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Translocation of Protein Kinase Cγ Occurs During the Early Phase of Acquisition of Food Rewarded Spatial Learning

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This study describes the translocation of the brain specific protein kinase C gamma isoenzyme (PKCγ) in the hippocampus during food rewarded spatial learning. The holeboard test was used for spatial orientation, and immunoblot analysis was used for assessment of PKCγ in cytosolic, membrane-inserted and membrane-associated fractions. Membrane-associated PKCγ was increased during early acquisition of spatial learning, but not in a later phase of training. This transient and apparently temporary intracellular PKCγ translocation was only observed in the posterior but not in the anterior hippocampus, and was only detected within 10 min after termination of the learning trial. This study supports the idea that PKCγ is significantly involved in the biochemical events underlying learning and memory, notably during the period of novel information processing. The results further promote the hypothesis that the hippocampus is specifically involved in temporal information processing, which requires the engagement of PKCγ.

Protein kinase C (PKC) comprises a family of serine–threonine protein kinases with at least 12 members (Nishizuka, 1995; Pears, 1995). PKC isoenzymes are classified into three groups, conventional, novel, and atypical, with different distributions, substrates, and cofactor specificity. This suggests that different isoforms may control different physiological events (Hug & Sarre, 1993; Nishizuka, 1995; Tanaka & Saito, 1992). It is generally accepted and well documented that PKC is involved in a variety of processes, including growth, neural development, synaptic transmission, and muscle contraction (Liu, 1996; Nishizuka, 1995; Sunayashiki-Kusuzaki, Lester, Schreurs, and Alkon (1993), whereas the brain specific PKC gamma isoenzyme (PKCγ) is particularly involved in spatial learning and memory processes (Bowers et al., 1995; Olds, Anderson, McPhie, Staten, & Alkon, 1989; Routtenberg, 1985; Van der Zee, Compaan, De Boer, & Luiten, 1992; Van der Zee & Douma, 1997; Van der Zee, Kronforst-Collins, Maizels, Hunzicker-Dunn, & Disterhoff, 1997). The putative role of PKCγ in learning was substantiated by striking increases in PKCγ-immunoreactivity in rat, mouse, and rabbit hippocampus (Beldhuis, Everts, Van der Zee, Luiten, & Bohus, 1992; Douma, Van der Zee, & Luiten, 1997; Van der Zee et al., 1992, 1997).

There is ample evidence that PKC becomes activated by association with increased levels of diacylglycerol (DAG) in the membrane (Bell & Burns, 1991; Newton, 1995; Nishizuka, 1986). DAG is produced as a result of hydrolysis of inositol phospholipids by agonist-induced activation of phospholipase C. The activation of calcium- and phospholipid-dependent PKC (conventional PKC) is associated with a shift in the subcellular distribution of this enzyme from a cytosolic localization to a membrane-bound state (Nishizuka, 1986). Data regarding such translocation of PKC, however, are somewhat inconsistent throughout the literature and suggest that the intracellular shifts of the enzyme are related to temporal characteristics of the learning paradigm that is used. Akers, Lavinger, Colley, Linden, and Routtenberg (1986) proposed that hippocampal PKC is translocated from the cytosol to cell membrane after the induction of long-term potentiation (LTP), an activity-dependent enhancement of synaptic efficacy that is used extensively as a physiological model of learning. However, in contrast to these findings, a rapid and transient increase in cytosolic PKC activity was described after induction of LTP in hippocampal slices (Otani & Ben-Ari, 1993). In addition, Angenstein, Riedel, Reymann, and Staak (1994) reported that the γ isoenzyme in rat hippocampus was increased in the cytosolic fraction after tetanic stimulation of the perforant pathway in vivo.

