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Opposite modulation of glutamate uptake by nicotine in cultured astrocytes with/without cAMP treatment

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Abstract

Cultured cerebellar astrocytes were exposed to nicotine with or without dibutyryl cAMP (dBcAMP) for 2 to 10 days in situ in order to determine the effects of nicotine exposure on glutamate uptake in differently conditioned astrocytes. After acute nicotine exposure, glutamate uptake was lower and higher in the naive and the preconditioned astrocytes, respectively. After subacute nicotine exposure, the inhibitory effect of L-*trans*-pyrollidine-2,4-dicarboxylic acid (PDC) on the glutamate uptake in the naive and the conditioned cells was decreased and increased, and the IC_{50}'s for PDC were higher and lower, respectively. In addition, glutamine synthetase activity after subacute nicotine exposure was lower in the naive and higher in the conditioned astrocytes; the change in Na^+/K^+ ATPase activity was the opposite to that in glutamine synthetase activity. These results suggest that nicotine modulates the characteristics of glial glutamate transporters and glutamine synthetase activity in astrocytes. Furthermore, nicotine might differently affect the state of glial cells during development.

Keywords: Nicotine; Glutamate uptake; Glutamine synthetase; Na^+/K^+ ATPase

1. Introduction

Glutamate is a major excitatory neurotransmitter in the mammalian brain and has various important roles in neurotoxicity and neuronal plasticity (Collingridge and Lester, 1989; Meldrum and Garthwaite, 1990). The glutamate level in the synapses is maintained through glutamate transporters (Fairman et al., 1995; Mennerick and Zorumski, 1994). In the brain, most glutamate is taken up by astroglia, rather than by neurons (Nicholis and Attwell, 1990; Rothstein et al., 1996). It is well known that excessive glutamate in the synapse can induce neuronal cell death. It has been reported that glutamate transporter expression is changed after exposure to various substances as well as to pathogens (Gegelashvili and Schousboe, 1997). Incubation of astrocyte cultures with dibutyryl cAMP (dBcAMP) caused changes in the uptake of glutamate as well as in cell morphology (Swanson et al., 1997; Won and Oh, 2000). This suggests that glutamate uptake is altered by cAMP. In addition, neurons and glia mutually influence their development, structure and function during neuroembryogenesis (LoPachin and Aschner, 1993). Therefore, changes in the glutamate uptake properties of glial cells might affect glutamatergic activity.

Nicotine is a well-known psychostimulant drug with both reinforcing and dependence-producing actions in animals as well as in humans. Nicotine also plays an important role in the development and flexibility of synapses (Aramakis and Metherate, 1998). Recently, it was reported that nicotine rendered the glutamate uptake sub-sensitive to glutamate uptake inhibitor in glial cells cultured in the absence of cAMP (Lim et al., 2000). However, super-sensitivity of glutamate uptake to glutamate uptake inhibitor in glial cells prepared from perinatal nicotine-exposed pups has also been reported (Lim and Kim, 2001). This suggests that nicotine might affect the development of glial cells in addition to that of neurons. Therefore, in order to understand the actions of nicotine in glial cells, it is essential to investigate the effects of nicotine on the different states of glial cells. However, there are few reports on the changes in cAMP-treated cells after nicotine exposure.

This study was designed to determine if the response to nicotine, the response to glutamate uptake inhibitor, and

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glutamine synthetase activity are altered in glial cells after acute and subacute nicotine exposure with or without cAMP treatment.

