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Effects of *l*-stepholidine on synaptosomal Ca^{2+}-ATPase and subcellular calmodulin in rat striatum[1]

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ABSTRACT *Our results showed that l-stepholidine (l-SPD) inhibited basal* Ca^{2+}-ATPase activity in rat striatal synaptosomes with an IC_{50} of 31.5 $\mu mol \cdot L^{-1}$, suggesting its interaction with Ca^{2+} transport. *l*-SPD inhibited also calmodulin (CaM)-activated basal Ca^{2+}-ATPase in a concentration-dependent manner. A complete reversal of CaM activation of Ca^{2+}-ATPase was observed with *l*-SPD 10 $\mu mol \cdot L^{-1}$. The activity of synaptosomal Ca^{2+}-ATPase and membrane-bound CaM level were decreased in haloperidol (1 $mg \cdot kg^{-1} \cdot d^{-1}$, ip) and *l*-SPD (5, 10, and 30 $mg \cdot kg^{-1} \cdot d^{-1}$, ip) treated rats for 7 and 14 d, respectively. But the activity of Ca^{2+}-ATPase and membrane CaM level were increased after treatment with the same of doses haloperidol and *l*-SPD for 21 d. During the treatments with haloperidol and *l*-SPD cytosolic and nuclear CaM levels were not altered. These results suggest that *l*-SPD may modulate the release and synthesis of dopamine (DA) and the negative feedback regulation of presynaptic DA receptors by altering Ca^{2+} and CaM regulating processes in the central dopaminergic nervous system.

KEY WORDS berbines; *l*-stepholidine; corpus striatum; synaptosomes; calcium adenosine triphosphatase; calmodulin

l-Stepholidine (*l*-SPD), a novel dopamine (DA) receptor antagonist, inhibits the feedback regulation of presynaptic DA receptors and increases the activity of tyrosine hydroxylase (TH), resulting in the increase of the DA biosynthesis[1]. We have carried out a series of studies of the pharmacological effects of *l*-SPD on the central dopaminergic nervous system[1,2]. However, there are no studies available to show the mechanism of *l*-SPD regulating the presynaptic receptor functions. Owing to the important role of Ca^{2+} / calmodulin (CaM) in the synaptic function[3-5], studies were initiated to investigate the effect of *l*-SPD on the activity of synaptosomal Ca^{2+}-ATPase and subcellular CaM levels in rat striatum. CaM activates Ca^{2+}-ATPase activity in a concentration-dependent manner[4]. TFP is known as an antagonist of CaM and has no effect on the basal Ca^{2+}-ATPase activity[6]. We also investigated the effect of TFP on the basal Ca^{2+}-ATPase activity so as to assay the degree of depleting endogenous CaM in synaptosomes.

MATERIALS AND METHODS

Drugs and reagents *l*-SPD was isolated from *Stephania intermedia* Lo, $[\alpha]_D = -440°$ in pyridine; haloperidol (Hal, Shanghai First Pharmaceutical Factory, China); trifluoperazine (TFP) and Na_2ATP (Sigma Chemical Co, USA); dithiothreitol (DTT, Serva Feinbio- chemical, Heidelberg). The sample of CaM (isolated from bovine brain) and CaM assay kit were purchased from the Department of Biochemistry, Xuzhou Medical College. All chemicals were AR.

Rats Sprague-Dawley ♀ rats weighing 227 ± *s* 18 g were used.

Preparation of synaptosomes The striatum were immediately taken into ice-cold homogenizing medium (sucrose 0.32 $mol \cdot L^{-1}$ and imidazole-HCl 10 $mmol \cdot L^{-1}$, pH 7.45) after decapitation of rat, each preparation was pooled from 10 rats. Synaptosomes were prepared using a slightly modified procedure of Gordon-Weeks[7]. The tissue was homogenized in a 9 volumes of homogenizing medium. The homogenate was centrifuged at 750 × *g* at 4℃ for 10 min. The supernatant was centrifuged at 17 000 × *g* at 4℃ for 20 min and the pellet was suspended in homogenizing

