological diseases, including Parkinson's disease. Nicotine administration accelerates the utilization of brain DA‡ [2], and facilitates its release in the rat striatum [3]. DA neurons in the substantia nigra pars compacta possess nicotine-binding sites, not only on the somata, but also on terminals [4]. Studies using rat striatal slices and synaptosomal preparations show that nicotine inhibits DA uptake by slices but not by synaptosomes, suggesting an indirect effect of nicotine on DA uptake, possibly via the trans-synaptic regulation of DA accumulating terminals [5]. However, the effect of nicotine on DA uptake is not completely understood. In the present study, the effects of nicotine on DA uptake were investigated in COS cells transiently expressing the rat DA transporter and in PC12 cells, in view of its possible direct action on the transporter or via an indirect mechanism of nicotinic receptor/transporter cross talk within the same cells.

Materials and Methods

COS cells were cultured in DMEM with 10% fetal calf serum at 37° under an atmosphere of 5% CO_2. COS cells were transfected with rat DA transporter complementary DNA (DAT1) subcloned into a eukaryotic cell expression vector, pcDNAI (pcDNADAT1), as described previously [6]. Cells (1×10^7) were electroporated with 6.7 pmol of

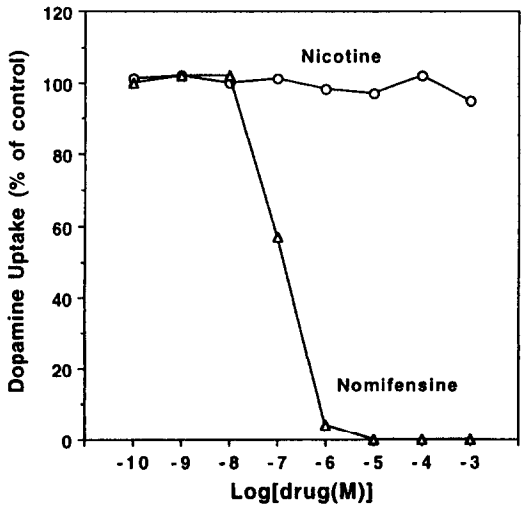

Fig. 1. Effect of nicotine (○) or nomifensine (△) on [^{3}H]-dopamine uptake in COS cells expressing the rat dopamine transporter. Values are the mean of six independent experiments, each performed in triplicate and expressed as a percentage of the control uptake. SEM was less than 5.8% of the mean. K_m for [^{3}H]dopamine uptake was 1.8 ± 0.2 μM and V_{max} was 8.8 ± 0.9 pmol/min/10^5 cells.

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‡ Abbreviations: DA, dopamine; ACh, acetylcholine; nACh, nicotinic acetylcholine; DMEM, Dulbecco's modified Eagle's medium; NGF, nerve growth factor; KRH, Krebs-Ringer-HEPES.

