Pharmacology Biochemistry and Behavior, Vol. 46, pp. 553–560, 1993

0091-3057/93 $6.00 + .00

Environmental Stimulation Promotes Changes in the Distribution of Phorbol Ester Receptors

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Received 28 September 1992

SPIELER, K., P. SCHOCH, J. R. MARTIN AND W. HAEFELY. *Environmental stimulation promotes changes in the distribution of phorbol ester receptors.* PHARMACOL BIOCHEM BEHAV 46(3) 553–560, 1993.—The translocation of protein kinase C (PKC) from the cytosol to the membrane might be functionally involved in learning and memory. Using [^{3}H]-phorbol 12,13-dibutyrate (^{3}H-PDBu) binding three pools of binding sites could be distinguished in tissue preparations: Pool a comprised the soluble receptors which bound phorbol ester with low affinity in the absence of calcium. Pool b was composed of high-affinity phorbol ester binding sites identified in the soluble fraction upon addition of calcium. Pool c represented stably membrane-bound receptors binding phorbol ester independently of calcium. ^{3}H-PDBu binding was then measured in the cortices and hippocampi of rats trained in an eight-arm radial maze. A progressive training-dependent increase of membrane-bound binding activity with a concomitant decrease in the soluble fraction was detected independent of learning the maze task.

These results suggest that it is the experience of an enriched environment by the repeated behavioral stimulation in a maze rather than the acquisition of a memory task that leads to enhanced incorporation of phorbol ester receptors (PKC) into the cell membrane.

Protein kinase C Phorbol ester binding Behavioral stimulation Learning Subcellular distribution Calcium

IT has been hypothesized that persistent changes in protein phosphorylation that outlast an initiating signal may be an important early molecular event in the acquisition and storage of memory (28,34,43). The relevance of such changes is supported by the finding that nonassociative (22) and associative learning (5,7), as well as long-term potentiation (LTP) of synaptic transmission (29), are all associated with enhanced protein phosphorylation. There is evidence from a number of studies that one of the early key events in memory formation is the activation of protein kinases C (PKC). This family of ubiquitous calcium- and phospholipid-dependent enzymes exists in the cell in both soluble and membrane-bound forms. Catalytic activation appears to involve a transfer of the enzyme from the cytoplasm to the plasma membrane. This intracellular redistribution of PKC occurs in response to the second messengers diacylglycerol (DAG) and calcium (36,45) and is therefore part of the signal transduction cascade triggered by receptors that couple to phospholipase C. Protein phosphorylation by PKC affects among many other functions membrane conductivity and neurotransmitter release (31,41). PKC has been implicated in the neuronal plasticity underlying, for example, associative conditioning of the marine snail Hermissenda with a paired light-rotation training protocol (6,16) and tone–air puff classical conditioning of the rabbit nicitating membrane reflex (9), as well as in LTP of synaptic transmission in hippocampal slices (3,8,27) and in memory formation of the chick (15). Ligand labeling of PKC with tritiated phorbol 12,13-dibutyrate (PDBu) in the hippocampus of conditioned rabbits suggested that translocation of PKC in the CA1 and CA3 regions is a time- and information processing–dependent process (38). PDBu binding activity co-chromatographs with enzymatic activity of PKC through several purification steps (23,35,40). PDBu binding, however, was found to be less affected by the experimental conditions than the catalytic activity (11) and, therefore, represents a more convenient measure for detecting the presence of the enzyme. Reports on co-factor requirements for binding of phorbol ester to PKC are contradictory. In particular, the calcium requirement appears to be dependent on assay conditions (21,42) and on the phospholipid composition of the membranes (25). The interaction of PKC with phospholipids was proposed to occur in two steps (20,45): a calcium-dependent loose association of PKC with phospholipids followed by its stable insertion into the hydrocarbon region of the bilayer whereby the enzyme

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acquires properties of a membrane protein. This latter form of PKC displayed, on the one hand, constitutive in vitro kinase activity (calcium- and phorbol ester–independent) and, on the other hand, was able to bind phorbol ester independently of calcium (12). The first part of the present study explores the experimental conditions that allow the measurement of the cellular distribution of phorbol ester receptors between particulate and soluble fractions. Tissue fractionation was performed in the presence of EGTA to prevent translocation of shiftable phorbol ester receptors to the membrane during homogenization. Under these conditions phorbol ester binding to the particulate fraction was calcium-independent. In the soluble fraction, however, two pools of phorbol ester binding activity, calcium-independent and calcium-dependent, were detected.

