

Single and Repeated Stress-Induced Modulation of Phospholipase C Catalytic Activity and Expression: Role in LH Behavior

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PI-PLC, a critical enzyme of the phosphoinositide (PI) signaling pathway, mediates many physiological functions in the brain, including cellular plasticity. Stress-induced learned helplessness (LH) in animals serves as a model of behavioral depression. Recently, we observed that repeated stress prolongs the duration of LH behavior in rats, enabling us to compare neurobiologic abnormalities in acute and chronic depression. Here we examine whether LH behavior is associated with alterations in phospholipase C (PLC), and whether repetition of inescapable shock has similar or dissimilar effects on PLC to those of the single-stress paradigm. Rats were exposed to inescapable shock either once on day 1, or twice, on days 1 and 7. Rats were tested for escape latency on days 2 and 4 after day 1 inescapable shock or on days 2, 8, and 14 after day 1 and 7 inescapable shock. PI-PLC activity and mRNA and protein expression of three different PLC isozymes were determined in the frontal cortex and hippocampus. Higher escape latencies were observed in LH rats tested on day 2 after single inescapable shock and on day 14 after repeated inescapable shock. Single inescapable shock reduced PI-PLC activity in the frontal cortex and hippocampus of LH rats. On the other hand, repeated inescapable shock not only reduced PI-PLC activity in these brain areas of LH rats but also selectively decreased the expression of PLC β_1 and PLC γ_1 isozymes. Our results suggest different responsiveness at the level of PI-PLC after single vs repeated stress, and that reductions in PLC may be critical in the pathophysiology of depression and other stress-related disorders.

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INTRODUCTION

Depression is a major public health concern, afflicting about nine million people each year in the United States alone. Despite many years of research, the molecular and cellular mechanisms associated with depression are not clear.

The learned helplessness (LH) model is considered one of the most valid and reliable animal models of depression. It represents the theoretical basis of the origin and development of depression, as the model is a combination of cognitive and neurovegetative abnormalities and genetic susceptibility (Jesberger and Richardson, 1985; Henn *et al*, 1993; Nestler *et al*, 2002). In this animal model, however, the behavioral deficits can be seen for only a few days (2–3 days). Very recently, we developed an animal model of

depression in rats using an LH paradigm in which repeated stress prolongs the duration of LH behavior (Dwivedi *et al*, 2004). This is critical, since the neurobiology and the response to chronic/recurrent depression may be different from that in acute depression.

An emerging hypothesis suggests that depression may be associated with neuroplasticity and cellular adaptation (Duman *et al*, 2000), which may be due to the convergence of downstream events beyond the receptors at the level of intracellular signaling molecules (Nestler *et al*, 2002). Although several neurotransmitter systems have been studied in LH animals, including norepinephrine (Weiss *et al*, 1970; Minor *et al*, 1988; Petty *et al*, 1993), dopamine (Anisman and Zacharko, 1992), GABA (Drugan *et al*, 1989; Petty *et al*, 1992), serotonin (5HT) (Petty and Sherman, 1983; Maier *et al*, 1995; Maswood *et al*, 1998), adenosine (Minor *et al*, 2001), NMDA (Grahn *et al*, 2002), and opiate (Drugan and Maier, 1983), the role of signaling molecules beyond the receptors in LH behavior has not been fully investigated. In this regard, recently we showed that protein kinase A, a phosphorylating enzyme in the adenylyl cyclase-cAMP signaling mechanism, is significantly altered in the frontal cortex and hippocampus of LH rats, the effect being

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greater in the repeated-stress paradigm than in the acute-stress paradigm (Dwivedi *et al*, 2004).

PLC is a crucial enzyme of the phosphoinositide (PI) signaling mechanism that hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) (reviewed by Rebecchi and Pentylä, 2000). IP₃ mobilizes Ca²⁺ from intracellular sources after binding with IP₃ receptors; DAG activates the phosphorylating enzyme protein kinase C (Dekker *et al*, 1995). These events mediate cellular activation and subsequent biological responses, including neurotransmitter release, cell growth, differentiation, neuronal development, and plasticity. Recently, we showed that antidepressants and anxiolytics differentially modulate PLC in rat brain (Dwivedi *et al*, 2002) and that PLC is involved in suicidal behavior (Pandey *et al*, 1999). It is therefore of interest and important to examine whether LH behavior is associated with a modulation of PLC and whether these effects are similar or dissimilar in acute- and repeated-stress paradigms.

To characterize the role of PLC in LH behavior, we systematically determined PI-PLC catalytic activity and the expression of PLC isozymes at transcriptional and translational levels in the frontal cortex and hippocampus of rats subjected to two different stress paradigms. In addition, to investigate whether the changes in PLC are specifically associated with LH behavior, in the present study we included those rats in which the LH behavior had dissipated after the single stress.

