Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 4286–4289, May 1993
Neurobiology

Associative learning potentiates protein kinase C activation in synaptosomes of the rabbit hippocampus

(classical conditioning)

KOSAKU SUNAYASHIKI-KUSUZAKI*, DAVID S. LESTER, BERNARD G. SCHREURS, AND DANIEL L. ALKON

Neural Systems Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892

Communicated by Philip Siekevitz, November 12, 1992

ABSTRACT **Using electrophysiological, biochemical, and autoradiographic techniques, changes in protein kinase C (PKC) activity in specific regions of the hippocampus have been previously implicated in classical conditioning of the nictitating membrane response of the rabbit. Here we report that activation of PKC is potentiated 2- to 3-fold in synaptosomes of the hippocampal CA1 and CA2 to -3 regions in rabbits that have undergone classical conditioning of the nictitating membrane response. This potentiation is apparently due to a change in the biochemical properties of PKC within the synaptosomes and is not a result of an increase in total PKC activity. This observation correlates a subcellular biochemical change with classical conditioning of a mammal.**

Several studies have suggested that the activation of protein kinase C (PKC) influences normal synaptic functioning (1, 2) and plays a role in long-term potentiation (3, 4) and associative learning (1, 5–8). Although PKC activity has been measured in synaptic plasma membranes (SPMs) and postsynaptic densities (PSDs) of the cerebral cortex (9) and SPMs of the hippocampus (10), there are no reports documenting learning-induced changes in PKC activity in synaptosomes from specific brain regions. Our laboratory has previously shown that there are changes in PKC location and function in the hippocampus of rabbits that have undergone a classical conditioning paradigm (5, 7, 8). In the present report, we further investigate the nature of learning-specific changes in PKC by monitoring synaptosomal PKC activity from specific microdissected regions of the hippocampus of rabbits that have demonstrated associative learning. Using this approach, we provide evidence that there is a change in the activation properties of PKC in the synaptosomal fraction obtained from classically conditioned animals and not in samples from control animals. This change in activation properties was indicative of a change intrinsic to the SPMs, although it does not rule out postsynaptic involvement.

MATERIALS AND METHODS

Associative Learning. Adult male albino rabbits (*Oryctolagus cuniculus*) weighing 1.5–3.0 kg were individually housed, given free access to food and water, and maintained on a 12-hr light/12-hr dark cycle. Animals were allocated randomly to one of three groups, which consisted of (*i*) paired stimulus presentations, (*ii*) unpaired stimulus presentations, or (*iii*) no stimulus presentations (naive). Paired and unpaired subjects received 1 day of preparation and 3 consecutive days of stimulus presentation. The procedure was identical to that of Bank *et al.* (8). Naive subjects received 1 day of adaptation and 3 consecutive days of restraint. On adaptation day, the rabbits were prepared for periorbital electrical stimulation and recording of nictitating membrane movement and then adapted to the training chambers for the length of time of subsequent training sessions (80 min). Training sessions for paired subjects consisted of 80 presentations of a 400-ms, 100-Hz, 82-decibel tone conditioned stimulus (CS) that coterminated with a 100-ms, 50-Hz periorbital electrical pulse unconditioned stimulus (US). The presentations were delivered, on average, every 60 s. Sessions for unpaired subjects consisted of 80 CS-alone and 80 US-alone presentations, which occurred in an explicitly unpaired manner delivered approximately every 30 s. Naive subjects were placed in the training chamber but received no stimulus presentations. Stimulus delivery and data collection were accomplished by using a Compaq/ASYST computer system as described (11, 12).

Preparations of the SPM and PSD Fractions. Twenty-four hours after the final day of training, rabbits were anesthetized with sodium pentobarbital (30 mg/kg) and decapitated. A rapid craniotomy allowed the forebrain to be removed and chilled in 95% O_2/5% CO_2-saturated Krebs–Ringer solution within 60–90 s. Next, the right and left hippocampi (300–400 mg) were isolated and the CA1 and CA2 to -3 regions were microdissected. The isolated tissue was then homogenized in 0.8 ml of ice-cold 0.32 M sucrose solution containing 25 mM dithiothreitol and 1 mM EDTA. The entire isolation procedure was carried out at 4°C. SPMs were isolated from the supernatant on Percoll/sucrose gradients (13) (15% layer; Pharmacia) and were rinsed twice. From each preparation, a portion of SPMs was kept for protein determination, a portion was kept for fractionation of soluble (cytosolic) and particulate (membrane) PKC activities, and half was kept for preparation of PSDs. Hippocampal PSDs were separated from the SPMs on sucrose gradients (1.5 M layer) as described (9, 14). Six preparations were treated with K^+ (30 mM) and phorbol 12-myristate 13-acetate (PMA, 100 nM; Sigma) in Krebs–Ringer buffer to stimulate the synaptic particles at 30°C for 5 min (10). Under comparable conditions, total SPM–PSD PKC activity in the fractions was also derived for animals of all groups.