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medium and again centrifuged at 17 000 × *g* at 4℃ for 20 min. The pellet was then resuspended in 10 ml of sucrose 0.32 mol · L^{-1} and carefully layered on a two-step discontinuous Ficoll-sucrose gradient, consisting of 13 and 7.5% (wt / vol) Ficoll in sucrose 0.32 mol · L^{-1}. After centrifugation at 65 000 × *g* at 4℃ for 45 min, the synaptosomal fractions were obtained at the interface of the 7.5–13% Ficoll-sucrose layer. The synaptosome band was carefully removed, diluted with 9 volumes of sucrose solution and centrifuged at 17 000 × *g* at 4℃ for 30 min. The synaptosomal pellet was suspended in Tris-HCl 10 mmol · L^{-1} (pH 7.45) buffer containing EGTA 1 mmol · L^{-1} and centrifuged at 105 000 × *g* for 60 min to deplete the endogenous CaM[8]. The process was repeated once again with Tris-HCl 10 mmol · L^{-1} (pH 7.45) buffer containing EGTA 0.1 mmol · L^{-1}. After centrifugation the synaptosomal fraction was suspended in Tris-HCl 10 mmol · L^{-1} (pH 7.45), rapidly divided into small aliquots and stored at −25℃.

For *in vivo* studies, rats were divided into 3 groups and treated with saline, *l*-SPD, and Hal, respectively. After the rats were killed, the striatum were used for the preparations of the synaptosome and the subcellular fractions[9], which were used for the estimations of Ca^{2+}-ATPase activity and CaM levels, respectively.

Ca^{2+}-ATPase assay Ca^{2+}-Mg^{2+}-ATPase activity was determined by measuring the inorganic phosphate (P_i) liberated during the hydrolysis of ATP[10]. The reaction medium contained imidazole-HCl buffer 135 mmol · L^{-1} (pH 7.45), $MgCl_2$ 5 mmol · L^{-1}, $CaCl_2$ 0.05 mmol · L^{-1}, ATP 4 mmol · L^{-1}, and synaptosomal protein 50–100 μg. The mixture was incubated at 37℃ for 30 min and stopped by the addition of 0.1 ml of 50% trichloroacetic acid (TCA). The P_i liberated was estimated by the colorimetric method[10]. Mg^{2+}-ATPase activity was measured in the presence of EGTA 0.5 mmol · L^{-1} and this value was subtracted from total ATPase activity to get Ca^{2+}-ATPase activity. Enzyme activity was expressed as P_i μmol · mg^{-1} · h^{-1}.

For assessing the effect of *l*-SPD on basal Ca^{2+}-ATPase activity, the synaptosomes were preincubated with *l*-SPD for 2 min prior to the reaction with ATP. For assessing the effect of *l*-SPD on CaM-activated Ca^{2+}-ATPase, the synaptosomes were preincubated for 2 min with CaM, and then *l*-SPD was added.

Calmodulin assay The total homogenate and subcellular fractions were suspended in imidazole-HCl buffer (pH 7.45) 10 mmol · L^{-1} containing DTT 3 mmol · L^{-1}, $MgCl_2$ 10 mmol · L^{-1}, and NaCl 0.15 mmol · L^{-1}. The protein concentrations of all fractions were diluted into 0.5–1.0 mg · ml^{-1}. Each fraction was heated at 95–100℃ for 5 min and centrifuged at 5000 × *g* for 15 min. The clear supernatant was used for the determination of CaM using the enzyme-linked immunoassay method[12].

Protein assay Proteins of total homogenate, synaptosome and subcellular fraction were determined with Folin phenol reagent[13]. Bovine serum allumin was used as standard.

RESULTS

Effect of *l*-SPD on basal Ca^{2+}-ATPase activity *l*-SPD inhibited the basal Ca^{2+}-ATPase activity with IC_{50} of 31.5 μmol · L^{-1}. The inhibition was concentration-dependent and was significant at 10 μmol · L^{-1} or higher (Fig 1). TFP failed to inhibit the

