目的：用大鼠脑对 ω-conotoxin (ω-CTX)及氨氯地平与N型钙通道的内在关系进行分析. **方法**：将大鼠全脑匀浆于 HEPES 50 mmol·L^{-1}(pH 7.4)缓冲液中，经40 000 × *g* 离心后，收集膜区域. 以 ^{125}I-ω-conotoxin (CTX)作为放射配体测定. **结果**：^{125}I-ω-CTX与冷冻标本及新鲜标本结合的 B_{max} 无区别. N型钙通道的 K_d 和 B_{max} 值分为 0.02 ± 0.01 mmol·L^{-1}和 1029 ± 108 pmol/g 蛋白质. ω-CTX 及氨氯地平的 pK_i 值分别为 9.57 以及 < 4，普萘洛尔、哌唑嗪、阿托品、组胺的 pK_i 值也非常低. **结论**：L型钙离子拮抗剂氨氯地平与N离子通道的亲和性很低.

BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1998 Mar; 19 (2): 100 - 103

Enhancement of (−)-stepholidine on protein phosphorylation of a dopamine- and cAMP-regulated phosphoprotein in denervated striatum of oxidopamine-lesioned rats[1]

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KEY WORDS stepholidine; phosphoproteins; phosphorylation; dopamine D_1 receptors; oxidopamine; corpus striatum; SK&F-38393; Sch-23390

AIM: To study effects of (−)-stepholidine (SPD) on the phosphorylation of a dopamine- and cAMP-regulated phosphoprotein (DARPP-32) in the striatum of oxidopamine-lesioned rats. **METHODS**: The amount of dephospho-DARPP-32 was measured by a back-phosphorylation assay. **RESULTS**: In the striatum of control rats, SPD *per se* had no effect on the phosphorylation of DARPP-32, but it antagonized the decrease by 28 % of dephospho-DARPP-32 induced by the D_1 agonist SK&F-38393. In the denervated striatum of oxidopamine-lesioned rats, SPD decreased the amount of dephospho-DARPP-32 by 44 %. The effect of SPD was completely counteracted by the concomitant administration of the D_1 antagonist Sch-23390. **CONCLUSION**: SPD exhibits D_1 agonistic action on DARPP-32 phosphorylation in the denervated striatum of oxidopamine-lesioned rats, but it acts as a D_1 antagonist in normal striatum.

(−)-Stepholidine (SPD), an alkaloid isolated from Chinese herb *Stephania intermedia* Lo, is a tetrahydroprotoberberine. SPD has high affinities for both dopamine (DA) D_1 and D_2 receptors with a preference for D_1 receptors, and low affinities for non-DA receptors[1]. SPD possesses the characteristics of a D_2 antagonist[2, 3].

As for D_1 action of SPD, previous studies reported controversial observations. In rats with 6-d reserpine treatment, SPD reduced D_1 agonist SK&F-38393-induced inhibition of firing activity of nigral DA cells although SPD *per se* had no action, indicating a D_1 antagonistic action[4]. In rats with unilateral nigral lesions by oxidopamine, SPD induced a contralateral rotation in the manner similar to SK&F-38393, indicating a D_1 agonistic action[5]. SPD bound to high and low affinity sites (R_H and R_L) of D_1 receptors and the R_H could be regulated by GTP, indicating an intrinsic activity to D_1 receptors[6]. After blockade of D_2 receptors, SPD stimulated striatal cAMP formation[7]. In nigral lesioned rats, SPD induced a firing inhibition of substantia nigra pars reticular

[1] Project supported by the National Natural Science Foundation of China, No 39130091, 39600179; the Foundation of State Key Laboratory of Nuclear Medicine, No 9403; and the Foundation of State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Science, No K016.
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Received 1997-04-23 Accepted 1997-11-27

neurons as SK&F-38393 did, but partially reduced SK&F-38393-induced firing inhibition[8]. Based on these observations, a D_1 partial agonistic action of SPD is proposed.

There existed a DA- and cAMP-regulated phosphoprotein (DARPP-32, $m = 32$ kDa) in striatal neurons containing D_1 receptors. DA activated adenyl cyclase (AC) through D_1 receptors. The increased cAMP level stimulated the activity of cAMP-dependent protein kinase (PKA) which phosphorylated DARPP-32. Phospho-DARPP-32 represented a positive feedback signal through which some of the actions of DA might be amplified[9]. The pharmacological modulation of DARPP-32 was detected *in vivo*. The phosphorylation state of DARPP-32 was increased by *in vivo* administration of D_1 receptor agonists, and their effects were counteracted by the concomitant administration of D_1 (but not of D_2) antagonists[10].

To elucidate the action of SPD on D_1 receptors, we evaluated effects of SPD on DARPP-32 phosphorylation in striatum of drug-naïve and oxidopamine-lesioned rats.

