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Cellular Mechanism of Nicotine-mediated Intracellular Calcium Homeostasis in Primary Culture of Mouse Cerebellar Granule Cells

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ABSTRACT

Intracellular calcium concentration ($[Ca^{2+}]_i$) may play a crucial role in a variety of neuronal functions. Here we report that in primary culture of mouse cerebellar granule cells nicotinic acetylcholine receptors (nAChRs) are expressed in a specific developmental stage and involved in the regulation of intracellular calcium homeostasis. Nicotine-mediated calcium responses were measured using $^{45}Ca^{2+}$ or fluorometrically using the calcium-sensitive fluorescent dye fura-2. Maximal uptake of $^{45}Ca^{2+}$ evoked by nicotine in mouse cerebellar granule cells were revealed 8~12 days in culture. In contrast, nicotine did not alter the basal $^{45}Ca^{2+}$ uptake in cultured glial cells. In cerebellar granule cells nicotine-evoked $^{45}Ca^{2+}$ uptake was largely blocked by the NMDA receptor antagonists. Glutamate pyruvate transaminase (GPT), which removes endogenous glutamate, also prevented nicotine effects, implying the indirect involvement of glutamate in nicotine-mediated calcium responses. Fluorometric studies using fura-2 showed two phases of nicotine-evoked $[Ca^{2+}]_i$ rises: the initial rising phase and the later plateau phase. Interestingly, the NMDA receptor antagonists and GPT appeared to inhibit only the later plateau phase of nicotine-evoked $[Ca^{2+}]_i$ rises.

The present results imply that nicotine mediated $^{45}Ca^{2+}$ uptake and $[Ca^{2+}]_i$ rises are attributed to the calcium fluxes through both nAchRs and NMDA receptors in a time-dependent manner. Consequently, nAChRs may play an important role in neuronal development by being expressed in a specific developmental stage and regulating the intracellular calcium homeostasis.

Key Words: Nicotinic acetylcholine receptor (nAchR), Glutamate, Cerebellar granule cell, Primary culture, $[Ca^{2+}]_i$, $^{45}Ca^{2+}$

INTRODUCTION

In the central nervous system, cholinergic neurons have been shown to involve in the brain function. Acetylcholine-mediated synaptic transmission in brain occurs through the activation of two subtypes of receptors such as the muscarinic and nicotinic acetylcholine receptors (nAChRs). A significant amount of neuronal nicotinic acetylcholine receptor subunits have been revealed in various regions of the brain owing to the molecular biological techniques (Duvoisin *et al.*, 1989; Wada *et al.*, 1990, Sequela *et al.*, 1993). These nicotinic acetylcholine receptor subunits are known to constitute ligand-gated cation channels (Heinemann *et al.*, 1991) and may be involved in the rapid control of fluxes of cations including calcium (Mulle *et al.*,

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1992; Sequela *et al.*, 1993; Vernino *et al.*, 1992). Although the existence of nAchRs in the central nervous system has been revealed by many investigators, however, little is known of their functions.

Intraneuronal calcium concentration plays a critical role in synaptic development, maintenance, and plasticity in the mammalian central nervous system. *N*-methyl-D-aspartate (NMDA) receptors and voltage-dependent calcium channels (VDCCs) have been shown to regulate neuronal functions such as neuronal differentiation and neuronal cell death by increasing the intracellular calcium level (Collins and lile, 1989; Didier *et al.*, 1992; Komuro and Rakic, 1992; McDonald and Johnston, 1990). nAChRs are expressed during the neuronal development (Balazs *et al.*, 1988; Daubas *et al.*, 1990; Margiotta *et al.*, 1987; Matter *et al.*, 1990), and can promote the permeability of calcium. Thus, it is possible that in the central nervous system nAchRs can regulate the intracellular calcium homeostasis in a complementary manner with NMDA receptors and VDCCs.

Currently, however, it is not clear how the activation of nAchRs can increase $[Ca^{2+}]_i$ in neuronal cells. The present study was, therefore, undertaken to characterize nAchRs and to illucidate the mechanism of nicotine-mediated increases in $[Ca^{2+}]_i$.