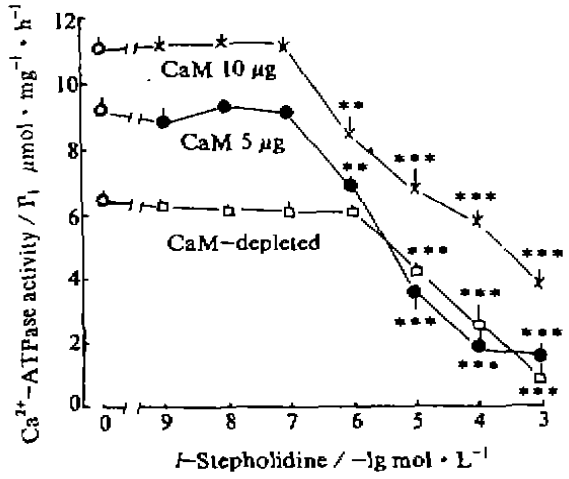

Fig 1. Effects of *l*-SPD on calmodulin-depleted or -activated Ca^{2+}-ATPase activities from rat striatal synaptosomes. (○) control. $n=3$ homogenates (each was pooled from 10 rats and assayed in triplicate). $\bar{x} \pm s$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* corresponding control.

basal Ca^{2+}–ATPase activity (6.50± 0.23 P_i $\mu mol \cdot mg^{-1} \cdot h^{-1}$) at the concentrations of 12.5 (5.95± 0.43 P_i $\mu mol \cdot mg^{-1} \cdot h^{-1}$) and 25 $\mu mol \cdot L^{-1}$ (5.95± 0.35 P_i $\mu mol \cdot mg^{-1} \cdot h^{-1}$), suggesting the endogenous CaM in synaptosomes was well depleted.

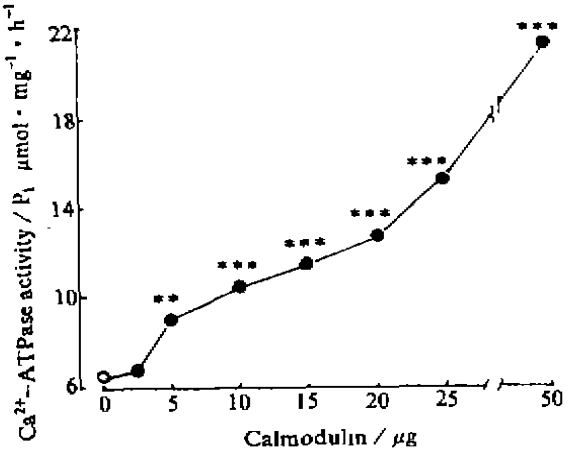

Fig 2. Activation of calmodulin on CaM–depleted Ca^{2+}–ATPase from rat striatal synaptosomes. (○) control. $n=3$ homogenates (each was pooled from 10 rats and assayed in triplicate). $\bar{x} \pm s$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* control.

Effect of *l*–SPD on CaM–activated basal Ca^{2+}–ATPase activity The Ca^{2+}–ATPase activity was assayed by incubating the synaptosomes with CaM 2.5–50 μg. CaM activated the synaptosomal Ca^{2+}–ATPase in a concentration–dependent manner and an elevation of 100% was seen with CaM15 μg (Fig 2). *l*–SPD decreased the CaM–activated Ca^{2+}–ATPase in a concentration–dependent manner. A complete reversal of CaM–activation was observed with *l*–SPD 10 $\mu mol \cdot L^{-1}$. *l*–SPD 1 $\mu mol \cdot L^{-1}$, having no significant effect on basal Ca^{2+}–ATPase. decreased the Ca^{2+}–ATPase activities activated by CaM 5, and 10 μg by 75% and 44%, respectively. (Fig 1). TFP 12.5 and 25 $\mu mol \cdot L^{-1}$ inhibited the activation of CaM on basal Ca^{2+}–ATPase (Tab 1).

Tab 1. Effect of trifluoperazine on CaM–activated Ca^{2+}–ATPase from rat striatal synaptosomes. $n=3$ homogenates (each was pooled from 10 rats and assayed in triplicate). $\bar{x} \pm s$, $^{***}P<0.01$ *vs* control. $^{+++}P<0.01$ *vs* corresponding CaM.