The second part addresses the question of whether maze learning affects the distribution of phorbol ester receptors in the rat brain. For this reason soluble and particulate tissue fractions from the hippocampus and cortex were investigated for phorbol ester binding. We found that maze learning itself had no effect on phorbol ester binding activity and distribution in either fraction and brain region. However, the prolonged behavioral stimulation of experimental and control rats within the defined experimental paradigm led to an increase of the tightly membrane-associated and a concomitant reduction of the calcium-dependent soluble pool of phorbol ester receptors independently from maze learning. These results are discussed within the context of an involvement of PKC in neuronal plasticity in response to behavioral manipulations.

MATERIALS AND METHODS

Materials

[^{3}H]-phorbol-12,13-dibutyrate ([^{3}H]PDBu) in ethanol solution (13.2 Ci/mmol) was purchased from New England Nuclear (Boston), evaporated under nitrogen, and redissolved in dimethyl sulfoxide (DMSO). Polyethyleneimine, EGTA, leupeptin, phorbol 12-myristate 13-acetate (PMA), benzamidine, and aprotinin (A-6279) were from Sigma (St. Louis); sodium-tetrathionate dihydrate, 1,4 dithio-DL-threitol (DTT), and phenylmethansulfonylfluoride (PMSF) from Fluka (Buchs, Switzerland); and $MgCl_2$, $CaCl_2$, and Titriplex III (EDTA) from Merck (Darmstadt, Germany).

Methods

[^{3}H]PDBu binding to membrane and cytosolic fractions of tissue homogenates.

Preparation and fractionation of tissue homogenates. Female RORO SPF rats (a registered strain derived from Wistar rats, Biological Research Laboratories, Füllinsdorf, Switzerland) were sacrificed by decapitation. Brain tissue samples (0.05–0.1 g) of the hippocampus and cortex were dissected on ice and homogenized by hand in 300 μl ice-cold buffer I (25 mM Tris, 100 mM NaCl, 3.2 mM $MgCl_2$, 10 mM EGTA, 1 mM EDTA, 2 mM DTT, 5 mM sodium tetrathionate, 2.5 mM benzamidine, 1 mM PMSF, leupeptin 2 mg/100 ml, aprotinin 0.1 ml/100 ml, pH 7.5) for 10 s using a Teflon pestle especially manufactured for Eppendorf tubes. Each homogenate was diluted (to a final concentration of 3.3 mM EGTA) with ice-cold buffer II (25 mM Tris, 100 mM NaCl, 3.2 mM $MgCl_2$, pH 7.5) before it was separated into soluble and particulate fractions by centrifugation at 4°C for 30 min. Small samples (0.9 ml) to be used for binding experiments at a single [^{3}H]PDBu concentration were centrifuged at 10 000 × *g*. Pooled samples (5.4 ml) for the determination of B_{max} and K_d were fractionated at 50 000 × *g*. The resulting supernatant was adjusted to 0.3 mM EGTA by appropriate dilution with ice-cold buffer II. The pellet was usually washed once with the homogenization buffer and resuspended in ice-cold buffer II. Protein content was determined with the BCA (bicinchoninic acid) protein assay supplied by PIERCE (Rockford, IL).

[^{3}H]PDBu binding. The incubation was carried out under continuous shaking at room temperature for 30 min in a total volume of 1 ml containing 0.05–0.11 mg tissue. The concentration of [^{3}H]PDBu was either fixed at 2.5 nM or varied between 1 nM and 100 nM in the case of saturation binding experiments. "Basal binding" was measured in the absence of exogenous calcium and "calcium-stimulated" binding in the presence of 1 mM $CaCl_2$. Nonspecific binding was determined in the presence of an excess of unlabeled PMA (20 μM), and specific binding was calculated for each sample as the difference between total and nonspecific binding. All measurements were done as duplicates. The separation of bound from free [^{3}H]PDBu was performed by filtration (using a Brandel cell harvester) through Whatmann GF/C filters (Maidstone, Kent, UK) presoaked in 0.3% polyethyleneimine. The filters were rinsed with 8 ml ice-cold washing buffer (25 mM Tris-HCl, 100 mM NaCl, 0.3% polyethyleneimine, pH 7.0) and dispersed in 10 ml of scintillation fluid (Ultima Gold, LKB, Duebendorf, Switzerland). The radioactivity associated with the filters was measured in a Packard scintillation counter.

Data analysis. [^{3}H]PDBu binding was analysed according to the law of mass action using the curve-fitting program LIGAND (33) or by weighted, least-square regression to the Scatchard formula using the program RS1/1 Release 4.3.1 (1991) by Bolt Beranek and Newman Inc. (Cambridge, MA).

Behavioral training.

Maze learning. Female RORO SPF rats weighing 150–200 g were used. The animals were housed individually in a colony room with a 12-h light/dark cycle. Water was freely available at all times. Beginning four days prior to behavioral training the animals were reduced to 85% of their free-feeding weight by limiting their daily supply of food. Food deprivation was maintained throughout the experiment.