For these studies primary cultures of cerebellar granule cells were used. Cerebellar granule cells express both ionotropic NMDA and non-NMDA receptors (Aronica *et al.*, 1993; Gallo *et al.*, 1987; Drejer *et al.*, 1986) as well as a metabotropic glutamate receptor coupled to phospholipase C which catalyzes phosphoinositide hydrolysis (Aronica *et al.*, 1993; Nicoletti *et al.*, 1986). Thus, this culture system allowed us to study the interaction between nAchRs and glutamate receptor subtypes.

MATERIALS AND METHODS

Materials

Nicotine, NMDA, tetrodotoxin (TTX), glycine, poly-D-lysine, para-aminobenzoic acid, glutamate, glutamine, Dulbecco's minimum essential media (DMEM) were purchased from Sigma Chemical Company (St. Louis, MO). D-2-amino-5-phosphovalerate (APV), 7-chloro-kynurenate (7-Cl-KYN) were purchased from Research Biochemicals International (RBI; Natick, MA). Trypsin, DNAase, fetal calf serum, penicilline, streptomycin were obtained from Gibco (BRL Life Technologies, Inc. NY). $^{45}Ca^{2+}$ were purchased from Dupont NEN Research product (Boston, MA). Fura-2/AM and fura-2 pentaphosphate were purchased from Molecular Probes (Eugene, OR). All other chemicals were obtained from standard commercial sources.

Cell Culture

Primary culture of cerebellar granule cells was prepared essentially as described by Drejer and Schousboe (1988). In brief, cerebella from 2- to 4-day old mice (Charles River) were freed of meninges and minced into small pieces in Dulbecco's minimum essential medium (DMEM). Cells were dissociated by 0.1% trypsin/0.05% DNAase-containing DMEM and incubated for 10 min at 37℃. After removal of supernatant, cerebella pieces were washed thrice with DMEM, resuspended, and triturated thoroughly in the growth media (DMEM supplemented with 24.5 mM KCl, 50 mM glucose, 1 μg/ml para-aminobenzoic acid, 10% heat-inactivated fetal calf serum). The dissociated cells were passed through 135 μm nylon mesh, and preplated onto the culture flask coated with poly-D-lysine (10 μg/ml) for 15 min at 37℃ to remove astrocytes. The medium containing unattached neurons was then passed through two sizes of sterile nylon sieves (80 and then 25 μm). Cells (about 2 million cells/ml) were then plated on poly-D-lysine (100 μg/ml)-coated 12 mm coverslips sitting in 35 mm culture dishes. Cells were treated for 24 h with 10 μM cytosine arabinoside after 1 day in culture in order to remove any residual astroglia and other proliferating cells. In the cells grown on the 12 mm coverslips five to eight days after cytosine arabinoside treatment, less than 5% of the cells were immunoreactive for glial fibrillary acidic protein (GFAP), an astrocyte-specific marker.

For the primary culture of non-neuronal cells, prefrontal cortices from 2- to 4-day old

mice were dissociated by mild trypsinization (0.1% trypsin/0.05% DNAase-containing DMEM) and passed through sterile nylon sieves (80 μm pore size) into DMEM containing 10% heat-inactivated fetal calf serum. Cells were then counted and plated (about 50,000 cells/ml) on poly-D-lysine (2 μg/ml)-coated 12 mm coverslips sitting in 35 mm culture dishes. For non-neuronal cell culture, cells were not treated with cytosine arabinoside.

$^{45}Ca^{2+}$ uptake

$^{45}Ca^{2+}$ uptake was measured using cells attached to 35-mm culture dishes. At somewhere-indicated days in culture, cells were rinsed twice with 1.5 ml of Mg^{2+}-free KRH buffer (125 mM NaCl, 1.2 mM KH_2PO_4, 6 mM glucose, and 1 mM $CaCl_2$, 25 mM HEPES, pH 7.4) and were preincubated for 20 min at room temperature in 1.5 ml of the fresh buffer. $^{45}Ca^{2+}$ uptake was measured by stimulating cells with agonists at room temperature in 0.8 ml of Mg^{2+}-free KRH buffer containing 1 μCi $^{45}CaCl_2$. Cells were incubated for 2.5 min at room temperature and then washed twice with ice-cold Mg^{2+}-free KRH buffer. Radioactivity in neutralized 0.5 N NaOH cell digests was counted by liquid scintillation spectroscopy. An aliquot was used to determine the protein amount by the method of Lowry *et al.* (1951). Data were expressed as the amount of calcium influx (nmoles) per mg protein for 2.5 min.

