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Stimulation of dopamine receptors inhibited Ca^{2+}-calmodulin-dependent protein kinase Ⅱ activity in rat striatal slices[1]

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KEY WORDS dopamine; Ca^{2+}-calmodulin-dependent protein kinase; anoxia; calcium; reserpine; dopamine agonists; dopamine antagonists; corpus striatum

ABSTRACT

AIM: To investigate the mechanism underlying dopaminergic neurotoxicity in the striatum during anoxia. **METHODS**: Using rat striatal slices as an *in vitro* model, the activity of Ca^{2+}-calmodulin-dependent protein kinase Ⅱ (CCDPK Ⅱ) was examined by the method of substrate phosphorylation ^{32}P-incorporation. **RESULTS**: Anoxia for 30 min greatly reduced CCDPK Ⅱ activity by about 75 %. Reserpinization by repeated reserpine administration (1 $mg \cdot kg^{-1} \cdot d^{-1}$ for 7 d, sc) preserved CCDPK Ⅱ activity against the anoxia-induced decrease (about 40 % of control). The activity of CCDPK Ⅱ was reduced significantly by exposure of rat striatal slices to micromolar concentrations of dopamine in the presence of extracellular Ca^{2+}. Omission of Ca^{2+} in the incubation medium (with addition of 1 mmol/L egtazic acid) diminished the dopamine-induced decrease of the kinase activity. Application of apomorphine, a non-selective dopamine receptor agonist, produced a similar concentration-related decrease of CCDPK Ⅱ activity. Exposure to SKF38393 (selective D_1-like receptor agonist) or quinpirole (selective D_2-like receptor agonist) also inhibited the kinase activity. The dopamine-induced decrease of CCDPK Ⅱ activity was attenuated by preincubation with Sch-23390 (selective D_1-like receptor antagonist) or domperidone (selective D_2-like receptor antagonist). **CONCLUSION**: Dopamine is involved in the anoxia-induced inhibition of CCDPK Ⅱ activity by activation of both D_1-like and D_2-like receptors and influx of Ca^{2+}, which may contribute to dopamine-mediated striatal neuronal damage.

INTRODUCTION

Ca^{2+}-calmodulin-dependent protein kinase Ⅱ (CCDPK Ⅱ), remarkably abundant in the mammalian brain and composing up to 1 % of total forebrain protein, is a multifunctional protein kinase critical to Ca^{2+}-mediated signal transduction and synaptic plasticity. Significant inhibition of CCDPK Ⅱ activity in the hippocampus, striatum, and cortex has been observed in various models of ischemia. The inhibition of CCDPK Ⅱ activity has been implicated in the biochemical processes leading to ischemic neuronal damage[1,2].

It has been indicated that glutamate, by acting on *N*-methyl-*D*-aspartate (NMDA) receptor ion channels, increases Ca^{2+} influx and mediates the inhibition of CCDPK Ⅱ activity[3,4]. However, preadministration of NMDA receptor antagonists could only partially preserve the ischemia-induced loss of the kinase activity[1,4], which suggested that additional factors may also be involved in the inhibition resulting from ischemia. Direct stimulation of dopamine receptors may fall into this category.

Dopamine receptors are classified as "D_1-like" (D_1 and D_5) or "D_2-like" (D_2, D_3, and D_4) based upon both molecular biological and pharmacological similarities. Numerous studies performed in rat brain and in cell lines transfected with rat or human dopamine receptor cDNA have shown that stimulation of D_1 or D_2-like receptors appears to increase intracellular Ca^{2+} levels[5]. Therefore, dopamine may decrease the activity of CCDPK Ⅱ and be involved in the ischemia-induced inhibition of CCDPK Ⅱ activity through a D_1 or D_2-like receptors coupled to Ca^{2+} signaling transduction pathway, which may partially contribute to dopaminergic neurotoxic effect during cerebral ischemia. In this paper, the

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hypothesis was examined using rat striatal slices.