2. Materials and methods

2.1. Animals and materials

Male and female Sprague–Dawley rats weighing 200–250 g were housed at room temperature (22–24 °C) under a 12-h light/12-h dark cycle with access to food and water ad libitum. L-*trans*-pyrollidine-2,4-dicarboxylic acid was purchased from Research Biochemical (Natick, MA, USA) and [^{14}C]L-glutamate (specific activity, 261.6 mCi/mmol) was obtained from New England Nuclear (Boston, MA, USA). The ion exchange chromatography supports, AG1-X8 (acetate form), were purchased from BIO-RAD (Hercules, CA, USA). Both fetal bovine serum and bovine calf serum were purchased from Hyclone (Logan, UT, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

2.2. Cerebellar glial cell cultures

The cells were prepared using a slight modification of the method reported by McCaslin and Morgan (1987). Seven-day-old rat pups were decapitated and the heads were partially sterilized by dipping them in 100% ethanol. The cerebella were excised and placed in a culture medium lacking serum and bicarbonate. The cells were then mechanically dissociated. The growth medium used (5 ml/60-mm dish) was Dulbecco's modified Eagle's medium (DMEM) supplemented with 40 mM $NaHCO_3$, 0.15 mM $CaCl_2$, 66 μM $MgSO_4$, 0.44 mM KCl, 6% fetal bovine serum and 6% bovine calf serum. After 2 days of stabilization, the growth medium was aspirated from the cultures and replaced with a new growth medium with/without 0.15 mM dBcAMP and 100 μM nicotine. The cells were then returned to the CO_2 incubator. All the cells were used 10 days after plating. Although the applied nicotine concentration was relatively high, it did not affect glial cell viability.

2.3. Determination of cell viability

The viable cells were quantified using the method reported by Mosmann (1983). The mitochondrial enzymes in normal cells have the capacity to transform the 3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyl tetrazolium (MTT) salt into water-insoluble MTT formazan. Ten days after plating, the growth medium was separated by filtration and washed with 0.1 M phosphate-buffered saline (pH 7.6). The MTT salt was dissolved in serum-free DMEM at a concentration of 0.5 mg/ml and incubated with the cells at 37 °C for 4 h. The MTT formazan produced was dissolved in 0.04 N HCl in isopropanol and scanned using an enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 570 nm.

2.4. Determination of glutamate uptake in glial cells

Cerebellar glial cells, which were grown for 10 days after plating, were used. At the end of the growing period, the growth medium was removed and replaced with physiological saline HEPES (PSH) buffer containing 5 mM HEPES, 135 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2$, 10 mM glucose and 44 mM $NaHCO_3$ (pH 7.4). After being washed with the PSH buffer for 1 h, the cells were incubated in the presence of 10 μM glutamate and/or various L-*trans*-pyrollidine-2,4-dicarboxylic acid (PDC) concentrations at 37 °C for 30 min. After incubation, the buffer was collected and the amount of glutamate remaining in solution was determined using the method reported by Schmid et al. (1980). The buffer collected from the cultures was treated with an *o*-phthaldialdehyde derivatizing agent according to the method reported by Shoup et al. (1984). Fifty microliters was injected into a high-performance liquid chromatography–electrochemical detector (HPLC–ECD). Separation was achieved using a C18 reverse type column (Rainin instrument 15 cm in length). A 0.1 M sodium phosphate buffer (pH 5.4) containing 37% methanol, with a flow rate of 1 ml/min, was used as the mobile phase. The glutamate concentrations were determined by a direct comparison of the sample peak heights to those of an external standard.

2.5. Determination of glutamine synthetase activity in glial cells

Glutamine synthetase activity was determined using a slight modification of the method reported by Caldani et al. (1982). The cerebellar glial cells, which were grown for 10 days after plating, were mechanically scraped off with a 10 mM imidazole–HCl buffer (pH 6.8) including EDTA, and then sonicated. The enzyme samples were incubated with an assay buffer at 37 °C for 20 min. The buffer was composed of 10 mM [^{14}C]L-glutamate (0.8 mCi/mmol), 15 mM $MgCl_2$, 4 mM NH_4Cl, 1 mM 2-mercaptoethanol, 50 mM imidazole–HCl, 1 mM ouabain and 10 mM ATP. The reaction was quenched by adding 1 ml of ice-cold deionized water and immediately loaded onto a column (Dowex AG1-X8, acetate form). The column was washed with 5 ml of ice-cold deionized water and the eluate was mixed with scintillation solution. Radioactivity was determined using a liquid scintillation spectrophotometer.

