Research report

Degree of immediate early gene induction in striatum by eticlopride determines sensitivity to N-methyl-d-aspartate receptor blockade

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Abstract

Cortical afferents excite striatal efferent neurons through activation of N-methyl-d-aspartate (NMDA) receptors, which can be modulated by D2 dopamine receptors. It is suggested that activation of PKA by D2 receptor blockade leads to NMDA receptor phosphorylation in the dendrites or phosphorylation of transcription factors in the nucleus. Thus, the levels and cellular localization of activated PKA may determine if D2 antagonist-mediated gene expression is dependent on NMDA receptor activation. We have previously demonstrated that NMDA receptor antagonists block gene expression induced by a high dose of eticlopride in medial and central but not lateral striatum. Here, we examined the effects of NMDA receptor antagonists on striatal gene expression after administration of a low dose of eticlopride. The results showed that NMDA receptor antagonists blocked gene induction by eticlopride throughout striatum. Less PKA activation by the low dose of eticlopride might explain why the expression was more sensitive in the lateral striatum to NMDA receptor blockade than in our previous study. To increase levels of PKA activation to the extent that NMDA receptor blockade would have less effect on eticlopride-mediated gene induction in all regions of striatum, we administered the phosphodiesterase inhibitor IBMX to animals treated with eticlopride. The combined administration of IBMX and eticlopride induced gene expression that was only partially attenuated (c-fos) or unaffected (zif268) by NMDA receptor blockade. These data support the suggestion that the degree of second messenger activation by D2 receptor blockade determines whether D2 dopamine receptor antagonist-mediated gene expression is dependent on NMDA receptor activation. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The striatum is the main input nucleus of the basal ganglia and is involved in motor and cognitive functions. The striatum receives afferent glutamate projections from the cerebral cortex and thalamus, which act on N-methyl-d-aspartate (NMDA) and non-NMDA receptors [13,20–22]. It also receives dopamine input from the substantia nigra pars compacta [16]. Calcium entry via the NMDA receptor and L-type channels presumably activates a kinase cascade that leads to the phosphorylation of the transcription factor CREB (cAMP response element binding pro-
receptor blockade attenuates D2 dopamine receptor antagonist-mediated immediate early gene expression [6,12,19,43]. Such data suggest strongly that there are intracellular interactions between these two transmitter receptor systems leading to the regulation of gene expression. However, the signaling pathways responsible for the modulatory effects between these two neurotransmitter systems have not yet been fully explored, particularly in vivo.

Konradi and colleagues [24,26,32] have proposed that the levels of activated PKA may determine whether striatal immediate early gene expression is dependent on NMDA receptor activation. In primary striatal cell culture, the NMDA receptor antagonist MK-801 had no effect on phosphorylation of CREB or the induction of c-fos after the application of a high concentration of the cAMP inducer forskolin [24,32]. However, MK-801 did block CREB phosphorylation and c-fos expression induced by a low concentration of forskolin [24,32]. The model proposed by Konradi and colleagues for D2 dopamine/NMDA receptor interactions in the regulation of gene induction is thus based on the level of activation of PKA by the D2/cAMP pathway [24,26]. Low levels of activated PKA are thought to remain localized to the dendrites and to phosphorylate the NMDA receptor [26,32,36], leading to greater influx of calcium and a strong depolarization of the dendritic spine. This depolarization is thought to promote opening of voltage-sensitive L-type calcium channels. The resultant calcium influx is then thought to activate a signaling pathway that propagates to the nucleus, leading to the phosphorylation of CREB and the induction of c-fos [26,31]. Therefore, MK-801 is thought to effectively block c-fos induction in response to low levels of PKA activation by inhibiting Ca\(^{2+}\) influx-mediated activation of signaling kinases. However, high levels of cAMP accumulation are proposed to activate a more widespread distribution of PKA throughout the neuron. In this case, PKA activated within the nucleus is thought to directly phosphorylate CREB, leading to c-fos induction that is independent of NMDA receptor activation. Thus, this effect is not blocked by NMDA receptor antagonist administration [26,32].