The SPM fraction was centrifuged at 15,000 × *g* for 20 min at 4°C; the pellets were resuspended and homogenized in 1.0 ml of ice-cold buffer [20 mM Tris·HCl/10 mM EGTA/2 mM EDTA/0.5 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride (Sigma)/leupeptin (1 μg/ml), pH 7.5], and sonicated for 10 s (Kontes; dial setting, 5). The homogenate was centrifuged at 100,000 × *g* for 30 min. The supernatant was the cytosolic fraction. The pellet was suspended in 1.0 ml of buffer containing 0.5% Triton X-100 at 4°C for 45 min. The centrifugation step was repeated and the supernatant represented the membrane fraction (15).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PKC, protein kinase C; SPM, synaptic plasma membrane; PSD, postsynaptic density; PMA, phorbol 12-myristate 13-acetate; DAG, diacylglycerol.

*To whom reprint requests should be addressed.

Determination of PKC Activity. Lipids were prepared as described (16). The standard reaction solution for PKC contained 20 mM Tris·HCl buffer (pH 7.5), 2.5 mM MgCl, 0.5 mM dithiothreitol, 50 μg of histone III-S per ml, 0.2 mg of phosphatidylserine per ml, 0.8 mg of phosphatidylcholine per ml (Avanti Polar Lipids), 50 μg of diacylglycerol (DAG) per ml (Avanti Polar Lipids), 18 μM [γ-^{32}P]ATP (Amersham; 0.2 μCi per assay; 1 Ci = 37 GBq), 1 μM leupeptin (Boehringer Mannheim), and 100 μM Ca^{2+} or 0.5 mM EGTA. These are accepted levels of Ca^{2+} and ATP for PKC activity measurements (17, 18). The PKC-specific activity was the difference between the activities measured in the presence and absence of Ca^{2+}. The measures for Ca^{2+}-independent activity were similar to those obtained for Ca^{2+}-dependent, phospholipid-independent activity (data not shown). This procedure has been shown to specifically distinguish PKC activity from other protein kinases (17, 19). It provides an estimation of the maximally activated PKC (15); thus, additional PMA or Ca^{2+} would have no significant effect on the determined absolute rates (19).

The reaction was started by addition of 30 μl of tissue extract to 0.3 ml of reaction solution and incubated at 30°C for 16 min. The reaction was stopped by the addition of 1 ml of 25% trichloroacetic acid (TCA) solution, and cooled at 4°C for 60 min. The solution was filtered through nitrocellulose filters (0.45 μm; Millipore) with a filtration manifold (Millipore) and was washed with 2 ml of 5% TCA three times.

Determination of Protein Concentration. Protein concentration was determined with a Pierce protein assay kit (Pierce).

RESULTS

Associative Learning. Paired subjects showed levels of conditioning in excess of 90% conditioned responses by the end of training, whereas unpaired and naive subjects showed only baseline levels of responding (i.e., <2% responses; $P < 0.001$). More detailed accounts of comparable behavioral results have been described (12).

Biochemical Analysis. Measurements were made of PKC activity of SPMs and PSDs from hippocampal regions of individual rabbits. Table 1 presents actual counts to demonstrate the sensitivity of this assay in monitoring membrane-associated PKC activity derived from SPMs and PSDs that have not been exposed to stimulating conditions. In addition, these data demonstrate the variability of the activity between animals and the reproducibility obtained from the same animal.

Analyses of preparations from naive controls and from unpaired and paired subjects showed no significant difference in total PKC activity between the different groups ($P > 0.1$; data not shown). This observation agrees with a previous report in which total PKC activity was determined for hippocampus of conditioned (i.e., paired) and unconditioned (i.e., unpaired) rabbits (8). Soluble and particulate fractions were prepared from SPMs in the present experiment in order to monitor the subcellular distribution of PKC activity—i.e., cytosol or membrane. The soluble fractions ("cytosolic" activity) obtained from fractionated SPMs were too dilute and activity was lost upon concentration of the sample. PKC activity was measured in the detergent-solubilized membrane fractions, considered to be representative of membrane-associated PKC (15). Although, there was considerable variability among the membrane preparations, there was no significant difference among naive, unpaired, and paired subjects in total PKC activity in the synaptosomal membrane preparations of the CA1 or CA2 to -3 region or in the PSDs obtained from CA1 (Table 2).