MATERIALS AND METHODS

Animals

Virus-free male Holtzman rats (Harlan Sprague-Dawley Laboratories, Indianapolis, IN, USA) were housed in individual cages under standard laboratory conditions (temperature 21 ± 1°C, humidity 55 ± 5%, 12-h light/dark cycle). Animals were provided free access to food and water. Animals were housed for 3 weeks before the experiment, and the body weight was 325–375 g at the start of the experiment. All the experiments were performed between 0800 and 1000 h. Experimental procedures were approved by the Animal Care Committee of the University of Illinois at Chicago.

Behavioral Procedures

Learned helplessness induction by inescapable shock (IS) and escape testing were performed by the procedures described earlier by us (Dwivedi *et al*, 2004). Rats were divided into three groups designated as group A, group B, and group C. Group A rats were given IS on day 1 and were tested for escape latency on day 2. Group B rats were given IS on day 1 and tested for escape latency on day 2; the rats were tested again on day 4. Group C rats were given IS on day 1 and tested for escape behavior on day 2; these animals were given another IS on day 7 and tested for escape behavior on day 8 and on day 14. All the animals were decapitated 24 h after the last escape testing.

Inescapable Shock Treatment

The rats were placed in Plexiglas tubes with the rat's tail extending from the rear of the tube. The tail was attached

with tape to a Plexiglas rod. Shocks were delivered by means of a computer-controlled constant-current shock generator (Model ENV-410B, Med Associates, Lafayette, IN, USA) to electrodes augmented with electrode paste to the rat's tail. The inescapable shock consisted of 100 random shocks delivered for 5 s at the intensity of 1.0 mA, with a mean interval of 60 s. Another group of rats (to be used as tested control) was placed in Plexiglas tubes, but was not subjected to shocks.

Shuttle Box Escape Testing

This was conducted in a shuttle box (70 × 20 × 20 cm³) (Med Associates, Lafayette, IN, USA). The shuttle box contained an electrified grid floor and was divided into two equal chambers with an arched doorway in the center (5 × 7 cm²). Footshock was delivered through the grid floor by a shock generator (Model ENV-413, Med Associates, Lafayette, IN, USA). The shuttle box was contained in a soundproof cubicle with an exhaust fan, and a 6-W lamp located in the center of the roof provided illumination. The shuttle box escape testing began with five trials (FR-1), during which a single crossing would terminate the shocks. This was followed by 25 trials (FR-2) in which a rat had to cross from one side of the shuttle box to the other and come back to terminate the shocks. Shocks were terminated automatically after 30 s if there was no response within that time. The intensity of the shocks was 0.6 mA. The shocks were presented on a variable schedule. Shuttle escape latencies and escape failures were recorded automatically by a computer attached to the generator and shuttle box. An FR-2 trial with escape latency > 20 s was defined as an escape failure. Rats having 10–25 failures to escape were considered to be deficient in the escape response.

Rats were divided into two groups based on the mean latency observed after FR-2: (1) those rats in which the mean latency was ≥ 20 s (termed learned helpless (LH)) and (2) those in which the mean latency was < 20 s (termed as non-learned helpless (NLH)). In our study, we found that about 50% of all the rats tested became LH rats. In group C, in which IS was given on day 1 and again on day 7, most of the rats who initially did not show an escape deficit (NLH) remained NLH even after the second shock paradigm; only a few showed a mean latency ≥ 20 s after the second paradigm. These rats were not included in the present study. The rats that were confined to Plexiglas tubes but were not shocked were also tested and termed as the tested control (TC). Groups A, B, and C contained eight LH, seven NLH, and eight TC rats.

Rats were decapitated 24 h after the last escape testing, as mentioned earlier. Trunk blood was collected on ice, centrifuged, and the plasma was stored at –80°C. Plasma corticosterone levels were measured by a commercially available radioimmunoassay kit (ICN Biomedical Inc., Cleveland, OH, USA). Brains were removed quickly. The various brain areas were dissected on ice and immediately stored at –80°C until analyzed.

Determination of PI-PLC Activity

PI-PLC activity was measured in membrane and cytosol fractions of the cortex and hippocampus by the enzymatic

assay procedures described earlier (Dwivedi *et al*, 2000) in the presence of 5 μ g protein and phosphatidylinositol 4,5 bisphosphate (PIP₂) as substrate (50 μ M unlabeled PIP₂, 2.0 μ Ci/ml [³H]PIP₂, and 0.5 mg/ml cetrimide). After termination of the reaction, radioactivity was counted. PI-PLC activity is expressed as the amount of [³H]IP₃ formed (dpm)/min/mg protein.