The apparatus for maze learning was an elevated eight-arm radial maze made of grey plastic material. The eight maze arms extended outward from an octagonal central arena. Removable barriers of the same material were used to close selected arms, preventing entry from the central arena. All eight arms of the maze were baited by placing a 45-mg food pellet in a small cup located at the end of an arm. In a first trial, access to only four baited arms was permitted. Any subsequent visit (visit of an arm = run) to these arms (they were not rebaited) during this trial was defined as a working memory error. The trial was completed when each of the four arms was visited at least once, and the rat was returned to the home cage. The formerly closed doors were opened, providing access to those arms which were previously denied (and which were still baited). The rat was then placed in the central arena for the subsequent second trial. It had now to discriminate the arms entered during the previous trial from those that still contained food to complete the session. Any visit to the arms accessible in the first trial which were no longer baited was counted as reference memory error, any repeated visit to the other arms was again counted as working memory error. Animals were trained twice daily until they performed the task at least twice successively without any error. Training to the criterion of two successive sessions without error required

from 5 to 14 sessions. Control animals were also placed into the maze with all doors open but with the arms unbaited. Thus, the control rats were able to explore the apparatus as many times as the experimental group of rats, but were not forced to learn the memory task.

RESULTS

Phorbol Ester Binding to Particulate and Soluble Brain Fractions

Cellular distribution and phorbol ester binding activity of PKC are regulated (among others) by calcium and phospholipids (mainly phosphatidyl serine [PS]). This has to be taken into consideration when studying its localization in membrane and cytosolic tissue fractions by [^{3}H]-PDBu binding. Therefore, hippocampal and cortical tissues were homogenized in the presence of EGTA to prevent calcium-dependent "loose" binding of PKC to the membrane during homogenization or to reverse it if it had occurred prior to this procedure. Binding was then measured in the absence (basal) or presence (calcium-stimulated) of added calcium at a single [^{3}H]PDBu concentration of 2.5 nM.

Binding to the membrane-bound phorbol ester receptor was only slightly higher in the presence than in the absence of calcium (Fig. 1A). This phorbol ester binding activity probably represents the stably membrane-inserted pool of PKC which is only poorly sensitive to exogenously added calcium (12).

The cytosolic fraction, in contrast to the particulate fractions, showed low basal binding activity. It could, however, be significantly enhanced by the addition of 1 mM calcium (Fig. 1A).

To distinguish between effects of calcium on B_{max} and K_d, saturation [^{3}H]-PDBu binding experiments were performed. It was found to be saturable and to increase in proportion to the amount of protein in the assay (data not shown). Scatchard plots were linear under all experimental conditions.

In the *particulate* fraction, the number of binding sites (B_{max}) was found to be similar in the presence and absence of added calcium (Fig. 1B). However, in the presence of calcium they exhibited a slightly higher affinity (Fig. 1C).

In the *soluble* fraction two distinct pools of phorbol ester receptors could be detected: One was calcium-independent, the other needed calcium for binding to occur. Addition of calcium to cytosolic fractions increased the binding activity by augmenting the number of binding sites (Fig. 1B) as well as the affinity for [^{3}H]PDBu (Fig. 1C). Comparison of [^{3}H]PDBu binding in the presence or absence of calcium allows one therefore to monitor two pools of PKC in the soluble fraction.

To exclude the possibility of loosing low-affinity phorbol ester binding during filtration of soluble fractions we did the following control experiment: Tissue homogenates were prepared in parallel in the presence of EGTA (as usual) and in its absence. The soluble fractions were analyzed for ^{3}H-PDBu binding without adding calcium to the assay medium. They displayed a considerable difference of K_d: high affinity (~10 nM) in preparations obtained in the absence of EGTA and low affinity (~46 nM) in preparations obtained in the presence of the calcium chelator. No difference, however, was found for B_{max}. This indicates that the soluble, calcium-independent pool of PKC can be accurately measured using a filtration technique even if PKC displays decreased affinity for phorbol esters.

Purified PKC was shown earlier to bind calcium only in the presence of acidic phospholipids (13). We found, however, that [^{3}H]PDBu binding did not increase upon addition of PS in either fraction (data not shown), indicating that enough phospholipids remained associated with the enzyme, even in the soluble fraction. This is in line with similar findings obtained by others (26).

