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Delay fear conditioning modifies phospholipase C-β1a signaling in the hippocampus and frontal cortex

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Abstract

The use of the single-trial fear conditioning paradigm allows for control over the exact moment when an animal is exposed to a learning event, making it possible to study both the initial neurobiological changes that are associated with learning and changes that take place over long periods of time. In the present study, we performed detailed analyses of the alterations in phosphatidylinositol-specific phospholipase C-β1a (PLC-β1a) levels and enzyme activities in subcellular fractions prepared from the hippocampal formation (HPF) and medial frontal cortex (MFC) 1, 3, 5, 7, 24, and 72 h following single-trial fear conditioning. We observed tissue- and time-dependent changes in both PLC-β1a enzyme activity and anti-PLC-β1a immunoreactivity in each subcellular fraction. Based on these observations, we hypothesize that changes in PLC-β1a catalytic activity and subcellular distribution play important roles in neuronal signaling processes that are required for fear-conditioned learning and memory.

Keywords: Fear conditioning; Phospholipase C; Subcellular fractionation

1. Introduction

Phosphatidylinositol-specific phospholipase C (PLC) isozymes hydrolyze phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], yielding two intracellular second messengers: inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and 1,2-diacylglycerol (Majerus et al., 1990; Williams, 1999). Production of Ins(1,4,5)P_3 results in the release of Ca^{2+} from intracellular storage sites, while 1,2-diacylglycerol directly activates the conventional and novel isoforms of protein kinase C (Berridge, 1993; Nishizuka, 2001). Complementary DNA clones have been isolated for at least 11 distinct mammalian PLC isozymes (Rhee, 2001), which are grouped into four types: β, δ, γ, and ε. For each of these types, multiple subtypes have been identified. For example, there are four known PLC-β isoforms designated as PLC-β1–4. PLC-β1 is the predominant PLC isozyme in the brain, accounting for approximately 50% of the total PLC activity in this tissue (Takenawa et al., 1991). PLC-β1 exists as two splice variants consisting of the 150-kDa PLC-β1a, which represents the preponderance of the PLC-β1 complement, and the 140-kDa PLC-β1b (Bahk et al., 1994).

There are a growing number of studies examining the roles of protein kinases in the induction and maintenance of long-lasting memories. Much of this research is focused on the four major protein kinase families present in the post-synaptic density: protein kinase C, protein kinase A, Ca^{2+}-calmodulin-dependent protein kinase, and extracellular signal-regulated protein kinase. Members of each of these families have been shown to be necessary for memory formation and synaptic plasticity in various capacities. In contrast, the role of phospholipases in learning and memory has often been overlooked. Support for a role for one or more PLC isozyme in fear conditioning comes from studies showing that the turnover rates of inositol phosphates and 1,2-diacylglycerol in the dentate gyrus and areas CA1 and CA3 of the hippocampus are increased following fear conditioning. (Laroche et al., 1990). Despite the presence of most, if not all, of the known members of the PLC family in the brain (Homma et al., 1989; Kelley et al., 2001; Lee

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and Rhee, 1996; Watanabe et al., 1998), PLC-β1 stands out as a potentially important player in neuronal signaling (see Weeber et al., 2001, for discussion) due to its highly elevated expression in the CNS (Bahk et al., 1994; Ross et al., 1989; Smrcka and Sternweis, 1993; Takenawa et al., 1991). This hypothesis is supported by our earlier studies showing changes in hippocampal formation (HPF) and cortical PLC-β1a regulation following fear conditioning (Weeber et al., 2001).

The present experiments were aimed at investigating whether there are time-dependent changes in PLC-β1a enzyme activity and protein level associated with learning and memory formation in the rat HPF and medial frontal cortex (MFC). For these studies, we employed single-trial fear conditioning, which makes it possible to identify the exact moment that learning is initiated (Bevins and Ayres, 1994; Fanselow and Bolles, 1979), and thus facilitates the identification of biochemical changes that occur in conjunction with in vivo learning and memory processes.

Fear-induced long-term memory is dependent on de novo RNA and protein synthesis (Barrientos et al., 2002; Davis and Squire, 1984; Lattal and Abel, 2001; Stiedl et al., 1999). Recent studies have demonstrated that under certain conditions, there are two temporal phases that are critical for long-term consolidation of fear memory: one period exists for the first few minutes up to 1 h following training, while the other period occurs between 1 and 6 h after training, with the period from 2 to 4 h most commonly being reported as important (Bernabeu et al., 1997; Bourtchuladze et al., 1998; Igaz et al., 2002; Quevedo et al., 1999). The ability to observe these two phases depends on the strength of the training procedure that has been employed. Bourtchuladze et al. (1998) reported that a weak fear conditioning procedure, such as one in which the test subject experiences only a single pairing of a conditioned stimulus (CS) and an unconditioned stimulus (US), is associated with two periods of protein-synthesis-sensitive memory formation—immediately after training and 4 h after training. In contrast, if a strong training procedure (e.g., one in which the subject experiences multiple pairings of the CS and US) is used, inhibition of protein synthesis affects long-term memory only if the inhibitor is administered immediately after training. These studies indicate that long-term memory formation occurs at different rates, depending on the intensity of the training experience. One interpretation of these results is that strong training procedures regulate multiple signal transduction pathways, which in turn regulate transcriptional and translational processes required for long-term memory formation. Weaker training procedures can either regulate these same pathways less robustly or regulate fewer signal transduction pathways, which, as a result, causes a second period of protein-synthesis-dependent consolidation to occur. There is an astonishingly incomplete picture of the upstream signaling events that may control pathways leading to transcription- and translation-dependent memory formation.