Immunolabeling of PLC β_1 , γ_1 , and δ_1 Isozymes

The procedure and characterization of the immunolabeling of PLC isozymes have been described earlier by us (Dwivedi *et al*, 2000). Equal volumes of protein samples (membrane or cytosol fractions; 20 μ l containing 30 μ g protein) were resolved onto 7.5% (w/v) polyacrylamide gel and subsequently transferred to an ECL nitrocellulose membrane (Amersham, Arlington Heights, IL, USA). Membranes were incubated for 1 h with primary monoclonal antibody (anti-PLC β_1 , γ_1 , or δ_1) at a dilution of 1:1000 and subsequently with horseradish-peroxidase-linked secondary antibody (anti-mouse IgG; 1:3000) for 1 h at room temperature. To normalize the data, β -actin level was measured in the same immunoblot using β -actin as the primary monoclonal antibody (1:5000 for 2 h) and anti-mouse IgG (1:5000 for 2 h) as the secondary antibody. The optical densities of the bands on the autoradiograms were quantified using the Loats Image Analysis System (Westminster, MD, USA), and the optical density of each PLC isozyme band was corrected by the optical density of the corresponding β -actin band.

Determination of mRNA Levels of PLC β_1 and γ_1 Isozymes by Competitive Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The procedure for determining the mRNA levels of PLC isozymes in rat brain has been described in detail earlier (Dwivedi *et al*, 2002). The primer pairs were designed to allow amplification of 532–847 base pairs (bp): forward, 5'-TTT TCG GCA GAC CGG AAG CGA, and reverse, 5'-TGC TGT TGG GCT CGT ACT TCT (GenBank accession #M20636) for PLC β_1 ; and 506–877 bp: forward, 5'-AGG ATC GTA TAT CAG CCA AGG A, and reverse, 5'-AGT ATG GCT CCT CAA TCT CTC G (GenBank accession #NM013187) for PLC γ_1 . The specificity of the PLC β_1 and γ_1 mRNA products was checked by sequencing the amplified area with the Sequenase Version 2.0 DNA Sequencing Kit using *Hind*III and *Eco*RI, which produced fragments of the expected size.

The detailed procedures for the preparation of internal standards (cRNA) have been described earlier (Dwivedi *et al*, 2002). Each internal standard template was generated by site-directed mutagenesis to introduce a *Bgl*III restriction site. The internal primer sequences for PLC β_1 and γ_1 were as follows: PLC β_1 (645–668 bp): 5'-CCT GAA AGAT CTT CCC CGA CC; PLC γ_1 (689–706 bp): 5'-TGG ACC TTC AGA TCT TGG AAA CCA. The underlined bases indicate the *Bgl*III restriction site, whereas bold and italicized bases indicate the mutation sites.

Decreasing concentrations of PLC β_1 or γ_1 internal standard cRNA were added to 1 μ g of total RNA isolated from cortices or hippocampi. After termination of the RT

reaction, cDNA aliquots containing reverse transcribed materials were amplified with Hot *Tub* DNA polymerase in the Thermal Cycler (9600, Perkin-Elmer). Trace amounts of [³²P]dCTP (0.5–1 μ Ci/sample) were included during the PCR step for subsequent quantification. The PCR mixture was amplified for 26 cycles. Following amplification, aliquots were digested with *Bgl*III in triplicate and run on to 1.5% agarose gel electrophoresis. The results are expressed as attomoles/ μ g of total RNA.

RESULTS

Escape Latencies and Escape Failures

We observed no significant differences in mean escape latencies after the FR-1 trials in all experimental paradigms (data not shown). Significant differences were observed in escape latencies among TC, NLH, and LH rats after the FR-2 trials. In group A (IS on day 1 and escape test on day 2), LH rats showed a significantly higher mean escape latency ($P < 0.001$) than NLH or TC rats, whereas the escape latency of NLH rats was not significantly different from that of TC rats ($P = 0.86$). The escape latencies (in seconds) in group A rats were as follows: TC = 6.4 ± 1.8 ; NLH = 7.2 ± 3.7 ; LH = 23.6 ± 3.3 . Similarly, in group B (IS on day 1 and escape test on day 2 and again on day 4), the mean escape latency was significantly higher on day 2 in LH rats ($P < 0.001$) as compared with TC or NLH rats (TC = 5.7 ± 2.8 ; NLH = 4.2 ± 2.1 ; LH = 25.3 ± 3.5); however, on day 4, the escape latency of the LH rats was similar to that of NLH or TC rats (TC = 5.2 ± 1.2 ; NLH = 4.5 ± 1.9 ; LH = 8.2 ± 3.3). In group C (IS on days 1 and 7 and escape latency determined at three different time intervals: days 2, 8, and 14), the mean escape latency was significantly higher in LH ($P < 0.001$) rats as compared with the NLH group at all time intervals. The mean latencies at the different time intervals in group C rats were as follows: day 2: TC = 6.1 ± 3.0 ; NLH = 4.9 ± 1.8 ; LH = 25.1 ± 3.5 ; day 8: TC = 6.4 ± 2.7 ; NLH = 4.8 ± 2.1 ; LH = 25.2 ± 3.8 ; and day 14: TC = 4.3 ± 2.2 ; NLH = 4.9 ± 2.4 ; LH = 26.3 ± 3.2 . None of the groups showed any significant differences in mean escape latency between TC and NLH rats.