We previously observed regional differences in striatal immediate early gene expression after administration of the D2 dopamine receptor antagonist eticlopride and the NMDA receptor antagonists CGS 19755 and MK-801 in intact rats [19]. Eticlopride administered at a dose of 1.0 mg/kg induced the immediate early genes c-fos and zif268 throughout striatum, with the highest levels apparent in lateral striatum. Pretreatment with CGS 19755 or MK-801 attenuated eticlopride-induced c-fos and zif268 induction in medial striatum, while having no significant effect in the lateral third of striatum. We think that the regional differences occurred as a result of higher numbers of D2 dopamine receptors in the lateral striatum [17,33], and thus a more widespread activation of PKA throughout the neurons in that region. Presumably, less nuclear PKA was activated in the medial and central thirds of striatum, leading to a lesser degree of direct phosphorylation of CREB by PKA and greater dependence on NMDA receptor activation [4,14,26,41]. By blocking the NMDA receptor with CGS 19755 or MK-801, the D2 dopamine receptor-mediated induction of c-fos and zif268 in the medial and central striatum was therefore attenuated.

The present study was designed to further evaluate the hypothesis that the level of activation of signaling pathways by D2 receptor blockade determines whether eticlopride-induced gene expression is dependent on NMDA receptor activation. We hypothesized that treatment with a low dose of eticlopride (0.5 mg/kg) would render immediate early gene expression susceptible to NMDA receptor blockade throughout the striatum due to lower levels of PKA activation, whereas a higher dose of eticlopride (1.0 mg/kg) had previously induced gene expression independent of NMDA receptor activation in the lateral striatum [19]. In addition, we hypothesized that administration of the phosphodiesterase inhibitor IBMX to animals treated with eticlopride would increase the amount of PKA activation to the extent that NMDA receptor blockade would no longer have an effect on D2 antagonist-mediated gene induction in any region of striatum.

2. Materials and methods

2.1. Animals and housing

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing 225 to 300 g were used in all experiments. Rats were housed in groups of four in hanging wire-mesh cages in a temperature-controlled room on a 12:12 light:dark cycle. Rats had free access to food and water. All studies were approved by the Institutional Animal Care and Use Committee at the University of Utah and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

CGS 19755 was donated by Ciba-Geigy Corporation (Summit, NJ, USA). Eticlopride hydrochloride and MK-801 were obtained from Research Biochemicals International (Natick, MA, USA) and IBMX was from Calbiochem (San Diego, CA, USA). All drug doses were calculated as free bases with the exception of eticlopride hydrochloride, which was calculated as the salt. CGS 19755 and MK-801 were dissolved in phosphate-buffered saline, eticlopride in normal saline, and IBMX in dimethyl sulfoxide (DMSO). All drugs were administered intraperitoneally in a volume of 1 ml/kg.
2.3. Pharmacological manipulations

2.3.1. Determining the effects of MK-801, CGS 19755, and IBMX on eticlopride-induced gene expression

Rats were taken from their home cages and placed in plastic tube cages (four per cage). Rats were weighed and then injected with either MK-801 (1 mg/kg), CGS 19755 (10 mg/kg), IBMX (5.0–25.0 mg/kg), or the appropriate vehicle solution. Sixty minutes (CGS 19755), 30 min (MK-801), or 15 min (IBMX) later, rats were injected with the D2 dopamine receptor antagonist eticlopride (0.5 mg/kg, i.p.). Doses and times between injections were chosen based on previously published studies showing effective NMDA receptor blockade at these doses and within these time ranges [7,8,11,23]. Animals were sacrificed 40 min after the injection of eticlopride. Previous studies have demonstrated that the expression of immediate early genes in response to eticlopride administration is significant at this time point (H. Steiner, personal communication). Control animals received two vehicle injections.

2.3.2. Determining the effect of CGS 19755 on gene expression induced by the combined administration of eticlopride and IBMX

Rats were taken from their home cages, placed in plastic tube cages (four per cage), and weighed. Rats were injected with CGS 19755 (10 mg/kg) or vehicle 45 min prior to the administration of IBMX (25 mg/kg) or vehicle. Fifteen minutes later, eticlopride was administered (1.0 mg/kg). Rats were sacrificed 40 min after the eticlopride injection.