MATERIALS AND METHODS

Chemicals and reagents SPD (Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China) was dissolved in H_2SO_4 0.1 mmol·L^{-1}, then diluted and adjusted to pH 5 with NaOH 0.1 mmol·L^{-1}. Apomorphine (Shenyang Pharmaceutical Co, China). SK&F-38393, Sch-23390 and oxidopamine-HCl (RBI, USA). Phenyl methyl sulphonyl fluoride (PMSF), Pepstatin and PKA (Sigma, USA). [γ-^{32}P]ATP (185 PBq·mol^{-1}, Beijing Yahui Biomedical Co, China).

Rats and pretreatment Sprague-Dawley rats (♂, 180 ± s 29 g, Shanghai Experimental Animal Center, Shanghai. Certification № 005 conferred by Animal Management Committee, Chinese Academy of Sciences) were used. Rats were anesthetized with pentobarbital (40 mg·kg^{-1}, ip), and injected into unilateral medial forebrain bundle (MFB) with the saline solution (4 μL) containing oxidopamine-HCl 9.7 μg and ascorbic acid 1 μg. After 1 – 4 weeks, lesioned rats were screened in a bowl and the turns in a given time were rec orded. Only the rats showing contralateral rotation at a speed of > 5 turns ·min^{-1} in response to apomorphine (0.2 mg·kg^{-1}, ip) and SPD (4 mg·kg^{-1}, ip) were used.

Preparation of the striatal protein extracts Rats were decapitated 30 min after the intraperitoneal injection of SPD and other DA agents. Striata were dissected and rapidly homogenized in ice-cold Tris-HCl buffer (pH 7.4) 10 mmol·L^{-1} containing edetic acid 2 mmol·L^{-1}, PMSF 0.1 mmol·L^{-1} using ten strokes in a teflon-glass Potter homogenizer.

DARPP-32 was extracted[11]. Immediately after homogenization, proteins were precipitated by adding homogenate 200 μL to 5 mL of ice-cold zinc acetate 5 mmol·L^{-1} and spun at 4000 × g for 15 min. The pellet was resuspended in citric acid 1 mL (pH 2.8) 10 mmol·L^{-1} containing 0.1 % Triton X-100 and pepstatin A 2 μg. After centrifugation at 28 000 × g for 15 min, the supernatant was adjusted to pH 6.5 with Na_2HPO_4 0.5 mol·L^{-1} and left on ice for 10 min. After centrifugation at 16 000 × g for 15 min, the final supernatant was kept on ice. Protein concentration was measured[12].

Back-phosphorylation assay Phosphorylation was carried out at 30 ℃ for 60 min in a final volume of 100 μL containing HEPES (pH 7.4) 50 mmol·L^{-1}, $MgCl_2$ 10 mmol·L^{-1}, egtazic acid 1 mmol·L^{-1}, edetic acid 1 mmol·L^{-1}, catalytic subunit of PKA 10 nmol·L^{-1}, [γ-^{32}P]ATP 5 nmol·L^{-1}, and striatal proteins 40 μg. Phosphorylation was started by the addition of [γ-^{32}P]ATP. The reaction was stopped by adding the stopping solution 100 μL containing 3 % sodium dodecylsulphate (SDS), 5 % 2-mercaptoethanol, 10 % glycerol, and 0.002 % bromophenol blue in Tris-HCl (pH 6.8) 0.12 mmol·L^{-1}.

Samples were boiled for 2 min and then subjected to one-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) using 10 % acrylamide and 0.3 % bis-acrylamide in the resolving gel. Electrophoresis was carried out at 60 V for 15 min, then 120 V until the dye reached the bottom of the gel. Resulting gels were stained with Coomassie blue, destained, and dried. The radioactivity retained on the gel was visualized by phosphoimage analysis (Bio-Rad GS250 Molecular Imaging System, USA). The amount of [γ-^{32}P]phosphate incorporated in the 32 kDa protein band was determined using Phosphor Analyst Software. Data were expressed as percentage of incorporation compared to the saline control.

RESULTS

DARPP-32 phosphorylation in the striatum of normal rats SK&F-38393 (3 mg·kg^{-1}) lowered [^{32}P]phosphate incorporated in the 32 kDa protein band to 72 % of the saline group, showing that the amount of dephospho-DARPP-32 was decreased by 28 %. The simultaneous injection of Sch-23390 (0.01 mg·kg^{-1}) antagonized this effect of SK&F-38393. These results were in line with previous reports[10].

After the injection of SPD (4, 10, and 20 mg·kg^{-1}), ^{32}P incorporation was not changed. When

rats were treated with SK&F-38393 and SPD (4 $mg \cdot kg^{-1}$) concomitantly, SPD counteracted the decrease in ^{32}P incorporation induced by SK&F-38393 (Tab 1). This indicated that the D_1 action of SPD was similar to the D_1 antagonist Sch-23390.

Tab 1. Incorporation of [^{32}P]phosphate in DARPP-32 in the striatum of normal rats and the denervated striatum of oxidopamine-lesioned rats. $n = 3$ (each was pooled from 2 rats), $\bar{x} \pm s$.