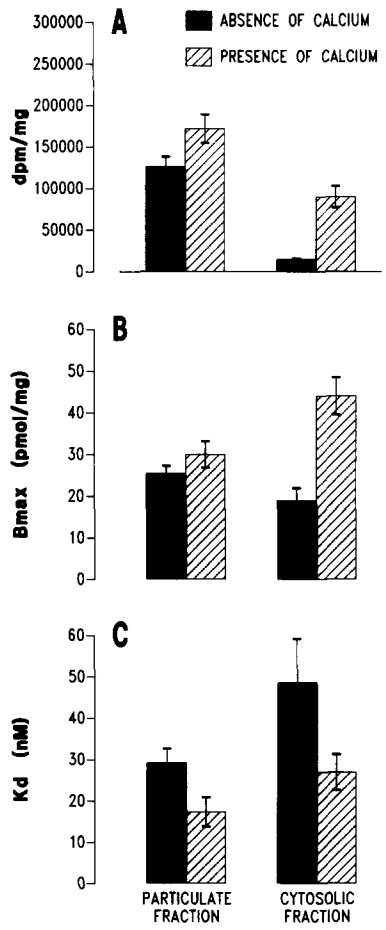

FIG. 1. (A) Basal and calcium-stimulated [^{3}H]PDBu binding in the particulate and soluble fractions of hippocampus. Single hippocampi were homogenized in the presence of EGTA as described in Methods. [^{3}H]PDBu (2.5 nM) binding was performed in the presence or absence of 1 mM $CaCl_2$, corresponding to calcium-stimulated and basal binding conditions, as described in Methods. Values represent means ± SE of 11 independent experiments. (B and C) B_{max} and K_d values of [^{3}H]PDBu binding to particulate and soluble fractions of cortical tissue. Single cortical hemispheres were homogenized and separated into particulate and soluble fractions as described in Methods. Binding was measured at six different concentrations of [^{3}H]PDBu, and B_{max} and K_d were determined by Scatchard plot analysis. The experiment was repeated three times. Similar results were obtained with corresponding fractions from hippocampal and striatal tissue (data not shown). Black columns: basal binding (without addition of calcium). Hatched columns: calcium-stimulated binding (in the presence of 1 mM calcium).

In conclusion, with EGTA in the homogenization buffer a particulate fraction was obtained that carried membrane-associated phorbol ester binding sites which were more or less calcium-independent. In contrast, the soluble fraction contained two pools of phorbol ester receptors: a calcium-

independent one that bound phorbol ester with low affinity and a second one that was revealed only upon addition of calcium, but which then displayed high affinity.

[^{3}H]PDBu Binding in Brain Fractions of Radial Maze Performing Animals

Rats were trained daily for radial maze performance until they fulfilled the criterion of accomplishing the task without error in two subsequent sessions as described in the Methods section (Fig. 2). Controls were allowed to explore the entire maze but none of the arms were baited. When an experimental rat had successfully reached criterion performance in the maze task it was sacrificed together with a control rat. The cortex and the hippocampus were dissected out and further processed into membrane and cytosolic fractions as described above. [^{3}H]PDBu binding was measured in both the absence and the presence of 1 mM calcium to determine the distribution of PKC under basal and stimulating conditions, respectively. Comparison of binding data between maze learners and controls revealed no statistically significant difference in either brain region investigated (Fig. 3). In addition, binding activity did not correlate with the number of errors committed by experimental rats during the acquisition period. Since some of the experimental rats learned rapidly whereas others needed more time to accomplish the memory task (Fig. 2), the phorbol ester binding data for each individual rat were also evaluated in relation to the total number of runs performed to the maze arms during all the sessions until fullfillment of the criterion. For control rats the total number of visits into arms during all the sessions was used in this analysis. This revealed interesting correlations between [^{3}H]-PDBu binding activity and total number of runs. Since control and maze animals did not differ in this respect, their data were pooled for all further evaluations. In the particulate fractions of the two brain areas investigated the levels of phorbol ester binding correlated strongly with the total number of runs (Figs. 4 and 5). This increase in [^{3}H]PDBu binding with increasing number of runs was found under basal ($p < 0.001$) as well as under calcium-stimulated conditions ($p < 0.01$) (Table 1). In the cytosolic fractions the calcium-independent sites were not affected by the number of runs in any of the areas investigated, whereas the calcium-dependent phorbol ester binding in the soluble fraction of the hippocampus correlated negatively with the number of runs (Fig. 4). A weaker, nonsignificant correlation was observed for the soluble fraction of the cortex (Fig. 5, Table 1).

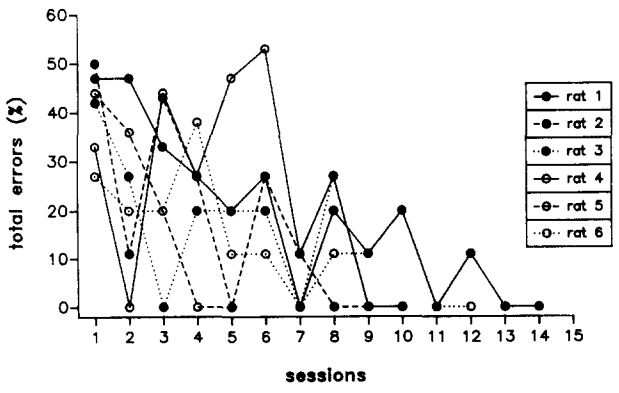

FIG. 2. Learning curves of six maze learners. Animals were trained until they performed the task of maze learning in at least two subsequent sessions without any error. Total of errors (sum of working and reference memory errors) is expressed as percentage of total number of runs.