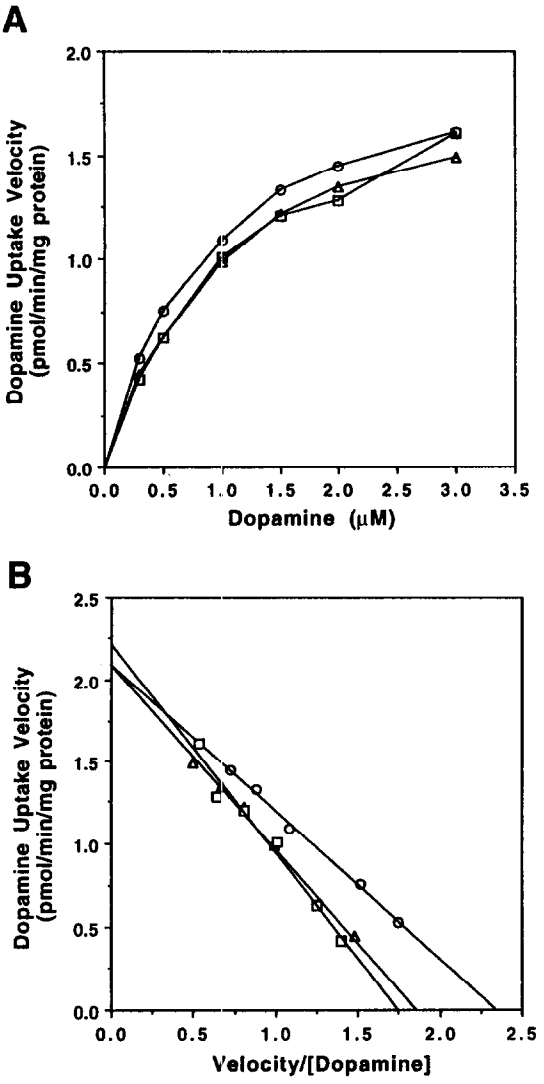

Fig. 2. Kinetic studies of [^{3}H]dopamine uptake in PC12 cells untreated (○) or treated with βNGF, 50 ng/mL, for 3 days (△) and 7 days (□). (A) Cells were incubated for 15 min with various concentrations of [^{3}H]dopamine in KRH buffer. Results are means of triplicate incubations. (B) Eadie–Hofstee plot of the concentration dependence of [^{3}H]dopamine uptake.

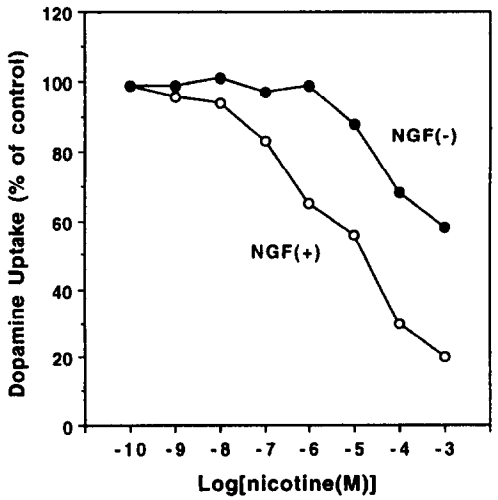

Fig. 3. Effect of nicotine on [^{3}H]dopamine uptake in PC12 cells untreated (●) or treated with βNGF, 50 ng/mL, for 7 days (○). Each value was the mean of six experiments and was calculated in the same manner as in Fig. 1. Control values of [^{3}H]dopamine uptake into βNGF-untreated and -treated PC12 cells were 14.2 and 14.9 fmol/10 min/10^5 cells in the absence of nicotine, respectively.

pcDNADAT1 with 200 V at 1600 μF. Cells were cultured on a 24-well, collagen-coated plastic plate for 2 days, just prior to confluence. PC12 cells were grown on a 24-well, collagen-coated plastic plate at 37° under an atmosphere of 10% CO_2 in DMEM containing 5% fetal calf serum and 10% horse serum. For the treatment with NGF, PC12 cells were grown in the presence of βNGF, 50 ng/mL (Toyobo Co., Japan) for 7 days. PC12 cells were exposed to 10 μM reserpine (Sigma Chemical Co., U.S.A.) for 16 hr prior to experiments to deplete intracellular DA. Following this treatment, nicotine stimulation (10^{-4} M) for 10 min did not evoke significant DA release, as determined by HPLC-ECD.

To assess DA uptake, cells were washed twice with 2 mL of Krebs-Ringer-HEPES buffer (KRH buffer), consisting of 5.6 mM glucose, 125 mM NaCl, 4.8 mM KCl, 1.2 mM potassium phosphate, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM sodium ascorbate and 25 mM HEPES, pH 7.3, and then incubated with 15 nM [^{3}H]DA (46 Ci/mmol; Amersham, U.K.) in 1 mL of KRH buffer for 10 min at 37°. This was followed by two washes with 2 mL of ice-cold KRH buffer. Radioactivity remaining in the cells was extracted and measured by liquid scintillation counting. NaCl in KRH buffer was replaced by 125 mM choline chloride to assess non-specific uptake.