Changes in subcellular distribution of PKC have been shown to occur after classical conditioning of the nictitating membrane–eyelid response (Bank, Deweer, Kuzirian, Rasmussen, & Alkon, 1988). In this study, translocation of hippocampal PKC from the cytosol to the cell membrane was reported 24 hr after the rabbits were overtrained. However, Sunayashiki-Kusuzaki et al. (1993), using the same associative learning paradigm in the rabbit, did not find PKC translocation at the 24 hr posttraining time point. Likewise, Van der Zee et al. (1997) demonstrated the absence of an altered PKCγ distribution 24 hr after trace eyeblink conditioning, a more complex and hippocampally dependent learning task.

The results described above clearly demonstrate that PKC is involved in memory processes (see Van der Zee & Douma,

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1997, for review), but that the translocation cascade of the enzyme may be more complex than previously thought and dependent on a variety of experimental conditions. In particular, time-related parameters appeared to be highly relevant determinants for the intracellular location of PKC. More specifically, it may well be that the reported lack of translocation of PKCγ in the previous reports may be due to the choice of the 24 hr posttraining time point in fully trained subjects. In this report, we therefore further investigated the nature of behaviorally induced translocation of PKCγ at different time points during the acquisition in a spatial learning task.

Material and Method

The findings presented here were obtained from 18 young adult male Wistar rats (±330 g body weight). The rats were group housed (6 per cage) and kept on a 12-hr light–dark cycle (lights on between 0700 and 1900 hr). All rats had free access to standard rat chow and tapwater. The rats were divided into three groups: C (controls; $n = 6$), T4 (trained to Day 4; $n = 6$), and T11 (trained to Day 11; $n = 6$). This division between T4 and T11 was based on their rate of acquisition during training. Rats with a sharp rise in the acquisition of spatial orientation on Day 4 were selected as T4. The behavioral training as described below was carried out during the light period (between 0900 and 1600 hr). The rat experiments were approved by the Committee on Animal Bio-Ethics of the University of Groningen.

Spatial Orientation Task

All rats of the T4 and T11 groups were trained in a spatial discrimination task using a holeboard described originally by Oades and Isaacson (1987). The holeboard is composed of a square arena (70 × 70 × 45 cm) made of polyvinyl chloride (PVC), containing four rows of four equidistant holes (14 cm apart, 3.5 cm diameter, depth of 3 cm) in the floor plate (Oades, 1981). A start box was attached to one of the PVC walls of the holeboard. Four holes arranged in a fixed pattern were baited with chocolate chips. In addition, each hole was supplied with chocolate chips covered by a replaceable, perforated false bottom to mask potential odor cues emanating from the reward in the baited holes. Thus, the rats were unable to discriminate between baited and unbaited holes by orientating on olfactory cues in the holeboard in the training phase of the test. The holeboard was placed in a dimly illuminated room that contained a number of distinctive extravisual cues to enhance spatial orientation. Prior to training, the rats were familiarized with the holeboard in two 3-min trials on 5 consecutive days. During this habituation period, all holes were baited with an accessible chocolate chip. A trial was started by placing the rat in the start box. A guillotine door was lifted after 10 s, giving the rat free access to the holeboard. Once the rat had entered the arena, the guillotine door was lowered. If a rat had not left the startbox within 2 min, it was gently pushed into the arena. The floor of the start box and holeboard arena was cleaned with a wet and a dry cloth between two trials. After the habituation period, the rats of the T4 and T11 groups were subsequently exposed to two training trials, one in the morning and one in the afternoon. The rats of the T4 group were anesthetized and decapitated 10 min after the morning trial on Day 4 and the rats of the T11 group were anesthetized and decapitated 10 min after the morning trial on Day 11. In the training trials, a fixed set of four holes arranged in a symmetrical pattern were baited (Oades, 1981). A hole visit was scored when the rat entered its nose in a hole. Hole visits were registered manually using a computer assisted recording device. A trial was terminated either when 3 min had elapsed or when the rat had found and consumed all food pellets in the baited holes. From these data, the reference memory scores were calculated. Results of the morning and afternoon trials were averaged. The reference memory ratio was defined as the number of visits and revisits to the baited set of holes divided by the total number of visits and revisits to baited and nonbaited holes.