DISCUSSION

The high-affinity phorbol ester binding site on PKC offers a convenient way to study the cellular distribution of this enzyme in soluble and particulate tissue fractions and hence translocation between cytoplasma and membrane. The unique role of PKC in neuronal signal transduction resides in its property to link two signaling pathways by responding to calcium and diacylglycerol with a translocation from the cytosol to the plasma membrane associated with an increase in its catalytic activity. This ability to associate two different stimuli and to transform them into a long-lasting change of the phosphorylation status of membrane proteins led to the hypothesis that PKC is involved in neuronal plasticity by affecting, for example, excitability and/or neurotransmitter release (7,10,38). Of particular interest is the involvement of PKC in learning and memory. A critical issue when studying translocation of PKC is the preservation of its in vivo distribution during homogenization and fractionation of the tissue. In the present study we used EGTA in the homogenization buffer to minimize exposure of intracellular PKC to the high extracellular calcium concentration and to the calcium released from intracellular stores upon rupture of the cells. This procedure should, on the one hand, prevent artifactual calcium-dependent membrane-association of PKC during homogenization. On the other hand, it might strip off the PKC loosely attached to the membranes in vivo prior to homogenization. The PKC recovered in the particulate fraction under our conditions is therefore likely to represent the tightly membrane-bound enzyme. This is supported by the independence of [^{3}H]-PDBu binding from exogenous calcium, a property typical for membrane-inserted PKC (12). In the soluble fraction two pools of phorbol ester receptors could be distinguished on the basis of their distinct requirement for calcium. Measurement of [^{3}H]-PDBu binding in the absence and presence of calcium therefore allowed an estimate of changes in the size of each pool.

PKC is known to be subject to regulation not only by calcium and lipids, but also by proteolysis (19). Artifacts due to proteolytic degradation were minimized by the inclusion of EGTA in the homogenization process, which should prevent activation of calcium-dependent proteases, as well as by the use of PMSF, an irreversible serine protease inhibitor.

PKC represents a growing family of related kinases which all bind phorbol esters with high affinity. They are heterogenous, however, with regard to their calcium dependence, cellular localization, and ability to translocate between cytosolic and membraneous compartments (4,14,18,37,39). No attempt was made in this study to assign phorbol ester binding activities in the different pools to a specific PKC isoform described in the literature. Nevertheless, since the β-isoform is the major PKC expressed in the brain (44) and since the change of phorbol ester binding upon behavioral stimulation is so substantial, it is tempting to speculate that the β-isoform is part of the process.

In addition to PKC isoforms, a phorbol ester binding protein, N-chimaerin, has recently been identified that does not belong to the PKC family (1,2). It binds phorbol ester independent of calcium, which suggests that the major calcium-

