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The Regulation of AP-1 DNA Binding Activity by Long-term Nicotine Stimulation in Bovine Adrenal Medullary Chromaffin Cells: Role of Second Messengers

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The signal pathways involved in the regulation of AP-1 DNA binding activity in long-term nicotine stimulated bovine adrenal medullary chromaffin (BAMC) cells have not been well characterized. To understand the involvement of second messengers in the regulation of AP-1 DNA binding activity, the present study was designed to define the time-course for inhibition of nicotine-induced responses by cholinergic antagonists, Ca^{2+} and calmodulin (CaM) antagonists, and calcium/calmodulin-dependent protein kinase (CaMK) II inhibitor using electrophoretic mobility shift assay. Nicotine (10 μM) stimulation increased AP-1 DNA binding activity at 24 hr after treatment. Posttreatment with hexamethonium (1 mM) plus atropine (1 μM) (HA), nimodipine (1 μM), or calmidazolium (1 μM) at 0.5, 3, and 6 hr after the nicotine treatment significantly inhibited the AP-1 DNA binding activity increased by long-term nicotine stimulation. However, posttreatment with HA, nimodipine, or calmidazolium at 9 or 12 hr after the nicotine treatment did not affect the nicotine-induced increase of AP-1 DNA binding activity. The pretreatment of BAMC cells with various concentrations of KN-62 inhibited the increase of AP-1 DNA binding activity induced by nicotine in a concentration-dependent manner. KN-62 (10 μM) posttreatment beginning at 0.5, 3, or 6 hr after the nicotine treatment significantly inhibited the increase of AP-1 DNA binding activity. However, KN-62 posttreatment beginning at 9 or 12 hr after the nicotine treatment did not affect the increase of AP-1 DNA binding activity. This study suggested that stimulation (for at least 6 hr) of nicotinic receptors on BAMC cells was necessary for increase of AP-1 DNA binding activity, and activation of Ca^{2+}, CaM, and CaMK II up to 6 hr at least seemed to be required for the increase of nicotine-induced AP-1 DNA binding activity.

Key Words: AP-1 DNA binding activity, Bovine adrenal medullary chromaffin cells, Nicotine, Second messenger

INTRODUCTION

[Met5]enkephalin (ME) in adrenal chromaffin cells is co-stored and co-released with catecholamines (Viveros et al, 1979; Livett et al, 1981). The secretion of ME or catecholamines can be regulated by activity of splanchnic nerve, which releases acetylcholine as the major neurotransmitter in the adrenal medulla. The existence of nicotinic receptors on adrenal medullary chromaffin cells has raised the possibility that the cholinergic neural input to the adrenal medulla regulates ME and catecholamine secretion. Indeed, the effects of nicotine on ME and catecholamine secretion have been investigated *in vitro* as well as *in vivo* (Harish et al, 1987; Khalil et al, 1988; Watkinson et al, 1990; Suh et al, 1995). In addition, the regulation of the proenkephalin (proENK) and tyrosine hydroxylase genes in the adrenal medulla has been studied under the stress or insulin-induced hypoglycemia (Stachowiak et al, 1985; Harish et al, 1987; Weisinger et al, 1990) as well as *in vitro* using nicotine (Stachowiak et al, 1990; Suh et al, 1995).

The c-Fos and Fos-related antigens (Fra) are inducible transcriptional factors that dimerize with the family of Jun transcriptional factors to form AP-1 transcription complexes (Morgan & Curran, 1991). In adrenal medullary chromaffin cells, these transcriptional factor complexes recognize the AP-1 DNA element in the promoter regions of target genes, such as tyrosine hydroxylase, phenylethanolamine N-methyltransferase and proENK, and modulate gene transcription through the expression of AP-1 proteins (Stachowiak et al, 1990). In addition, cyclic AMP (cAMP) response element binding protein (CREB) would also be expected to alter the expression of specific target gene in adrenal medulla, such as tyrosine hydroxylase (Tinti et al, 1996), through interacting with CRE or CRE-like DNA element without altering its DNA binding activity through the phosphorylation by the protein kinases such as protein kinase A (PKA), calcium/calmodulin dependent protein kinase II, and IV (Gonzalez & Montminy, 1989; Sheng et