MATERIALS AND METHODS

Materials Dopamine hydrochloride, (±)-SKF38393 hydrochloride, (−)-quinpirole hydrochloride, *R*(+)-Sch23390 hydrochloride, and domperidone (RBI, USA); apomorphine (Sigma, USA). They were dissolved in a small amount of distilled water, except quinpirole was dissolved in H_2SO_4 (0.1 mol/L) and domperidone in 1 % lactate, and diluted with glucose-free Krebs-Ringer solution immediately before use.

[γ-^{32}P]ATP was purchased from Yahui Biological and Medical Engineering Co, Beijing; reserpine (dissolved in 1 % glacial acetic acid), sodium metabisulphite, and ATP were from Sigma; phenylmethanesulfonylfluorid (PMSF), and mercapto ethenol (β-ME) were from E Merck. Other reagents were of AR grade.

Preparation and preincubation of striatal slices Male Sprague-Dawley rats (160 − 200 g, Grade Ⅱ, Certificate № D02-49-2, purchased from Sippr-BK Experimental Animal Ltd Co, Shanghai), were killed by decapitation and the brains were quickly transferred to an ice-cold Krebs-Ringer buffer (NaCl 122, KCl 3.1, KH_2PO_4 0.4, $MgSO_4$ 1.2, $CaCl_2$ 1.3, $NaHCO_3$ 25, and glucose 10 mmol/L; pH 7.4) that had been equilibrated with 95 % O_2 + 5 % CO_2. The right and left striatum were dissected and sliced into 300-μm sections with a McIlwain tissue chopper. The slices from both striatum were preincubated in fresh Krebs-Ringer buffer for 90 min at 36 ℃, and incubation medium was continuously bubbled with 95 % O_2 + 5 % CO_2.

Anoxia and drug application Following 90 min preincubation, striatal slices were washed rapidly three times with glucose-free Krebs-Ringer buffer. For anoxic treatments, slices were incubated in this buffer continuously bubbled with 95 % N_2 + 5 % CO_2. For drug application, slices were incubated for indicated time in this buffer containing the following drugs: dopamine, apomorphine, SKF38393, or quinpirole. The effect of extracellular Ca^{2+} was examined by removing Ca^{2+} and adding egtazic acid 1 mmol/L to the incubation buffer. During whole course of drug application, incubation buffer was continuously bubbled with 95 % O_2 + 5 % CO_2. The control groups were subjected only to a change of incubation buffer for the same time as drug application or anoxic groups. To assess the effect of dopamine receptor antagonization, Sch-23390 or domperidone was added to the incubation buffer 15 min before exposure to dopamine.

Sodium metabisulphite (200 μmol/L) was added to the dopamine-containing incubation buffer to prevent oxidation of dopamine. In pilot experiments, a single treatment with sodium metabisulphite, Sch-23390, or domperidone did not affect CCDPK Ⅱ activity of striatal slices.

At the end of incubation, the buffer was rapidly aspirated and the slices were immediately frozen in liquid nitrogen until assay.

Reserpinization To prepare models of reserpinization rats, male Sprague-Dawley rats (250 − 300 g) received repeated injection of reserpine (1 mg·kg^{-1}·d^{-1} for 7 d, sc). Sham-operated rats were injected with vehicle only. After reserpine administration, rats were killed to prepare striatal slices.

CCDPK Ⅱ activity assay Frozen slices were quickly homogenized at 4 ℃ in ice-cold buffer containing Tris-HCl 20, edetic acid 2, NaF 2, PMSF 0.5, and β-ME 10 mmol/L; pH 7.5. The homogenate was centrifuged at 10 000 × *g* for 5 min at 4 ℃, and the supernatant was assayed immediately for Ca^{2+}-calmodulin-dependent activity of CCDPK Ⅱ by the method of substrate phosphorylation ^{32}P-incorporation as previously de-scribed[6,7]. $CaCl_2$ 1 mmol/L and calmodulin 0.5 g/L were added to activate CCDPK Ⅱ. The radioactivity was measured by a liquid scintillation spectrometer (LS 6500, Beckman). CCDPK Ⅱ activity was calculated from the amount of ^{32}P transferred in the presence of activators minus that measured without activators (in the presence of egtazic acid 1 mmol/L). Protein concentration of the supernatant was determined by the Lowry method with bovine serum albumin (BSA) as standard[8].