We show that the temporal profiles of changes in subcellular PLC-β1a enzyme activity and protein levels associated with fear-conditioned learning and memory are remarkably dynamic with prominent changes 3 to 5 h following conditioning. Based on these results and the known dependence of learning and memory on molecules known to be controlled by PLC-β1a signaling, we speculate on the role that PLC-β1a may play in fear-conditioned learning and memory.

2. Materials and methods

2.1. Animals

Female Sprague–Dawley rats (90–150 days of age) obtained from Harlan Industries (Indianapolis, IN) were used in these studies. Following delivery from the distributor, all experimental rats were housed in groups of two or three per cage and allowed to adjust to the animal housing room for at least 7 days prior to being assigned to an experimental group and housed individually. Animals were housed in the same room, which was maintained at 22 °C on an 8-h dim light–16-h dark cycle (lights on from 0930 to 1730 h), and were given 24-h access to standard rat chow and tap water. All procedures and methods for housing, fear conditioning, and sacrificing rats were approved by the University of New Mexico Health Science Center Animal Care and Use Committee.

2.2. Fear conditioning

The procedures used for fear conditioning and treatment of control groups were performed as previously described in Weeber et al. (2001). Briefly, rats were randomly assigned to one of three experimental groups: single-trial fear-conditioned [paired tone–shock (PTS)], unpaired control (UPC), or unhandled (UH). Fear-conditioned rats were exposed to a 30-s tone (the CS), which terminated with a 2-s, 1-mA foot shock (the US). UPC rats received all of the same stimuli as the fear-conditioned rats (i.e., transportation, handling, context, tone, and shock), but the context, CS, and US experiences were separated by a minimum interval of 60 min, with the US being delivered in the animal's home cage. The UPC procedure allowed us to control for the unconditioned effects of foot shock, as well as handling, tone, and context exposures. The third experimental group consisted of naive, UH control rats, which did not receive any of the experiences associated with the fear conditioning paradigm prior to sacrifice.

At the appropriate time following exposure to the foot shock, PTS and UPC rats were taken directly from the housing room to an adjacent room and were sacrificed by decapitation without anesthesia. UH rats were sacrificed in the same manner. The brains were rapidly removed, and the HPF and MFC (identified as all areas rostral to the fornix and optic chiasm with the olfactory region previously removed) were dissected.

2.3. Analyses of PLC-β1a enzyme activity and immunoreactivity in subcellular fractions

All procedures for the preparation of HPF and MFC subcellular fractions, isolation of PLC-β1a by immunoprecipitation, and quantification of PLC-β1a enzyme activity and anti-PLC-β1a immunoreactivity were as described in Weeber et al. (2001). Immunoreactivity was determined using 20, 25, and 5 μg, respectively, of HPF S2 (200,000 × *g* soluble), P2 (200,000 × *g* postnuclear particulate), and P1 (1000 × *g* particulate) fractions, and 40, 40, and 10 μg of MFC S2, P2, and P1 fractions.

2.4. Calculation of PLC-β1a enzyme specific activity

Specific activities were calculated as the quotient of PLC-β1a enzyme activity divided by anti-PLC-β1a immunoreactivity in the subcellular fraction being analyzed. Both enzyme activity [pmol Ins(1,4,5)P_3 product formed/min/μg protein] and immunoreactivity (Unit; "units" were calculated as the immunoreactivity/μg protein fraction) were expressed relative to the total protein content of the subcellular fraction being analyzed, and thus, specific activity was expressed as pmol Ins(1,4,5)P_3 product formed/min/"unit" of enzyme.

2.5. Statistical analyses

For ease of presentation and clarity of results, graphs are presented at the 95% confidence level (95% CL). Data analysis using two-way ANOVA was performed on UPC and PTS animal groups to determine significant differences with variables of behavioral treatment and time. In addition, a Tukey multiple comparisons test was performed to verify statistical significance at experimental times where error bars do not overlap ($P < .05$), and these are the only time points that are marked with an asterisk.

2.6. Materials

Polyclonal antibodies for PLCβ-1a were purchased from Santa Cruz Biotechnology. PtdIns(4,5)P_2 and Triton X-100 were purchased from Boehringer-Mannheim. [H^3] PtdIns(4,5)P_2 was purchased from NEN. Protein A–Sepharose CL4B was purchased from Pharmacia. All other chemicals and supplies were obtained from commercial sources.

3. Results and discussion

3.1. Overview of experimental design

To determine the time dependence of the effects of fear conditioning on PLC-β1a, we measured the catalytic activity and level of the enzyme in HPF and MFC subcellular fractions (nuclear, cytosolic, and postnuclear membrane) at six times (1, 3, 5, 7, 24, and 72 h) following CS delivery. Because rodent brain contains multiple PLC isozymes, we chose to isolate PLC-β1a from other PLC isoforms present in HPF and MFC subcellular fractions by affinity capture using isozyme-specific antibodies. Specificity of the antibody has been previously established (Weeber et al., 2001). Immune complex-associated PLC activity was determined by measuring the in vitro hydrolysis of PtdIns(4,5)P_2. Subcellular PLC-β1a levels were assessed by semiquantitative immunoblotting. From these two measures, we calculated enzyme specific activity (i.e., PLC catalytic activity per unit of enzyme). Changes in PLC-β1a specific activity revealed alterations in the catalytic activity of the enzyme that could not be accounted for by changes in PLC-β1a levels, thus implicating the regulation of enzyme activity during memory formation. This method of analysis was employed by Wahl et al. (1992) to demonstrate that tyrosine phosphorylation increases the catalytic activity of PLC-γ1.