The PKC activity of the synaptosomal fractions prepared from the hippocampal regions of animals from each of the groups was also tested under conditions that activate the cytosolic PKC—i.e., high K^+ with phorbol dibutyrate (10). Exposure to these activating treatments dramatically elevated membrane PKC activity in SPMs–PSDs of paired animals (Fig. 1), whereas naive and unpaired subjects (i.e., control groups) showed no such elevation. The increase was highly significant in the CA1 region (Fig. 1*A*; 215–676 pmol of γ-^{32}P incorporated per min per mg of protein; $P < 0.01$) and in the CA2 to -3 region (Fig. 1*B*; 182–539 pmol of γ-^{32}P incorporated per min per mg of protein; $P < 0.01$). There was no significant change in the amount of PKC activity associated with the isolated PSDs of the CA1 regions among the three groups (Fig. 1*C*). It is interesting to note that the variability in the membrane-associated PKC activities was considerably lower in stimulated preparations and was probably due to the addition of PKC activators (i.e., Ca^{2+} and

Table 1. PKC activity of defined regions of the hippocampus

	Histone 1 phosphorylation, cpm								
	N			UP			P		
	1	2	3	1	2	3	1	2	3
SPM									
CA1	2142	1275	1846	2283	3218	1527	3096	2838	4358
	2879	1567	1359	2120	2996	1666	3139	2903	4332
	2159	1518	1506	2239	3155	1621	3208	2950	4298
Mean ± SEM	(2393 ± 297)	(1453 ± 111)	(1570 ± 177)	(2214 ± 60)	(3123 ± 81)	(1605 ± 50)	(3148 ± 40)	(2897 ± 40)	(4329 ± 21)
CA2 to -3	1675	1253	1420	1823	1329	1406	1771	1624	2301
	1602	1275	1389	1488	1314	1441	2245	1800	2492
	2203	1233	1710	1455	1247	1406	2112	1875	3018
Mean ± SEM	(1827 ± 232)	(1254 ± 15)	(1506 ± 125)	(1589 ± 144)	(1297 ± 31)	(1418 ± 14)	(2043 ± 173)	(1766 ± 91)	(2604 ± 263)
PSD									
CA1	1406	2878	1423	3022	2603	3108	2598	2575	2425
	1532	2871	1515	2840	2521	3219	2787	2595	3015
	1628	3223	1456	3305	2959	3260	2864	2955	2569
Mean ± SEM	(1522 ± 79)	(2991 ± 142)	(1465 ± 33)	(3056 ± 166)	(2694 ± 165)	(3196 ± 56)	(2750 ± 97)	(2708 ± 151)	(2670 ± 218)

Ca^{2+}/DAG-specific histone III-S phosphorylation activity is presented as triplicates of membrane fractions of SPMs of CA1 and CA2 to -3 and PSDs of CA1 regions for three different animals that have undergone three behavioral regimens. N, naive sit control; UP, unpaired conditioning; P, paired conditioning.

Table 2. PKC activity in membrane extracts of hippocampus SPMs and PSDs from animals in the three behavioral groups

	PKC activity, pmol per min per mg of protein		
	SPM		PSD
Group	CA1	CA2 to -3	CA1
N	78.0 ± 22.6	139 ± 30.6	17.1 ± 3.2
UP	133 ± 24.0	70.7 ± 15.7	27.1 ± 6.3
P	161 ± 27.3	106 ± 28.3	28.8 ± 3.2

Characterization of PKC activity (histone 1 phosphorylation) in the membrane of rabbit hippocampus regions at the level of subcellular fractions in three groups. N, naive sit control; UP, unpaired conditioning; P, paired conditioning. Membrane-associated activity was extracted as described. Values presented are means ± SEM (n = 4 or 5).

phorbol dibutyrate). Finally, there was no significant difference in the total activities in stimulated SPM (containing the PSD fragments) preparations among the three behavioral conditions (Fig. 2). The total activities for the three groups under stimulated conditions (K^+/PMA-treated) were similar to those obtained under nonstimulated conditions (data not shown). This provides evidence that the increase in membrane-associated PKC under stimulated conditions was not due to an increase in the total PKC activity of the SPMs.