2.6. Determination of Na^+/K^+ ATPase activity in glial cells

Na^+/K^+ ATPase activity was determined using a slight modification of the method reported by Vasarhelyi et al. (1977). The cerebellar glial cells, which were grown for 10 days after plating, were mechanically scraped off with a 10 mM Tris–HCl buffer (pH 6.8) including 1 mM EDTA, and then sonicated. The enzyme samples were added to the incubation buffer, which was composed of 100 mM NaCl,

20 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM EGTA, 50 mM Tris–HCl, 1 mM ATP, 1 mM phosphoenolpyruvate, 0.16 mM NADH, 5 kU pyruvate kinase, and 12 kU lactate dehydrogenase (final concentration). The change in absorbance was monitored at 340 nm. After 300 s, 10 mM ouabain was added to inhibit the ouabain-sensitive ATPase and the change in absorbance was monitored continuously. Ouabain-sensitive Na^+/K^+ ATPase activity was calculated from the difference between the two slopes.

2.7. Determination of protein concentration

The protein concentration of the cultured cells was determined by the method reported by Lowry et al. (1951), using bovine serum albumin as the standard.

2.8. Statistics

The Student's *t*-test was used to determine statistical significance. The dose–response curve was analyzed by using appropriate computer programs.

3. Results

3.1. Effects of acute nicotine exposure on glutamate uptake in cerebellar glial cells

The inhibitory response of PDC to glutamate uptake in the differently conditioned glial cells is shown in Fig. 1. After growing in the different conditions, each glial cell had a comparable basal glutamate uptake. However, after exposure to 50 μM PDC, glutamate uptake was inhibited more (56%) in the cells that were not treated with dBcAMP than in those (21.5%) that were treated ($P<0.01$). Fig. 2 shows changes in glutamate uptake of the differently conditioned glial cells after exposure to the various nicotine concentrations. Glutamate uptake in the glial cells not treated with dBcAMP decreased dose-dependently, while that in the dBcAMP-treated glial cells increased.

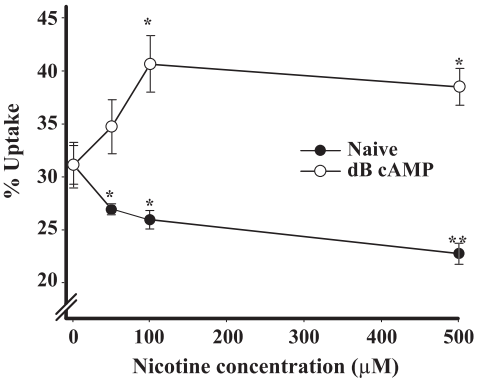

Fig. 2. Effects of acute nicotine exposure on glutamate uptake in the differently conditioned glial cells. Preparation and treatment of the cerebellar glial cells are the same as Fig. 1. Ten days after plating, the cells were incubated with various nicotine concentrations for 30 min. The buffer was then collected and analyzed by HPLC–ECD. The values represent means ± S.E.M. of four or five determinations. *$P<0.05$, **$P<0.01$ means significantly different from the control.

3.2. Effects of subacute nicotine exposure on glutamate uptake in cerebellar glial cells

No significant changes in cell viability were observed when the cultured cerebellar glial cells were exposed to 500 μM nicotine for between 2 and 10 days. Fig. 3 shows changes in glutamate uptake after nicotine exposure (100 μM) in subacutely nicotine-exposed glial cells cultured

