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European Journal of Pharmacology 441 (2002) 151–155

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Effect of chronic treatment with the protein kinase C inhibitor staurosporine on the acquisition and expression of contextual fear conditioning

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Received 14 January 2002; received in revised form 18 February 2002; accepted 22 February 2002

Abstract

The present study investigated the effects of acute and chronic administration of the protein kinase C inhibitor, staurosporine, on the acquisition and expression of conditioned freezing behavior, an index of anxiety induced by conditioned fear stress. Results revealed that acute staurosporine (0.01 and 0.1 mg/kg, i.p.) did not affect either acquisition or expression of conditioned freezing. Chronic staurosporine administration (0.01 or 0.1 mg/kg, i.p., for 14 days) significantly reduced the acquisition of conditioned freezing at a dose of 0.1 mg/kg, but failed to affect the expression of conditioned freezing at any dose. These results suggest the involvement of protein kinase C in synaptic and cellular plasticity underlying emotional learning and memory.

Keywords: Protein kinase C inhibitor; Staurosporine; Conditioned fear stress; Anxiety

1. Introduction

Contextual fear conditioning is known to be a reliable behavioral task for the investigation and understanding of the neuronal basis of fear and anxiety (Fanselow, 2000). In contextual fear conditioning, a neutral conditioned stimulus, such as the experiment chamber, which provides various elements of background or contextual stimuli, is paired with an aversive unconditioned stimulus, usually footshocks. The conditioned stimulus then rapidly acquires aversive properties and elicits a series of motor and autonomic responses when the animal is again placed into the environment (Fanselow, 2000). This design has also been used as a useful model for the pharmacological screening of anxiolytic and antidepressant agents predicted to be effective clinically (Hashimoto et al., 1996; Inoue et al., 1996a). In our previous studies, the anxiolytic-like effect of selective serotonin (5-hydroxytryptamine, 5-HT) reuptake inhibitors, 5-HT_{1A} receptor agonists, 5-HT precursor and monoamine oxidase inhibitors on freezing behavior was shown using this contextual fear conditioning set up (Hashimoto et al., 1996; Inoue et al., 1996a; Maki et al., 2000; Li et al., 2001). These results are consistent with the recent clinical evidence showing that a number of serotonergic agents, such as selective 5-HT reuptake inhibitors and 5-HT_{1A} receptor agonists, are effective in the treatment of human anxiety disorders (Erikkson and Humble, 1990).

The amygdala, the most important brain structure for contextual fear conditioning, has been considered to be the interface between the primary sensory system that carries information about the conditioned stimulus and unconditioned stimulus, and the motor and autonomic systems that control the conditioned reactions (for review, see Fendt and Fanselow, 1999). The cellular mechanism underlying this emotional learning is suggested to be NMDA-receptor-dependent long-term potentiation (Collingridge and Bliss, 1987; Rison and Stanton, 1995; Rogan and LeDoux, 1995; Rogan et al., 1997; Maren, 1999). Previous studies have found that long-term potentiation was induced in the basolateral amygdala after high-frequency stimulation of afferents, which carry either contextual (Maren and Fanselow, 1995) or auditory (Clugnet and LeDoux, 1990; Rogan and LeDoux, 1995; Huang and Kandel, 1998) information to amygdala neurons during fear conditioning. In addition,

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0014-2999/02/$ - see front matter
PII: S0014-2999(02)01441-3

fear conditioning also induces long-term potentiation-like changes in the basolateral amygdala (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997). Moreover, the involvement of amygdaloid long-term potentiation in the emotional learning and memory was also shown in a transgenic mouse study (Brambilla et al., 1997).

Over the last decade, protein kinases have received growing attention regarding their important role in the synaptic and cellular plasticity underlying learning and memory (for review, see Van der Zee and Douma, 1997). It is suggested that cAMP-dependent protein kinase (protein kinase A) activation is involved in the expression of an early phase of amygdaloid long-term potentiation (Huang and Kandel, 1998). Transcriptional activation mediated by cAMP response element (CRE), which is thought to reflect protein kinase A activity, has been demonstrated in the amygdala during contextual fear conditioning (Impey et al., 1998). In addition, inhibitors for protein kinase A and mitogen-activated protein kinase have been shown to interfere with the long-term memory for contextual and auditory fear in rats or mice (Bourtchouladze et al., 1998; Schafe et al., 1999). Moreover, shock-induced stress has been demonstrated to potentiate protein kinase C activity in the amygdala (Shors et al., 1997). Recently, Weeber et al. (2000) reported that the protein kinase C beta (the β isoform of protein kinase C) knockout mouse had a deficiency in both cued and contextual conditioning. Furthermore, infusion of 1-(5′-isoquinolinesulfonyl)-2-methylpiperazine (H-7), a potent inhibitor of both protein kinase C and protein kinase A, into the amygdala interfered with the long-term conditional fear in rats (Goosens et al., 2000). In addition, the effect of H-7 and another protein kinase C inhibitor, melittin, on rat learning memory tests (one trial passive avoidance response and spatial learning model) indicated that protein kinase C inhibitors markedly impaired the learning and memory of rats (Takashima et al., 1991).