$[Ca^{2+}]_i$ measurement

Intracellular calcium concentration was analyzed with fura-2 acetoxymethyl ester (fura-2/AM; Molecular Probes) using a protocol for digital Ca^{2+} imaging that has been detailed previously by this laboratory (Kim *et al.*, 1994). In brief, cells on the 12-mm glass coverslips were twice rinsed with KRH buffer and were loaded with 10 μM fura-2/AM for 30 min at 37°C. Cells were washed thrice with KRH buffer and were left in KRH for 20 min at room temperature before measuring $[Ca^{2+}]i$. $[Ca^{2+}]_i$ was measured every 10 seconds before and during the drug treatment. Nicotine and NMDA were always coapplied with glycine (1 μM) and TTX (1 μM). Changes in fluorescence intensity of fura-2 at excitation wavelengths of 340 and 380 nm were determined at room temperature using an image processing system (Quantex QX7-210, Sunnyvale, CA) interfaced to an IBM personal computer. $[Ca^{2+}]_i$ was determined as described by Grynkiewicz *et al.* (1985).

Statistical Analysis

Data are presented as mean ± standard error of the mean and were analyzed for statistical significance using a paired-t test or analysis of variance [ANOVA] and Dunnetts multiple comparison test.

RESULTS

Nicotine-stimulated $^{45}Ca^{2+}$ uptake

In 8~12 days old cerebellar granule cells, nicotine increased $^{45}Ca^{2+}$ uptake in a concentration-dependent manner (data not shown). But, at high concentration (>500 μM) nicotine induced $^{45}Ca^{2+}$ uptake in a bell-shaped pattern

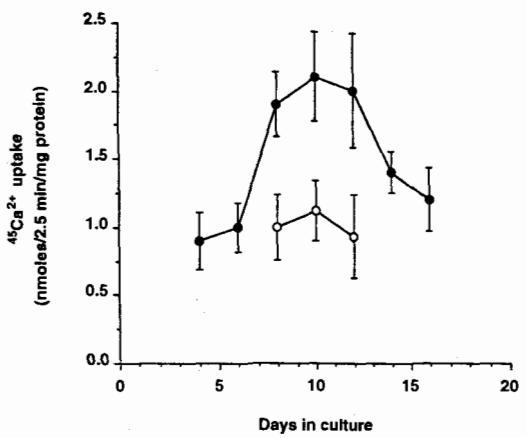

Fig. 1. Nicotine-stimulated $^{45}Ca^{2+}$ uptake during the development of cerebellar granule cells in culture. Cerebellar granule cells cultured for the indicated days were incubated for 2.5 min in Mg^{2+}-free KRH buffer containing 1 μCi $^{45}Ca^{2+}$. Nicotine-stimulated $^{45}Ca^{2+}$ uptake was measured in the absence (●) and presence (○) of 100 μM d-tubocurarine. Data were mean±standard error from 6 (without d-tubocurarine) or 2 separate experiments (with d-tubocurarine).

probably due to the desensitization of the nicotine receptor. 200 μM nicotine increased the $^{45}Ca^{2+}$ uptake by 2.4±0.41 fold during 2.5-min incubation (Fig. 1). Thus, further studies were performed using 200 μM nicotine to obtain maximal nicotine response. Nonneuronal cells were not responsive to 200 μM nicotine (data not shown). The nicotine-mediated calcium responses were completely inhibited by d-tubocurarine (100 μM; Fig. 1). To study whether the nicotine receptors are functionally expressed during the developmental stages, we investigated the nicotine-induced calcium flux at specific culture period. Nicotine-mediated calcium responses were observed to change during the culture period (Fig. 1). At 4 and 6 days in culture, 200 μM nicotine did stimulate a negligible flux of $^{45}Ca^{2+}$ into the cultured cells. However, nicotine responses rapidly increased and the large calcium responses evoked by nicotine were detected 8~12 days in culture. The nicotine responses gradually decreased in later stages of cell culture.