The escape failures in LH rats were significantly more numerous in group A and C rats, whereas no significant change was observed in group B rats. The number of escape failures in the different groups of rats was as follows: group A rats: TC = 2.1 ± 1.1 ; NLH = 2.7 ± 1.4 ; LH = 18.1 ± 2.5 (df = 2,20, $F = 192$, $P < 0.001$); group B rats: TC = 3.0 ± 1.3 ; NLH = 3.4 ± 1.7 ; LH = 3.6 ± 0.9 (df = 2,20, $F = 0.47$, $P = 0.64$); and group C rats: TC = 2.2 ± 1.0 ; NLH = 3.3 ± 1.1 ; LH = 19.0 ± 3.5 (df = 2,20, $F = 137$, $P < 0.001$).

Corticosterone Levels

Plasma corticosterone levels were measured in rats of groups A and C and were as follows (ng/ml): group A rats: TC = 257 ± 86 , NLH = 245 ± 99 , LH = 342 ± 100 ; group C rats: TC = 269 ± 116 , NLH = 253 ± 169 , LH = 384 ± 85 . Plasma corticosterone levels did not differ among TC, NLH, and LH rats either in group A (df = 2, 20, $F = 2.38$, $P = 0.11$) or in group C (df = 2, 20, $F = 2.49$, $P = 0.10$).

PI-PLC Activity

PI-PLC activity was determined in membrane and cytosol fractions of frontal cortex and hippocampus obtained from TC, LH, and NLH rats of group A (IS on day 1 and escape test on day 2), group B (IS on day 1 and escape test on day 2 and again on day 4), and group C (IS on days 1 and 7 and escape test on days 2, 8, and 14). As can be seen in Figure 1, PI-PLC activity was higher in both membrane and cytosol fractions of hippocampus than of the frontal cortex. A comparison of TC, LH, and NLH rats of group A showed that PI-PLC activity was significantly decreased in membrane and cytosol fractions of the frontal cortex and hippocampus of LH rats as compared with TC or NLH rats, whereas no significant differences were observed between NLH and TC rats (Figure 1). On the other hand, PI-PLC activity was not significantly different among TC, NLH, and LH rats of group B, either in the frontal cortex or hippocampus (Figure 1). When rats were given repeated shocks and tested on day 14 (group C rats), it was observed that PI-PLC activity was significantly decreased in mem-

brane and cytosol fractions of the frontal cortex and hippocampus of LH rats, the magnitude of the decrease being greater than that observed with group A rats (Figure 1).

Immunolabeling of PLC Isozymes

The immunolabeling of the various PLC isozymes (PLC β_1 , δ_1 , and γ_1) was determined in the same membrane and cytosol fractions of frontal cortex and hippocampus in which PI-PLC activity was determined. As in our earlier reports (Dwivedi *et al*, 2000, 2002), in the present study it was observed that PLC β_1 migrated to 150 kDa, whereas PLC δ_1 and γ_1 migrated to 85 and 145 kDa, respectively (Figure 2). The molecular mass of β -actin, used as a housekeeping protein, was 46 kDa. As we reported recently (Dwivedi *et al*, 2004), the immunolabeling of β -actin in the frontal cortex and hippocampus did not change after shock treatment.

The LH behavior-associated effects on the immunolabeling of PLC isozymes in membrane and cytosol fractions of