Data are reported as statistical analyses utilizing ANOVA and Student's *t*-test. A level of $P < 0.05$ was accepted as statistically significant.

Results

Effect of nicotine on DA uptake in COS cells possessing DA transporter. The effect of nicotine on [^{3}H]DA uptake was investigated in COS cells possessing the rat DA transporter. Nicotine (10^{-10}–10^{-3} M) failed to inhibit DA uptake by transfected COS cells with pcDNADAT1, while nomifensine, a potent inhibitor of DA uptake, inhibited DA uptake in a concentration-dependent manner (Fig. 1).

Kinetic studies of DA uptake in PC12 cells. The effect of nicotine on [^{3}H]DA uptake was investigated in PC12 cells. Kinetic study showed that Na^+-dependent DA transport is a saturable process in both βNGF-treated and -untreated PC12 cells (Fig. 2A). An Eadie–Hofstee plot of the concentration dependence of [^{3}H]DA uptake revealed no significant differences in Michaelis constant (K_m) or maximum velocity (V_{max}) of uptake between βNGF-treated and -untreated cells (Fig. 2B). K_m was 0.9, 1.1 or 1.2 μM, and V_{max} was 2.1, 2.1 or 2.2 pmol/min/mg of protein in untreated cells and in cells treated with βNGF for 3 or 7 days, respectively.

Effect of nicotine on DA uptake in PC12 cells. An inhibitory effect of nicotine on DA uptake occurred in a concentration-dependent fashion (10^{-5}–10^{-3} M) in βNGF-untreated PC12 cells (Fig. 3). Treatment of PC12 cells with βNGF for 7 days increased the inhibitory effect of nicotine in a concentration-dependent fashion (10^{-8}–10^{-3} M).

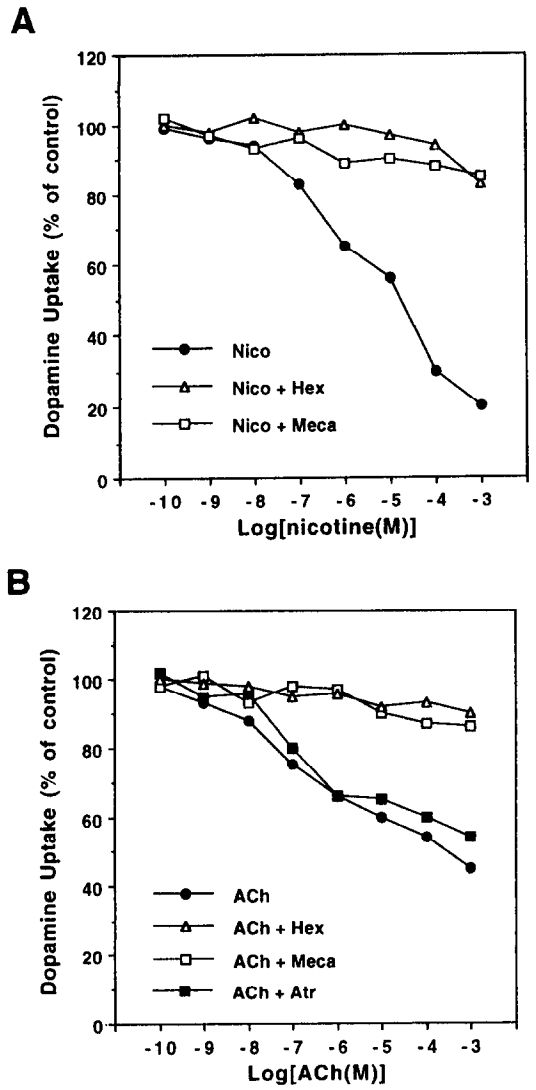

Fig. 4. Effect of cholinergic inhibitors on inhibition of [^{3}H]dopamine uptake by nicotine and acetylcholine in βNGF-treated PC12 cells. Mecamylamine (Meca; 3 μM), hexamethonium (Hex; 500 μM), or atropine (Atr; 100 μM) was added to KRH buffer with nicotine or acetylcholine. PC12 cells were treated with βNGF, 50 ng/mL, for 7 days. Each value was the mean of six experiments and calculated in the same manner as in Fig. 1.