2.4. In situ hybridization histochemistry

Rats were euthanized by exposure to CO₂ (1 min) and decapitated. The brains were rapidly removed and frozen in isopentane chilled on dry ice. Brains were stored at −20°C until they were cut into 12-μm thick sections in a cryostat (Cryocut 1800, Cambridge Instruments, Germany) and thaw-mounted onto gelatin-chrome alum-subbed slides. Slides were stored at −20°C. Once all brains from an experiment were sectioned, slides were postfixed in 4% paraformaldehyde/0.9% NaCl, acetylated in fresh 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl (pH 8.0), dehydrated in an ascending series of alcohols, delipidated in chloroform, and rehydrated in a descending series of alcohols. Slides were air-dried and stored at −70°C.

For detection of c-fos and zif268 mRNAs, full-length ribonucleotide probes complementary to the mRNAs for c-fos [10] and zif268 [27] were synthesized from the cDNAs using [35S]-UTP and SP6 (c-fos) or T7 (zif268) RNA polymerase (Boehringer Mannheim, Indianapolis, IN, USA). Labeled probes were diluted in hybridization buffer to obtain 2×10⁶ cpm/100 μl buffer. The ribonucleotide probe was mixed with nuclease-free water and RNA mix (final concentrations: 100 μg/ml salmon sperm DNA; 250 μg/ml yeast total RNA; 250 μg/ml yeast tRNA). The mixture was heated to 65°C for 5 min and then cooled on wet ice for 1 min. Dithiothreitol (100 mM, final concentration), sodium dodecyl sulfate (0.2% w/v, final concentration), sodium thiosulphate (0.1% w/v, final concentration), and hybridization buffer were added to the ribonucleotide mixture. The hybridization buffer contained (final concentrations): Tris buffer (23.8 mM, pH 7.4), EDTA (1.2 mM, pH 8.0), NaCl (357 mM), dextran sulfate (11.9%, w/v), Denhardt’s solution (1.2×), and formamide (59.5%, v/v). Ninety microliters of probe in hybridization buffer was applied to each slide containing four sections. Slides were coverslipped and hybridized overnight in humid chambers at 55°C. Slides were then washed at room temperature four times in 1× saline-sodium citrate (SSC, 0.15 M NaCl/0.015 M sodium citrate, pH 7.2), incubated in ribonuclease A (RNase A; 5–20 μg/ml; Boehringer Mannheim) in buffer containing 0.5 M NaCl, 10 mM Tris (pH 8.0), and 0.25 mM EDTA (pH 8.0) for 15 min at room temperature, and washed four times in 0.2× SSC at 60°C. Slides were rinsed briefly in deionized water, air dried, and apposed to Kodak Biomax X-ray film (Kodak Biomax MR, Eastman Kodak Co., NY, USA) for 4 days to 2 weeks to obtain film autoradiograms.

2.5. Data analysis

Film autoradiograms were analyzed using the Macintosh-based image analysis program, Image (Wayne Rasband, NIH). Images of brain sections were captured with a video camera, digitized, and stored on computer. Images were captured under constant lighting conditions and within the linear range of the system response. Mean gray values were analyzed in medial, central, and lateral thirds of the right striatum from its dorsal aspect to the anterior commissure ventrally. The average gray value of the white matter was subtracted from the average gray value of the regions of interest to correct for background labeling. Data from film autoradiograms were analyzed with a one-way analysis of variance for medial, central, and lateral thirds of the striatum. Post hoc analysis was performed with the Tukey–Kramer test. Statistical significance was set at P ≤ 0.05.

3. Results

3.1. CGS 19755 and MK-801 block immediate early gene expression throughout striatum after administration of a low dose of eticlopride

As noted in the Introduction, the induction of c-fos and zif268 in striatum by 1 mg/kg eticlopride was not blocked in the lateral striatum by NMDA receptor antagonists [19]. In the present study, a lower dose of eticlopride (0.5
mg/kg) also induced c-fos and zif268 expression throughout the striatum, with greater expression in the lateral region. However, in this case the NMDA receptor antagonists CGS 19755 (10 mg/kg) and MK-801 (1 mg/kg) effectively attenuated eticlopride-induced gene expression in all regions of the striatum ($P < 0.05$) (Fig. 1).

