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Pharmacology, Biochemistry and Behavior 75 (2003) 749–754
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
www.elsevier.com/locate/pharmbiochembeh

The effect of calcineurin activator, extracted from Chinese herbal medicine, on memory and immunity in mice

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Received 4 November 2002; received in revised form 5 February 2003; accepted 5 May 2003

Abstract

Calcineurin (CN) is a highly abundant phosphatase in the brain and it is the only Ca^{2+}- and calmodulin-dependent protein serine/threonine phosphatase. There is considerable evidence to suggest that CN plays an essential role in activity-dependent modulation of synaptic efficacy. It has been shown recently that inhibitors of CN, such as CsA or FK506, impair memory formation in day-old chicks. In our present study, extract of *Fructus cannabis* (EFC) with activation of CN, extracted from Chinese traditional medicine, was used to determine the effects on memory and immunity. In the step-down-type passive avoidance test, the plant extract (0.2 g/kg) significantly improved amnesia induced by chemical drugs in mice, and greatly enhanced the ability of cell-mediated type hypersensitivity and nonspecific immune responses in normal mice. The present study provided pharmacological evidence for Chinese herbal medicine screening from molecular model.

Keywords: Calcineurin; Activate; EFC; Step-down-type passive avoidance; Clearance of charcoal particles; Delayed-type hypersensitivity

1. Introduction

Protein phosphorylation and dephosphorylation are major mechanisms for controlling the activities of enzymes and other proteins (Rusnak and Mertz, 2000), and they are involved in regulating many life processes, such as memory formation and immune responses. The phosphorylation of kinases, such as the cAMP-dependent protein kinase A (PKA), the protein kinase C (PKC), the Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) and the mitogen-activated protein kinase (MAPK), is critical for many forms of synaptic plasticity and for learning and memory (Abel et al., 1998; Kornhauser and Greenberg, 1997; Lee et al., 2000; Lisman, 1994). Although many studies have focused on the kinases in synaptic plasticity, relatively few reports have been conducted concerning phosphatases. But phosphorylation is a reversible process, and phosphatases are also essential enzymes to memory formation. Current neural network models of learning and memory formation also support that bidirectional modifications of synaptic plasticity is particularly essential to store information more effectively (Linden, 1994; Sejnowski, 1991).

Calcineurin (CN) is a highly abundant phosphatase in the brain and it is the only Ca^{2+}- and calmodulin-dependent protein serine/threonine phosphatase. CN has a narrower range of substrates than those of other phosphatases. CN dephosphorylates some important neuronal substrates, including cytoskeletal proteins such as tau (Wei et al., 2002; Kayyali et al., 1997), *N*-methyl-*D*-aspartate (NMDA) channel constituents (Lieberman and Mody, 1994) and the PKC substrates such as neurogranin and GAP-43 (neuromodulin) (Liu and Storm, 1989; Schrama et al., 1989; Seki et al., 1995). CN also dephosphorylates inhibitor 1 and DARPP32 (dopamine and cAMP-regulated phosphoprotein) (Hemmings and Greengard, 1986).

The studies on biological roles of CN have progressed to the important discovery that it is the common target of the immunosuppressant drugs cyclosporin A (CsA) and FK506 (Liu et al., 1991). They are pharmacological reagents that have been used to demonstrate CN as a major player in Ca^{2+}-dependent eukaryotic signal transduction pathways. During T cell activation, upon binding of antigen to T cell receptor (TCR), intracellular Ca^{2+} is elevated through the action of protein tyrosine kinases and phospholipase C. CN is activated through binding of Ca^{2+}/CaM and dephosphor-

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doi:10.1016/S0091-3057(03)00148-5

ylate the cytosolic forms of the NF-ATc transcription factors. The dephosphorylated NF-ATc proteins translocate to the nucleus, where they bind, either alone or in cooperation with AP-1 family members, to specific *cis* elements in the promoter/enhancer regions of cytokine genes, such as IL-2. This pathway makes an important contribution to the induction of IL-2 gene transcription, a hallmark of T cell activation (Crabtree and Clipstone, 1994). CsA and FK506 inhibit CN activity after forming complexes with cytoplasmic immunophilins, cyclophilins and FKBP12, respectively (Kissinger et al., 1995; Mulkey et al., 1994; Clipstone et al., 1994; Trushin et al., 1994). These immunophilin–immunosuppressant complexes bind CN and inhibit its function by sterically hindering the access of substrates to the catalytic site (Luo et al., 1996; Griffith et al., 1995). The inhibition of CN is related to the adverse side effects of CsA and FK506.