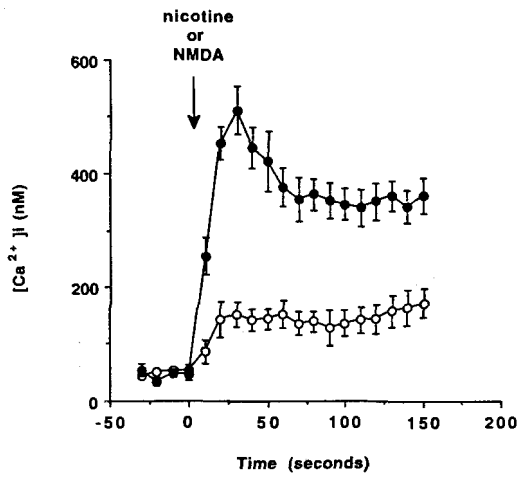

Fig. 2. Nicotine-stimulated increases in $[Ca^{2+}]_i$. Cerebellar granule cells cultured for 10 days were treated with 200 μM nicotine (○) and 300 μM NMDA (●). $[Ca^{2+}]_i$ was measured from 5~10 cells in one optical field, every 10 seconds before and during the 2.5-min incubation period. Data are expressed as mean±standard error for a representative from 5 separate experiments.

Nicotine-stimulated rises in $[Ca^{2+}]_i$

Similar to the findings in $^{45}Ca^{2+}$ uptake studies, nicotine increased $[Ca^{2+}]_i$ in a time-dependent manner during the culture period (Fig. 2). Preliminary experiments showed that at 4 and 6 days in culture, 200 μM nicotine did not stimulate noticeable increases of $[Ca^{2+}]_i$ and that the maximal $[Ca^{2+}]_i$ rises by nicotine were revealed 8~12 days in culture. For further studies on nicotine-mediated $[Ca^{2+}]_i$ rises, therefore, 10-day cultured cells were used. $[Ca^{2+}]_i$ evoked by 200 μM nicotine was much smaller than that by 300 μM NMDA, which at this concentration also causes maximal calcium influx in cultured cerebellar granule cells (Fig. 2).

Effects of NMDA receptor antagonists on nicotine-evoked calcium responses

Nicotinic receptors are known to be more permeable to monovalent cations than divalent cations. Thus, we hypothesized that nicotinic stimulation of calcium influx is at least in part due to release of endogenous glutamate and consequent activation of the NMDA receptor which is one of glutamate receptor subtypes and promotes calcium permeability. To test this hypothesis we used several different glutamate receptor antagonists including the NMDA receptor antagonist D-2-amino-5-phosphovalerate (APV) and 7-chloro-kynurenate (7-Cl-KYN). Nicotine-stimulated $^{45}Ca^{2+}$ uptake was antagonized 73±11.3% and 67±11.4% by NMDA receptor antagonists APV and 7-Cl-KYN, respectively (Fig. 3A). Nicotine-evoked $[Ca^{2+}]_i$ measured using fura-2 was also found to be antagonize by APV and 7-Cl-KYN. Interestingly, however, with little change of the initial peak $[Ca^{2+}]_i$ stimulated by nicotine only the plateau level of $[Ca^{2+}]_i$ was decreased by the addition of APV (Fig. 3B; for this study, 7-Cl-KYN was not used).

Effects of removal of glutamate from extracellular milieu

If nicotine-induced calcium response was mediated indirectly by the release of endogenous glutamate, the nicotine response could be removed by the addition of glutamate pyruvate