Statistical analysis All values were shown as the $x \pm s$. Statistical analysis was performed by *t*-test, for comparison of more than two groups, by analysis of variance (ANOVA) followed by Duncan's new multiple range comparison test with $P < 0.05$ as statistical difference.

RESULTS

Effect of reserpinization by repeated reserpine treatment on anoxia-induced inhibition of CCDPK Ⅱ activity After preadministration of reserpine, CCDPK Ⅱ activity in striatal slices had no clear changes compared with vehicle-treated groups.

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Following 30-min anoxia, CCDPK Ⅱ activity in striatal slices from vehicle-treated rats decreased by about 75 %, by contrast, the activity in striatal slices of reserpine-treated rats decreased only by about 40 % (Tab 1).

Tab 1. Protective effect of reserpinization by repeated reserpine treatment against anoxia-induced inhibition of CCDPK Ⅱ activity in rat striatal slices. $n = 5$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* the respective values of vehicle-treated groups.

Treatment	CCDPK Ⅱ activity/nmol·min^{-1}·g^{-1}	
	Control	Anoxia
Vehicle	110 ± 10	27 ± 7
Reserpine	101 ± 13^a	59 ± 5^c

Effect of dopamine exposure on CCDPK Ⅱ activity Exposure of rat striatal slices to dopamine (10 μmol/L) decreased the kinase activity in a time-dependent manner. Statistically significant ($P < 0.01$) reduction was observed 5 min after the addition of dopamine, and the enzyme activity was minimal 30 min after exposure to dopamine (Tab 2).

Tab 2. Time-dependent inhibitory effect of dopamine (10 μmol/L) on CCDPK Ⅱ activity in rat striatal slices. $n = 5$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Time/min	CCDPK Ⅱ activity/nmol·min^{-1}·g^{-1}
0	115 ± 5
5	97 ± 10^c
10	74 ± 10^c
20	74 ± 6^c
30	60 ± 6^c

Effect of extracellular Ca^{2+} on dopamine-induced inhibition of CCDPK Ⅱ activity After 90 min preincubation, rat striatal slices were transferred into a glucose-free medium containing various concentrations of dopamine and incubated for 30 min in the presence of extracellular Ca^{2+}. CCDPK Ⅱ activity was reduced by dopamine at concentrations of 5 – 50 μmol/L. The inhibitory effect was maximal with dopamine at 10 – 20 μmol/L, and the enzyme activity decreased by about 48 % (Tab 3).

To determine the relationship between dopamine-induced inhibition of the kinase activity and Ca^{2+} influx, striatal slices were washed three times with a Ca^{2+}-free/egtazic acid and glucose-free buffer after preincubation, and then exposed to various concentrations of dopamine for 30 min in the absence of extracellular Ca^{2+}. Such an application of dopamine resulted in a little (approximate 12 %) decrease of CCDPK Ⅱ activity only at concentration of 20 μmol/L. At other concentrations, dopamine did not induce any significant changes ($P > 0.05$) in the kinase activity (Tab 3). Control was subjected to a change of medium only in the Ca^{2+} and glucose-free buffer/egtazic acid without dopamine for the same time, and the kinase activity had no significant change.

Tab 3. Inhibitory effect of dopamine on CCDPK Ⅱ activity in rat striatal slices in the presence (+)/absence (–) of extracellular Ca^{2+} ($[Ca^{2+}]_o$). $n = 5$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* respective control ([dopamine] = 0). $^dP > 0.05$, $^fP < 0.01$ *vs* the respective concentrations of dopamine in the presence of $[Ca^{2+}]_o$.