3.2. The non-selective phosphodiesterase inhibitor IBMX potentiates immediate early gene expression induced by eticlopride

As is shown in Fig. 2, eticlopride (1 mg/kg) increased c-fos (A) and zif268 (B) expression in the striatum, with greater induction in the lateral striatum. Combined administration of eticlopride and IBMX increased the expression of both immediate early genes throughout all regions of the striatum. This effect was significantly greater than the induction produced by eticlopride alone at the IBMX dose of 25 mg/kg ($P < 0.05$, Fig. 2).

3.3. CGS 19755 only partially attenuates c-fos expression and does not affect zif268 expression after the combined administration of eticlopride and IBMX

As previously observed, the NMDA receptor antagonist CGS 19755 significantly reduced the induction of c-fos and zif268 by 1.0 mg/kg eticlopride alone (Fig. 3). However, it had less effect on the induction produced by
the combined administration of eticlopride and IBMX (Fig. 3). The expression of c-fos was only partially attenuated by CGS 19755 in the medial and central regions of the striatum, whereas zif268 expression was completely unaffected by NMDA receptor blockade with CGS 19755 in all regions of the striatum.

4. Discussion

Our previous data [19] demonstrated that NMDA receptor blockade selectively attenuated immediate early gene expression induced by a high dose of eticlopride (1.0 mg/kg) in the medial, but not the lateral aspect of the striatum. This was presumably due to greater numbers of D2 receptors and a more widespread activation of PKA in the lateral striatum. The results presented here demonstrate the susceptibility of c-fos and zif268 expression to NMDA receptor blockade throughout striatum after the administration of a lower dose of eticlopride (0.5 mg/kg). We propose that the lower dose of eticlopride activated less nuclear PKA than did the higher dose, rendering gene expression dependent on NMDA receptor activation even in the lateral third of the striatum. These data are consistent with the recently published findings of Leveque et al. [26]. In this study, we have expanded these findings, showing that the non-selective phosphodiesterase inhibitor IBMX (25 mg/kg) potentiated eticlopride-induced gene expression to such an extent that NMDA receptor blockade no longer affected the expression of zif268 and only partially suppressed c-fos expression. Although these studies do not directly assess the role of PKA in D2/NMDA receptor interactions, they do indirectly support the model proposed by Konradi and colleagues [24,26] that the levels of activated PKA determine whether striatal gene expression is dependent on NMDA receptor activation.

It has been demonstrated that D2 dopamine receptor stimulation leads to the activation of an inhibitory G protein (G) [38], presumably inhibiting cAMP formation and the activation of PKA. Thus, D2 receptor blockade induced in striatum by the combined administration of eticlopride and should disinhibit cAMP formation and PKA activation. IBMX. The NMDA receptor antagonist CGS 19755 (`CGS'; 10 mg/kg) and the non-selective phosphodiesterase inhibitor IBMX (25 mg/kg) were administered intraperitoneally to animals treated with the D2 dopamine receptor antagonist eticlopride (`Etic'; 1.0 mg/kg, i.p.). CGS 19755 or vehicle was administered 45 min prior to IBMX or vehicle, which was administered 15 min before the administration of eticlopride. Animals receiving eticlopride alone received two vehicle injections, 45 min and 15 min prior to eticlopride administration. Control animals received three vehicle injections. Animals were sacrificed 40 min after the last injection. Values are mean gray values (±S.E.M.; arbitrary units) obtained from densitometric analysis of the medial, central, and lateral thirds of the mid-striatum (approximately 0.5 mm anterior to bregma). Numbers in parentheses indicate the number of animals per group. *Significantly different from control, P < 0.05. +Significantly different from eticlopride alone, P < 0.05. #Significantly different from IBMX + eticlopride, P < 0.05.