In the present study, we investigated the effect of staurosporine, a protein kinase C inhibitor, on the acquisition and expression of contextual fear conditioning in rats. The time course and staurosporine dose were based on those of the study of Nabeshima et al. (1991).

2. Method and materials

2.1. Animals

Male Sprague–Dawley rats obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan), weighing 240–260 g at the time of testing, were housed in groups of four per cage and maintained on a 12-h light–dark cycle (light phase: 0630–1830 h), temperature-controlled environment (22 ± 1 °C) with free access to food and water. Acute staurosporine experiments began after 10 days and chronic staurosporine experiments began after a 2-day period of acclimatization. All experiments were performed between 0800 and 1300 h.

2.2. Drugs

Staurosporine (Asahikasei Japan) was suspended in 0.5% sodium carboxymethyl cellulose in saline. Staurosporine was administered intraperitoneally (i.p.) as a volume of 1 ml/kg.

2.3. Procedures

2.3.1. Conditioned fear stress-induced freezing

As described previously (Inoue et al., 1996b), the rats were individually subjected to inescapable electric footshock for a total of 2.5 min [five footshocks (2.5-mA scrambled shock, 30-s duration) that were delivered at intershock intervals of 35–85 s (mean 60 s)] in a shock chamber with a grid floor ($19 \times 22 \times 20$ cm, Medical Agent, Japan). Electric shocks were administered with a Model SGS-02D Shock Generator (Medical Agent). This provides a high-voltage, high-resistance circuit with resistance controlled by dial settings calibrated by the manufacturer in a short circuit current. At the setting of 2.5 mA, this generator actually gave a 0.2-mA shock intensity to the rats (Inoue et al., 1996b). In acute treatment experiments, 24 h after footshocks, the rats were placed individually in a shock chamber without shocks and were observed for 5 min. In a chronic treatment experiment for the acquisition of conditioned fear, 24 h after footshocks, and in a chronic treatment experiment for the expression of conditioned fear, 14 days after footshocks, the rats were individually placed in a shock chamber without shocks and were observed for 5 min. With these procedures, conditioned fear, as measured by freezing, develops to the contextual stimuli of the conditioned chamber (Fanselow, 1980). During the observation period, freezing behavior was recorded using a modification (Inoue et al., 1996b) of a time-sampling procedure (Fanselow, 1980). Freezing was defined as the absence of any observable movement of the skeleton and the vibrissae, except those related to respiration. All other behavior was scored as activity. The animal was classified as showing either freezing or active behavior according to its behavior throughout the entire 10-s period. The percentage score (%freezing) represented the number of 10-s periods during which the animal froze for the entire 10 s. These procedures were approved by the Hokkaido University School of Medicine Animal Care and Use Committee.

2.3.2. Effect of acute staurosporine on the acquisition of conditioned freezing

Thirty minutes before footshock, the rats were given a single intraperitoneal injection of staurosporine (0.01 and 0.1 mg/kg). Twenty-four hours after footshock, the rats were placed individually in the shock chamber without shocks and were observed for 5 min.