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ABBREVIATIONS: BAMC, bovine adrenal medullary chromaffin; EMSA, electrophoretic mobility shift assay; CaM, calmodulin; CaMK, Ca^{2+}/calmodulin-dependent protein kinase.

al, 1991). For example, the proENK gene contains enkephalin cAMP response element like sequence (ENKCRE-2) in its promoter region, which may play a role in its own gene expression through interacting with proto-oncoproteins such as Fos and Jun as well as CREB (Comb, et al, 1986; Sonnenberg et al, 1989; Konradi et al, 1993). Several groups have reported that AP-1 DNA binding activity was significantly increased after nicotine treatment. Pandy & colleagues (1999) observed increase of AP-1 DNA binding activity in the rat cortex 1 hr after single injection of nicotine. Similarly, a marked increase has been reported after nicotine treatment in rat adrenal glands (Pelto-Huikko, et al, 1995). They also have demonstrated that stimulation of both nicotinic receptors may mediate the induction of immediate early genes, resulting in increased AP-1 DNA binding activity. However, the signal pathways, especially second messengers, involved in the regulation of AP-1 DNA binding activity in long-term nicotine stimulated bovine adrenal medullary chromaffin (BAMC) cells have not been well characterized. Therefore, to understand the involvement of second messengers in the regulation of AP-1 DNA binding activity, the present study was designed to define the time-course for inhibition of nicotine-induced responses by cholinergic antagonists, calcium and calmodulin antagonists, and calcium/calmodulin-dependent protein kinase II inhibitor, using electrophoretic mobility shift assay (EMSA).

METHODS

Materials

Nicotine HCl, hexamethonium HCl, atropine HCl, calmidazolium HCl, and nimodipine were purchased from Sigma chemical Co. (St. Louis, MO). KN-62 was purchased from Research Biochemicals International (Natick, MA). Fetal calf serum was purchased from Life Technologies (GIBCO BRL Research Products, Grand Islsnd, NY.). Collagenase type B and leupeptin are products of Boehringer-Mannheim Biochemicals (Indianapolis, IN). [γ-^{32}P] ATP [3000Ci (111TBq/mmol)] was purchased from Du Pont NEN products (Wilmington, DE).

Cell culture

Primary cultures of BAMC cells were prepared according to the method of Wilson & Viveros (1981) with minor modifications. In brief, bovine adrenal glands were retrogradely perfused with collagenase type B (3×15 min, 37°C), and medullae were dissected and digested further in a conical flask (20 min, 37°C). Then, the dispersed BAMC cells were isolated by centrifugation through a Percoll gradient. After subsequent washings, the cells were mixed in DMEM/F12 medium containing 10% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and gentamicin (45 μg/ml). The cells were plated onto 30-mm wells (5×10^6 cells/well; for catecholamine assay). Two days after plating, the medium was changed to serum-free medium. The cells were kept in an incubator with humidified air/5% CO_2 atmosphere at 37°C for 3~5 days after plating. The drug treatments were initiated after cells were in serum-free medium for 1~3 days.

Isolation of total protein

Total cellular protein was extracted from BAMC cells by a rapid guanidine thiocyanate-water saturated phenol/chloroform extraction and subsequent precipitation with acidic sodium acetate (Chomczynski & Sacchi, 1987). The separated organic layer was extracted twice with an equal volume of sterilized (Millipore) water and proteins were precipitated by adding 2 volumes of absolute ethanol to the water-extracted organic phase. The protein pellets were washed twice with cold absolute ethanol and dried. The dried pellets were dissolved in a denaturing buffer [6 M guanidium chloride, 20 mM Tris-HCl (pH 8.0), and 1 mM EDTA]. The protein samples were dialyzed against a renaturing buffer [20 mM Tris-HCl (pH 8.0) 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 5 mM $MgCl_2$, 0.4 mM phenylmethylsulfonyl fluoride, and 20% glycerol] at 4°C. The concentration of protein was determined with the Coomassie blue protein assay reagent (Pierce Chemical Co., Rockford, IL), using bovine serum albumin as the standard.