Two groups of control animals were employed in these studies: UPC and UH. UPC animals were exposed to each of the stimuli that comprise the single-trial fear conditioning paradigm without the pairing of the US with either the CS or the context. UH animals did not experience the CS, context, or US. Importantly, there were no significant differences between PLC-β1a enzyme activities or concentrations in subcellular fractions prepared from UH tissues and UPC tissues at any of the times tested (Figs. 1–3). The consistent measurements from UPC samples indicate that the changes in PLC-β1a recorded following fear conditioning (see below) are not the result of handling, exposure to the context, auditory cue, or foot shock.

3.2. HPF postnuclear membrane and cytosolic fractions

Contextual fear-conditioned learning is dependent upon normal HPF function (Sanders et al., 2003). We have previously shown that changes in the subcellular distribution and catalytic activity of HPF PLC-β1a occur after fear conditioning (Weeber et al., 2001). However, as our previous study assessed changes at 1 and 24 h following conditioning, it gave a rather incomplete picture of the time dependence of changes in PLC-β1a signaling associated with fear conditioning. Therefore, we sought to profile these changes with time.

Measurements of PLC-β1a enzyme activities in the HPF membrane and cytosolic fractions revealed a biphasic profile. The membrane fraction showed a significant decrease in PLC-β1a activity 3 h after fear conditioning, followed by an increase 5 and 7 h after training (Fig. 1A). Interestingly, this appears to be a mirror image of the biphasic activity profile observed for PLC-β1a isolated from the cytosolic fraction (Fig. 1D). By 72 h following conditioning, PLC-β1a enzyme activity returned to baseline levels both in the membrane and cytosolic fractions.

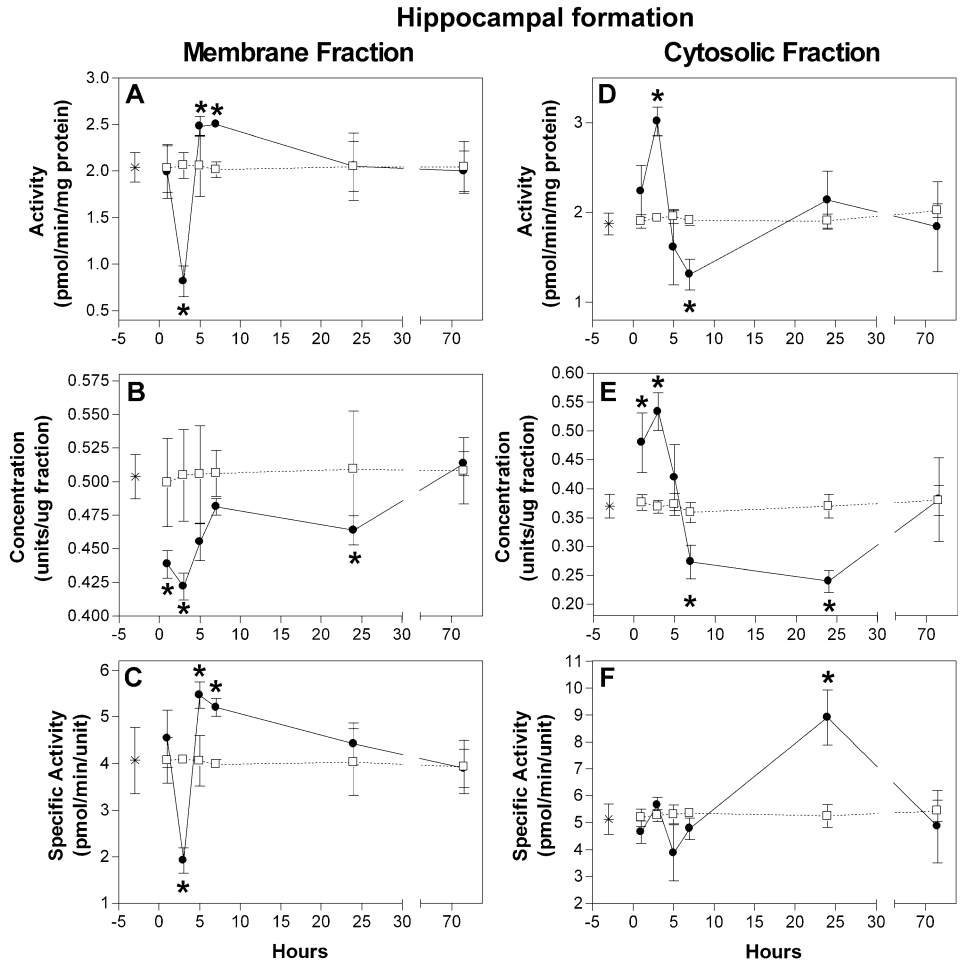

Fig. 1. Changes in HPF membrane- and cytosolic-associated PLC-β1a following single-trial fear conditioning. PLC-β1a enzyme activities were measured in the membrane (A) or the cytosolic fraction (D). Anti-PLC-β1a immunoreactivities were used to quantify PLC-β1a concentrations in the membrane (B) and cytosolic (E) fractions. Measurements were made from HPF subcellular fractions derived from fear-conditioned [PTS (□); $n=6$] and unpaired control [UPC (□); $n=6$] animals that were sacrificed 1, 3, 5, 7, 24, or 72 h after fear conditioning. In addition, enzyme activities and concentrations were measured in fractions derived from naive rats, not behaviorally manipulated [UH (*); $n=4$]. Activity and concentration measurements were used to calculate PLC-β1a specific activities for membrane-associated (C) and cytosolic-associated (F) enzyme. Data are shown as mean ± S.E.M., 95% CL. Data were analyzed by two-way ANOVA (see table for results) followed by post hoc analysis using Tukey multiple comparisons test. Asterisks signify significance determined by both nonoverlapping error bars and by Tukey multiple comparisons test ($P>.05$).
Results of two-way ANOVA:

	Membrane fraction			Cytosolic fraction		
	Activity	Concentration	Specific activity	Activity	Concentration	Specific activity
Time $F(5,60)$, P	22.48, <.001	4.48, .0016	26.76, .0001	16.22, <.0001	18.46, <.0001	14.46, <.0001
Treatment $F(1,60)$, P	1.72, NS	42.18, <.0001	4.76, .033	2.09, NS	2.44, NS	.70, NS
Interaction $F(5,60)$, P	23.92, <.0001	3.49, .0078	27.93, <0.0001	16.76, <.0001	20.42, <.0001	15.24, <.0001

We found that, like the activity profiles, anti-PLC-β1a immunoreactivity changed with time in the membrane and cytosolic subcellular fractions. In both compartments, the greatest change occurred 3 h following fear conditioning, with a decrease in PLC-β1a protein associated with the membrane fraction and an increase in the cytosolic fraction; 72 h following conditioning PLC-β1a levels had returned to baseline concentrations (Fig. 1B and E). In the initial few hours after conditioning, changes in HPF PLC-β1a concentrations in the membrane fraction generally were equal to, and in an opposite direction to, changes in concentrations in the cytosolic fractions. This observation indicates that the reductions in membrane PLC-β1a concentrations were the result of translocation of the enzyme to the cytosol and that this translocation event was initiated by fear conditioning. However, it should be noted that the membrane fraction isolated in these studies is in fact composed of all cellular membranes, excluding nuclear membranes. Thus, we are unable to identify unequivocally the membrane type(s) [e.g., plasma, Golgi]

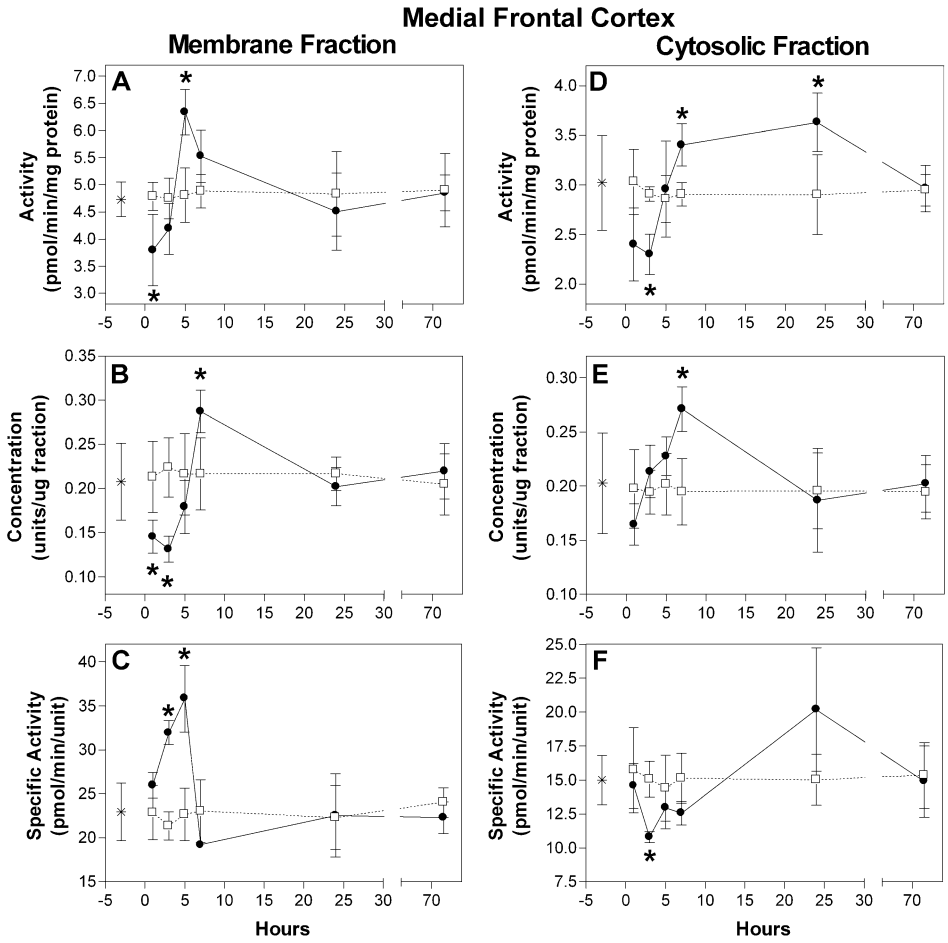

Fig. 2. Changes in MFC membrane- and cytosolic-associated PLC-β1a following single-trial fear conditioning. PLC-β1a enzyme activities were measured in the membrane (A) or the cytosolic fraction (D). Anti-PLC-β1a immunoreactivities were used to quantify PLC-β1a concentrations in the membrane (B) and cytosolic (E) fractions. Measurements were made from MFC subcellular fractions derived from fear-conditioned [PTS (□); $n=6$] and unpaired control [UPC (□); $n=6$] animals that were sacrificed 1, 3, 5, 7, 24, or 72 h after fear conditioning. In addition, enzyme activities and concentrations were measured in fractions derived from naive rats, not behaviorally manipulated [UH (*); $n=4$]. Activity and concentration measurements were used to calculate PLC-β1a specific activities for membrane-associated (C) and cytosolic-associated (F) enzyme. Data are shown as mean ± S.E.M., 95% CL. Data were analyzed by two-way ANOVA (see table for results) followed by post hoc analysis using Tukey multiple comparisons test. Asterisks signify significance determined by both nonoverlapping error bars and by Tukey multiple comparisons test ($P>.05$).
Results of two-way ANOVA:

	Membrane fraction			Cytosolic fraction		
	Activity	Concentration	Specific activity	Activity	Concentration	Specific Activity
Time $F(5,60)$, P	10.94, <.001	10.22, <.0001	15.14, <.0001	10.31, <.0001	5.32, .0004	6.40, <.0001
Treatment $F(1,60)$, P	0.13, NS	9.18, .0036	32.69, <.0001	0.08, NS	5.03, .0286	2.21, NS
Interaction $F(5,60)$, P	9.98, <.0001	11.86, <.0001	20.04, <.0001	12.66, <.0001	5.4, .0004	15.24, <.0001

from which the enzyme dissociated. The idea of PLC translocation following a signaling event is not novel. Redistribution of PLC-β isozymes between subcellular fractions has been reported following cell activation in various nonneuronal cells and cell lines. For example, PLC-β1 (Zini et al., 1996) and PLC-β2 (Bertagnolo et al., 1997) have been reported to translocate from the cytosol to the nucleus; PLC-β2 and PLC-β3 have been reported to move from a cytosolic to a cytoskeletal fraction (Banno et al., 1996; Coburn et al., 1997). However, to our knowledge, our studies are the first evidence of the subcellular translocation of a PLC-β isozyme in brain tissue following behavioral activation.

In the cytosolic compartment at times up to 7 h following conditioning, the profile of concentration changes is similar to that of the changes in enzyme activity, suggesting that most of the activity differences are due to concentration changes (Fig. 1D and E). This conclusion is supported by a lack of change in enzyme specific activity (Fig. 1F). In contrast, visual inspection indicated that in the membrane

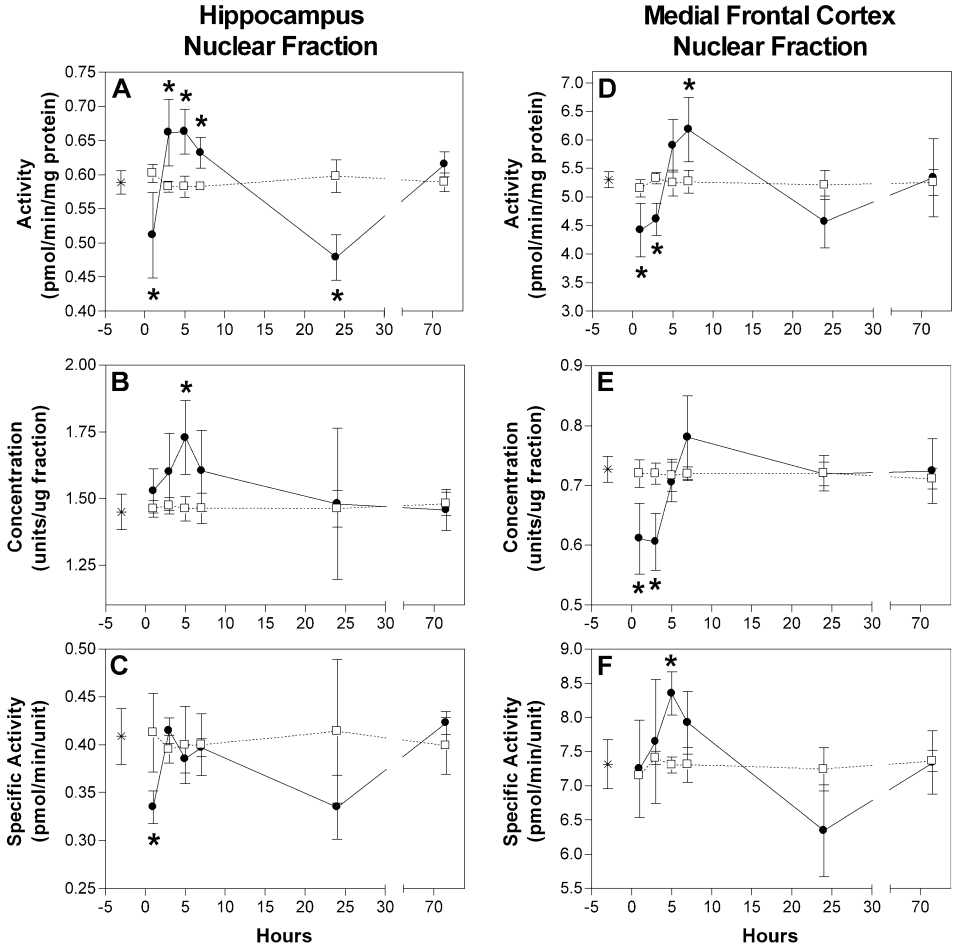

Fig. 3. Changes in nuclear-associated PLC-β1 isolated from the hippocampus and MFC following single-trial fear conditioning. HPF nuclear fraction PLC-β1a activity (A), anti-PLC-β1a immunoreactivity (B), and specific activity (C) were determined as described in the Methods section. Similarly, MFC nuclear fraction PLC-β1a enzyme activity (D), anti-PLC-β1 immunoreactivity (E), and specific activity (F) were determined as described. Measurements were made from fear-conditioned [PTS (□); $n=6$] and unpaired control [UPC (□); $n=6$] animals that were sacrificed 1, 3, 5, 7, 24, or 72 h after receiving a fear conditioning. Control rats not behaviorally manipulated are also shown [UH (*); $n=4$]. Data are shown as mean ± S.E.M., 95% CL. Data were analyzed by two-way ANOVA (see table for results) followed by post hoc analysis using Tukey multiple comparisons test. Asterisks signify significance determined by both nonoverlapping error bars and by Tukey multiple comparisons test ($P>.05$).
Results of two-way ANOVA:

	Membrane fraction			Cytosolic fraction		
	Activity	Concentration	Specific activity	Activity	Concentration	Specific activity
Time $F(5,60)$, P	11.32, <.001	1.49, NS	1.57, NS	7.60, <.0001	5.40, .0004	5.58, .0003
Treatment $F(1,60)$, P	0.25, NS	9.35, .0033	4.65, .0351	0.42, NS	5.05, .0283	2.04, NS
Interaction $F(5,60)$, P	17.06, <.0001	1.68, NS	3.43, .0083	6.97, <.0001	5.73, .0002	4.60, .0013

fraction, changes in concentration were not of sufficient magnitude to account for the changes in enzyme activities at these same times. Thus, the profile of the calculated specific activity of the enzyme (Fig. 1C) was similar to the enzyme activity profile (Fig. 1A) for this fraction. This result indicates that enzyme activity in the membrane fraction is subject to regulation (see below). Finally, the significant decrease in cytosolic fraction PLC-β1a concentration 24 h following conditioning resulted in a prominent increase in the specific activity of the enzyme associated with the fraction (Fig. 1F).

3.3. MFC postnuclear membrane and cytosolic fractions

In the rodent, the MFC has extensive neuronal connectivity with limbic structures and is important in the acquisition and consolidation of fear-conditioned learning (Morgan and LeDoux, 1995; Morrow et al., 1999; Sacchetti et al., 2002). We previously reported that 1 and 24 h after single-trial fear conditioning, PLC-β1a activity and concentration in MFC subcellular fractions were altered (Weeber et al., 2001). In the present study, we more fully assessed the temporal pattern of these changes.

The profiles generated from activity (Fig. 2A) and concentration (Fig. 2B) measurements of membrane PLC-β1a in the MFC were, in general, similar to those of the cytosolic fraction (Fig. 2D and E). However, there appears to be a temporal delay of PLC-β1a activity changes in the cytosolic fraction, being 1 to 3 h behind those of the membrane fraction. In addition, PLC-β1a levels were decreased in the membrane fraction, but not the cytosolic fraction, at early (1–5 h) times. Interestingly, this is in contrast to the mirror-like profiles seen in the membrane and cytosolic fractions isolated from the HPF. The PLC-β1a enzyme activity and concentration profiles for the membrane fraction in the MFC were similar to those observed in the HPF, except that there was not a significant decrease in PLC activity associated with the MFC fraction 3 h following conditioning. For the first 7 h posttraining, the profile of cytosolic PLC-β1a enzyme activity (Fig. 2D) was similar to changes in PLC-β1a levels (Fig. 2E) in the MFC, except at the 3-h time point, where activity is still decreased but concentrations are on the rise. This observation is supported by a minimal change in enzyme specific activity in this fraction at these times, except again for the 3-h time point, which was the only time a statistically significant change in PLC specific activity was seen (Fig. 2F). A striking difference between HPF and MFC was seen at the 24-h post-fear-conditioning time, at which time we observed that both the MFC membrane and cytosolic concentrations of PLC-β1a had returned to baseline levels (Fig. 2B, E), whereas the HPF membrane and cytosolic PLC-β1a concentrations were significantly downregulated at the 24-h time point (Fig. 1B and E).

Another major difference between the MFC and HPF was seen in the profile of the calculated PLC-β1a specific activities of the membrane fraction (Fig. 2C). We observed a significant increase in specific activity in the MFC 3 to 5 h after training, then a sharp decrease in specific activity by 7 h. Although the calculated specific activity at the 7-h time point was not statistically different from UPC or UH specific activities, there was a significant reduction of approximately 50% in specific activity between the 5- and 7-h time points. The appearance of this profile is quite different from that recorded in the HPF (Fig. 1C). Interestingly, the specific activity graphs generated from the MFC cytosolic fraction (Fig. 2F) closely resembles that of the HPF cytosolic PLC-β1a specific activity graph (Fig. 1F).

Taken together, these differences in the activity, concentration, and specific activities of cortical PLC-β1a suggest that the mechanisms controlling PLC-β1a in the MFC may be similar to, but distinctly different from, those in the HPF. Many of these differences appear to be temporal differences, with MFC increases in concentrations and activities occurring later than in the hippocampus. Beyond the temporal divergence is the significant increase in the percent change in PLC-β1a activity and concentration between the HPF and the MFC at time points up to 5 h.

3.4. Nuclear-associated PLC-β1a

There are several reports examining the possible role of PLC-β1 in signal transduction in the nucleus of various cells and cell lines (Bertagnolo et al., 1995; Divecha et al., 1993; Manzoli et al., 1997; Marmiroli et al., 1996). This research suggests that PLC-β1 translocation and involvement in nuclear signaling events are important in normal cellular function and signaling from the membrane to the nucleus. However, there are no reports of PLC-β1 involvement in nuclear signaling correlated with synaptic transmission, plasticity, or learning and memory processes. We reasoned that analysis of the effects of fear conditioning on nuclear PLC-β1a might assist in interpreting the results obtained in the analyses of cytosolic, as well as membrane, PLC-β1a.