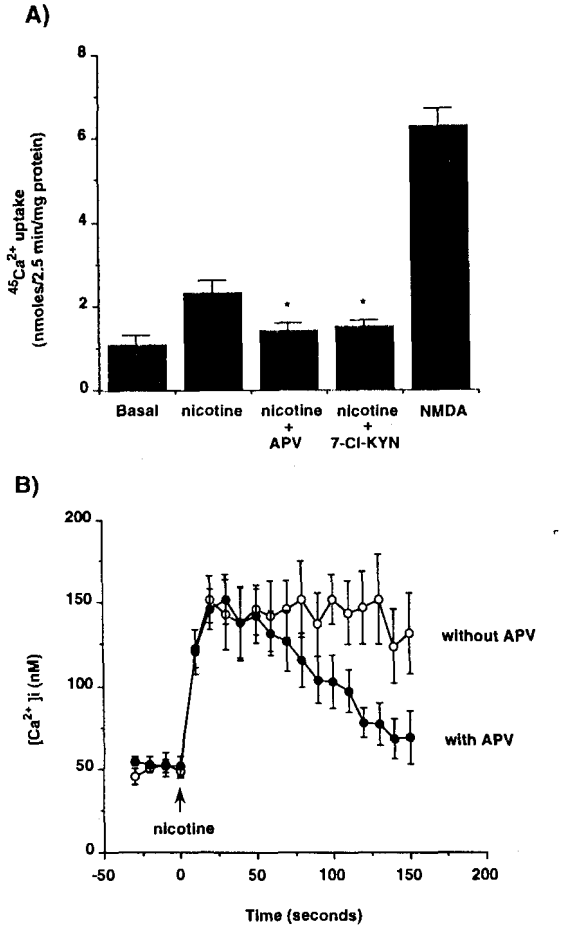

Fig. 3. NMDA receptor antagonists decreased nicotine-stimulated calcium responses Nicotine-mediated $^{45}Ca^{2+}$ uptakes (A) and $[Ca^{2+}]_i$ rises (B) were measured in the presence and absence of the NMDA receptor antagonists APV (300 μM; A and B) or 7-Cl-KNY (20 μM; only A). $[Ca^{2+}]_i$ was measured from 5~10 cells in one optical field, every 10 seconds before and during the 2.5-min incubation period. Data are expressed as mean±standard error from 5 (A) and 6 separate experiments (B).

* $P<0.05$; using Dunnetts multiple comparison test, compared with the response evoked by nicotine alone.

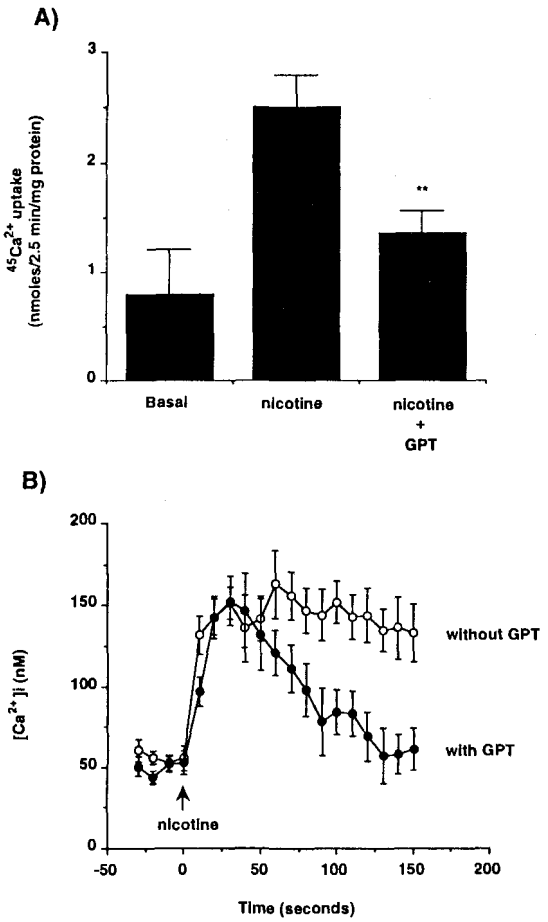

Fig. 4. Removal of endogenous glutamate prevented nicotine-stimulated calcium responses Nicotine-mediated $^{45}Ca^{2+}$ uptakes (A) and $[Ca^{2+}]_i$ rises (B) were measured in the presence and absence of GPT (200 μM). $[Ca^{2+}]_i$ was measured from 5~10 cells in one optical field, every 10 seconds before and during the 2.5-min incubation period. Data are expressed as mean±standard error from 5 (A) and 5 separate experiments (B). ** $P<0.01$; using Dunnetts multiple comparison test, compared with the response evoked by nicotine alone.