In addition, CsA and FK506 are powerful probes in the research of CN's role in brain. Using CsA or FK506, most physiological studies including those conducted in our laboratories, show long-term potentiation (LTP) and long-term depression (LTD) are surveyed in hippocampal slices (Luo and Wei, 1998; Luo et al., 2002). But convincing pharmacobehavioural evidence for a role of CN in learning and memory formation is rather sparse. Up to now, there are few reports on single-trial, passive avoidance task in day-old chicks that have applied either CsA or FK506 as antagonists of CN. These reports indicate that the intracranial administration of CsA and FK506 disrupts memory formation. The effects were dose-dependent, most prominent when administered between 10 min before and 40 min after learning, and did not reach significance until at least 85 min posttraining whether 5 nm CsA was given immediately or 30 min posttraining, or 50 nm CsA was given immediately after training. Importantly, the memory impairment induced by CsA persisted for at least 24 h posttraining and was not apparent in chicks trained on 1 day with the drug administered 2 h prior to test on the second day. Hence, it is speculated that CsA selectively and permanently disrupts a relatively late stage of memory formation in the chick, possibly at least partially through inhibition of CN activity (Bennett et al., 1996; Bennett and Schmidt, 2002).

Except for some metal ions, such as Ca^{2+}, Mg^{2+} and Zn^{2+}, relatively little attention has been paid to the activator of CN. Based on the characters of CN, an effective molecular screening model was established in our laboratory (Yan and Wei, 1999). Using CN as a target enzyme, it is possible to determine its activity and its interaction with drugs. We then tested a number of traditional Chinese medicines (Yan et al., 2000), including *Fructus cannabis*.

F. cannabis is the seed of *Cannabis sativa* L. A number of major components have been identified in it, such as fat oil, lignanamide, campesterol stigmasterol, cannabinoid liganol, aminopeptidase, etc. It is a traditional remedy used to treat geriatric constipation. *F. cannabis* is reported to have a broad range of therapeutic effects including antitumor, antihypertensive and antihyperlipidaemic. In our laboratory, we extracted a component of *F. cannabis* that activates CN. The effects of this extract of *F. cannabis* (EFC) on memory were investigated in one-trial passive avoidance step-down test in mice. Scopolamine, sodium nitrite and ethanol were chosen to inhibit memory. The muscarinolytic scopolamine only affects learning (Zhang and Saito, 1986). Sodium nitrite, which can induce anoxia in the brain, only affects retention (Kumar et al., 2000). The central nervous system depressant, 45% alcohol, disrupts the retrieval process (Vohra and Hui, 2000). The three chemicals can induce amnesia in step-down test, which is recommended as a simple, rapid and sensitive method in the study of learning and memory.

2. Materials and methods

2.1. Animals and housing conditions

Male Kunming mice, weighing 20 ± 2 g at the beginning of the experiment, were purchased from Experimental Animals Center of Beijing University. Animals were housed in group under the following laboratory conditions: temperature 20 ± 1 °C, humidity 40–60%, 12:12 L/D cycle, lights on at 08:00 h. Mice had free access to food and water, except during behavioral experiments. Animals were treated in accordance with the current law and the NIH Guide for Care and Use of Laboratory Animals.

2.2. Preparation of EFC

F. cannabis (500 g) was purchased from Beijing Tongrentang Pharmaceutical Group. Materials were extracted two times with 90% ethanol for 24 h and filtrated. The filtrate was concentrated to dry. The ethanol extract was subjected to column chromatography under reduced pressure on silica gel, eluted with a chloroform–methanol solvent system. The elutions that can activate CN were collected and dried (EFC), the total yield was 5 g (1%) in terms of starting materials.

Bovine brain CN and calmodulin were isolated from frozen tissues as previously described (Wei et al., 1993). CN activity was assayed using the method of our laboratory (Yan and Wei, 1999). The phosphatase activity of CN was defined as 100%. EFC activated the phosphatase activity of CN and induced a 35 ± 5% increase.

2.3. Drug administration

EFC was dissolved in saline prior to administration at doses of 0.2, 0.4 and 0.8 g/kg. Scopolamine hydrobromide (1.5 mg/kg) and sodium nitrite ($NaNO_2$, 120 mg/kg) were dissolved in sterile 0.9% saline prior to injection. The injection volume was kept constant at 10 ml/kg irrespective of dose; 45% ethanol was diluted in saline and orally administered in a volume of 20 ml/kg. Dinitrochlorobenzene (DNCB) was dissolved in acetone.

