

# Inhibitory effect of dopamine on $Ca^{2+}$ -calmodulin-dependent protein kinase II activity in rat hippocampal slices<sup>1</sup>

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**KEY WORDS** dopamine;  $Ca^{2+}$ -calmodulin dependent protein kinase; cerebral ischemia; calcium; dopamine agonists; dopamine antagonists; hippocampus

## ABSTRACT

**AIM:** To study the effect of dopamine (DA) on  $Ca^{2+}$ -calmodulin dependent protein kinase II (CCDPK II) activity in rat hippocampus. **METHODS:** Using rat hippocampal slices as an *in vitro* model, the activity of CCDPK II was examined by the method of <sup>32</sup>P-incorporation. **RESULTS:** Exogenous DA reduced CCDPK II activity in hippocampal slices in a concentration- and time-dependent manner. Removal of extracellular calcium antagonized the DA-induced inhibition of CCDPK II activity, partially or completely. The activity of CCDPK II was markedly decreased by apomorphine (a nonselective DA receptor agonist), SKF38393 (a selective D<sub>1</sub>-like DA receptor agonist), or quinpirole (a selective D<sub>2</sub>-like DA receptor agonist). The inhibition of CCDPK II activity induced by exogenous DA was abolished by preincubation with Sch-23390, a selective D<sub>1</sub>-like DA receptor antagonist, or domperidone, a selective D<sub>2</sub>-like DA receptor antagonist. **CONCLUSION:** DA has an inhibitory effect on CCDPK II activity in rat hippocampus, related to stimulation of D<sub>1</sub>-like and D<sub>2</sub>-like receptors and calcium influx.

## INTRODUCTION

The hippocampus was the most vulnerable brain

region to ischemic injury. The excessive release of DA in the hippocampus during cerebral ischemia played an important role in the development of ischemic cerebral injury<sup>(1,2)</sup>. However, the mechanism underlying dopaminergic neurotoxicity remained unclear.

Overload of intracellular calcium was thought to be a pivotal factor in ischemic cerebral injury. Elevation of intracellular calcium concentration led to activation of calcium-dependent lipases, nucleases, proteases, nitric oxide synthases, and protein kinases such as CCDPK II, etc., and consequent neuronal cell death. CCDPK II, which was highly enriched in neural tissues and composed up to 2% of total proteins in the hippocampus, was a multifunctional protein kinase and regulated many physiological responses to calcium mobilization including gene expression, neurotransmitter synthesis and exocytosis, postsynaptic responses and cytoskeletal interactions, etc. Previously, we reported that hypoxia could result in the significant inhibition of CCDPK II activity in rat hippocampus<sup>(3)</sup>. The inhibition of the enzyme activity had been implicated in contributing to neuronal cell damage<sup>(4)</sup>. Therefore, elucidating the effect of DA on CCDPK II activity would provide insight into the molecular mechanism of dopaminergic neurotoxicity in cerebral ischemic injury. In this paper, the effect of DA on CCDPK II activity in rat hippocampus was firstly investigated.

## MATERIALS AND METHODS

**Materials** Dopamine hydrochloride, *d,l*-SKF38393 hydrochloride, *l*-quinpirole hydrochloride, R(+)-Sch-23390 hydrochloride, and domperidone (RBI, USA); apomorphine (Sigma, USA). They were dissolved in a small amount of distilled water, except quinpirole was dissolved in H<sub>2</sub>SO<sub>4</sub> (0.1 mol · L<sup>-1</sup>) and domperidone in 1% lactate. They were

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diluted with glucose-free Krebs-Ringer solution immediately before use.

[ $\gamma$ - $^{32}$ P]ATP was purchased from Yahui Biological and Medical Engineering Co, Beijing; sodium metabisulfite and ATP from Sigma; phenylmethane-sulfonylfluorid (PMSF), and mercapto ethenol ( $\beta$ -ME) from E Merck. Other reagents were of AR.

**Hippocampal slice preparation and drug application** Male Sprague-Dawley rats (Grade II, Certificate No 02-49-02, purchased from Sippr-BK Experimental Animal Ltd Co, Shanghai) weighing 160 - 200 g were decapitated and the brains were placed in ice-cold Krebs-Ringer solution: NaCl 122, KCl 3.1,  $\text{KH}_2\text{PO}_4$  0.4,  $\text{MgSO}_4$  1.2,  $\text{CaCl}_2$  1.3,  $\text{NaHCO}_3$  25, glucose 10  $\text{mmol}\cdot\text{L}^{-1}$ ; pH 7.4 equilibrated with 95 %  $\text{O}_2$  + 5 %  $\text{CO}_2$ . Parasagittal hippocampal slices (350  $\mu\text{m}$ ) were prepared with a McIlwain tissue chopper and preincubated in normal Krebs-Ringer solution at 36  $^\circ\text{C}$  for 90 min. Following preincubation, slices were transferred to glucose-free Krebs-Ringer solution and incubated with or without drugs for 30 min or as indicated. During whole course of preincubation and drug application, incubation medium was continuously bubbled with 95 %  $\text{O}_2$  + 5 %  $\text{CO}_2$ . Sodium metabisulfite (200  $\mu\text{mol}\cdot\text{L}^{-1}$ ) was added to the dopamine-containing solution to prevent oxidation of dopamine. In pilot experiments, sodium metabisulfite (200  $\mu\text{mol}\cdot\text{L}^{-1}$ ) did not affect the activity of CCDPK II. DA receptor antagonists were added 15 min before exposure to DA. After experiments, the slices were quickly frozen in liquid nitrogen until use.