Preparation of the Fractions

The rats were anesthetized with ether and decapitated 10 min after the final training trial. The brains were rapidly removed and placed in ice-cold phosphate-buffered saline (0.01 M, pH = 7.4) for 1 min. Next, the two hippocampi were dissected and divided into an anterior and a posterior part (at intra-aural 4.2 coordinate according to the atlas of Paxinos & Watson, 1982). Subcellular fractionation into cytosolic, membrane-associated and membrane-bound fractions was based on the method used by Angenstein et al. (1994) and Staak, Behnisch, and Angenstein (1995). In short, the isolated tissue was then homogenized by sonification in 18 μl ice-cold buffer A per 1 mg hippocampal tissue (18 μl/mg). The homogenate buffer A composed of 25 mM Tris-HCl (pH = 7.6), 250 mM sucrose, 1 mM ethylene glycol-bis(β-aminoethylether)-N,N,N′,N-tetraacetic acid (EGTA), 5 mM dithiothreitol, 1 mM $CaCl_2 \cdot 2H_2O$ (pH = 7.6), 1 mM sodiumorthovanadate, and 0.04 g/ml protease inhibitor cocktail tablet (Boehringer; Mannheim, Germany). The homogenate was centrifuged at 100 000 × g for 30 min to yield cytosolic fractions (CF) and pellet fractions. The pellet was resuspended by sonification in 3 μl/mg ice-cold homogenate buffer A containing 10 mM EGTA (final concentration) and 2 mM ethylenediamine tetraacetic acid (EDTA), and was again centrifuged at 100,000 × g (gravity) for 30 min to obtain a supernatant of EGTA-extractable membrane-associated protein fraction (AF). The resulting pellet was resuspended in buffer B (17 μl/mg) containing 0.5% Tween-20 and was incubated for 45 min at 4 °C to yield the membrane-bound fraction (MF). Aliquots of these fractions were prepared for Western-blot analysis and protein determination (Bio-Rad Protein Assay, Veenendaal, The Netherlands). The entire isolation procedure was carried out at 4 °C. Proteins were separated by SDS-PAGE (sodium docedylsulfate polyacrylamide gel electrophoresis; 8% gel) and transferred to Nytran membrane (Schleicher & Schuell, 's Hertogenbosch, The Netherlands). Immunoblot analysis was carried out with the primary antibody mouse anti-PKCγ immunoglobulin, (IgG)–(36G9; 1:100). The 36G9 monoclonal IgG antibody raised against purified bovine PKCγ protein (Cazaubon, Marais, Parker, & Strasberg, 1989) was donated by A. D. Strosberg and S. Cazaubon, Paris. Next, the blots were exposed for 2 hr at room temperature to Goat antimouse horseradish peroxide–(HRP); 1:800, Bio-Rad Protein Assay. Finally, immunolabeling was visualized by reacting with 0.03% diaminobenzidine and 0.01% H_2O_2.

Data Analysis

The immunoblots were scanned by image analysis on a Leica on the Quantimet 500 system (Rÿswÿk, The Netherlands) and were quantified with Sigmagel (Jandel Scientific Software). Analyses of the optical density (OD) were performed blind to the training condition of the rats. The OD was expressed in arbitrary units corresponding to gray levels. Relative amount of total PKCγ was calculated by the formula $\{[(OD_{CF} + OD_{AF} + OD_{MF})/(OD_{CF(control)} + OD_{AF(control)} + OD_{MF(control)})] \times 100\%\}$. Immunoblot data were analyzed with the Mann–Whitney U test. Correlation coefficients

between the amount of translocation and performance were calculated using the Spearman correlation test. A probability level of $p < .05$ was taken as statistical significance. All data are presented as means with their standard errors (*SEM*s).