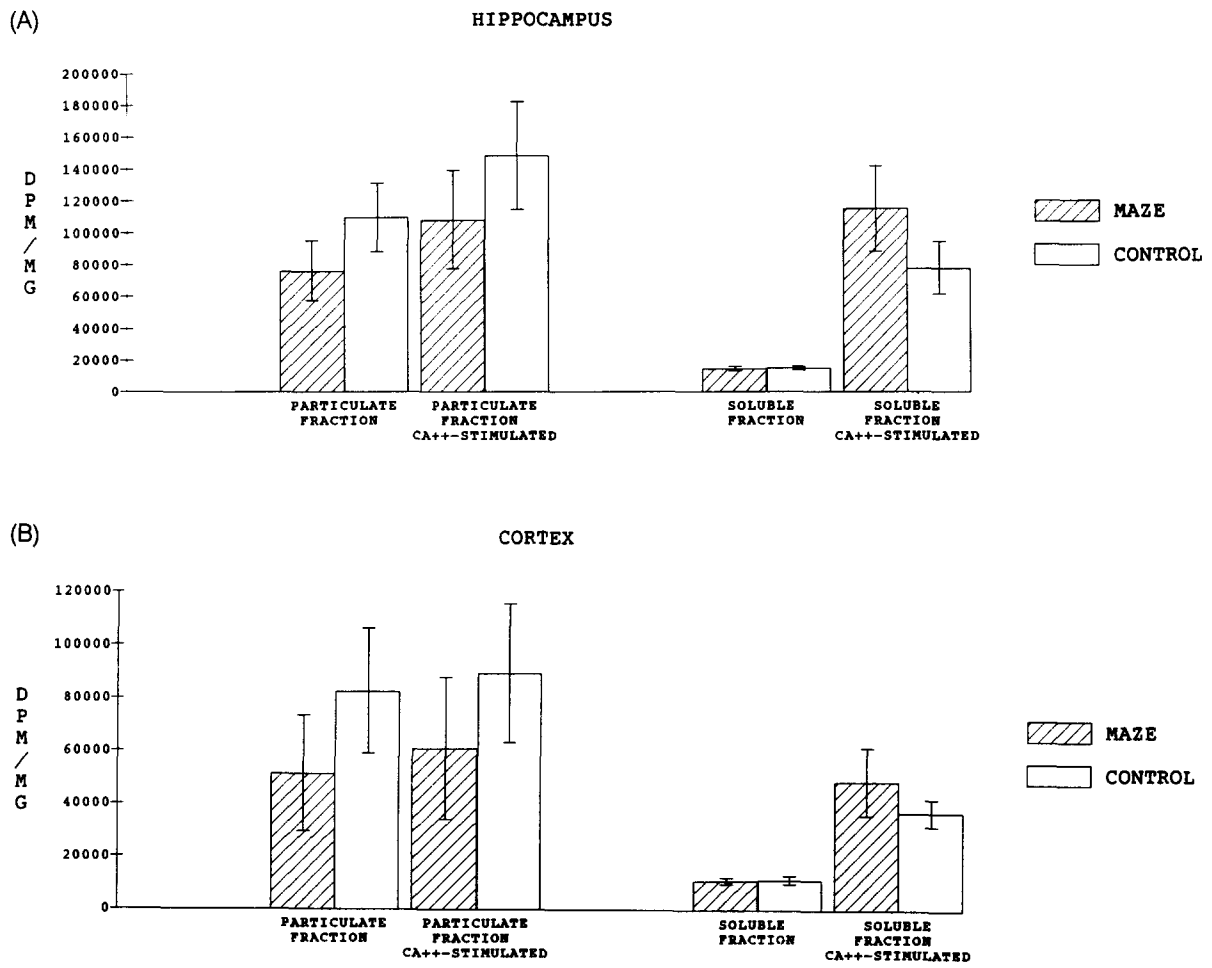

FIG. 3. Binding to soluble and particulate fractions of hippocampal (A) and cortical (B) tissue of maze learners and their controls (N = 6). No statistical difference was found. The large standard errors of the means (SE) result from the large range of individual values of rats requiring vastly differing numbers of runs to reach criterion (see Figs. 4 and 5). The tendency of lower binding in particulate and higher, calcium-dependent binding in soluble fractions of learners versus controls is due to the fact that learners performed on average somewhat fewer runs than their individual controls. It therefore has no bearing on a specific maze learning effect.

regulated phorbol ester binding protein still is PKC. It cannot be excluded, however, that proteins which bind phorbol ester in the absence of calcium ("basal binding") consist also of N-chimaerin in addition to PKC isoforms lacking the calcium-binding regulatory domain. Our findings and conclusions with respect to calcium-dependent phorbol ester binding should therefore not be affected by this new class of phorbol ester binding proteins.

As a behavioral paradigm to study learning-associated changes in phorbol ester binding and, hence, PKC localization (and activation) we used radial maze performance. To reduce interindividual variations we trained all animals until they performed the maze task without error in two successive sessions. To reach this criterion we had to train the animals twice a day during a total of 5 to 14 sessions. Some animals were therefore handled much less, requiring only about 60 runs to acquire correct behavior. Others needed up to 160 runs until they achieved criterion performance. In parallel, for each experimental rat trained for maze performance, a control animal was exposed to the same environment for the same time without closing and baiting any maze arms (i.e., without having to learn the memory task).

A careful comparison of individual pairs of learners and control animals did not reveal any difference in phorbol ester binding in the soluble or particulate fractions of the hippocampus or cortex (Fig. 3), either in the absence or in the presence of calcium. It is concluded from these results that spatial learning per se does not involve an intracellular shift or activation of PKC, at least not to the extent that it could be detected by radioligand binding in homogenized tissue fractions. Therefore, results from learners and control animals were pooled and analyzed for dependence of phorbol ester binding on the amount of behavioral stimulation produced by the repeated handling and exposure of the animals to the maze environment. This environmental stimulation is represented by the parameter runs, which also depend on curiosity and activity of the individual rats. A strong positive correlation between phorbol ester binding in the membrane fractions of the cortex and hippocampus and the duration of environmental stimulation was found. This was accompanied by a corresponding decrease of binding activity in the calcium-dependent, soluble pool, suggesting that the experience of repeated handling and exploration of the maze led to an enhanced incorporation of translocatable PKC into the membrane. This