**Assay for CCDPK II activity** The slices were homogenized in a glass homogenizer on ice with ice-cold homogenization buffer: Tris-HCl 20, edetic acid 2, NaF 2, PMSF 0.5,  $\beta$ -ME 10  $\text{mmol}\cdot\text{L}^{-1}$ ; pH 7.5. The homogenate was spun at 10 000  $\times g$  at 4  $^\circ\text{C}$  for 5 min, and the supernatant was assayed for  $\text{Ca}^{2+}$ -calmodulin-dependent activity of CCDPK II by the method of  $^{32}\text{P}$ -incorporation<sup>(3)</sup>. The radioactivity was measured by a liquid scintillation spectrometer (LS 6500, Beckman). Protein concentration of the supernatant was determined with bovine serum albumin (BSA) as standard<sup>(5)</sup>. The CCDPK II activity was expressed in the amount of  $^{32}\text{P}$ -incorporation ( $\text{nmol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ ).

**Statistical analysis** Values were expressed as  $\bar{x} \pm s$  and compared by ANOVA followed by the

Duncan's new multiple range method or Newman-Keuls test.

## RESULTS

**Inhibitory effect of DA on CCDPK II activity** After exposure of rat hippocampal slices to exogenous DA at 100  $\mu\text{mol}\cdot\text{L}^{-1}$ , the activity of CCDPK II decreased gradually with incubation time. Much inhibition of CCDPK II activity was observed 20 min after the addition of DA (Fig 1).

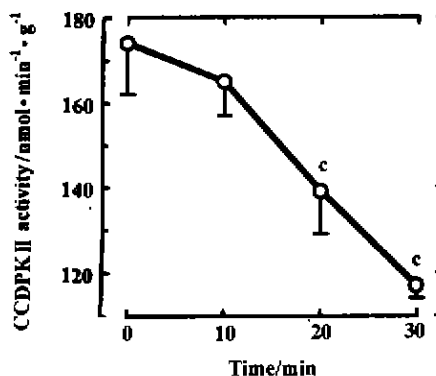


Fig 1. Inhibitory effect of DA (100  $\mu\text{mol}\cdot\text{L}^{-1}$ ) on CCDPK II activity in rat hippocampal slices.  $n = 4$  rats.  $\bar{x} \pm s$ . <sup>c</sup>  $P < 0.01$  vs control.

Incubation with DA at 30 - 2000  $\mu\text{mol}\cdot\text{L}^{-1}$  for 30 min markedly depressed CCDPK II activity. The maximal inhibition was observed with DA at 500  $\mu\text{mol}\cdot\text{L}^{-1}$ , with the enzyme activity falling to 54.4 % of the control. Incubation of slices in calcium-free medium (with 1  $\text{mmol}\cdot\text{L}^{-1}$  egtazic acid) containing DA at the same concentrations antagonized the DA-induced inhibition of CCDPK II activity, partially or completely (Tab 1).

**Effect of DA receptor agonists on CCDPK II activity** Incubation with apomorphine (1 - 20  $\mu\text{mol}\cdot\text{L}^{-1}$ ), a nonselective DA receptor agonist, for 30 min resulted in an inhibition of CCDPK II activity. The maximal inhibition was observed with apomorphine at 10  $\mu\text{mol}\cdot\text{L}^{-1}$ , with the enzyme activity falling to 53.5 % of the control. Incubation for 30 min with SKF38393, a selective  $\text{D}_1$ -like DA receptor agonist, or quinpirole, a selective  $\text{D}_2$ -like DA receptor agonist, reduced the activity of CCDPK II. SKF38393 at 1 - 200  $\mu\text{mol}\cdot\text{L}^{-1}$  and quinpirole at 20 - 200  $\mu\text{mol}\cdot\text{L}^{-1}$

**Tab 1. Inhibitory effect of exogenous DA on CCDPK II activity in rat hippocampal slices with or without extracellular calcium ( $[Ca^{2+}]_o$ ).  $n=6$  rats.  $\bar{x} \pm s$ .  $^aP > 0.05$ ,  $^cP < 0.01$  vs control.  $^fP < 0.01$  vs the respective values obtained with  $[Ca^{2+}]_o$ .**