Results

Behavioral Performance

The rats displayed primarily exploratory behavior during the first days of the habituation period. During exploration, most of the time was spent in the outer zones of the holeboard arena. These location preferences gradually disappeared, whereafter the holes were randomly visited without a clear preference pattern. The rats were increasingly able to distinguish baited holes from nonbaited holes during the following training period. Rats with a strong increase in reference memory (RM) ratio (≥.05) on Day 4 of the training period were selected (T4 group) and used for analysis of PKCγ translocation. The remaining rats were further trained up to Day 11 (T11 group; see Material and Method section for details). Development of RM for the T4 and T11 groups is presented in Figure 1. The averaged increase in RM on Day 4 of the T4 group was .07 (±.015 *SEM*) and .02 (±.04 *SEM*) on Day 11 of the T11 group. The latter rats, however, did not yet reach the maximal reference memory ratio possible (which is .90 on average), as was shown by Beldhuis et al. (1992).

Analysis of PKCγ Immunoblotting

To determine the subcellular distribution, we biochemically analyzed hippocampal PKCγ by Western blotting. Tissue samples to be analyzed were taken from the anterior and posterior part of the hippocampus and contained all subregions of the hippocampus. The immunoblots showed a band at approximately 80 kDa, representing the migration position of PKCγ. Furthermore, the immunoblots revealed no evidence for proteolytic activity, because no immunoreactive bands appeared around 30 kDa, which indicates absence of proteolytic splicing of PKC into single regulatory domains. The optical densities of the immunoreactive bands in the immunoblots were measured and converted to percentaged cytosolic, membrane-associated and membrane-bound fraction of PKCγ, respectively. Moreover, the relationship between protein concentration and the optical density was determined for the membrane-bound fraction of 1 rat on Day 4. The sigmoidal Boltzman curve clearly indicates that from 10 μg to 55 μg of total protein, a lineair increase was present between protein concentration and optical density of PKCγ blots (see Figure 2). The data of the immunoblots (see Figure 3) showed that there were no differences in the relative amount of total PKCγ between C, T4, and T11 rats.

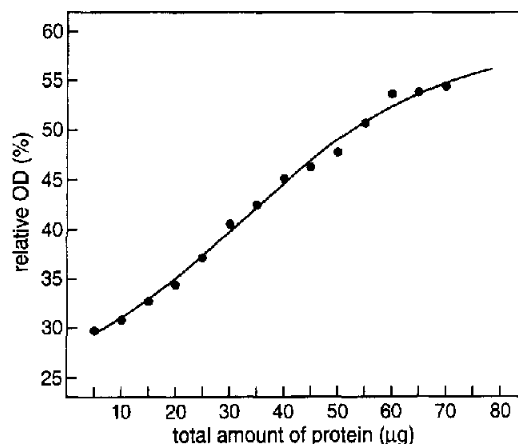

Figure 2. Relationship between total protein concentration of the membrane-bound fraction and the optical density (OD) for protein kinase C gamma isoenzyme expressed as a sigmoidal Boltzman curve. The figure clearly indicates that in the range from 10 μg to 55 μg, protein concentration is linearly related to the measured OD of the blotted protein.

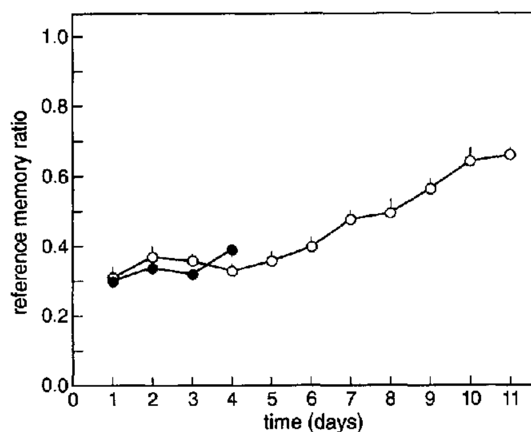

Figure 1. Development of reference memory ratio of rats trained for 4 days (T4; filled circles) and 11 days (T11; open circles). The rats in the T4 group showed a strong increase in reference memory ratio on Day 4, whereas the rats in the T11 group showed a more gradual increase in level of spatial orientation.