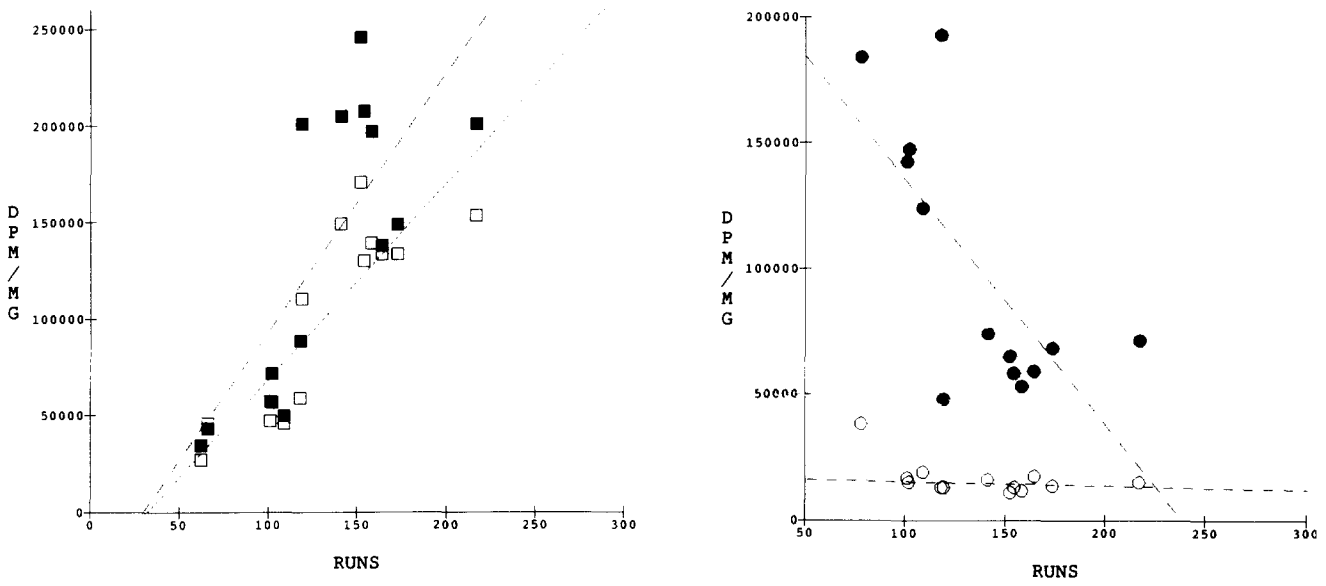

FIG. 4. [^{3}H]PDBu binding obtained in the particulate (left diagram) and in the soluble (right diagram) fraction of the hippocampus is plotted against the total number of runs in the maze. Each pair of a filled and open symbol represents the values of one rat. ■: binding in the presence of 1 mM calcium, □: no calcium added (N = 14). The number of runs (i.e., visits to baited and unbaited maze arms) was correlated with [^{3}H]PDBu binding by linear regression analysis.

incorporation apparently occurred in a quasi-irreversible way, since it could not be reversed by subsequent calcium sequestration during homogenization. Translocation of PKC is thought to be a graded response to enzyme activation (21,45): Mild activation leads to reversible membrane association, whereas strong activation, triggered by persistent and high intracellular calcium (and DAG) levels, leads to stable insertion of the enzyme into the membrane (11). Based on the current knowledge, brief and weak behavioral stimulation might therefore lead to reversible activation and translocation of PKC, which after repeated experience of such an enriched environment eventually results in persistent insertion of PKC into the membrane. Alternative interpretations, however, are possible. The additional membrane-associated enzyme might also be recruited from de novo synthesis of PKC, and the loss of soluble enzyme might be due to calcium-dependent