DA/ $\mu\text{mol} \cdot \text{L}^{-1}$	CCDPK II activity/ $\text{nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ + $[Ca^{2+}]_o$	$\text{nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ - $[Ca^{2+}]_o$
0	169 ± 12	169 ± 11
30	138 ± 11 <sup>c</sup>	179 ± 22 <sup>af</sup>
100	116 ± 5 <sup>c</sup>	148 ± 7 <sup>cf</sup>
500	92 ± 8 <sup>c</sup>	150 ± 6 <sup>cf</sup>
1000	132 ± 9 <sup>c</sup>	161 ± 4 <sup>af</sup>
2000	124 ± 8 <sup>c</sup>	171 ± 16 <sup>af</sup>

resulted in an inhibition of the enzyme activity (Tab 2).

**Tab 2. Effect of apomorphine, SKF38393, and quinpirole on CCDPK II activity in rat hippocampal slices.  $n=6$  rats.  $\bar{x} \pm s$ .  $^cP < 0.01$  vs control.**

Treatment	Concentration/ $\mu\text{mol} \cdot \text{L}^{-1}$	CCDPK II activity/ $\text{nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$
Control	-	170 ± 11
Apomorphine	1	130 ± 11 <sup>c</sup>
	5	110 ± 11 <sup>c</sup>
	10	91 ± 6 <sup>c</sup>
	20	123 ± 7 <sup>c</sup>
SKF38393	1	140 ± 12 <sup>c</sup>
	10	136 ± 10 <sup>c</sup>
	100	123 ± 8 <sup>c</sup>
	200	103 ± 10 <sup>c</sup>
Quinpirole	10	171 ± 17
	20	147 ± 8 <sup>c</sup>
	50	107 ± 9 <sup>c</sup>
	100	111 ± 9 <sup>c</sup>
	200	134 ± 11 <sup>c</sup>

### Effect of DA receptor antagonists on DA-induced inhibition of CCDPK II activity

Preincubation of slices with specific DA receptor antagonists, Sch-23390 ( $D_1$ -like DA receptor antagonist) or domperidone ( $D_2$ -like DA receptor antagonist), antagonized the inhibition of CCDPK II activity induced by exogenous DA ( $500 \mu\text{mol} \cdot \text{L}^{-1}$ ). Sch-23390 at  $50 \mu\text{mol} \cdot \text{L}^{-1}$  and domperidone at  $10 \mu\text{mol} \cdot \text{L}^{-1}$  abolished the DA-induced inhibition of CCDPK II activity (Tab 3).

**Tab 3. Protection of Sch-23390 and domperidone against inhibition of CCDPK II activity induced by exogenous DA in rat hippocampal slices.  $n=6$  rats.  $\bar{x} \pm s$ .  $^cP < 0.01$  vs DA ( $500 \mu\text{mol} \cdot \text{L}^{-1}$ ).  $^dP > 0.05$  vs control.**

Treatment	Concentration/ $\mu\text{mol} \cdot \text{L}^{-1}$	CCDPK II activity/ $\text{nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$
Control	-	169 ± 12
DA	500	92 ± 8
DA + Sch-23390	1	106 ± 15
	5	121 ± 13 <sup>c</sup>
	20	136 ± 6 <sup>c</sup>
	50	163 ± 16 <sup>cd</sup>
DA + domperidone	0.1	114 ± 8 <sup>c</sup>
	1	132 ± 11 <sup>c</sup>
	5	152 ± 5 <sup>c</sup>
	10	169 ± 14 <sup>cd</sup>

### DISCUSSION

For the first time, the present study shows that DA has an inhibitory effect on CCDPK II activity in the hippocampus, depending on both duration of DA exposure and DA concentration. This DA-mediated effects were blocked by removal of extracellular calcium. The result suggests that the inhibitory effect of DA on CCDPK II activity is partially related to influx of extracellular calcium. Maybe there is release of intracellular calcium storage because the inhibitory effects of DA at some concentrations were not antagonized completely.

It has been demonstrated that the inhibition of CCDPK II activity is concerned with calcium-stimulated autophosphorylation of the enzyme at a threonine residue, Thr<sup>266</sup>. The autophosphorylation converts the kinase from the  $Ca^{2+}$ -calmodulin-dependent form to  $Ca^{2+}$ -calmodulin-independent form, which results in the inhibition of  $Ca^{2+}$ -calmodulin-dependent activity and the enhancement of  $Ca^{2+}$ -calmodulin-independent activity. Excessive activated CCDPK II can potentiate the presynaptic release of glutamate by phosphorylating synapsin I<sup>[6]</sup>, but also phosphorylate postsynaptic *N*-methyl-*D*-aspartate (NMDA) receptors or non-NMDA receptors and promote their subsequent responsiveness to released glutamate<sup>[7,8]</sup>. Excessive stimulation of glutamate receptors causes massive calcium influx and results in

neuronal cell damage.