Fig. 3. Effect of CGS 19755 on (A) c-fos and (B) zif268 expression induced in striatum by the combined administration of eticlopride and IBMX. The NMDA receptor antagonist CGS 19755 (‘CGS’; 10 mg/kg) and the non-selective phosphodiesterase inhibitor IBMX (25 mg/kg) were administered intraperitoneally to animals treated with the D2 dopamine receptor antagonist eticlopride (‘Etic’; 1.0 mg/kg, i.p.). CGS 19755 or vehicle was administered 45 min prior to IBMX or vehicle, which was administered 15 min before the administration of eticlopride. Animals receiving eticlopride alone received two vehicle injections, 45 min and 15 min prior to eticlopride administration. Control animals received three vehicle injections. Animals were sacrificed 40 min after the last injection. Values are mean gray values (±S.E.M.; arbitrary units) obtained from densitometric analysis of the medial, central, and lateral thirds of the mid-striatum (approximately 0.5 mm anterior to bregma). Numbers in parentheses indicate the number of animals per group. *Significantly different from control, P < 0.05. +Significantly different from eticlopride alone, P < 0.05. #Significantly different from IBMX + eticlopride, P < 0.05.
induced by a low dose of eticlopride not only in the lateral striatum, but also in the medial and central regions. The expression of zif268 also was completely blocked by administration of MK-801 and CGS 19755 throughout striatum after administration of this lower dose of eticlopride. Furthermore, our findings extend those of previous reports in that we have shown that D2 dopamine receptor antagonist-mediated gene expression is differentially susceptible to NMDA receptor blockade depending on the dose of antagonist administered and the region of striatum examined (see Fig. 1). This is presumably due to greater activation of PKA in the lateral striatum after administration of the higher dose of eticlopride. Interestingly, administration of a higher dose of haloperidol (1 mg/kg) in the Boegman and Vincent study [6] had a greater effect on the number of Fos-positive nuclei than did the lower dose, but data were not reported for the effect of MK-801 on Fos induction by the high dose of haloperidol.

The model of Konradi and colleagues [24,26] suggests that the intracellular kinase mediating the interactions between NMDA and dopamine receptors is PKA. Therefore, we administered the non-selective phosphodiesterase inhibitor IBMX to animals treated with eticlopride in an attempt to potentiate the eticlopride-induced gene expression, presumably through an increase in PKA activation. The combined administration of IBMX plus eticlopride produced significantly greater gene induction than did eticlopride alone. Pretreatment with the NMDA receptor antagonist CGS 19755 had no effect on the zif268 expression induced by the combined IBMX and eticlopride administration, whereas c-fos expression was partially attenuated.

Although IBMX potentiated eticlopride-induced immediate early gene expression, the mechanism for this potentiation is unknown. It has been previously demonstrated that IBMX administration alone can lead to the expression of c-fos in the striatum [37]. However, the induction by IBMX in that study was attributed to the antagonistic effect of IBMX on the adenosine A2a receptor [15], due to the lack of effect of other selective phosphodiesterase inhibitors on striatal c-fos expression [37]. Adenosine A2a receptors are colocalized on striatopallidal neurons with D2 dopamine receptors [35] and are positively coupled to adenylyl cyclase [30,39]. However, it has been demonstrated that A2a receptor antagonist, rather than agonist, administration leads to the induction of immediate early genes in striatum, presumably by promoting the release of calcium from microsomes of the endoplasmic reticulum [37,40]. Therefore, we cannot absolutely attribute the increase in immediate early gene expression reported herein to the phosphodiesterase properties of IBMX. Further experiments are necessary to differentiate these two possibilities.

At present, the basis for the differential effect of CGS 19755 on c-fos and zif268 expression induced by IBMX and eticlopride is unknown. These two immediate early genes can be induced through activation of various signaling pathways and thus may be differentially regulated by intracellular second messengers [3,14]. Furthermore, the number and types of transcriptional regulatory elements within the promoter regions differ between c-fos and zif268 genes [3,28,34]. Therefore, the expression of c-fos may be more dependent on activation of the NMDA receptor, whereas zif268 expression is more heavily dependent on activation of other intracellular signals. Further experiments are necessary to address this possibility. However, our data strongly suggest that the degree of gene induction and, presumably, intracellular signaling activity by the combined administration of eticlopride and IBMX determines the extent to which D2 antagonist-induced gene expression is dependent on NMDA receptors, especially in the case of zif268 expression.

In conclusion, our data support the model proposed by Konradi and colleagues [24,26] that the degree of activation of intracellular signaling pathways determines whether D2 dopamine receptor antagonist-mediated immediate early gene expression is dependent on NMDA receptor activation. It is important to note, however, that the contribution of PKA to the induction of immediate early genes by eticlopride has not been directly tested in our experiments. Therefore, it will be necessary to examine the effects of PKA inhibition on eticlopride-induced c-fos and zif268 expression in vivo.

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