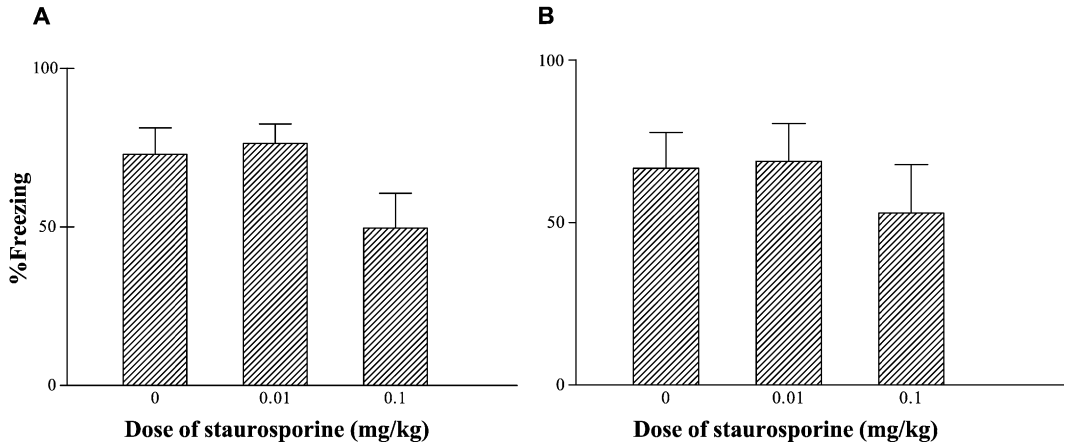

Fig. 1. Effect of acute staurosporine (0.01 and 0.1 mg/kg) on the acquisition (A) and expression (B) of conditioned freezing. (A) Staurosporine was given intraperitoneally 30 min before footshock. Twenty-four hours after footshock, freezing behavior was observed. (B) Staurosporine was given intraperitoneally at 23.5 h after footshock and 30 min before testing. Mean percentages ± S.E.M. of freezing scored for a 5-min observation period are shown. Behavior was sampled at 10-s intervals. The number of rats per group for each experiment was 8.

2.3.3. Effect of acute staurosporine on the expression of conditioned freezing

Rats received a single intraperitoneal injection of staurosporine (0.01 and 0.1 mg/kg) 23.5 h after footshock. Thirty minutes after the injection, rats were placed individually in the shock chamber without shocks and were observed for 5 min.

2.3.4. Effect of chronic staurosporine on the acquisition of conditioned freezing

Rats received repeated intraperitoneal injections of staurosporine (0.01 and 0.1 mg/kg) once per day for 14 days. Thirty minutes after the last injection, the rats received footshocks. Twenty-four hours after footshock, rats were placed individually in the shock chamber without shocks and were observed for 5 min.

2.3.5. Effect of chronic staurosporine on the expression of conditioned freezing

Twenty-four hours after footshock, staurosporine (0.01 and 0.1 mg/kg) or saline was administered i.p. once per day for 14 days. Thirty minutes after the last treatment, rats were placed individually in the shock chamber without shocks and were observed for 5 min.

2.3.6. Pain

The effect of chronic treatment (0.1 mg/kg, i.p., for 14 days) with staurosporine on footshock-induced pain was examined as previously described by Inoue et al. (1996b). Three behaviors, vocalization, limb withdrawal of the forepaw and limb withdrawal of the hindpaw, were used as being indicative of nociception. These have generally been used as endpoints in the hot plate procedure (Carter, 1991).

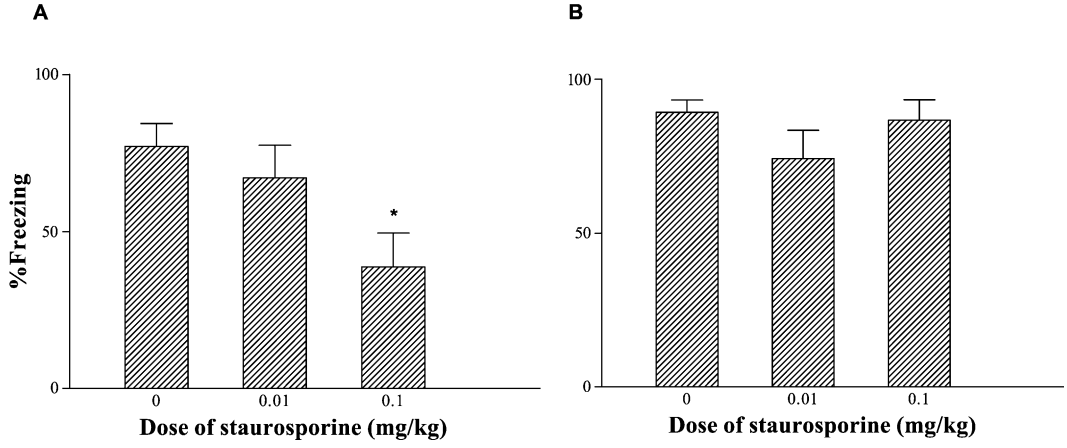

Fig. 2. Effect of chronic staurosporine (0.01 and 0.1 mg/kg) on the acquisition (A) and expression (B) of conditioned freezing. (A) Staurosporine was given intraperitoneally once a day for 14 days, and the last injection was given 30 min before footshock. Twenty-four hours after footshock, freezing behavior was observed. (B) Twenty-four hours after footshock, staurosporine was given intraperitoneally once a day for 14 days. Twenty-four hours after the last injection, freezing behavior was observed. Mean percentages ± S.E.M. of freezing scored for a 5-min observation period are shown. Behavior was sampled at 10-s intervals. $*P<0.05$. In (A), $N=7-8$, in (B), $N=8$ rats.