Electrophoretic Mobility Shift Assay (EMSA)

The AP-1 oligo nucleotide (5'-CTA-GTG-ATG-AGT-CAG-CCG-GAT-C-3'/ 3'-GAT-CAC-TAC-TCA-GTC-GGC-CTA-G-5') used in this study was purchased from Stratagene (La Jolla, CA). Annealing was achieved by incubating an equal molar concentration of each single stranded oligo in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 200 mM NaCl for 10 min at 65°C, and then the mixture was allowed to cool to room temperature gradually.

The AP-1 DNA binding assay was based essentially on the procedures described by Mar et al (1992). Binding reactions (20 μl) were carried out at room temperature and the reaction mixtures contained 20 μg of nuclear protein, 20mM Tris-HCl (pH 7.8), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 2~5 mM DTT, 50 μg/ml BSA, 100 μg/ml poly (dI-dC), 5~10% glycerol, and approximately 0.1 ng (5×10^4 cpm, Cherenko counts) of the specified probe labeled with [γ-^{32}P] ATP by using T4 polymerase. Protein-DNA complexes were separated from protein-free DNA by nondenaturing electrophoresis in polyacrylamide (30 : 1, acrylamide : bis-acrylamide) gels. Gels were run at room temperature in 50 mM Tris, 50 mM borate, and 1 mM EDTA (pH 8.3) for 2hr at a constant voltage (150 V), dried, and autoradiographed.

RESULTS

Effects of cholinergic antagonists on the AP-1 DNA binding activity induced by nicotine

The effect of continuous stimulation of BAMC cells with nicotine on the AP-1 DNA binding activity was examined by EMSA. The effect of long-term (up to 24 hr) stimulation of BAMC cells with nicotine (10 μM) and the temporal effects of cholinergic antagonists, hexamethonium (1 mM) plus atropine (1 μM), on the AP-1 DNA binding activity are characterized in Fig. 1. Nicotine stimulation increased AP-1 DNA binding activity 24 hr after the treatment. Hexamethonium plus atropine pretreatment for 30 min almost completely blocked the nicotine-induced increase of AP-1 DNA binding activity (data not shown). Posttreatment with hexamethonium plus atropine at 0.5, 3, and 6 hr after the nicotine treatment dramatically inhibited the increase of AP-1 DNA binding activity. However, posttreatment with hexamethonium plus atropine beginning at 9 or 12 hr after

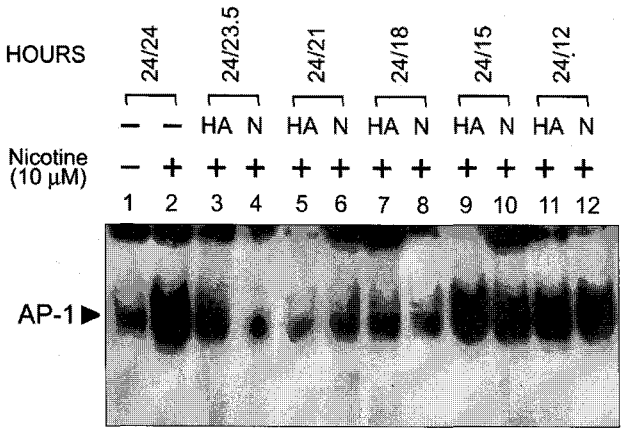

Fig. 1. Effects of cholinergic antagonists and nimodipine on AP-1 DNA binding activity induced by nicotine. After BAMC cells (10×10^6 cells) were treated with nicotine, either hexamethonium (1mM) plus atropine (1 μM) (HA) or nimodipine (N; 1 μM) were added at 0.5, 3, 6, 9, or 12 hr. The cells were harvested after 24 hr of incubation with nicotine and the electrophoretic mobility-shift assays were then performed to measure AP-1 DNA binding activity.

the nicotine treatment did not affect the nicotine-induced increase of AP-1 DNA binding activity (Fig. 1).