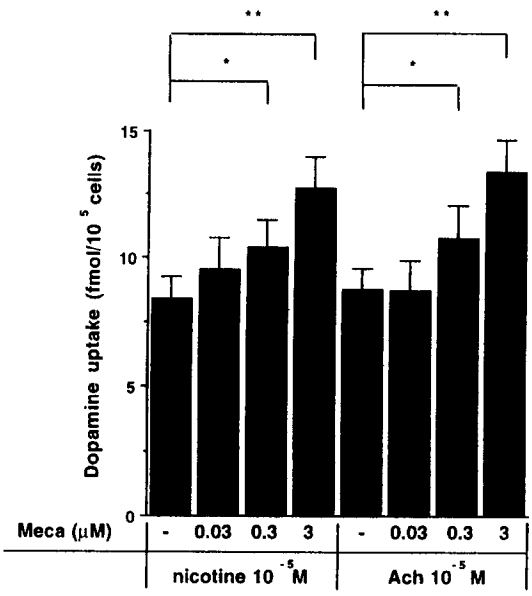

Fig. 5. Effect of mecamylamine (Meca) on inhibition of [^{3}H]dopamine uptake induced by nicotine (10^{-5} M) or ACh (10^{-5} M). Each value represents [^{3}H]dopamine uptake from triplicate determinations in five independent experiments. Vertical bar represents SD, statistical significance differences (Student's t-test): $^{**}P < 0.01$, $^{*}P < 0.05$.

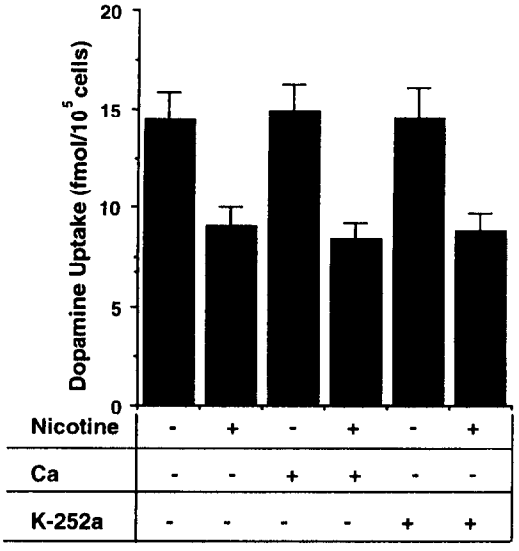

Fig. 6. Effect of Ca^{2+} deprivation and K-252a (100 nM) on inhibition of [^{3}H]dopamine uptake induced by nicotine (10^{-5} M). Each value represents mean ± SD, N = 6.

Maximal inhibition of DA uptake by nicotine was 81%, with an IC_{50} of 8.0×10^{-6} M in βNGF-treated PC12 cells.

When mecamylamine, an antagonist of nACh receptor, was added to KRH buffer at a concentration of 3 μM, the inhibitory effect of nicotine on DA uptake was abolished in βNGF-treated PC12 cells (Fig.4A). The inhibitory effect of nicotine in βNGF-treated PC12 cells was also prevented by 500 μM hexamethonium, another nACh receptor antagonist. Mecamylamine (3 μM) or hexamethonium (500 μM) alone had no effect on DA uptake.

ACh (10^{-8}–10^{-3}M) also inhibited DA uptake maximally by 55%, with an IC_{50} of 2×10^{-7} M, similar to nicotine (Fig. 4B). Mecamylamine (3 μM) or hexamethonium (500 μM) attenuated the inhibitory effect of ACh on DA uptake, whereas 100 μM atropine, an antagonist of muscarinic ACh receptors, failed to affect the inhibitory effect of ACh. The effect of mecamylamine on the inhibition of DA uptake induced by nicotine and ACh was concentration dependent as shown in Fig. 5.