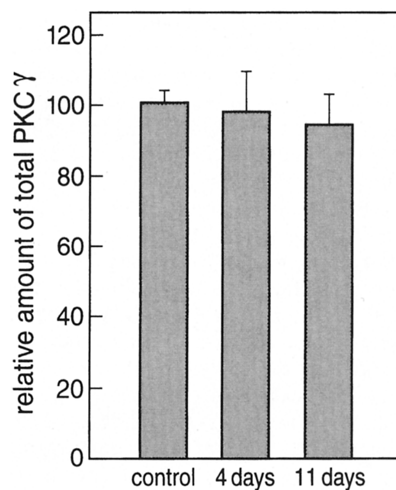

Figure 3. Optical density measures of immunoblots of posterior hippocampal tissue homogenates of control rats, 4 days-trained rats, and 11 days-trained rats reveal no difference in relative amount of total protein kinase C gamma isoenzyme (PKCγ)-immunostaining relative to untrained controls. Bars represent *M* ± *SEM*.

Moreover, no differences were found between the cytosolic, membrane-associated and membrane-bound PKC fractions in the anterior hippocampus (see Figure 4, top). In contrast, there was a significant difference between those fractions in the posterior part of the hippocampus after 4 days, but not after 11 days of training. Ten minutes after the last trial on Day 4, PKCγ was redistributed from the cytosolic to the membrane-associated fraction when compared to untrained controls ($p < .039$). So the increase in membrane-associated PKCγ was paralleled by a decrease in the amount of cytosolic PKC protein ($p < .039$; see Figure 4, bottom). No significant translocation was seen at the 10-min time point on Day 11 in either the anterior or the posterior part of the hippocampus. These findings suggest that there was translocation of PKCγ from the cytosol to the membrane compartment of the cell during the early acquisition phase only. A comparison of immunoblots is shown in Figure 5.

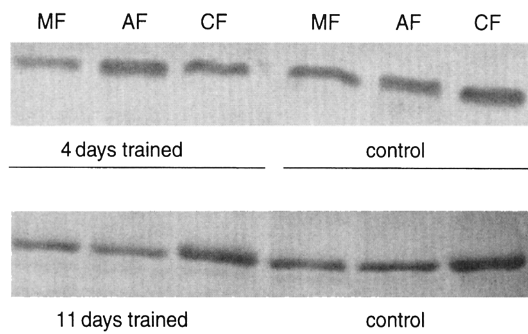

Figure 5. Example of the subcellular distribution of protein kinase C gamma isoenzyme (PKCγ) on immunoblots of the posterior hippocampus on Day 4 (top) and Day 11 (bottom). A clear redistribution of PKCγ to the membrane-associated fraction was found on Day 4 relative to controls (top). MF = membrane-bound fraction, AF = membrane-associated fraction, CF = cytosolic fraction.

Discussion

The present results clearly demonstrate that PKCγ is subject to a shift from cytosol to the membrane-associated fraction in relation to (spatial) learning. This translocation was seen during the early acquisition in the food-rewarded holeboard learning task 10 min after the trial on Day 4, but not in a later phase of the training (Day 11). In addition, no PKCγ translocation was found 24 hr after the last training trial in the later phase in this learning paradigm (unpublished observation). This latter finding is in agreement with Sunayashiki-Kusuzaki et al. (1993) and Van der Zee et al. (1997), who reported a lack of translocation in, respectively, delay and trace eyeblink conditioning at this time point in fully trained rabbits. It is possible that hippocampal PKCγ plays an important role in the acquisition of specific information (e.g., on the complex environment in spatial learning) during the initial phase of the training period.