When PLC-β1a activity was measured in isolated nuclei from HPF and MFC, a strikingly similar pattern of activity was seen over time. However, unlike the temporal patterns seen in the membrane and cytosolic fractions, the changes in nuclear-associated PLC-β1a activity appeared to have a triphasic profile (Fig. 3A and D). In both tissues, activities were reduced (relative to behavioral controls) 1 h after fear conditioning, followed by a significant increase in the 5- to 7-h time range, then another decrease 24 h after training, albeit significant only in the hippocampus, and a return to baseline levels 72 h after conditioning. This unexpected activity profile suggests a great degree of temporal control of nuclear phosphatidylinositols and PtdIns(4,5)P-derived second messenger production. HPF nuclear PLC-β1a concentrations were statistically different at the 5-h time point only (Fig. 3B). However, only the significant decrease in HPF activity at the 1-h time point resulted in a major change in the calculated enzyme specific activity at that time (Fig. 3C). In the MFC, the only significant increase in specific activity was seen at the 5-h time point after conditioning.

Changes in the amount of PLC-β enzyme associated with the nuclear compartment may result from (1) altered metabolism of enzyme already associated with the nucleus, (2) altered targeting of newly synthesized enzyme, or (3) translocation of preexisting enzyme from a nonnuclear compartment (e.g., the cytosol) to the nucleus. We found that the total amount of PLC-β1 in the nuclear fraction is 8- to 10-fold greater than that associated with the cytosolic and membrane fractions, which contain approximately equal amounts of enzyme (data not shown). Further purification of nuclei from the nuclear fractions revealed that, indeed, PLC-β1a is present in nuclei (Weeber et al., 2001). This suggests that a significant increase in the amount of PLC-β1a in the nuclear fraction is not likely to result from translocation of enzyme from the cytosolic or membrane fraction; rather, it may in part be due to altered turnover (translation and catabolism) and/or targeting of newly synthesized protein at early times and the same events, as well as transcriptional events, at later times. This does not discount the possibility of translocation of cytosolic or membrane PLC-β1 to the nucleus; however, it is unlikely

that it would be an amount capable of causing significant changes in nuclear PLC-β1 concentrations.

The amount of data presented here makes an overview of the dynamic temporal-dependent changes and overall trends of subcellular PLC-β1a activities and concentrations difficult to visualize. This becomes a greater challenge when differences in brain tissues are also introduced. To address this limitation, we graphed (Fig. 4) fear-conditioning-dependent changes in PLC-β1a concentrations and activities: [(PTS value/UPC value) × 100%]. This dramatically illustrates the acute increases and decreases in PLC-β1a activities and concentrations in the 0- to 7-h range after fear conditioning. Further, a correlation is seen between temporal fluctuations in PLC-β1a concentrations that are loosely synchronized with PLC-β1a activities. Fig. 4 also demonstrates the differences between HPC and MFC tissues. This may reflect the differences in the dependence on PLC-β1a for the induction of specific pathways for proper memory encoding in the HPF and MFC.

3.5. PLC-β1a may play a role in protein-synthesis-dependent fear-conditioned memory formation

It is a generally held belief that the formation of memories is due, at least in part, to changes in the synaptic strength of specific neuronal pathways (Bailey and Kandel, 1993). Changes in synaptic strength would undoubtedly involve transcription and translation of the necessary proteins to enhance synaptic connections. As noted in the Introduction, several studies have demonstrated that a protein-synthesis-dependent phase of fear-conditioned memory formation exists between 1 and 6 h after training. The demonstration that HPF and MFC PLC-β1a catalytic activity and subcellular distribution are significantly altered during this period (especially 3 and 5 h after conditioning) implicates PLC-β1a as an important component of the signaling pathway(s) involved in transcription- and/or translation-dependent consolidation of fear memories in these two brain regions. These effects of PLC-β1a could result from either direct effects on the translational and transcriptional machinery, or indirect effects mediated by alterations in the levels of the substrates [e.g., PtdIns(4,5)P_2] and products [e.g., Ins(1,4,5)P_3 and 1,2-diacylglycerols] of the reactions that it catalyzes.

It is important to note that two C-terminal splice variants of PLC-β1 exist: a and b (Bahk et al., 1994). In most studies published to date, effects of treatments (e.g., pharmacologic or molecular) on these two variants have not been distinguished. Thus, it cannot be unequivocally determined whether observed effects are attributable to one or both of these subtypes. In this context, we note that our studies analyzed the PLC-β1a isoform.