Thirty minutes after the last treatment, the rats were placed individually in a shock chamber. After a 5-min adaptation period, these rats were subjected to 15 series of scrambled electric footshocks. Each series had a 10-s duration and was spaced at 40-s intervals, ranging from 0.4 to 3.2 mA in 0.2-mA steps, presented in ascending order. The response of rats to each shock was recorded, and minimal intensities of electric footshocks at which each three behaviors appeared were determined.

2.4. Data analysis

All the data are presented as the means ± S.E.M. of the individual values for the rats from each group. The statistical significance of differences between two groups was analyzed using an unpaired *t*-test (two-tailed). Multiple group comparisons were performed using a one-way analysis of variance (ANOVA) followed by Duncan's test.

3. Results

3.1. Effect of acute staurosporine on the acquisition of conditioned fear

The protein kinase C inhibitor, staurosporine, did not significantly affect conditioned freezing compared with the vehicle group at the dose of either 0.01 or 0.1 mg/kg [one-way ANOVA, $F(2,21) = 2.77$, NS] (Fig. 1A).

3.2. Effect of acute staurosporine on the expression of conditioned freezing

Staurosporine did not significantly affect conditioned freezing compared with the vehicle group at the dose of either 0.01 or 0.1 mg/kg [one-way ANOVA, $F(2,21) = 0.48$, NS] (Fig. 1B).

3.3. Effect of chronic staurosporine on the acquisition of conditioned freezing

Staurosporine failed to affect conditioned freezing at a dose of 0.01 mg/kg, but at the dose of 0.1 mg/kg, it significantly attenuated conditioned freezing compared with the vehicle group [one-way ANOVA, $F(2,20) = 4.10$, $P < 0.05$] (Fig. 2A).

Table 1
Effect of staurosporine (0.1 mg/kg, i.p.) on pain-related behavior induced by footshock

Behavior	Saline group	Staurosporine group	
Withdrawal of forepaws	0.85 ± 0.06	1.05 ± 0.12	n.s.
Withdrawal of hindpaws	1.10 ± 0.10	1.33 ± 0.12	n.s.
Vocalization	1.55 ± 0.06	1.68 ± 0.05	n.s.

Data are represented as the means ± S.E.M. of pain threshold (mA), at which each behavior first appeared ($N = 8$; n.s., not significant).

3.4. Effect of chronic staurosporine on the expression of conditioned freezing

Staurosporine failed to affect conditioned freezing at the dose of either 0.01 or 0.1 mg/kg [one-way ANOVA, $F(2,21) = 1.35$, NS] (Fig. 2B).

3.5. Pain sensitivity

Chronic staurosporine (0.1 mg/kg) treatment did not change the minimal intensities of electric footshocks at which three pain-related behaviors first appeared, i.e. pain thresholds (Table 1).

4. Discussion

In this study, we investigated the effects of acute and chronic staurosporine administration on both acquisition and expression of conditioned freezing. The results revealed that chronic administration of staurosporine, at a dose of 0.1 mg/kg, attenuated the acquisition of conditioned freezing, but failed to affect the expression of conditioned freezing at either of the doses used. Acute staurosporine administration failed to affect the acquisition and expression of conditioned freezing.

Since chronic staurosporine treatment did not affect the threshold of footshock-induced pain, the blocking effect of chronic staurosporine treatment on the acquisition of conditioned fear cannot be attributed to a reduced sensitivity to electric footshock. In addition, the chronic staurosporine treatment after footshock left the freezing intact in the expression experiment, indicating that chronic staurosporine treatment interfered with the development or process of fear learning, but did not reduce either anxiety or fear-related behavior.