Effect of Ca^{2+} and K-252a on nicotine inhibition of DA

uptake. The effect of nicotine on DA uptake was investigated when calcium ions were removed from KRH buffer. The inhibitory effect of nicotine was still observed in the Ca^{2+}-free condition in βNGF-treated PC12 cells (Fig. 6). The possible involvement of phosphorylation in inhibition of DA uptake induced by nicotine was investigated by using K-252a, a broad and specific protein kinase inhibitor. K-252a (100 nM) did not alter the inhibitory effect of 10^{-5} M nicotine on DA uptake (Fig. 6).

Discussion

The functional interaction between nicotine and the nigrostriatal dopaminergic system has drawn attention for possible application in treating or preventing neurological diseases. The effect of nicotine on DA uptake was investigated to determine whether nicotine acts directly on the DA transporter in COS cells expressing rat DA transporter as well as in PC12 cells. Nicotine failed to inhibit DA uptake in the transfected COS cells, indicating a lack of direct action on the transporter.

An inhibitory effect of nicotine on DA uptake was observed in PC12 cells. This effect was abolished by hexamethonium and mecamylamine, competitive antagonists of nACh receptor. ACh also inhibited DA uptake in a hexamethonium- and mecamylamine-sensitive, but atropine-insensitive, manner. These results clearly indicate that nicotine exerts an inhibitory effect on DA uptake through nACh receptors.

An inhibitory effect of nicotine on DA uptake was observed in both βNGF-treated and -untreated PC12 cells. PC12 cells can take up DA and norepinephrine through a Na^+-dependent process [7]. Kinetic studies revealed that treatment with βNGF essentially did not change K_m or V_{max} (Fig. 2B). However, the present results showed a clear difference in inhibition of DA uptake by nicotine in the presence and absence of NGF. There is some evidence that NGF increases both the Na^+ conductance capacity of nACh receptors [8] and the number of PC12 cells expressing functional nACh receptor [9]. Taken together, they lead to the assumption that an increase in functional nACh receptors rather than the transporter properties might have brought about the more pronounced inhibitory effect of nicotine on the transporter. Although the exact mechanism of inhibition of DA uptake is still unknown, the decrease in Na^+ gradient resulting from the Na^+ influx through nACh receptors caused by nicotine might easily be achieved in βNGF-treated PC12 cells, resulting in pronounced inhibition of DA uptake.

Rapier *et al.* [3] reported that nicotine-evoked DA release arises from cation influx through nACh receptor channels in a Ca^{2+}-dependent manner. However, the inhibitory effect of nicotine was still observed in a Ca^{2+}-free condition. Ca^{2+} influx through nACh receptor or voltage-sensitive Ca^{2+} channels might promote the intracellular phosphorylation of various proteins including the transporter that takes up DA and norepinephrine in PC12 cells. It has been demonstrated that protein kinase C-mediated phosphorylation could play a significant role in functional regulation of the DA transporter [10]. Therefore, it is assumed that a possible phosphorylation of the transporter may participate in the inhibition of DA uptake induced by nicotine. K-252a, a broad, specific, and potent protein kinase inhibitor [11], failed to alter the inhibitory effect of 10^{-5} M nicotine on DA uptake, suggesting that phosphorylation of the transporter does not play a crucial role in the inhibition of DA uptake induced by nicotine in PC12 cells.

Izenwasser *et al.* [5] revealed that nicotine inhibits DA uptake maximally by ~50%, with an IC_{50} of ~5 pM in chopped rat striatal tissues. They suggested that an indirect effect of nicotine might possibly be mediated by release of an unidentified transmitter onto DA terminals, thereby influencing the transporter [5]. The present study demonstrated, however, that an nACh receptor/DA transporter cross-talk mechanism occurred within the same PC12 cell. There is evidence indicating the presence of nACh receptor on DA neuron terminals [4]. Although the concentration of nicotine required for inhibition in the present study greatly exceeded that observed in chopped striatal tissues, the micromolar range is comparable to the plasma concentration of nicotine observed in smokers [12]. The physiological relevance of this cross-talk in the same cell along with its mechanism remain to be elucidated.

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