PLC-β1 has been shown to couple to receptors (e.g., metabotropic glutamate receptors) known to regulate gene expression and protein synthesis (Mao and Wang, 2003; Raymond et al., 2000). Microinjection of PLC-β1 into

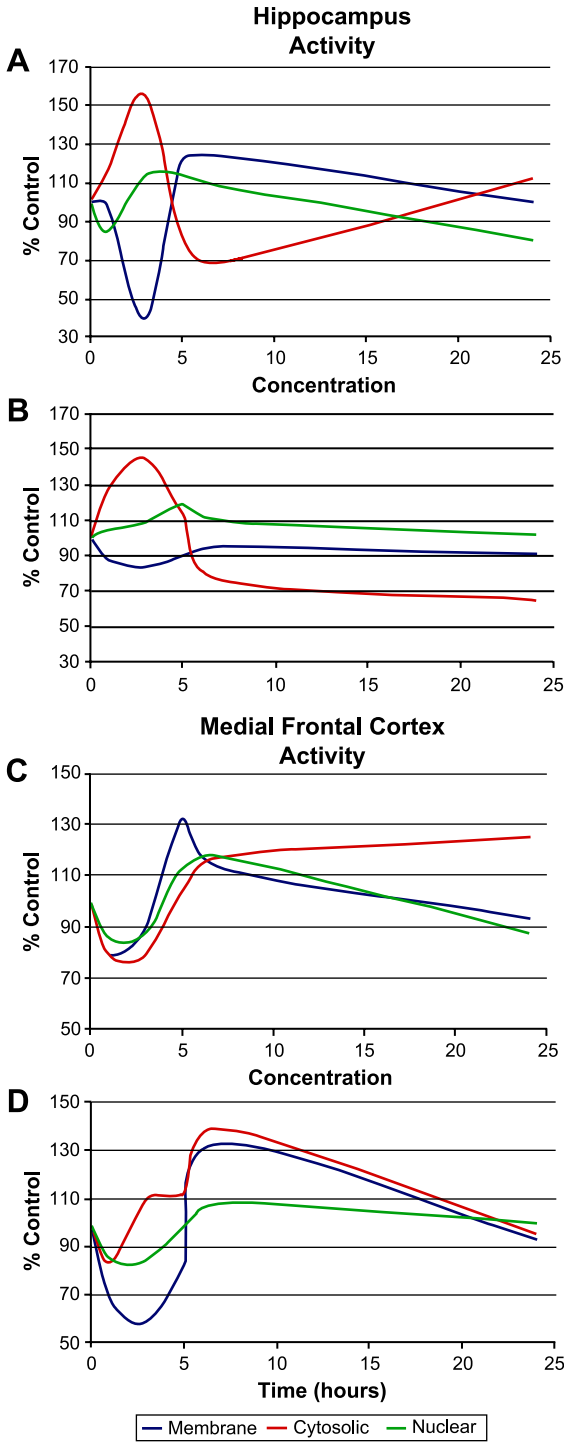

Fig. 4. Comparison of subcellular PLC-β1a activity and concentration profiles from the hippocampus and MFC over time. The percent change of PTS animals compared to the UPC animals at each time point was calculated and graphed. Best fit lines are drawn for changes in HPF PLC-β1a activity (A) and concentration (B), and MFC PLC-β1a activity (C) and concentration (D) for the membrane (blue), cytosolic (red), and nuclear (green) fractions. Time 0 was determined by the average of all time points determined from the UPC group.

quiescent NIH 3T3 cells induces DNA synthesis (Smith et al., 1989). Underexpression of PLC-β1 in Swiss 3T3 cells blocks IGF-1-stimulated DNA synthesis (Manzoli et al., 1997). Overexpression of both PLC-β1a and PLC-β1b reduces expression of the p45/NF-E2 transcription factor in murine erythroleukemia cells (Faenza et al., 2002). Finally, ultrastructural studies of human osteosarcoma Saos-2 cells have shown that PLC-β1 is present in the interchromatin domain and at the interheterochromatin border (Marmiroli et al., 1996; Zini et al., 1996), implicating it in the regulation of gene transcription.

PLC-β1a may regulate transcription and translation as the result of catalyzing the production of Ins(1,4,5)P_3 and 1,2-diacylglycerol, which are key regulators of Ca^{2+}-dependent and 1,2-diacylglycerol-dependent protein kinases, respectively. Both of these families of protein kinases have been shown to control transcription and translation (Angenstein et al. 2002; Ventura and Maioli, 2001; West et al., 2001). The potential importance of PLC-β1 in the regulation of gene transcription becomes more compelling in light of work establishing the existence of a nuclear PtdIns(4,5)P_2 signaling system (Divecha et al., 1991; Irvine, 2003; Sun et al., 1997).

3.6. Fear-conditioning-dependent regulation of PLC-β1a catalytic activity

Some of the most interesting results to arise from these studies were the cases where changes in enzyme activity could not be accounted for by concurrent changes in enzyme concentrations. In these instances, the calculated specific activity of PLC-β1a was altered. An association between the regulation of the specific activity of a PLC isoform and the formation of a specific memory has not been previously reported. Changes in the calculated enzyme specific activities seen following fear conditioning may be due to direct modification (e.g., phosphorylation) of PLC-β1a, association of the enzyme with an activity-modifying molecule (e.g., G-protein subunit), or changes in enzyme state (e.g., oligomerization). It has been shown that PLC-β1 activity is regulated by protein kinase C (Filtz et al., 1999; Litosch, 1997; Ryu et al., 1990), mitogen-activated protein kinase (Vitale et al., 2001), interaction with G-proteins (Blayney et al., 1996; Boyer et al., 1994; Jhon et al., 1993; Lee et al., 1994), and self-association (Morris and Scarlata, 1997; Paulssen et al., 1998). The mechanisms underlying the observed changes in PLC-β1a specific activity in response to fear conditioning are presently under investigation.

4. Conclusion

In summary, single-trial fear conditioning was found to initiate complex and dynamic temporal changes in PLC-β1a enzyme activities and concentrations associated with the HPF and MFC. These results extend our previous study (Weeber et al., 2001) and correlate the regulation of PLC-β1a enzyme activity and subcellular distribution and the processes involved in learning and memory formation.

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