Previous studies have shown that various protein kinase C inhibitors block long-term potentiation (Reymann et al., 1988; Malinow et al., 1989; Wang and Feng, 1992), and several studies have shown that protein kinase C is involved in contextual fear conditioning (Weeber et al., 2000). The present results are consistent with those of previous studies, indicating that protein kinase C has a prominent role in the synaptic and cellular plasticity underlying learning and memory. The study by Goosens et al. (2000) showed that intra-amygdala infusion of H-7, a potent protein kinase A/protein kinase C inhibitor, attenuates the acquisition of conditioned freezing, but does not affect the expression of conditioned freezing. These results are in agreement with the finding of this study that staurosporine affected only acquisition of contextual fear conditioning but not expression. However, the reason why a protein kinase A/protein kinase C inhibitor or a protein kinase C inhibitor affected only the acquisition but not the expression of conditioned freezing remains unclear.

In addition to the notion of the involvement of protein kinase C inhibitors in long-term potentiation, other mecha-

nisms may be related to the chronic effect of staurosporine on the acquisition of conditioned fear. Staurosporine also inhibits other kinases with weaker potencies (Ruegg and Burgess, 1989). Accordingly, further studies that examine the effects of more selective protein kinase C inhibitors on contextual fear conditioning will be necessary.

In conclusion, the present results suggest the involvement of protein kinase C in synaptic and cellular plasticity underlying fear learning and emotional memory.

Acknowledgements

We thank Asahikasei (Japan) for the generous gift of staurosporine and Professor Yasuharu Sasaki (Kitasato University) for helpful suggestions. This work was supported in part by grants-in-aid for Scientific Research No. 09470205 (T.K.) and No. 11670924 (T.I.) from the Japanese Ministry of Education, Science and Culture. The publishing cost of this article was supported by the memorial fund of the late psychiatrist, Dr. Kozo Ito.

References

Bourtchouladze, R., Abel, T., Berman, N., Gordon, R., Lapidus, K., Kandel, E.R., 1998. Different training procedures recruit either one or two critical periods for contextual memory consolidation, each of which requires protein synthesis and PKA. Learn. Mem. 5, 365–374.

Brambilla, R., Gnesutta, N., Minichiello, L., White, G., Roylance, A.J., Herron, C.E., Ramsey, M., Wolfer, D.P., Cestari, V., Rossi-Arnaud, C., Grant, S.G., Chapman, P.F., Lipp, H.P., Sturani, E., Klein, R., 1997. A role for the Ras signaling pathway in synaptic transmission and long-term memory. Nature 390, 281–286.

Carter, R.B., 1991. Differentiating analgesic and non-analgesic drug activities on rat hot plate: effect of behavioral endpoint. Pain 47, 211–220.

Clugnet, M.C., LeDoux, J.E., 1990. Synaptic plasticity in fear conditioning circuits: induction of LTP in the lateral nucleus of the amygdala by stimulation of the medial geniculate body. J. Neurosci. 10, 2818–2824.

Collingridge, G.L, Bliss, T.V.P., 1987. NMDA receptors—their role in long-term potentiation. Trends Neurosci. 10, 288–293.

Erikkson, E., Humble, M., 1990. Serotonin in psychiatric pathophysiology: a review of data from experimental and clinical research. In: Pohl, R., Gershon, S. (Eds.), Progress in Basic Clinical Pharmacology. The Biological Basis of Psychiatric Treatment, vol. 3. Karger, Basel, pp. 66–119.

Fanselow, M.S., 1980. Conditional and unconditional components of post-shock freezing. Pavlovian J. Biol. Sci. 15, 177–182.

Fanselow, M.S., 2000. Contextual fear, gestalt memories, and the hippocampus. Behav. Brain Res. 110, 73–81.

Fendt, M., Fanselow, M.S., 1999. The neuroanatomical and neurochemical basis of conditioned fear. Neurosci. Biobehav. Rev. 23, 743–760.

Goosens, K.A., Holt, W., Maren, S., 2000. A role for amygdaloid PKA and PKC in the acquisition of long-term conditional fear memories in rats. Behav. Brain Res. 114, 145–152.

Hashimoto, S., Inoue, T., Koyama, T., 1996. Serotonin reuptake inhibitors reduce conditioned fear stress-induced freezing behavior in rats. Psychopharmacology 123, 182–186.

Huang, Y.Y., Kandel, E.R., 1998. Postsynaptic induction and PKA-dependent expression of LTP in the lateral amygdala. Neuron 21, 169–178.

Impey, S., Smith, D.M., Obrietan, K., Donahue, R., Wade, C., Storm, D.R., 1998. Stimulation of cAMP response element (CRE)-mediated transcription during contextual learning. Nat. Neurosci. 1, 595–601.