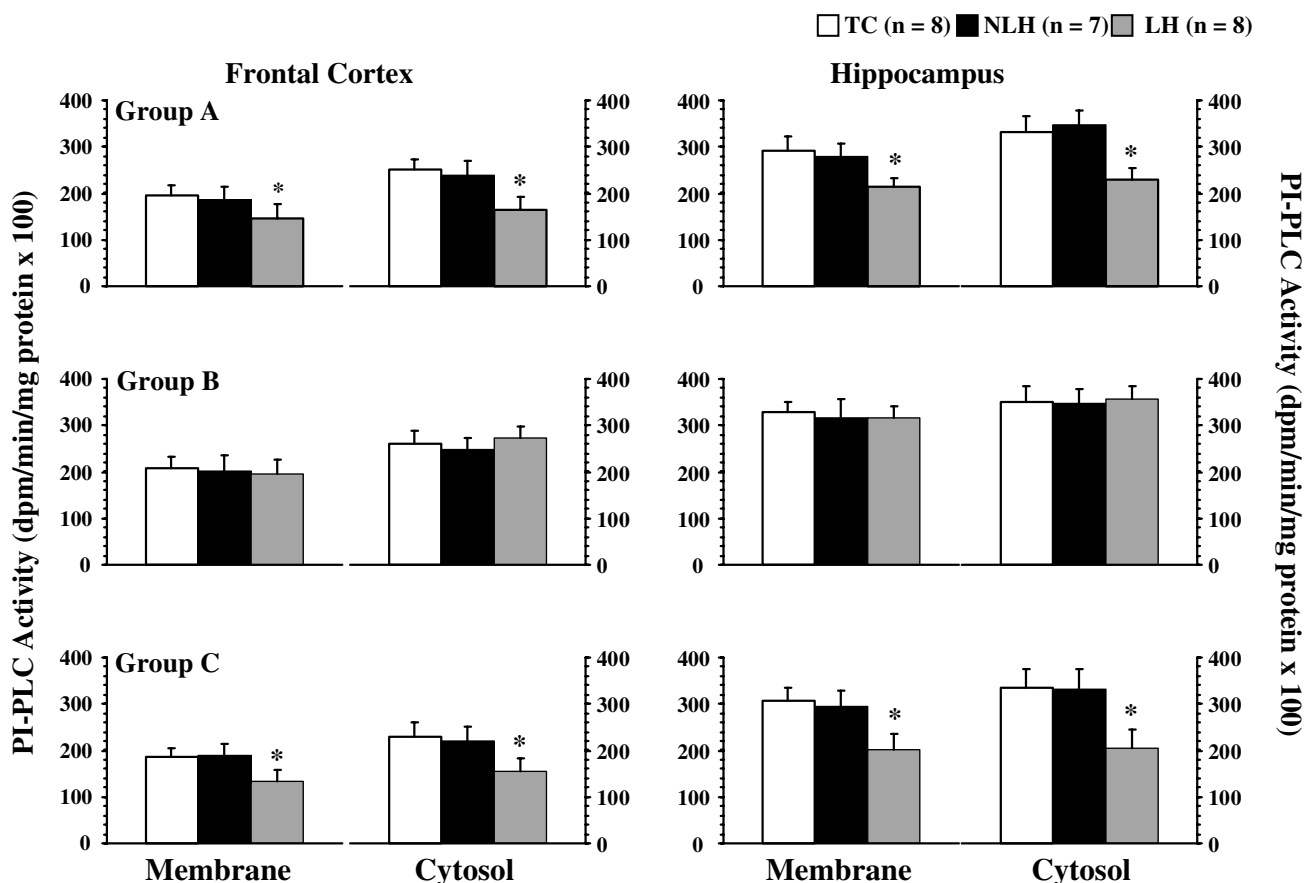


Figure 1 PI-PLC activity in membrane and cytosol fractions of the frontal cortex and hippocampus of groups A (given inescapable shock on day 1 and tested for escape latency on day 2), B (given inescapable shock on day 1 and tested for escape latency on day 4), and C (given inescapable shock on day 1 and tested for escape latency on day 2; rats were again given shock on day 7 and tested for escape latency on day 8, and re-tested on day 14); each comprised of TC, NLH, and LH rats. Rats were decapitated 24 h after the last testing. Values are means \pm SD. Overall group differences in PI-PLC activity in different groups were as follows: Group A (frontal cortex: membrane, $df=2, 20$, $F=9.2$, $P=0.001$; cytosol, $df=2, 20$, $F=22.59$, $P<0.001$; hippocampus: membrane, $df=2, 20$, $F=20.16$, $P<0.001$; cytosol, $df=2, 20$, $F=34.45$, $P<0.001$); Group B (frontal cortex: membrane, $df=2, 20$, $F=0.2$, $P<0.77$; cytosol, $df=2, 20$, $F=2.0$, $P=0.16$; hippocampus: membrane, $df=2, 20$, $F=0.48$, $P=0.62$; cytosol, $df=2, 20$, $F=0.19$, $P=0.82$); and Group C (frontal cortex: membrane, $df=2, 20$, $F=12.45$, $P<0.001$; cytosol, $df=2, 20$, $F=14.73$, $P<0.001$; hippocampus: membrane, $df=2, 20$, $F=24.47$, $P<0.001$; cytosol, $df=2, 20$, $F=26.89$, $P<0.001$). * $P<0.001$ as compared with TC.

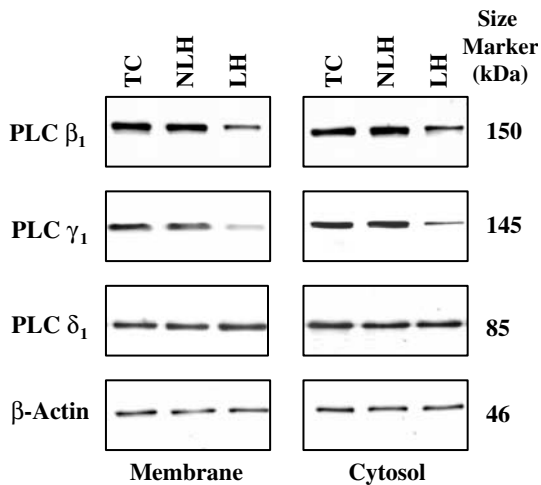


Figure 2 Representative Western blots of PLC isozymes in membrane and cytosol fractions of the frontal cortex obtained from group C rats (given inescapable shock on day 1 and tested for escape latency on day 2; rats were again given shock on day 7 and tested for escape latency on day 8, and re-tested on day 14), comprised of TC, NLH, and LH rats. Rats were decapitated 24 h after the last testing. Protein samples (30 μ g) were electrophoresed on a 7.5% polyacrylamide gel, followed by electrotransfer to a nitrocellulose membrane. The blots were probed with monoclonal anti-PLC β_1 , δ_1 , or γ_1 antibodies (1:1000 dilution). The molecular weights for the PLC β_1 , γ_1 , and δ_1 isozymes were 150, 145, and 85 kDa, respectively. The blots were stripped and reprobed with β -actin antibody (1:5000 dilution). β -Actin migrated to 46 kDa. Ratios of the optical densities of PLC β_1 , δ_1 , or γ_1 to the optical density of β -actin were calculated.