Dopamine/μmol·L^{-1}	CCDPK Ⅱ activity/nmol·min^{-1}·g^{-1}	
	+ $[Ca^{2+}]_o$	– $[Ca^{2+}]_o$
0	115 ± 5	112 ± 11^d
5	100 ± 4^c	116 ± 4^{df}
10	60 ± 6^c	99 ± 8^{af}
20	60 ± 4^c	98 ± 5^{bf}
50	79 ± 9^c	116 ± 9^{df}

Effect of dopaminergic agonists on CCDPK Ⅱ activity Treatment with apomorphine, a non-selective dopamine receptor agonist, led to a concentration-related inhibition of CCDPK Ⅱ activity similar to dopamine exposure (Tab 4).

Tab 4. Inhibitory effect of apomorphine, SKF38393, and quinpirole on CCDPK Ⅱ activity in rat striatal slices. $n = 5$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* control.

Treatment	Concentration/μmol·L^{-1}	CCDPK Ⅱ activity/nmol·min^{-1}·g^{-1}
Control	–	115 ± 8
Apomorphine	0.01	83 ± 10^c
	0.1	67 ± 4^c
	0.5	61 ± 7^c
	1	87 ± 6^c
SKF38393	0.1	113 ± 15^a
	1	73 ± 7^c
	5	88 ± 6^c
Quinpirole	0.1	123 ± 5^a
	1	63 ± 8^c
	5	66 ± 13^c

D_1-like receptor agonist SKF38393 (1 and 5 μmol/L) greatly ($P < 0.01$) decreased CCDPK Ⅱ activity. The kinase activity was minimal with SKF38393 1 μmol/L (falling by about 40 %), and the inhibitory effect subsequently was attenuated. The D_2-like receptor agonist quinpirole (1 and 5 μmol/L) also inhibited CCDPK Ⅱ activity and the kinase activity decreased by about 45 %, but no clear concentration-dependency was observed (Tab 4).

Effect of dopaminergic antagonists on dopamine-induced inhibition of CCDPK Ⅱ activity

The dopamine (10 μmol/L)-induced decrease of CCDPK Ⅱ activity was diminished significantly ($P < 0.01$) by preincubating slices with a D_1-like receptor antagonist Sch-23390 (20, 50, 100 μmol/L) or a D_2-like receptor antagonist domperidone (1, 5 μmol/L) (Tab 5).

Tab 5. Protective effect of Sch-23390 and domperidone against dopamine-induced inhibition of CCDPK Ⅱ activity in rat striatal slices. $n = 5$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* dopamine (10 μmol/L). $^fP < 0.01$ *vs* control.

Treatment	Concentration/ μmol·L^{-1}	CCDPK Ⅱ activity/ nmol·min^{-1}·g^{-1}
Control	-	115 ± 5
Dopamine	10	60 ± 6^f
Sch-23390	20	73 ± 6^{cf}
	50	98 ± 9^{cf}
	100	96 ± 6^{cf}
Domperidone	0.5	65 ± 4^{af}
	1	89 ± 8^{cf}
	5	91 ± 9^{cf}

DISCUSSION

The striatum, selectively vulnerable brain region to transient cerebral ischemia, receives dopaminergic projection from the substantia nigra pars compacta. Many experiments have demonstrated that striatal dopamine released to extracellular space can rise up to about 400 – 500 folds over preischemic level (micromolar level) and the excessive release of dopamine mediates a neurotoxic effect during cerebral ischemia[9,10]. However, the mechanism underlying dopaminergic neurotoxicity in the striatum remains unclear.

The results of the present paper showed that repeated reserpine treatment diminished anoxia-induced inhibition of CCDPK Ⅱ activity in the striatum. Reserpine is known to block the uptake of dopamine into the storage. The chronic and repeated reserpine treatment can result in depletion of dopamine stores by approximate 98 %[11]. It suggests that the excessive release of dopamine in the striatum may be involved in anoxia-induced decrease of CCDPK Ⅱ activity. Previously, we reported that dopaminergic antagonists (Sch-23390 and domperidone) protected the kinase activity against decrease induced by anoxia in the striatum[6]. Taken together, dopamine may decrease CCDPK Ⅱ activity by activation of D_1 and D_2-like receptors, which partially contribute to dopamine neurotoxic effect in the striatum during anoxia.