Effects of nimodipine and calmidazolium on the AP-1 DNA binding activity induced by nicotine

To determine if calcium (Ca^{2+}) and calmodulin were involved in the nicotine-induced increases of AP-1 DNA binding activity, the effect of nimodipine (an L-type Ca^{2+} channel blocker) and calmidazolium (a calmodulin antagonist) were examined. Nimodipine (1 μM) or calmidazolium (1 μM) pretreatments for 30 min almost completely blocked the increase of AP-1 DNA binding activity induced by nicotine (data not shown). Nimodipine or calmidazolium posttreatments beginning at 0.5, 3, or 6 hr after the nicotine treatment significantly inhibited the increase of AP-1 DNA binding activity (Figs. 1 and 2B left). However, nimodipine or calmidazolium posttreatments beginning at 9 or 12 hr after the nicotine treatment did not affect the increase of AP-1 DNA binding activity (Figs. 1 and 2B left).

Effect of KN-62 on the AP-1 DNA binding activity induced by nicotine

To determine if Ca^{2+}/calmodulin-dependent protein kinase II was involved in the increases of AP-1 DNA binding activity induced by nicotine, the effect of KN-62 (a Ca^{2+}/calmodulin-dependent protein kinase II inhibitor) on nicotine-induced response was examined. The concentration-dependent effect of KN-62 on AP-1 DNA binding activity is shown in Fig. 2A. The pretreatment of BAMC cells with various concentrations of KN-62 inhibited the increase of AP-1 DNA binding activity induced by nicotine in a concentration-dependent manner (Fig. 2A). KN-62 (10 μM) posttreatment beginning at 0.5, 3, or 6 hr after the nicotine treatment significantly inhibited the increase of AP-1 DNA binding activity (Fig. 2B right). However, KN-62 posttreatment beginning at 9 or 12 hr after the nicotine treatment did not affect the increase of AP-1 DNA binding activity (Fig. 2B right).