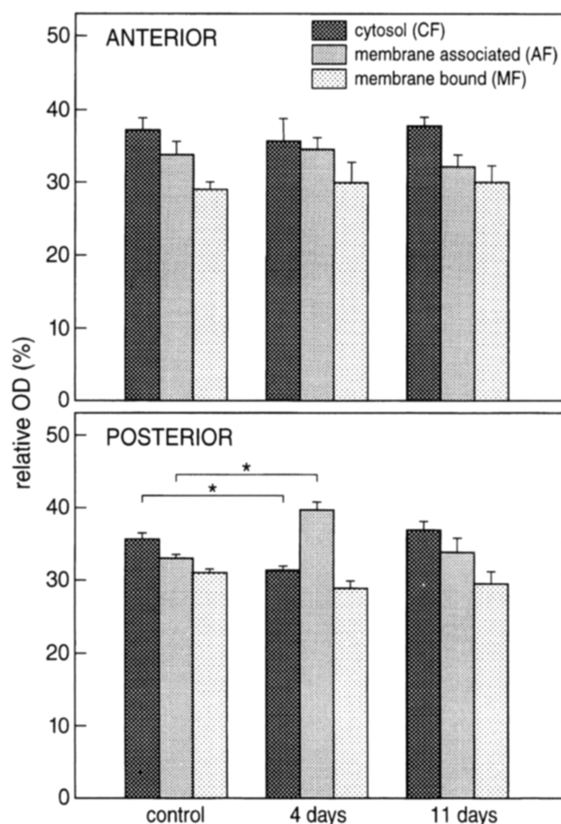

Figure 4. Immunoblot analysis of cytosolic fraction (CF), membrane-associated fraction (AF), and membrane-bound fraction (MF) protein kinase C gamma isoenzyme (PKCγ). No significant difference was found between those fractions in the anterior hippocampus (top), whereas the posterior part of the hippocampus showed a significant translocation of PKCγ to the MF on Day 4 (bottom). This increase in immunoreactivity was paralleled by a decrease in the relative amount of PKCγ in the CF. Bars represent $M \pm SEM$; $*p < .05$.

The contribution of PKCγ during spatial orientation learning seems to diminish once a more general knowledge of the task is acquired. In other words, during the later phase of the learning task used in the present study, possibly associated with consolidation and retrieval of the acquired environmental information, the role of PKCγ appears to be less prominent. It has been shown that the excitability of hippocampal neurons, which is partly regulated by PKC (Etcheberrigaray, Matzel, Lederhendler, & Alkon, 1992; Fagnou & Tuchek, 1995), remains unchanged once a hippocampally dependent task has been acquired (Moyer et al., 1996). Furthermore, recently it was reported that hippocampal CA1 pyramidal cells during trace eyeblink conditioning in the rabbit demonstrated several stages of learning-related activity (McEchron & Disterhoft, 1997). The most pronounced activity was found in pyramidal cells on the day of initial conditioned eyeblink responses, possibly representing a stage of activity that is critical for the initial events of learning. Interestingly, Noguès, Micheau, and Jaffard (1996) found a small but significant correlation between the decrease of cytosolic PKC activity and the level of performance at an early stage of training, but not in partially trained, well-trained, or overtrained animals. They concluded that because PKC activity is correlated with the early step of training irrespective of the number of sessions performed, this activity may be related to the ability to learn the task.