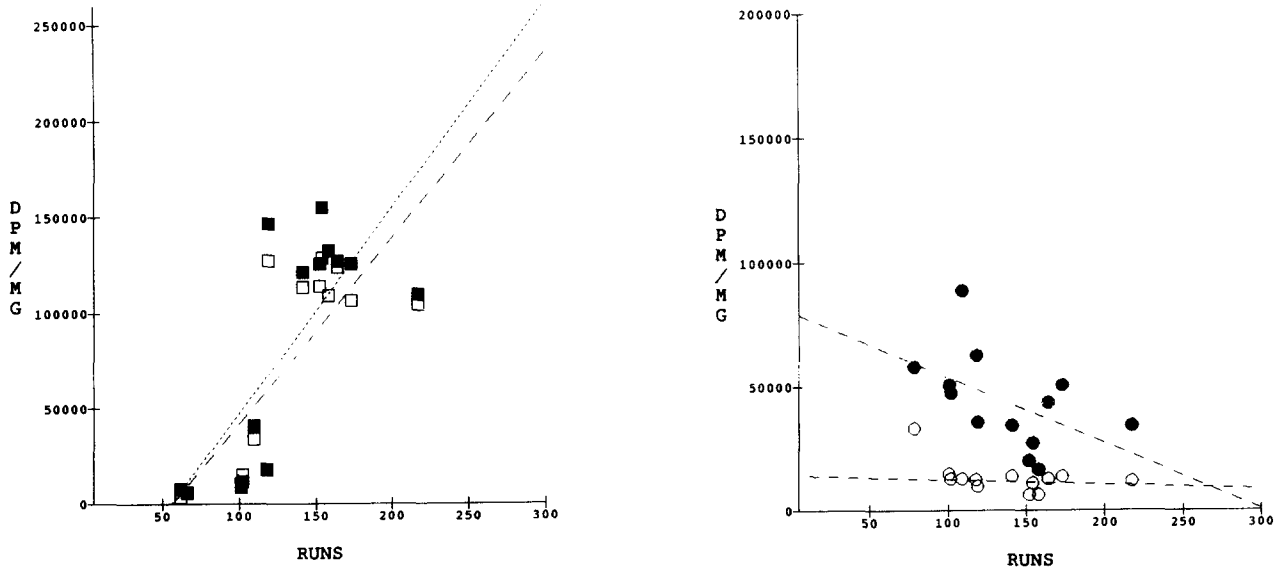

FIG. 5. [^{3}H]PDBu binding obtained in the particulate (left diagram) and in the soluble (right diagram) fraction of the cortex is plotted against the total number of runs in the maze. Each pair of a filled and open symbol represents the values of one rat. ■: binding in the presence of 1 mM calcium, □: no calcium added (N = 13). The number of runs (i.e., visits to baited and unbaited maze arms) was correlated with [^{3}H]PDBu binding by linear regression analysis.

TABLE 1

STATISTICAL PARAMETERS OF THE REGRESSION LINES IN FIGS. 4 AND 5

	Hippocampus		Cortex	
	p	*R*	*p*	*R*
Soluble Fraction				
Basal	0.49	−0.22	0.47	−0.23
CA^{++}-Stimulated	0.007	−0.71	0.07	−0.51
Particulate Fraction				
Basal	0.00008	0.86	0.0009	0.78
CA^{++}-Stimulated	0.0017	0.76	0.0018	0.76

proteolysis, as it was observed in vitro using purified enzyme (24).

Exposure of rats to a novel environment for several weeks was shown to result in enlarged dendritic fields and enhanced synaptic transmission in the hippocampal formation (17). The present findings of a redistribution of phorbol ester binding activity might be part of such a plastic response to environmental stimulation.

A reversible, EGTA-labile membrane-association of PKC, operative in the acquisition of cognitive responses, cannot be excluded however. Concomitant to the increase of membrane-bound phorbol ester receptors the soluble pool strongly decreased. Whether this is due to a translocation followed by a stable insertion of shiftable PKC into the membrane or to changed turnover of soluble and membrane-bound protein is not known. Furthermore, our study is limited to the subcellular translocation of PKC. It is, however, silent on the consequences of protein phosphorylation in learning and environmental stimulation. This may be important, since it is conceivable that under certain conditions PKC redistribution and enzyme activation might be regulated independently from each other. Moreover, our work is confined to the translocation of PKC in the hippocampus and cortex. While important associative phenomena occur in these locales, other learning-related areas (e.g., deep cerebellar nuclei) might well exhibit stronger coupling between associative learning and PKC translocation. It is also possible that the chosen approach of binding studies in tissue homogenates does not provide sufficient spatial resolution to detect PKC translocation in limited and specific cell populations. Autoradiographic studies of phorbol ester binding in tissue slices might therefore be appropriate to study this question. Another caveat relates to the fact that all experimental subjects were studied at behavioral criterion. There are, however, electrophysiological data from LTP studies in rat hippocampal slices showing that PKC translocation is involved in the induction but not in the expression of LTP (31,33). This might indicate that PKC translocation is more prominent during acquisition of memory than when criterion has been attained. Nevertheless, our findings of an intracellular redistribution of phorbol ester binding sites during repeated environmental stimulation, but not in response to learning a maze task, is consistent with an involvement of PKC in neuronal plasticity. Because it is difficult to separate the experience of an enriched environment from learning, these findings do not exclude the possibility that PKC is involved in certain forms of memory or behavioral adaptations. In addition, the results emphasize the importance of appropriate controls in studies on mechanisms of learning and memory.

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