transaminase (GPT), which catalyzes transamination of glutamate into α-ketoglutarate in the presence of pyruvate (O'Brien and Fischbach, 1986). For this study cells were thorougly washed and preincubated 2 min with the experimental media containing 2 mM pyruvate and 1 U/ml of GPT. Cells were stimulated with nicotine in the presence of 2 mM pyruvate and 1 U/ml of GPT. Under this condition, nicotine-

mediated uptake of $^{45}Ca^{2+}$ decreased 68±13.2% (Fig. 4A). Similarly in the presence of the NMDA receptor antagonists, the plateau level of $[Ca^{2+}]_i$ decreased near to the basal $[Ca^{2+}]_i$ during exposure to nicotine in the presence of GPT (Fig. 4B)

DISCUSSION

The present study demonstrated that nicotinic acetylcholine receptors were functionally expressed during the development of cerebellar granule cells in culture. In this developmental period, the activity of other neurotransmitter receptor types, such as NMDA and muscarinic AChRs, has also been shown to increase in primary culture of cerebellar granule cells (Alonso *et al.*, 1990; Didier *et al.*, 1993). The expression of nAchRs and possibly other neurotransmitter receptor types in cerebellar granule cells seems to be well correlated with the period of synaptogenesis (Van-Vliet *et al.*, 1989). A transient expression of nAchR during the development has previously been reported in several other brain regions. For example, nicotine binding increases in some post-natal mouse brain such as cerebellum and hindbrain, and then slowly decreases to the adult level (Fielder *et al.*, 1990). However, nicotine binding to other brain regions remained unchanged or increased between birth and adulthood (Fielder *et al.*, 1990). A transient expression of nAchR during the in vitro culture has also been recently reported in the cerebellar granule cells (Didier *et al.*, 1995).

Nicotine-evoked $^{45}Ca^{2+}$ uptake reached to the maximal level at 8~12 days in culture and then rapidly decreased. Thus, the nicotine-evoked $^{45}Ca^{2+}$ uptake at 16 day in culture was only about 25% of that at 10 days. However, nicotine-stimulated calcium responses measured fluorometrically using fura-2 displayed a slower decrease in the later developmental stage: the nicotine-evoked increase in $[Ca^{2+}]_i$ at 16 day of culture was about 60% of that at 10 days in culture (data not shown). This discrepancy could be due to the increase of the mass of proliferating non-neuronal cells such as astroglia, which might underestimate the $^{45}Ca^{2+}$ uptake by expressing the amount of $^{45}Ca^{2+}$ uptake per mg protein.

In the central nervous system nicotine may produce intracellular signals that may have important roles in synaptic development, maintenance, and plasticity. Presynaptic nAchRs are found to be abundant in the nervous system, where they are thought to regulate the release of various neurotransmitters (Lena *et al.*, 1993). In the in vivo striatum nicotine induced c-fos expression mostly by dopamine D1 receptor and also NMDA receptor (Kiba and Jayaraman, 1994). MK801 attenuated behavioral adaptation to chronic nicotine administration in rats, suggesting that NMDA receptor may be involved in the adaptive processes seen with chronic nicotine administration (Shoaib and Stolerman, 1992). In cultured cerebellar granule cells nicotine appeared to release glutamate, which was demonstrated by the following two reasons: 1) competitive NMDA antagonists prevented nicotine effects, and 2) removal of glutamate from the extracellular milieu by incubating the cells with glutamate pyruvate transaminase prevented nicotine effects. Thus, it may be plausible that the calcium influx measured in this study resulted at least in part from an endogenous glutamate release and a resultant NMDA receptor activation. The cellular mechanism for the glutamate release evoked by nAchRs remains unknown. Like the neuromuscular AchRs, neuronal AchRs constitute ligand-gated cation channels which facilitate the entry of cations, mainly Na^+, in neuronal cells. Thus, nAChR activation may depolarize cell membrane potential and consequently reverse the cellular glutamate influx, resulting in the increase of the extracellular glutamate concentration. nAchRs are also permeable to Ca^{2+} in rat central neurons (Mulle *et al.*, 1992). Ca^{2+} influx through the nAchRs may also facilitate the release of neurotransmitters including glutamate, which is a major neurotransmitter in the granule cells and also other neurons in cerebellum.