It is unlikely that the lack of significant PKCγ translocation on Day 11 may be due to the relatively low increase in

acquisition during the last preceding learning session, because individual data did not show a correlation between the improvement of reference memory and the degree of translocation at Day 4 or Day 11 (Spearman $r = .08$, $p = .91$; $r = .6$, $p = .17$, respectively). Because of the selection of good learners on Day 4, which are therefore no longer present in the group studied on Day 11 (i.e., the slow learners), it may be possible that good learners have a relatively higher concentration of membrane-associated PKCγ in comparison to slow learners. However, the absence of a correlation between improvement of reference memory and the degree of translocation at Day 4 or Day 11 argues against this possibility. This indicates that although PKCγ is significantly involved in spatial learning, the unraveling of its exact role in learning and memory processes requires further investigation.

This role of PKCγ in the hippocampus seems not to be essentially related to synaptic facilitation (Goda, Stevens, & Tonegawa, 1996), but more to temporary storage of newly acquired information in a postsynaptic cascade of events (Van der Zee et al., 1997), which may lead to morphological modifications of synapses or may induce effects on gene transcription (Fields, Tyler, Kraft, & May, 1990). Moreover, the data on Day 11 further suggest that membrane-associated PKCγ could have been redistributed back to the cytosol. It should be emphasized that PKC translocation is often found to be a relatively rapid and transient process (Nishizuka, 1995). As mentioned before, Bank et al. (1988) observed a change in subcellular PKC localization in conditioned rabbits. These changes in distribution were measured enzymatically 24 hr after the conditioning process had taken place, suggesting a sustained physiological response. However, the suggestion that prolonged or permanent transition of PKC from a soluble to an integral membrane protein form plays an important role in memory processes (Alkon & Rasmussen, 1988; Burgoyne, 1989) is questionable and not supported by most of the more recent findings on this subject. The doubt is primarily based on the observation of rapid redistribution of membrane-associated PKC to the cytosol after the dissociation of applied phorbol esters in vivo (Mosior & Newton, 1995; Szallasi, Smith, & Blumberg, 1994).

The molecular mechanisms underlying translocation of PKC are not yet clarified and are beyond the scope of this study, but increases in intracellular Ca^{2+} concentration and/or regional changes in Ca^{2+} concentration have been proposed as factors to promote PKC translocation (Liu, 1996; Liu, Powell, Sudhof, & Robinson, 1994; Newton, 1995; Sando, Maurer, Bolen, & Grisham, 1992). It is conceivable that acquisition of spatial orientation in the holeboard is accompanied by temporary rises of intracellular Ca^{2+}.

It still remains to be determined whether LTP is the molecular equivalent of the events occurring during spatial learning and memory processes. Akers et al. (1986) reported that PKC activity was increased twofold in membranes and decreased proportionately in cytosol at 1 hr after the induction of LTP, which suggests translocation of the activity. They theorized that LTP was accompanied by persistent activation of PKC, as this enzyme is known to bind to membranes when activated (Bell & Burns, 1991; Nishizuka, 1986). However, there is also evidence that translocation of PKC does not contemporaneously correlate with activation of the enzyme as measured by substrate phosphorylation (Liu, Engler, Funder, & Robinson, 1992; Liu et al., 1994; Sando et al., 1992; Trilivas, McDonough, & Brown, 1991). Interestingly, it has been shown recently that a brief application of 50 mM K^+ to CA1 hippocampal slices, which induced LTP, rapidly enhances PKCγ activity in the membrane fraction. This was quickly followed by a PKC activation in the cytosol, which lasts at least for 1 hr (Roisin, Leinekugel, & Tremblay, 1997). Furthermore, Angenstein et al. (1994) reported a translocation of PKCγ from the membrane to the cytosol following the induction of LTP in the rat hippocampus. These findings provide supporting evidence for our suggestion that on Day 11 PKCγ was partially translocated back to the cytosol.

In conclusion, the present data suggest that PKCγ translocation is a transient event that occurs during the first leap in holeboard learning only and that the degree of translocation is not directly coupled to the increase in correct behavioral performance at the individual level. These findings, therefore, promote the hypothesis that PKCγ is only one of many proteins that are involved in the biochemical events underlying learning and memory.

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