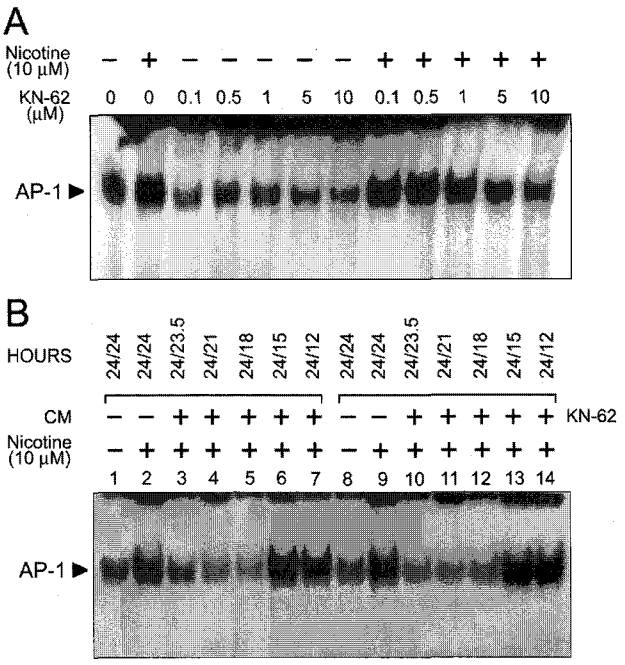

Fig. 2. Effects of KN-62 and calmidazolium on AP-1 DNA binding activity induced by nicotine. A. After the BAMC wells were pretreated with various concentrations of KN-62 (from 0.1 to 10 μM) for 30 min, nicotine (10 mM) was added into the medium. The cells were harvested after 24 hr of incubation with nicotine and then the electrophoretic mobility-shift assays were performed to measure AP-1 DNA binding activity. B. BAMC cells were treated with nicotine (10 μM), and either KN-62 (10 μM) or calmidazolium (CM; 1 μM) was then added at 0.5, 3, 6, 9, and 12 hr. The cells were harvested after 24 hr of incubation with nicotine and the electrophoretic mobility-shift assays were then performed to measure AP-1 DNA binding activity.

DISCUSSION

The present study demonstrated that the continuous stimulation of nicotine receptors of BAMC cells for up to 6 hr was required for the increase of AP-1 DNA binding activity. Calcium influx, calmodulin and Ca^{2+}/calmodulin-dependent protein kinase II pathways were founded to be involved in the increase of AP-1 DNA binding activity induced by nicotine in BAMC cells. However, blockade of these pathways by antagonists at 9 hr after the addition of nicotine failed to block the increase of AP-1 DNA binding activity induced by nicotine.

To determine optimum duration of the nicotinic receptors stimulation to elicit the increase of AP-1 DNA binding activity, BAMC cells were posttreated with hexamethonium plus atropine (HA) to inhibit the cholinergic receptors at various times after the nicotine treatment. We found that the blockade of cholinergic receptors with HA beginning at either 0.5, 3, or 6 hr after the nicotine treatment significantly inhibited the nicotine-induced AP-1 DNA binding activity. When added 9 or 12 hr after the nicotine treatment, these antagonists did not affect the increase of AP-1 DNA binding activity induced by nicotine, suggesting that continuous stimulation (for at least 6 hr) of nicotinic receptors was required for the increase of AP-1 DNA binding activity.

We also examined possible involvement of second messengers in the nicotine-induced increase of AP-1 DNA binding

activity. Posttreatments of BAMC cells with nimodipine, calmidazolium, or KN-62 up to 6 hr significantly inhibited the nicotine-induced increase in AP-1 DNA binding activity, indicating that Ca^{2+}, calmodulin, and Ca^{2+}/calmodulin-dependent protein kinase II were involved in the early (<6 hr) increase of AP-1 DNA binding activity induced by long-term stimulation of nicotinic receptors with nicotine. However, our results suggested that Ca^{2+}, calmodulin, and Ca^{2+}/calmodulin-dependent protein kinase II appeared to be not involved in the delayed increase of AP-1 DNA binding activity, because posttreatment of BAMC cells with nimodipine, calmidazolium or KN-62 beginning at 9 hr after the nicotine addition did not affect the nicotine-induced the increase of AP-1 DNA binding activity. These results suggested that other unknown mechanisms might have been involved in the delayed increase of AP-1 activity induced in BAMC cells by nicotine.

Results with the cholinergic blockers, the Ca^{2+} or calmodulin blockers, and the Ca^{2+}/calmodulin-dependent protein kinase II indicated a common time-course: perturbation of the nicotine treatment during the first 6 hr by these blockers prevented the increases of AP-1 DNA binding activity, however these blockers showed no effects on the long-term events, when they were added 9 hr after the nicotine treatment. Furthermore, the present study suggests a possibility that, whatever the intracellular events that are necessary for the nicotine-induced long-term effects (seen at 24 hr), they were completed during the first 9 hr of the nicotine treatment. It is not clear, however, why 6 to 9 hr of nicotine-treatment was required to complete the intracellular events necessary for the long-term effects. In conclusion, the present study showed that stimulation (for at least 6 hr) of nicotinic receptors on BAMC cells was necessary for the increase of AP-1 DNA binding activity, and that activation of Ca^{2+}, calmodulin, and Ca^{2+}/calmodulin-dependent protein kinase II up to 6 hr at least appeared to be required for the increase of nicotine-induced AP-1 DNA binding activity.

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