All above results may support our hypothesis that neurotoxic effects of DA would partially due to DA-induced the inhibition of CCDPK II activity, which enhances excitotoxicity of excitory amino acid and increases calcium influx.

Radioligand assay proves the distribution of DA receptors in the hippocampus. Direct dopamine receptor stimulation may mediate inhibitory effect of DA on CCDPK II activity. Apomorphine, a non-selective DA receptor agonist had an inhibitory effect on CCDPK II activity, similar to the effect of DA. The result suggests the effect of DA is mediated by stimulating DA receptors. The DA receptors are now classified as "D<sub>1</sub>-like" (D<sub>1</sub> and D<sub>5</sub>) and "D<sub>2</sub>-like" (D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub>). In order to assess the receptor subtypes mediating DA inhibitory effect, selective DA receptor agonists and antagonists were used. The results suggest stimulation of D<sub>1</sub>-like or D<sub>2</sub>-like receptor subtypes can both result in inhibition of the enzyme activity. The inhibition of CCDPK II activity is calcium-dependent. Therefore, our results demonstrate that stimulation of D<sub>1</sub>-like or D<sub>2</sub>-like receptor subtypes can elevate the intracellular calcium concentration in rat hippocampus. Intracellular calcium accumulation causes autophosphorylation of the enzyme, which makes the kinase convert to Ca<sup>2+</sup>-calmodulin-independent form and results in inhibition of Ca<sup>2+</sup>-calmodulin-dependent activity. However, The mechanism of D<sub>1</sub>-like or D<sub>2</sub>-like receptor mediating intracellular calcium accumulation remains to be elucidate.

At higher concentration, DA, apomorphine, quinpirole-induced inhibitory rates of CCDPK II activity decreased. This may result from activation of Ca<sup>2+</sup>-calmodulin-dependent protein phosphatases, such as calcineurin. Calcineurin is abundant in the hippocampus. Several proteins including synapsin I and NMDA receptors are common to CCDPK II and calcineurin as substrates for phosphorylation and dephosphorylation<sup>[6-10]</sup>. It had been reported that stimulation of D<sub>2</sub>-like receptors can activate calcineurin<sup>[11]</sup>. The activation of calcineurin leads to dephosphorylation of synapsin I and NMDA receptors<sup>[9,10]</sup> and consequent decrease of calcium influx induced by excessive stimulation of glutamate receptors. As a result of D<sub>2</sub>-like receptors stimulation

at higher concentration, the DA-induced inhibitory rates of CCDPK II activity is attenuated. The above results indicated that the inhibition of CCDPK II activity induced by exogenous DA might be mainly due to stimulation of D<sub>1</sub>-like receptors.

In conclusion, DA has an inhibitory effect on CCDPK II activity in rat hippocampus, related to stimulation of D<sub>1</sub>-like or D<sub>2</sub>-like receptor subtypes and calcium influx.

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**目的:** 研究多巴胺(DA)对大鼠海马脑片  $Ca^{2+}$ -钙调素依赖性蛋白激酶 II (CCDPK II)活性的影响。  
**方法:** 采用大鼠海马脑片体外培养模型,以 $^{32}P$ 掺入法测定CCDPK II的活性。  
**结果:** 外源性 DA 可显著降低大鼠海马脑片CCDPK II活性,并有一定的浓度依赖性和时间依赖性。去除胞外的  $Ca^{2+}$  对不同浓度 DA 诱导的CCDPK II 活性抑制有部分或完全保护作用。阿扑吗啡(非特异性 DA 受体激动剂)、SKF38393 (特异性  $D_1$  样 DA 受体激动剂)和哇吡罗(特异性  $D_2$  样 DA 受体激动剂)均可显著降低CCDPK II的活性。Sch-23390 (特异性  $D_1$  样 DA 受体拮抗剂)和多潘立酮(特异性  $D_2$  样 DA 受体拮抗剂)均可拮抗 DA 所诱导的酶活性抑制。  
**结论:** DA 抑制海马CCDPK II 的活性,其作用机制与  $D_1$  样和  $D_2$  样受体以及胞外  $Ca^{2+}$  的内流有关。

**多巴胺诱导大鼠海马脑片  $Ca^{2+}$ -钙调素依赖性蛋白激酶 II 活性的抑制作用<sup>1</sup>**

R972.1

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**关键词** 多巴胺;  $Ca^{2+}$ -钙调素依赖性蛋白激酶; 脑缺血; 钙; 多巴胺激动剂; 多巴胺拮抗剂; 海马

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CCDPK II 药理

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