TFP / $\mu mol \cdot L^{-1}$	CaM / μg	Ca^{2+}–ATPase activity / P_i $\mu mol \cdot mg^{-1} \cdot h^{-1}$
0	0	6.25 ± 0.21
0	5	9.05 ± 0.18***
12.5	5	6.83 ± 0.10+++
25.0	5	6.36 ± 0.11+++
0	10	11.40 ± 0.30***
12.5	10	8.85 ± 0.10+++
25.0	10	8.05 ± 0.31+++

Effects of *l*–SPD and Hal on subcellular CaM and Ca^{2+}–ATPase in rats The subcellular CaM levels and synaptosomal Ca^{2+}–ATPase activity in the rats treated with *l*–SPD and Hal for 1, 24, and 72 h had no changes. Ca^{2+}–ATPase activity in synaptosomes were inhibited after the rats were treated with *l*–SPD (5, 10, and 30 $mg \cdot kg^{-1} \cdot d^{-1}$, ip) and Hal (1 $mg \cdot kg^{-1} \cdot d^{-1}$, ip) for 7 and 14 d (Tab 2). Meanwhile, all the CaM levels of the total and membrane fractions in rats treated with *l*–SPD and Hal were decreased (Tab 3). On the contrary, when the rats were treated with the same doses of *l*–SPD and Hal for 21 d, the synaptosomal Ca^{2+}–ATPase activities and CaM levels of membrane fractions were

Tab 2. Effects of *l*–SPD and haloperidol (Hal) on activity (P_i $\mu mol \cdot mg^{-1} \cdot h^{-1}$) of synaptosomal Ca^{2+}–ATPase in striatum in rats 4 d after withdrawal. $n=5$, $\bar{x} \pm s$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* control.

Drugs / $mg \cdot kg^{-1} \cdot d^{-1}$		Days 7	14	21
l–SPD	0	8.84 ± 0.31	9.03 ± 0.21	8.44 ± 0.45
	5	6.03 ± 0.72**	6.92 ± 0.51***	9.59 ± 0.66**
	10	5.91 ± 0.19***	5.91 ± 0.3***	10.0 ± 0.7**
	30	4.66 ± 0.17***	5.24 ± 0.42***	10.6 ± 0.7**
Hal	1	6.63 ± 0.79**	6.22 ± 0.46***	11.3 ± 0.4***

Tab 3. Effect of chronic treatment with *l*-SPD and haloperidol (Hal) on striatal subcellular calmodulin contents ($\mu g \cdot mg^{-1}$) in rat 4 d after withdrawal. n = 3 homogenates (each was pooled from 10 rats and assayed in triplicate). $\bar{x} \pm s$. $^{**}P<0.05$, $^{***}P<0.01$ *vs* corresponding control.

Days	Drug / mg · kg^{-1}		Total	Membrane-bound	Cytosol	Nucleus
7	Control		5.5 ± 0.8	8.3 ± 0.6	9.2 ± 1.1	3.3 ± 0.3
	l-SPD	5	3.7 ± 0.7**	4.4 ± 0.4***	10.8 ± 1.1	3.5 ± 0.8
		10	2.6 ± 0.4***	4.1 ± 0.4***	10.4 ± 0.6	3.3 ± 0.5
		30	2.6 ± 0.5***	3.8 ± 0.3***	10.1 ± 2.1	3.5 ± 0.8
	Hal	1	2.2 ± 0.5***	2.70 ± 0.24***	10.2 ± 1.5	3.8 ± 0.6
14	Control		4.8 ± 0.4	8.96 ± 0.22	8.8 ± 0.8	3.7 ± 0.3
	l-SPD	5	3.15 ± 0.23**	2.49 ± 0.17***	8.8 ± 1.9	3.9 ± 0.4
		10	2.18 ± 0.16***	1.38 ± 0.22***	8.9 ± 1.3	3.8 ± 0.6
		30	2.18 ± 0.16***	1.19 ± 0.07***	8.8 ± 1.0	3.5 ± 0.3
	Hal	1	3.1 ± 0.4**	3.03 ± 0.18***	9.1 ± 1.8	3.9 ± 0.4
21	Control		6.0 ± 0.6	7.7 ± 0.4	9.2 ± 0.5	4.1 ± 0.5
	l-SPD	5	6.5 ± 0.8	9.7 ± 0.6**	9.4 ± 1.6	4.05 ± 0.24
		10	6.0 ± 0.8	10.1 ± 0.9**	9.6 ± 0.5	3.9 ± 0.8
		30	6.4 ± 1.4	10.1 ± 0.7**	9.4 ± 1.4	4.2 ± 1.0
	Hal	1	6.4 ± 0.6	11.6 ± 0.7***	9.6 ± 1.9	3.9 ± 0.4

increased dramatically. CaM levels of the total homogenate were restored to normal levels (Tab 3). During all the treatments with *l*-SPD and Hal, cytosolic and nuclear CaM levels were not altered.