Table 1
Effects of EFC on transient amnesia of acquired learning induced by scopolamine

Treatment (g/kg)[a]	Step-down latency (s)	Number of errors
Saline	230.7 ± 32.6 **	0.5 ± 0.2 **
Sco + saline	102.8 ± 30.5	1.4 ± 0.3
Sco + EFC (0.2)	217.1 ± 29.7*	0.6 ± 0.2*
Sco + EFC (0.4)	279.1 ± 12.1***	0.4 ± 0.2***
Sco + EFC (0.8)	257.0 ± 24.3***	0.3 ± 0.2***

All the values are expressed as mean ± S.E.M., $n = 11$.

[a] Mice were administrated saline or EFC (0.2, 0.4 and 0.8 g/kg ig) once daily for 7 days. Ten minutes before training on Day 6, saline or scopolamine was administered intraperitoneally in mice. The testing trial carried out on Day 7.

* $P < .05$.

** $P < .01$.

*** $P < .001$ vs. Sco + saline.

2.4. Step-down-type passive avoidance test

2.4.1. The effect of EFC on transient amnesia of acquired learning induced by scopolamine

After 7-day oral administration of EFC, long-term memory was examined using the step-down-type of passive avoidance test. The apparatus consisted of acrylic box with a stainless-steel grid floor. A platform was fixed in the end of the box. Electric shock (1 Hz, 1 ms, 36 V dc) was delivered to the grid floor with an isolated pulse stimulator. When mice were placed in the box, the electrical resistance varied between 100 and 250 kΩ. Therefore, each mouse received an electric shock varying between 0.14 and 0.36 mA. Ten minutes before training, saline or scopolamine was administered intraperitoneally in mice. At the beginning of training, mice were placed in the box to adapt for 3 min without electric shock. When electric shock was delivered, mice escaped from the grid floor back up onto the platform. The duration of training test was for 5 min and the shock was maintained for this period. Twenty-four hours after training, mice were placed on the platform for the retention test. The electric shocks were still delivered for 5 min. Step-down latency and the number of errors were recorded with improved retention reflected by increased latency and a reduction in errors.

2.4.2. The effect of EFC on transient amnesia of memory retention induced by sodium nitrite

After 7-day administration of EFC, saline or sodium nitrite was subcutaneously injected immediately after training. Twenty-four hours later, mice were placed on the platform for testing. Step-down latency and the number of errors were recorded within 5 min.

2.4.3. The effect of EFC on transient amnesia of memory retrieval induced by 45% ethanol

After 7-day administration of EFC, mice were trained. Thirty minutes before testing, saline or 45% ethanol was orally administered. Step-down latency and the number of errors were recorded within 5 min.

2.5. Clearance of charcoal particles

Mice were injected intravenously with 1:4 diluted Yidege ink 10 ml/kg. At 1 min (T_1) and 12 min (T_2), blood (20 μl) was taken from retro-ocular venous plexus and resolved in 0.1% Na_2CO_3 solution (2 ml). The absorbency (A) was measured at 675 nm in 720 spectrophotometer (Zhao et al., 1997). The clearance rate K and clearance index α were calculated:

$$K = (\lg C_1 - \lg C_2)/(T_1 - T_2) \quad \alpha = (W/\mathrm{WLS}) \cdot K^{1/3}$$

where W = weight of rat and WLS = liver weight + spleen weight.

2.6. Delayed-type hypersensitivity

Kunming mice were depilated using BaS (barium sulfide) on the nape skin. 50% DNCB diluted in acetone was used to induce hypersensitivity. After 10-day administration of EFC, mice were attacked on the abdomen skin by 2.5% DNCB. Twenty-four hours later, mice were injected intravenously with 1% Evans Blue (10 ml/kg) and killed after 30 min. The blue-dyed skin on the abdomen was removed and macerated for 24 h in 4-ml solutions of acetone–physiological saline (1:1) mixture. Then the mixture was centrifuged and absorbency of the supernatants was measured at 610 nm in 720 spectrophotometer.

2.7. Statistical analysis

The data were expressed as mean ± S.E.M. Statistical analysis of the data for multiple comparisons was performed by one-way ANOVA followed by the Dunnett's t test.