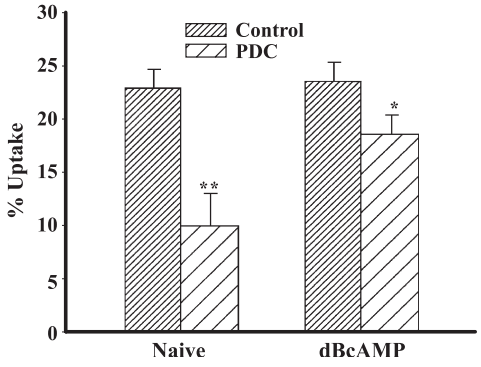

Fig. 1. Effects of PDC on basal glutamate uptake in the differently conditioned glial cells. Cerebellar glial cells obtained from 7-day-old rat pups were used. The cells were exposed with/without dBcAMP (0.15 mM) from 2 to 10 days in situ. Ten days after plating, the cells were incubated with 50 μM of PDC for 30 min. The buffer was then collected and analyzed by HPLC–ECD. The values represent means ± S.E. of five or nine determinations. *$P<0.05$, **$P<0.01$ means significantly different from the control.

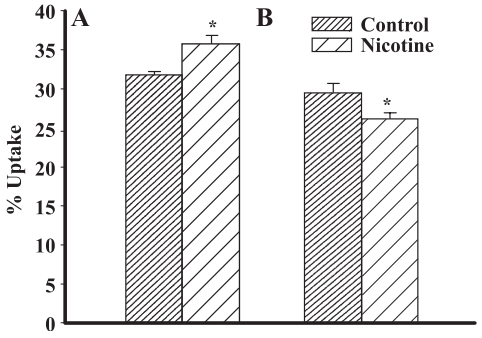

Fig. 3. Effects of nicotine exposure on glutamate uptake after subacute nicotine exposure in the naive (A) and dBcAMP-treated (B) cerebellar glial cells. Cerebellar glial cells obtained from 7-day-old rat pups were used. The cells were exposed with/without dBcAMP (0.15 mM) and nicotine (100 μM) from 2 to 10 days in situ. Ten days after plating, the cells were incubated with nicotine (100 μM) for 30 min. The buffer was then collected and analyzed by HPLC–ECD. The values represent means ± S.E.M. of four or five determinations. *$P<0.05$ means significantly different from the respective control.

under the different conditions. After nicotine exposure, glutamate uptake in the glial cells not treated with dBcAMP was higher (12.5%) and that in the dBcAMP-treated cells was lower (11.4%). The inhibitory effect of PDC on glutamate uptake in subacutely nicotine-exposed glial cells cultured under the different conditions is shown in Fig. 4. After subacute nicotine exposure of glial cells not treated with dBcAMP, the IC_{50} of PDC on glutamate uptake increased from 46.4 μM (lower and upper critical level: 42.0–51.4 μM) to 83.6 μM (68.9–102.7 μM). However, this level was decreased from 185.8 μM (119.6–288.6 μM) to 29.7 μM (23.3–37.9 μM) after subacute nicotine exposure in the dBcAMP-treated cells.

3.3. Effects of subacute nicotine exposure on Na^+/K^+ ATPase and glutamine synthetase activity in cerebellar glial cells

Table 1 shows changes in Na^+/K^+ ATPase activity and glutamine synthetase activity of the differently condition ed glial cells after subacute nicotine exposure. The Na^+/K^+ ATPase activity of the differently conditioned glial cells was similar. After subacute nicotine exposure, the Na^+/K^+ ATPase activity in the naive glial cells was increased by 64.1%, whereas that of dBcAMP-treated cells was decreased by 44.4%. However, glutamine synthetase activity was decreased (74.0%) in naive glial cells, but was increased (103.8%) in the dBcAMP-treated cells.