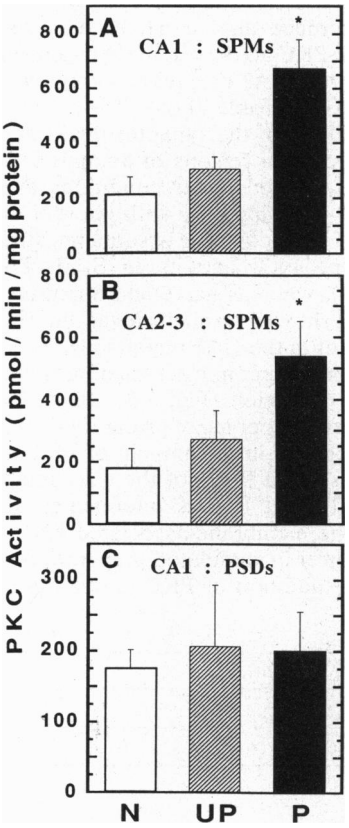

FIG. 1. Associative learning-related changes in membrane-associated PKC activity in synaptic fractions from microdissected regions of rabbit hippocampus. Synaptosomes from CA1 and CA2 to -3 regions and postsynaptic density fractions from the CA1 region were prepared from the hippocampus of rabbits that had undergone one of three behavioral paradigms: naive (N), unpaired (U), or paired (P) training. Synaptosomes were pretreated under stimulating conditions with phorbol dibutyrate (100 nM) and K^+ (40 mM) in Krebs–Ringer buffer for 5 min and then disrupted and separated into soluble and particulate fractions by high-speed ultracentrifugation. PSDs were prepared after stimulation of SPMs. (*A*) PKC activity in membranes derived from stimulated SPMs of the CA1 region. (*B*) PKC activity in membranes derived from stimulated SPMs of the CA2 to -3 region. (*C*) PKC activity in PSD fractions purified from stimulated SPMs of the CA1 region. Six different preparations were used for each condition and each preparation was measured in triplicate. SEs are represented by error bars and asterisks indicate a significant result as determined by two-tailed Student's t test ($P < 0.01$).

DISCUSSION

The potentiation of PKC activation in SPMs of classically conditioned animals presented in this report indicates that there is some intrinsic property in the synaptosomes of CA1 and CA2 to -3 regions that has changed as a function of associative learning. As there is no significant difference in the total PKC extractable activity of stimulated or nonstimulated SPMs, it is proposed that there is some unique biochemical property of the synaptic membrane that has changed as a function of classical conditioning. Subsequently, upon stimulation of the SPM, PKC has a greater tendency to form a membrane-associated PKC active species (19). The complexity of neuronal membrane-associated PKC activity has been documented (17, 19, 20).

Two distinct populations of membrane-associated PKC activity have been identified (17, 19). The relative concentration of each of these and the physical properties controlling their membrane association has been shown to depend on the lipid environment (17, 19). Recent studies have suggested that there are at least three components necessary in the neuronal long-term activation of PKC. They are the lipid activators, DAG and arachidonic acid, and the second messenger, Ca^{2+} (4, 6). One or more of these components may be changed after the animal has undergone conditioning. For example, the concentration of one of the lipid activators may be elevated. The K^+ depolarization would raise the Ca^{2+} concentration, and the phorbol ester may take the role of the other nonelevated lipid activator. Thus, formation of the membrane-associated form of PKC, which has been implicated as a key component in the associative learning process (8, 21), may require the combination of both lipid activators and Ca^{2+}. The lack of or lower basal concentrations of one of these cofactors—e.g., free arachidonic acid or DAG—could explain the inability to induce the tightly associated membrane PKC form (see ref. 19) in the SPMs derived from naive and unpaired animals; that is, the lipid environment of these SPM membranes may not be optimized for potential formation of this membrane PKC form. However, these types of problems only demonstrate our lack of understanding of the physical interactions between PKC and its mem-

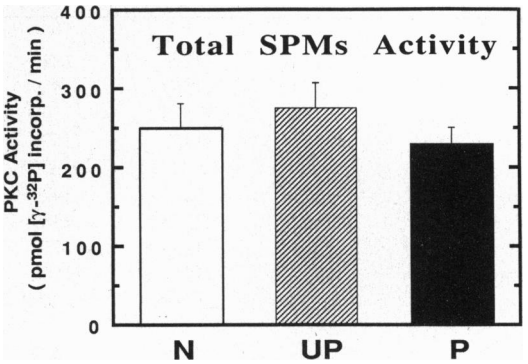

FIG. 2. Total activities of stimulated SPMs from hippocampal CA1 region of rabbits that have undergone the indicated behavioral paradigm. Conditions were as for Fig. 1, except that the intact stimulated SPM was solubilized to extract total PKC activity. Four animals were used for each condition and each preparation was done in triplicate. SEs are represented by error bars. Means were not statistically different.