the frontal cortex of group A rats (IS on day 1 and tested for escape latency on day 2) are given in Figure 3. In this group of rats, no significant differences among LH, NLH, and TC rats were observed in the immunolabeling of PLC β_1 , δ_1 , or γ_1 isozyme in membrane or cytosol fractions of the frontal cortex or hippocampus. Similarly, group B rats (given IS on day 1 and tested for escape latency on day 4) did not show significant differences in the immunolabeling of any of the PLC isozymes, either in the frontal cortex or hippocampus. When PLC isozyme protein levels were determined in group C rats (IS on days 1 and 7 and escape test on day 14), the immunolabeling of PLC β_1 and PLC γ_1 were significantly decreased in both membrane and cytosol fractions of the frontal cortex and hippocampus of LH rats as compared with NLH or TC rats (Figure 3).

mRNA Levels of PLC Isozymes

Since PI-PLC activity was decreased in LH rats after both single and repeated stress, but specific PLC isozymes were decreased only after repeated stress, we determined the LH behavior-associated effects on the transcriptional regulation of PLC β_1 and γ_1 isozymes in all groups of rats. Representative gel electrophoreses of PLC β_1 and γ_1 are given in Figure 4. It was observed that amplification products arise from the mRNA template at 316 and 372 bp and digestion products arise from the cRNA at 194 + 122 and 189 + 183 bp, respectively, for PLC β_1 and γ_1 isozymes. Comparative analysis showed that mRNA levels of PLC β_1 and γ_1 were higher in the hippocampus than in frontal cortex (Figure 5). As noted with the immunolabeling of PLC

isozymes, no significant differences were observed in mRNA levels of PLC β_1 or γ_1 isozyme either in the frontal cortex or hippocampus of groups A or B, LH rats as compared with NLH or TC rats (Figure 5). On the other hand, repeated shock (group C rats; IS on days 1 and 7, and tested on day 14) significantly decreased PLC β_1 and γ_1 mRNA levels in the frontal cortex and hippocampus of LH rats as compared with NLH or TC rats (Figure 5).

DISCUSSION

The behavioral findings and the observed effects on PLC were highly correlated and were stress paradigm-dependent. The escape latencies and escape failures were clearly different in LH rats as compared with TC or NLH rats. Whereas escape latencies and escape failures were higher in LH rats, they were similar in NLH and TC rats. A higher escape latency was noted in LH rats exposed to IS on day 1 when tested for escape latency on day 2, but not when these rats were tested on day 4, suggesting that this behavioral deficit does not persist for 4 days after a single-IS paradigm. On the other hand, when rats were exposed to another IS on day 7 in addition to the first IS and were tested on day 14, the escape latency was higher in LH rats as compared with NLH or TC rats. Our observations thus suggest that exposure to a second IS produces a longer-lasting behavioral deficit. This is an important observation, as this model may enable comparison of neurobiological phenomena in acute vs chronic/recurrent depression, which may have different responsive mechanisms.

Our studies of PLC in the three different groups of rats showed that the catalytic activity of PI-PLC was decreased in both membrane and cytosol fractions of the frontal cortex and hippocampus of LH rats subjected to a single stress. Similar results were noted after repeated stress; however, no change was observed when rats given single stress were tested on day 4. We further examined whether the effect of LH behavior on PI-PLC activity was related to the expression of any specific PLC isozyme. On the basis of sequence homology and localization of structural domains, PLC has been characterized into three major families: PLC β , δ , and γ (Rebecchi and Pentylala, 2000; Rhee, 2001). All PLC isozymes recognize PIP₂ as a substrate and carry out Ca²⁺-dependent hydrolysis of inositol lipids; however, these isozymes are differentially regulated and expressed. The PLC β family includes the 150-kDa products of four genes (β_1 – β_4). Of these β_1 , β_3 , and β_4 are present in the brain. PLC β_1 is highly expressed in the cortex and hippocampus (Ross *et al*, 1989), whereas expression of PLC β_2 is almost negligible in the cortex and hippocampus (Tanaka and Kondo, 1994). The expression of PLC β_3 is low throughout the brain (Tanaka and Kondo, 1994). The activity of these isoforms is regulated by hormone–receptor complexes through heterotrimeric G proteins (Kamat and Carpenter, 1997). Both G_q and G $\beta\gamma$ proteins activate PLC β . The PLC γ family consists of PLC γ_1 and γ_2 . PLC γ_1 is ubiquitously expressed (Rhee *et al*, 1991), whereas PLC γ_2 expression is confined to the spleen and thymus (Emori *et al*, 1989). PLC γ is regulated by receptors and nonreceptor tyrosine kinases (Rhee, 2001). Little is known about the regulation of PLC δ . Recent studies, however, suggest that