To further examine the hypothesis, we observed the effects of exogenous dopamine and dopaminergic drugs on CCDPK Ⅱ activity using rat striatal slices. The results suggest that micromolar concentrations of dopamine decrease CCDPK Ⅱ activity by stimulation of D_1-like and D_2-like receptors and Ca^{2+} influx. The inhibition of CCDPK Ⅱ activity depends on elevation of intracellular Ca^{2+} levels. Intracellular Ca^{2+} overload, by coupling to calmodulin which in turn binds to CCDPK Ⅱ, triggers autophosphorylation of CCDPK Ⅱ at Thr286, and the autophosphorylation converts the kinase from the Ca^{2+}-calmodulin-dependent form to the Ca^{2+}-calmodulin-independent form. As a result of transient Ca^{2+} mobilization, the Ca^{2+}-calmodulin-independent activity of CCDPK Ⅱ is persistently activated but the Ca^{2+}-calmodulin-dependent activity is inhibited[12]. Therefore, the results are in agreement with those that stimulation of D_1 or D_2-like receptors induces Ca^{2+} influx[5]. Ca^{2+} overload is thought to be crucial mechanism leading to ischemic neuronal damage through the subsequent initiation of Ca^{2+} dependent processes such as the activation of CCDPK Ⅱ, proteases, and endonucleases, *etc*. Thus, Ca^{2+} influx results from stimulation of D_1 and D_2-like receptors may be one of the mechanisms underlying striatal dopaminergic neurotoxicity during cerebral ischemia. It has been indicated that higher concentrations of dopamine have inhibitory effect on striatal neuron electrophysiological activities by stimulation of D_1-like receptors in the striatum[10]. This may be the major cause that the dopamine, apomorphine, and SKF38393-induced inhibition of CCDPK Ⅱ activity is attenuated at higher concentrations. This may be due to activation of calcineurin[13,14], an effect mediated through the intracellular Ca^{2+} increase. However, the exact mechanisms remain to be elucidated.

Taken together, dopamine inhibits CCDPK Ⅱ

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activity by stimulation of D_1-like and D_2-like receptors and Ca^{2+} influx, which is involved in ischemia-induced decrease of the enzyme activity and may be the major mechanism underlying striatal dopaminergic neurotoxicity during cerebral ischemia.

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激动多巴胺受体抑制大鼠纹状体脑片 Ca^{2+}-钙调蛋白依赖性蛋白激酶Ⅱ活性[1]

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关键词 多巴胺；Ca^{2+}-钙调蛋白依赖性蛋白激酶；低氧；钙；利血平；多巴胺激动剂；多巴胺拮抗剂；纹状体

目的：研究缺氧时纹状体多巴胺能神经毒性的机制. **方法**：采用大鼠纹状体脑片体外培养模型，以底物磷酸化^{32}P-掺入法测定 Ca^{2+}-钙调蛋白依赖性蛋白激酶Ⅱ（CCDPKⅡ）的活性. **结果**：缺氧 30 min，纹状体脑片 CCDPKⅡ活性降低 75 %，慢性利血平化使得缺氧诱导的酶活性降低程度减轻，与对照组相比大约降低 40 %. 外源性多巴胺显著降低纹状体脑片 CCDPKⅡ活性. 去除胞外 Ca^{2+} 后，多巴胺诱导的酶活性降低作用被削弱. 阿扑吗啡（非特异性多巴胺受体激动剂）、SKF38393（特异性 D_1 样受体激动剂）和喹吡罗（特异性 D_2 样受体激动剂）均可显著降低 CCDPKⅡ的活性. Sch-23390（特异性 D_1 样受体拮抗剂）和吗丁啉（特异性 D_2 样受体拮抗剂）均可拮抗多巴胺所诱导的酶活性的抑制作用. **结论**：多巴胺参与缺氧诱导的纹状体 CCDPKⅡ活性抑制，其作用机制与 D_1 样和 D_2 样受体的激活以及胞外 Ca^{2+} 的内流有关，从而导致多巴胺介导的纹状体神经损伤.

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