nAchR activation resulted in two phases of $[Ca^{2+}]_i$ rise in cerebellar granule cells. With no significant blockade of the initial phase of nicotine-stimulated $[Ca^{2+}]_i$, competitive NMDA antagonists or removal of glutamate by GPT from

the extracellular melieu prevented the later plateau level of $[Ca^{2+}]_i$ stimulated by nicotine. This may imply that the initial phase of calcium rise can be attributed to the calcium flux mainly through the nicotinic acetylcholine receptor channel opened by nicotine. In contrast, the later plateau level of $[Ca^{2+}]_i$ could be attributed by the sustained influx of calcium through the NMDA receptor-ion channel complex by glutamate released from the neuronal cells.

In contrast to its positive excitatory effects, nicotine has been also reported to inhibit whole cell NMDA-induced responses in rat cortical neurons in culture by interacting at the MK-801 binding sites of NMDA receptor (Aizenman *et al.*, 1991). Nicotine protected cortical neurons and striatal neurons in culture from NMDA-mediated neurotoxicity (Akaike *et al.*, 1994; Marin *et al.*, 1994). Therefore, it is possible that nicotine-mediated intracellular events are not so simple that nicotine can regulate NMDA receptor activity and glutamate release in separate mechanisms.

Several laboratories have demonstrated that nicotine controls neuritic outgrowth (Lipton *et al.*, 1988) and neuronal survival in several brain regions (Meriney *et al.*, 1987, Marin *et al.*, 1994). In general, however, nAchR action is less prominent in the central nervous system. In cerebellum, most neurons and nerve fibers employ excitatory amino acids as neurotransmitters. The modulation of the excitatory neuronal activity is considered to be important for the cerebellar development. Because nAchR activation by acetylcholine or nicotine can control the NMDA receptor activity by stimulating the glutamate release, therefore, nAchR may represent a key factor in cerebellar development. Further studies on the functional significance of nAchR expression in cerebellar granule cells are required to be done.

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=국문초록=

니코틴의 마우스 소뇌과립세포내 칼슘의 항상성 조절기전

이화여자대학교 의과대학 약리학교실, 의과학연구소 신경과학부

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세포내 칼슘농도는 신경세포의 다양한 기능에 매우 중요한 역활을 하고 있다. 본 연구에서는 일차배양한 마우스 소뇌과립세포에서 니코틴성 아세틸콜린 수용체가 특정 발생단계에 발현되고 세포내 칼슘의 농도조절에 관여하는 것을 관찰하였다. 니코틴에 의한 세포내 칼슘농도의 변화는 $^{45}Ca^{2+}$ 나 fura-2를 사용하여 형광법으로 측정하였다. 니코틴은 마우스 소뇌과립세포내 칼슘의 농도를 최대한 증가시키는 것으로 보인다. 반면에 일차배양한 Glia 세포들에서는 $^{45}Ca^{2+}$ 농도를 증가시키지 않았다. 세포내 칼슘농도에 미치는 니코틴의 효과는 NMDA 수용체에 대한 길항제에 의하여 억제되었다. 또한 Glutamate pyruvate transminase (GPT)를 사용하여 배양액의 글루타민산을 제거하면 니코틴효과가 소실되는 것이 관찰되었다. 이러한 결과는 니코틴에 의한 세포내 칼슘농도의 변화가 세포에서 유리된 글루타민산에 의한 간접적인 효과임을 암시한다. Fura-2를 사용한 형광법으로 실험한 결과 니코틴은 two phase로 세포내 칼슘농도를 증가시키는 것을 보여주었다. NMDA 수용체 길항제와 GPT는 단지 후기 plateau상만 억제하였다. 따라서 본 연구결과는 니코틴이 직접 니코틴성 아세틸콜린 수용체를 자극하여 일시적으로 세포내 칼슘농도를 증가시키고 글루타민산을 유리하여 NMDA 수용체를 활성화시킴으로써 세포내 칼슘농도를 지속적으로 증가시키는 것으로 보여진다. 이러한 결과는 니코틴성 아세틸콜린 수용체가 특정한 발생과정에 발현되어 세포내 칼슘농도 조절에 관여함으로써 신경발생과정에서 중요한 역활을 할 수 있음을 보여주고 있다.