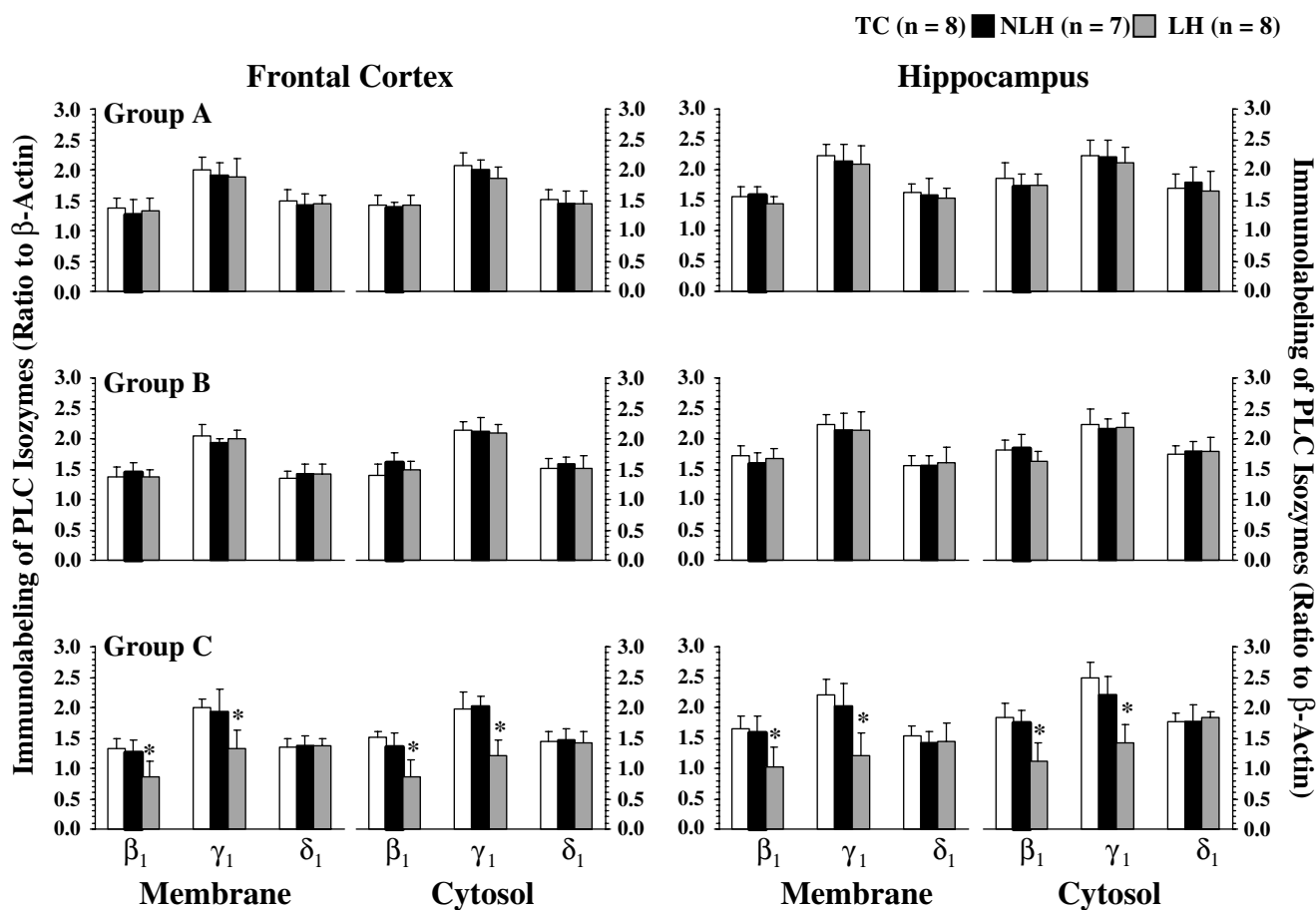


Figure 3 Immunolabeling of PLC isozymes in membrane and cytosol fractions of the frontal cortex and hippocampus of groups A (given inescapable shock on day 1 and tested for escape latency on day 2), B (given inescapable shock on day 1 and tested for escape latency on day 4), and C (given inescapable shock on day 1 and tested for escape latency on day 2; rats were again given shock on day 7 and tested for escape latency on day 8, and re-tested on day 14), comprised of TC, NLH, and LH rats. Rats were decapitated 24 h after the last testing. Values are means \pm SD. Overall group differences in different groups were as follows: Group A (frontal cortex: membrane, PLC β , df = 2, 20, $F = 0.55$, $P = 0.58$; PLC γ , df = 2, 20, $F = 0.59$, $P = 0.56$; PLC δ , df = 2, 20, $F = 0.08$, $P = 0.92$; cytosol, PLC β , df = 2, 20, $F = 0.18$, $P = 0.83$; PLC γ , df = 2, 20, $F = 2.4$, $P = 0.11$; PLC δ , df = 2, 20, $F = 0.25$, $P = 0.78$; hippocampus: membrane, PLC β , df = 2, 20, $F = 2.5$, $P = 0.10$; PLC γ , df = 2, 20, $F = 0.69$, $P = 0.51$; PLC δ , df = 2, 20, $F = 0.37$, $P = 0.69$; cytosol, PLC β , df = 2, 20, $F = 0.94$, $P = 0.40$; PLC γ , df = 2, 20, $F = 0.41$, $P = 0.67$; PLC δ , df = 2, 20, $F = 0.39$, $P = 0.68$); Group B (frontal cortex: membrane, PLC β , df = 2, 20, $F = 0.63$, $P = 0.54$; PLC γ , df = 2, 20, $F = 0.77$, $P = 0.48$; PLC δ , df = 2, 20, $F = 0.02$, $P = 0.59$; cytosol, PLC β , df = 2, 20, $F = 2.5$, $P = 0.11$; PLC γ , df = 2, 20, $F = 0.06$, $P = 0.94$; PLC δ , df = 2, 20, $F = 0.29$, $P = 0.75$; hippocampus: membrane, PLC β , df = 2, 20, $F = 1.13$, $P = 0.34$; PLC γ , df = 2, 20, $F = 0.4$, $P = 0.67$; PLC δ , df = 2, 20, $F = 0.10$, $P = 0.90$; cytosol, PLC β , df = 2, 20, $F = 3.05$, $P = 0.07$; PLC γ , df = 2, 20, $F = 0.16$, $P = 0.85$; PLC δ , df = 2, 20, $F = 0.08$, $P = 0.92$); and Group C (frontal cortex: membrane, PLC β , df = 2, 20, $F = 10.63$, $P = 0.001$; PLC γ , df = 2, 20, $F = 12.92$, $P < 0.001$; PLC δ , df = 2, 20, $F = 0.03$, $P = 0.97$; cytosol, PLC β , df = 2, 20, $F = 7.83$, $P = 0.003$; PLC γ , df = 2, 20, $F = 27.77$, $P < 0.001$; PLC δ , df = 2, 20, $F = 0.48$, $P = 0.95$; hippocampus: membrane, PLC β , df = 2, 20, $F = 18.33$, $P < 0.001$; PLC γ , df = 2, 20, $F = 19.18$, $P < 0.001$; PLC δ , df = 2, 20, $F = 0.49$, $P = 0.62$; cytosol, PLC β , df = 2, 20, $F = 18.55$, $P < 0.001$; PLC γ , df = 2, 20, $F = 28.98$, $P < 0.001$; PLC δ , df = 2, 20, $F = 0.19$, $P = 0.83$). * $P < 0.001$ as compared with TC.