Group	Normal striatum/%	Denervated striatum/%
Saline	100	100
SK&F-38393	72 ± 9	44 ± 6
SK&F-38393 + Sch-23390	101 ± 11	104 ± 18
SPD	97 ± 7	56 ± 14
SK&F-38393 + SPD	94 ± 15	
SPD + Sch-23390		104 ± 14

DARPP-32 phosphorylation in the denervated striatum of oxidopamine-lesioned rats

In the denervated striatum, SK&F-38393 (3 $mg \cdot kg^{-1}$) decreased [^{32}P]phosphate incorporated in the 32 kDa band to 44 % of the saline group, and Sch-23390 (0.01 $mg \cdot kg^{-1}$) counteracted the effect of SK&F-38393. The amplitude of ^{32}P incorporation change induced by SK&F-38393 was much larger than that in normal rats. It suggested the supersensitivity of DA receptors after the oxidopamine lesion.

In the denervated striatum, SPD (4 $mg \cdot kg^{-1}$) decreased [^{32}P]phosphate incorporated in the 32 kDa protein band to 56 % of the saline group. This reduction reflected the decrease of dephospho-DARPP-32 available *in vitro* for the back-phosphorylation and conversely indicated the increase of phospho-DARPP-32 *in vivo*. The simultaneous treatment with Sch-23390 counteracted the effect induced by SPD (Tab 1, Fig 1). These results indicated that after the oxidopamine lesion, the D_1 action of SPD was similar to the D_1 agonist SK&F-38393.

DISCUSSION

Previous binding assay and AC activity assay have demonstrated that SPD possessed the characteristics of a D_1 agonist[7-8]. The present results found that SPD increased the phosphorylation of DARPP-32 in the denervated striatum of the oxidopamine-lesioned rats. DARPP-32 has been proposed as a molecular marker of dopaminoceptive cells possessing D_1 receptors. The phosphorylation of DARPP-32 appears to be specificfor a D_1 agonist. Thus, the present results indicates that SPD acts as a D_1 agonist in the denervated striatum.

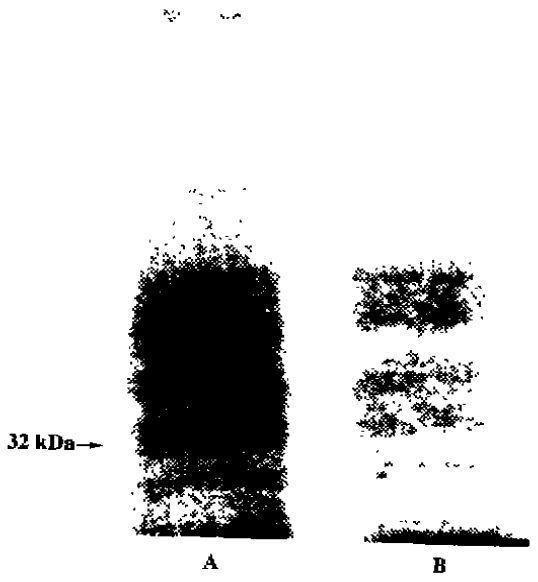

Fig 1. SDS-PAGE and phosphoimage of back-phosphorylation of proteins in acid extracts. SPD reduced [^{32}P]phosphate incorporated in the 32 kDa protein band of denervated striatum. Lane (a) saline; (b) SPD 4 $mg \cdot kg^{-1}$.

In the intact striatum, however, SPD antagonized the effect of SK&F-38393 on the phosphorylation of DARPP-32 while it *per se* had no effect, indicating a D_1 antagonist action. This is possibly due to the low intrinsic activity of SPD[8] and the existence of endogenous DA. The oxidopamine lesion upregulates the signal transduction mechanisms of D_1 receptors[13], increases the sensitivity of D_1 receptor to stimulation. Meanwhile, the endogenous DA is depleted by more than 90 %. Therefore, SPD shows the D_1 agonist action only after the lesion.

In summary, the agonistic action of SPD on DARPP-32 phosphorylation can be exhibited on supersensitive D_1 receptors in the denervated striatum, while SPD shows an antagonistic action on normal D_1 receptors.

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左旋千金藤立定增强大鼠损毁侧纹状体 DARPP-32 蛋白磷酸化的作用[1]

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关键词 千金藤立定; 磷蛋白; 磷酸化; 多巴胺 D_1 受体; 羟多巴胺; 纹状体; SK&F-38393; Sch-23390 侧纹状体

目的: 研究左旋千金藤立定(SPD)对羟多巴胺损毁大鼠纹状体中 DARPP-32 蛋白磷酸化程度的影响. **方法**: 反磷酸化测定脱磷 DARPP-32 的含量. **结果**: SPD 不改变正常大鼠纹状体中 DARPP-32 磷酸化的程度, 但能拮抗 D_1 激动剂的作用; 对羟多巴胺损毁大鼠的损侧纹状体, SPD 使脱磷 DARPP-32 的含量降低 44 %, 给予 D_1 拮抗剂可以拮抗这一作用. **结论**: 在损侧纹状体, SPD 显示 D_1 激动剂的作用特性, 增加 DARPP-32 蛋白的磷酸化, 而在正常纹状体, SPD 表现为 D_1 拮抗剂.