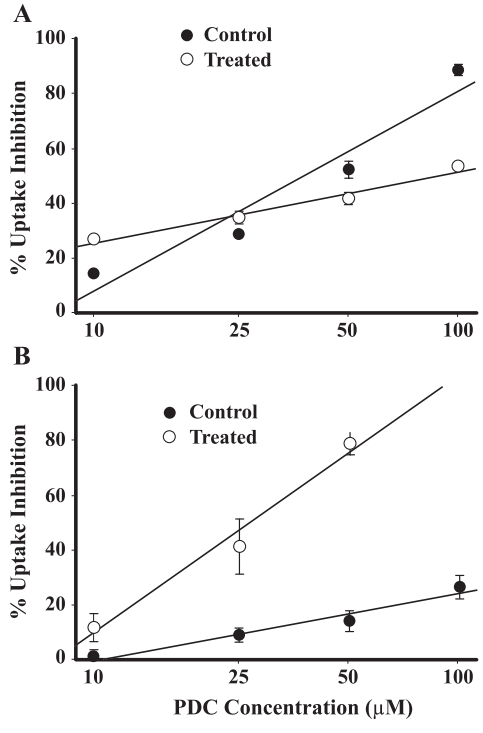

Fig. 4. Effect on glutamate uptake of PDC after subacute nicotine exposure in naive (A) and dBcAMP-treated (B) cerebellar glial cells. The preparation and treatment of cerebellar glial cells were the same as in Fig. 3. Ten days after plating, the cells were incubated with various concentrations of PDC for 30 min. The buffer was then collected and analyzed by HPLC–ECD. The values represent means ± S.E.M. for five determinations.

Table 1
The effects of subacute exposure to nicotine on the Na^+/K^+ ATPase and glutamine synthetase activities in the differently conditioned glial cells

Treatment	Na^+/K^+ ATPase	Glutamine synthetase
− cAMP	20.25 ± 1.98	2.04 ± 0.38
− cAMP + Nicotine	33.24 ± 2.84[a]	0.53 ± 0.14[a]
+ cAMP	24.61 ± 2.96	1.70 ± 0.18
+ cAMP + Nicotine	13.93 ± 1.50[a]	3.45 ± 0.24[a]

Cerebellar glial cells obtained from 7-day-old rat pups were used. The cells were incubated with or without dBcAMP (0.15 mM) and nicotine (100 μM) from 2 to 10 days in situ. Ten days after plating, the cells were scraped off and enzyme activity was measured. The units of the enzyme activities are μmol NADH oxidation/min/mg protein for Na^+/K^+ ATPase and nmol/min/mg protein for glutamine synthetase, respectively. The values represent means ± S.E.M. for four or six determinations.

[a] $P < 0.01$ means significantly different from the respective control.

4. Discussion

Glutamate uptake is the primary mechanism for inactivating synaptically released glutamate (Nicholis and Attwell, 1990). More than three types of glutamate transporters exist in the rat brain, namely, the glutamate/aspartate transporter (GLAST), glutamate transporter 1 (GLT-1) and excitatory amino acid carrier (EAAC1) (Seal and Amara, 1999). Two types of glutamate transporters (GLAST and GLT-1) exist in glial cells with a different affinity for PDC (Kondo et al., 1995), and with different patterns of expression in the brain during development (Furuta et al., 1997; Sutherland et al., 1996). The expression of the glial glutamate transporter subtype is influenced by dBcAMP (Swanson et al., 1997), which is used to mimic the neuronal influence on astrocyte cultures (Hertz, 1990). It has been reported that prolonged treatment with dBcAMP of astrocytes induces the new expression of specific potassium and chloride ion channels (Ferroni et al., 1995), and that glial cells cultured in the presence of dBcAMP have a higher glutamate uptake and a lower PDC sensitivity than those cultured in the absence of dBcAMP (Swanson et al., 1997). These results indicate that the effect of PDC-induced inhibition of glutamate uptake is greater in nontreated astrocytes than in dBcAMP-treated ones. It has been reported that Na^+/K^+ ATPase activity is involved in the glial glutamate uptake process and that glutamate uptake stimulates the activity of this enzyme (Pellerin and Magistretti, 1997). These results indicate that there are no changes in basal glutamate uptake and Na^+/K^+ ATPase activity in the differently conditioned glial cells. However, results showed a decreased sensitivity to PDC of dBcAMP-treated glial cells. Thus, exposure of glial cells to dBcAMP may affect the developmental characteristics of astrocytes, including the expression of glutamate transporter subtypes.