3. Results

3.1. Effects of EFC on transient amnesia of acquired learning induced by scopolamine

Effects of EFC on transient amnesia of acquired learning induced by scopolamine were shown in Table 1. Scopol-

Table 2
Effects of EFC on transient amnesia of memory retention induced by sodium nitrite

Treatment (g/kg)[a]	Step-down latency (s)	Number of errors
Saline	297.6 ± 2.4 **	0.1 ± 0.1*
Sodium nitrite + saline	168.3 ± 44.1	0.9 ± 0.3
Sodium nitrite + EFC (0.2)	283.4 ± 14.5*	0.2 ± 0.1*
Sodium nitrite + EFC (0.4)	278.6 ± 14.4*	0.3 ± 0.2
Sodium nitrite + EFC (0.8)	226.2 ± 33.4	0.4 ± 0.2

All the values are expressed as mean ± S.E.M., $n = 10$.

[a] Mice were administrated saline or EFC (0.2, 0.4 and 0.8 g/kg ig) once daily for 7 days. Saline or sodium nitrite was subcutaneously injected immediately after the training trial on Day 6. The testing trial carried out on Day 7.

* $P < .05$.

** $P < .01$ vs. sodium nitrite + saline.

Table 3
Effects of EFC on transient amnesia of memory retrieval induced by 45% ethanol

Treatment (g/kg)[a]	Step-down latency (s)	Number of errors
Saline	275.2 ± 26.0***	0.1 ± 0.1***
Ethanol + saline	49.5 ± 17.8	5.5 ± 1.3
Ethanol + EFC (0.2)	162.8 ± 42.0*	2.1 ± 0.8*
Ethanol + EFC (0.4)	98.0 ± 38.3	3.8 ± 0.8
Ethanol + EFC (0.8)	120.9 ± 32.5	1.6 ± 0.5**

All the values are expressed as mean ± S.E.M., $n = 11$.

[a] Mice were administrated saline or EFC (0.2, 0.4 and 0.8 g/kg ig) once daily for 7 days. After 7-day administration of EFC, mice were trained. Thirty minutes before testing, saline or 45% ethanol was orally administered in mice.

* $P < .05$.

** $P < .01$.

*** $P < .001$ vs. ethanol + saline.

amine (1.5 mg/kg) significantly shortened the latencies and increased the numbers of errors determined by step-down test. The extract (0.2, 0.4 and 0.8 g/kg) significantly improved dysmnesia mice performance.

3.2. Effects of EFC on transient amnesia of memory retention induced by sodium nitrite

Effects of EFC on transient amnesia of memory retention induced by sodium nitrite were shown in Table 2. Sodium nitrite impaired the step-down-type passive avoidance test performance of mice both in latencies and numbers of errors. EFC significantly prolonged the latencies at a dose of 0.2 and 0.4 g/kg, and decreased the numbers of error at a dose of 0.2 g/kg. The highest dose of extract (0.8 g/kg) also produced improvements but it was not statistically significant.

3.3. Effects of EFC on transient amnesia of memory retrieval induced by 45% ethanol

Effects of EFC on transient amnesia of memory retrieval induced by 45% ethanol were shown in Table 3; 45% ethanol impaired the step-down-type passive avoidance test performance of mice. EFC significantly prolonged the latency at a dose of 0.2 g/kg and decreased the number of errors at a dose of 0.2 and 0.8 g/kg. But a dose of 0.4 g/kg had no effects.

Table 4
Effect of EFC on clearance of charcoal particles in normal mice

Treatment (g/kg)[a]	Clearance rate (K)	Clearance index (α)
Saline	0.0245 ± 0.0021	4.5 ± 0.2
EFC (0.2)	0.0315 ± 0.0017*	5.3 ± 0.1***
EFC (0.4)	0.0238 ± 0.0009	4.7 ± 0.1
EFC (0.8)	0.0247 ± 0.0022	4.7 ± 0.1

All the values are expressed as mean ± S.E.M., $n = 11$.

[a] Mice were administrated saline or EFC (0.2, 0.4 and 0.8 g/kg ig) once daily for 7 days.

* $P < .05$.

*** $P < .001$ vs. saline.

Table 5
Effects of EFC on hypersensitive reaction in normal mice

Treatment (g/kg)[a]	Absorbency
Saline	0.155 ± 0.012
EFC (0.2)	0.209 ± 0.017
EFC (0.4)	0.202 ± 0.017
EFC (0.8)	0.219 ± 0.018*

All the values are expressed as mean ± S.E.M., $n = 10$.