Inoue, T., Tsuchiya, K., Koyama, T., 1996a. Serotonergic activation reduces defensive freezing in the conditioned fear paradigm. Pharmacol. Biochem. Behav. 53, 825–831.

Inoue, T., Hashimoto, S., Tsuchiya, K., Izumi, T., Ohmori, T., Koyama, T., 1996b. Effect of citalopram a selective serotonin reuptake inhibitor on the acquisition of conditioned freezing. Eur. J. Pharmacol. 311, 1–6.

Li, X.B., Inoue, T., Hashimoto, S., Koyama, T., 2001. Effect of chronic administration of flesinoxan and fluvoxamine on freezing behavior induced by conditioned fear. Eur. J. Pharmacol. 425, 43–50.

Maki, Y., Inoue, T., Izumi, T., Muraki, I., Ito, K., Kitaichi, Y., Li, X.B., Koyama, T., 2000. Monoamine oxidase inhibitors reduce conditioned fear stress-induced freezing behavior in rats. Eur. J. Pharmacol. 406, 411–418.

Malinow, R., Schulman, H., Tsien, R.W., 1989. Inhibition of postsynaptic PKC or CaMKll blocks induction but not expression of LTP. Science 245, 862–866.

Maren, S., 1999. Long-term potentiation in the amygdala: a mechanism for emotional learning and memory. Trends Neurosci. 22, 561–567.

Maren, S., Fanselow, M.S., 1995. Synaptic plasticity in the basolateral amygdala induced by hippocampal formation stimulation in vivo. J. Neurosci. 15, 7548–7564.

McKernan, M.G., Shinnick-Gallagher, P., 1997. Fear conditioning induces a lasting potentiation of synaptic currents in vitro. Nature 390, 607–611.

Nabeshima, T., Ogawa, S., Nishimura, H., Fuji, K., Kameyama, T., Sasaki, Y., 1991. Staurosporine facilitates recovery from the basal forebrain-lesion-induced impairment of learning and deficit of cholinergic neuron in rats. J. Pharmacol. Exp. Ther. 257, 562–566.

Reymann, K.G., Brodemann, R., Kase, H., Matthies, H., 1988. Inhibitors of calmodulin and protein kinase C block different phases of hippocampal long-term potentiation. Brain Res. 461, 388–392.

Rison, R.A., Stanton, P.K., 1995. Long-term potentiation and *N*-methyl-D-aspartate receptors: foundations of memory and neurological disease. Neurosci. Biobehav. Rev. 19, 533–552.

Rogan, M.T., LeDoux, J.E., 1995. LTP is accompanied by commensurate enhancement of auditory-evoked responses in a fear conditioning circuit. Neuron 15, 127–136.

Rogan, M.T., Staubli, U.V., LeDoux, J.E., 1997. Fear conditioning induces associative long-term potentiation in the amygdala. Nature 390, 604–607.

Ruegg, U.T., Burgess, G.M., 1989. Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. Trends Pharmacol. Sci. 10, 218–220.

Schafe, G.E., Nadel, N.V., Sullivan, G.M., Harris, A., LeDoux, J.E., 1999. Memory consolidation for contextual and auditory fear conditioning is dependent on protein synthesis, PKA, and MAP kinase. Learn. Mem. 6, 97–110.

Shors, T.J., Elkabes, S., Selcher, J.C., Black, I.B., 1997. Stress persistently increase NMDA receptor-mediated binding of [^{3}H]PDBu (a marker for protein kinase C) in the amygdala, and re-exposure to the stressful context reactivates the increase. Brain Res. 750, 293–300.

Takashima, A., Yokota, T., Maeda, Y., Itoh, S., 1991. Pretreatment with caerulein protects agonist memory impairment induced by protein kinase C inhibitors in the rat. Peptides 12, 699–703.

Van der Zee, E.A., Douma, B.R., 1997. Historic review of research on protein kinase C in learning and memory. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 21, 379–406.

Wang, J.H., Feng, D.P., 1992. Postsynaptic protein kinase C essential to induction and maintenance of long-term potentiation in the hippocampal CA1 region. Proc. Natl. Acad. Sci. U. S. A. 89, 2576–2580.

Weeber, E.J., Atkins, C.M., Selcher, J.C., Varga, A.W., Mirnikjoo, B., Paylor, R., Leitges, M., Sweatt, J.D., 2000. A role for the β isoform of protein kinase C in fear conditioning. J. Neurosci. 20, 5906–5914.