DISCUSSION

Synaptic membrane Ca^{2+}-ATPase is involved in the translocation of Ca^{2+} from intracellular to extracellular media. The results presented in this paper show that *l*-SPD is an inhibitor of synaptosomal Ca^{2+}-ATPase from rat striatum. The inhibition of Ca^{2+}-ATPase activity by *l*-SPD observed in the present study suggested its interaction with the calcium transport, which might affect other calcium dependent processes involved in neuronal functions. Ca^{2+}-ATPase is a trigger enzyme of CaM, which represents the activity of CaM[6]. Our results show that the inhibition of *l*-SPD on CaM-Ca^{2+}-ATPase activity was much more sensitive than on basal Ca^{2+}-ATPase. It was also noted that the inhibition of *l*-SPD on Ca^{2+}-ATPase was different from TFP which is known as a CaM antagonist and has no effect on basal Ca^{2+}-ATPase[6].

In brain two forms of CaM appear to be present[4]: a membrane-bound form and the other soluble form. The former functions with AC whereas the latter is required for phosphodiesterase (PDE)[4,14]. *In vivo* studies revealed that CaM levels in the membrane fractions were reduced in the striatum of the rats treated with *l*-SPD for 7 and 14 d, while nuclear and cytosolic CaM levels were not altered. This suggests that *l*-SPD may be altering the level of membrane-bound CaM. These results indicate that the *l*-SPD may regulate dopaminergic function by acting on AC rather than on PDE. It is supposed that *l*-SPD decrease membrane-bound CaM levels and subsequently attenuates the activation of DA on AC, resulting in the reduction of cAMP formation and affecting the physiological and biochemical responses mediated by cAMP-dependent protein kinase. The inhibition of *l*-SPD on the feedback regulation of presynaptic DA receptors may be also related to the above results. Because of the attenua-

tion of AC activation resulted from the reduction of membrane-bound CaM levels, *l*-SPD might in turn affect the phosphorylation of TH mediated by both cAMP-dependent protein kinase and Ca^{2+}/CaM-dependent protein kinase, altering the activity of TH and therefore affecting the biosynthis of DA. In addition, *l*-SPD may regulate the release of DA by altering the phosphorylation of the protein of synaptic membrane and vesicle.

The subcellular membrane-bound CaM levels of striatum in the rats treated with Hal (1 mg · kg^{-1} · d^{-1}, ip) for 21 d were increased significantly, which is considered as a biochemical index of supersensitivity of DA receptors[4,14]. The increase of membrane-bound CaM levels in the rats treated with *l*-SPD for 21 d may result from the supersensitivity of DA receptors induced by chronic treatment with *l*-SPD. This was further strengthened by the fact that the Ca^{2+}-ATPase activity was increased by chronic *l*-SPD treatment.

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左旋千金藤立定对大鼠纹状体突触体 Ca^{2+}-ATP 酶和亚细胞钙调素的影响

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提要 *l*-SPD 以浓度依赖的方式抑制大鼠纹状体 Ca^{2+}-ATP 酶活性和 CaM 对 Ca^{2+}-ATP 酶的激活. *l*-SPD (5, 10 和 30 mg · kg^{-1} · d^{-1})和 Hal (1 mg · kg^{-1} · d^{-1}) ip 7 和 14 d, 显著抑制 Ca^{2+}-ATP 酶活性, 降低膜结合型 CaM 的含量; 而相同剂量的 *l*-SPD 和 Hal ip 21 d, 却显著增加 Ca^{2+}-ATP 酶活性和膜结合 CaM 含量. 提示 *l*-SPD 可能通过对 Ca^{2+}-ATP 酶和 CaM 的影响而调节突触前 DA 受体的功能.

关键词 小檗因类; 左旋千金藤立定; 纹状体; 突触体; 钙腺苷三磷酸酶; 钙调素 S7D