[a] Mice were administrated saline or EFC (0.2, 0.4 and 0.8 g/kg ig) once daily for 10 days.

* $P < .05$, significant difference from saline treatment.

3.4. Effects of EFC on clearance of charcoal particles in normal mice

The effects of EFC on clearance of charcoal particles in normal mice were shown in Table 4. EFC (0.2 g/kg) produced significant increase on K and α.

3.5. The effects of EFC on hypersensitive reaction in normal mice

The effects of EFC on hypersensitive reaction in normal mice were shown in Table 5. EFC greatly increased the abilities of the hypersensitive reaction at a dose of 0.8 g/kg.

4. Discussion

In general, learning and memory are divided into three processes: learning acquisition, memory retention and retrieval. Chemical agents such as scopolamine, sodium nitrite and 45% ethanol impaired memory in mice trained on a step-down-type passive avoidance, which is used to measure the three stages of memory process depending on the drug-treated period. Scopolamine injected 10 min before training, sodium nitrite subcutaneously injected immediately after training trial and 45% ethanol orally administered 30 min before testing trial induced learning and memory impairment. The administration of EFC can improve the performance to some degree. EFC (0.2, 0.4 and 0.8 g/kg) produced an inverted bell-shaped dose effect on acquired learning of mice with scopolamine-induced dysmnesia. But the observed results of EFC at the same doses on other memory stages did not appear in the whole bell shape for retention and retrieval. Because EFC is an effective part of *F. cannabis* and contains a mixture of active components, EFC may be able to exert its improving effects through more than one component. It suggests that EFC might exert high effective action with an appropriate amount. Taking all these observations into account, including latencies and error numbers, we proposed that EFC can overcome amnesia in the three processes of learning and memory at a dose of 0.2 g/kg. Because CN potentially plays an essential role in activity-dependent modulation of synaptic efficacy, it is speculated that the improvement of EFC in memory is

related to activation of CN. It may be absorbed and activate CN. The activation of CN exerts subtle roles in brain via influencing LTP and LTD, which have been widely accepted as the synaptic models of learning and memory. LTP and LTD have been widely accepted as the synaptic models of learning and memory (Mulkey et al., 1994; Torii et al., 1995). In the CA1 region of the hippocampus, both LTP and LTD are induced by the increase of Ca^{2+} influx through the activated NMDA receptors (Tong et al., 1995). The high-frequency stimulation produces a great increase of Ca^{2+} influx, by which the PKC and Ca^{2+}/CaMK II are activated and the induction of LTP is triggered. The low-frequency stimulation results in a moderate elevation of postsynaptic Ca^{2+} level, by which the CN is activated and LTD is induced. It has been suggested that CN may be activated more rapidly and in response to lower levels of Ca^{2+} than is CaMK II. At the same time, it has been argued that activity-dependent alterations in intracellular Ca^{2+} concentrations may differentially affect the activity of CaMK II and CN, leading to changes in the phosphorylation state of key synaptic molecules, and hence to subtle and bidirectional changes in synaptic efficacy (Mansuy et al., 1998; Malleret et al., 2001; Winder et al., 1998; Lu et al., 1996, 2000; Zeng et al., 2001; Onuma et al., 1998). Furthermore, the activated CN may store memory more effectively. The CN-mediated effect of EFC on memory is just one possible explanation for the observed results. Other mechanism of action still cannot be excluded. The potential mechanism of EFC's improvement on memory may be a synergistic effect of active ingredients.

In our own laboratory, it had been found that immunosuppressive component from traditional Chinese drugs, ZIP1, could directly inhibit CN without drug-binding protein. At this stage, our experiment supports that EFC with activation of CN could increase the ability of immunity.

In sum, the administration of EFC significantly improves memory deficits in amnesic mice induced by chemical substance and increases the ability of immunity. At this stage, any conclusion that the improving effects of EFC are due to activation of CN must remain somewhat speculative. It is thus imperative to investigate the changes of activity and content of CN in brain. The precise mechanisms of action by which EFC improves memory and immunity also need further investigation but the present study provided direct pharmacobehavioural evidence for regulators screening from Chinese herbal medicine. More importance is that CN is an efficient enzyme for screening. The natural products we obtained from our screening offer the clues for further development of lead compounds.

Acknowledgements

This work was supported by the National Natural Science Foundation of China and the State Key Basic Research and Development Program.

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