Nicotine enhances the ion flux and the release of neurotransmitters, and elicits a variety of physiological and behavioral effects (Martin, 1986). Recently, glutamate uptake by cerebellar glial cells prepared from perinatally nicotine-exposed rat pups was reported to be altered in a different way depending on the duration of nicotine exposure (Lim and Kim, 2001). Accordingly, depending on the growth conditions, nicotine exposure might affect the glutamate uptake of cerebellar glial cells in a different manner. Although the changes in glial glutamate uptake after exposure to either or both dBcAMP and nicotine have not been explained, it has been reported that nicotine exposure can induce the phosphorylation of the cAMP-response element binding protein (Nakayama et al., 2001), and mecamylamine-precipitated nicotine withdrawal is reported to increase adenyl cyclase activity in a certain brain region (Tzavara et al., 2002). This suggests that nicotine may be involved in modifying the cyclic AMP pathway. This study showed that glial glutamate uptake after acute exposure to nicotine was either inhibited or enhanced in the naive or cAMP-treated glial cells, respectively. This suggests that nicotine may have different effects on glutamate uptake in glial cells, depending on the culture condition. These results also suggest that the nicotine-induced changes in glial glutamate uptake after subacute nicotine exposure are opposite to the effect of acute nicotine exposure on glial glutamate uptake. The opposite alterations following the acute and subacute treatments might reflect cellular adaptation. It was reported that the increase and the subsequent decrease in the basal uptake of glutamate from naive cerebellar glial cells were the result of the increasing concentrations of nicotine during in vitro subacute nicotine exposure (Lim et al., 2000). Therefore, the nicotine-induced decrease in glutamate uptake after concomitant exposure to dBcAMP and nicotine suggests that dBcAMP increases the effects of nicotine and/or that nicotine may affect the cAMP pathway during glial development. In addition, the results indicate that subacute nicotine exposure might induce changes in various glial functions, such as the PDC responsiveness of glutamate uptake and enzyme activity. Depending on the dBcAMP treatment, the subacute exposure to nicotine of glial cells modulated the effect of PDC on glutamate uptake and the activities of Na^+/K^+ ATPase and glutamine synthetase in an opposite manner. Following subacute nicotine exposure, dBcAMP-treated astrocytes were more sensitive than the nontreated ones to PDC-induced inhibition of glutamate uptake. It has been reported that the response to PDC of cerebellar glial cells prepared from nicotine-exposed pups is higher than that in controls (Lim and Kim, 2001). It has also been reported that glutamate transporter expression is down-regulated in prolonged thiamine-deficient rats (Hazell et al., 2001). Therefore, the increase in the PDC-induced inhibition of glutamate uptake after concomitant exposure to dBcAMP and nicotine suggests the possible induction of either conformational changes in glutamate transporters during development or the preferential development of PDC-sensitive glutamate transporters, such as GLT-1. Following subacute nicotine exposure, the parallel changes in nicotine-induced glutamate uptake and in Na^+/K^+ ATPase activity might reflect the glutamate uptake process, as described above. It has been reported that the activities of both the glutamate transporter and glutamine synthetase are coupled for neurotransmitter clearance (Derouiche and Rauen, 1995). However, opposite changes in glutamine synthetase activity and glutamate uptake activity were observed after subacute exposure to nicotine. Therefore, co-cultures are needed to further investigate the delicate changes in astrocyte glutamatergic activity because both glutamate uptake and glutamine synthetase activity in astrocytes are influenced by direct contact with neuronal cells.

In summary, nicotine affects glial cell properties, which are oppositely altered depending on the culture conditions. Subacute exposure to nicotine during development might affect glutamate transporters and the activity of enzymes and suggests that the different states of glial cells during aging and in some regions of the brain might be affected differently by exposure to subacute nicotine.

Acknowledgements

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