brane activation process (22). Interestingly, our laboratory has previously shown for the photoreceptor of the sea snail *Hermissenda crassicornis* that in order to obtain the biophysical state of the cell obtained from trained animals, PKC injection or phorbol ester application alone is insufficient. As observed for the SPMs in this study, phorbol ester and a Ca^{2+} load must also be applied (23, 24). This stimulation of the SPMs under physiologic conditions may represent a part of the process that occurs during storage and/or recall of the experience.

In summary, the potentiation of PKC activation in hippocampal synaptosomes of classically conditioned rabbits indicates that there is some intrinsic property in the membrane of the synaptic terminal of CA1 and CA2 to -3 that has changed. This paper provides biochemical evidence demonstrating the subcellular localization of an associative memory-related physiological change in a mammal.

We thank Mr. Jeffrey Hammer for help with computer graphics. Animal care complied with National Institutes of Health guidelines for care and use of laboratory animals.

1. Alkon, D. L. & Rasmussen, H. (1988) *Science* **239,** 998–1005.
2. Dekker, L. V., de Graan, P. N. E. & Gispen, W. J. (1991) *Prog. Brain Res.* **89,** 209–233.
3. Linden, D. J. & Routtenberg, A. (1989) *Brain Res. Rev.* **14,** 279–296.
4. Bramham, C. R., Lester, D. S. & Alkon, D. L. (1991) *Soc. Neurosci. Abstr.* **17,** 160.12.
5. LoTurco, J. J., Coulter, D. A. & Alkon, D. L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1672–1676.
6. Lester, D. S., Collin, C., Etcheberrigaray, R. & Alkon, D. L. (1991) *Biochem. Biophys. Res. Commun.* **179,** 1522–1528.
7. Olds, J. L., Anderson, M., McPhie, D. L., Staten, L. & Alkon, D. L. (1991) *Science* **245,** 866–869.
8. Bank, B., DeWeer, A., Kuzirian, A., Rasmussen, H. & Alkon, D. L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1988–1992.
9. Suzuki, T. & Siekevitz, P. (1989) *J. Neurochem.* **53,** 1751–1762.
10. Oda, T., Shearman, M. S. & Nishizuka, Y. (1991) *J. Neurochem.* **56,** 1263–1269.
11. Schreurs, B. G. & Alkon, D. L. (1990) *Psychobiology* **18,** 312–320.
12. Schreurs, B. G., Sanchez-Andres, J. V. & Alkon, D. L. (1991) *Brain Res.* **548,** 18–22.
13. Dunkley, P. R., Heath, J. W., Harrison, S. M., Jarvie, P. E., Glenfield, P. J. & Rostas, A. P. (1988) *Brain Res.* **441,** 59–71.
14. Carlin, R. K., Grab, D. J., Cohen, R. S. & Siekevitz, P. (1980) *J. Cell Biol.* **86,** 831–845.
15. Lester, D. S. (1989) *J. Neurochem.* **52,** 1950–1953.
16. Lester, D. S. (1990) *Biochim. Biophys. Acta* **1054,** 297–303.
17. Orr, N., Yavin, E., Shinitzky, M. & Lester, D. S. (1990) *Anal. Biochem.* **191,** 80–85.
18. Kitano, T., Go, M., Kikkawa, U. & Nishizuka, Y. (1986) *Methods Enzymol.* **124,** 349–352.
19. Orr, N., Yavin, E. & Lester, D. S. (1992) *J. Neurochem.* **58,** 461–470.
20. Burgoyne, R. G. (1989a) *Trends Biochem. Sci.* **14,** 87–88.
21. Lester, D. S. & Alkon, D. L. (1992) *Prog. Brain Res.* **89,** 235–248.
22. Zidovetzki, R. & Lester, D. S. (1992) *Biochim. Biophys. Acta* **1134,** 261–272.
23. Alkon, D. L., Kubota, M., Neary, J. T., Naito, S., Coulter, D. & Rasmussen, H. (1986) *Biochem. Biophys. Res. Commun.* **143,** 1245–1253.
24. Alkon, D. L., Naito, S., Kubota, M., Chen, C., Bank, B., Smallwood, J., Gallant, P. & Rasmussen, H. (1988) *J. Neurochem.* **51,** 903–917.