PLC δ is activated by an atypical G protein, that is, G_{Hh} , which acts as a transglutaminase (Murthy *et al*, 1999) and is a Ca^{2+} signal amplifier (Allen *et al*, 1997). In the present study, some interesting observations were noted when the expression of PLC β , γ , and δ isozymes was determined after single and repeated stress.

Whereas repeated stress was associated with a selective decrease in the protein expression of PLC β_1 and γ_1 in membrane and cytosol fractions of the frontal cortex and hippocampus of LH rats, no significant changes were noted in any of the PLC isozymes in LH rats after the single-stress paradigm. With the use of specific primers and quantitative RT-PCR, a concomitant decrease in mRNA levels of the PLC β_1 and γ_1 isozymes was observed in LH rats after repeated stress. Our findings that only PI-PLC activity is decreased in

LH rats after the single-stress paradigm and that not only PI-PLC activity but also the expression of specific isozymes of PLC are decreased in LH rats after the repeated-stress paradigm clearly demonstrate that PLC is differentially modulated in LH rats in response to single vs repeated stress.

The changes in PI-PLC activity and in expression of PLC β_1 and γ_1 appear to be specific to LH behavior, since these changes are very well correlated with the behavioral findings. For example, no changes in PI-PLC activity or in the expression of PLC isozymes were noted in those LH rats who were tested 4 days after a single-IS paradigm, and whose escape latencies were similar to those of TC or NLH rats. On the other hand, changes in PLC occurred only in those LH rats who showed higher escape latencies, whether

subjected to a single- or a repeated-IS paradigm. Furthermore, to rule out whether the changes in PLC are nonspecific, namely, due to the stress caused by restraint, tail shock, or testing, TC rats were handled similarly as NLH and LH rats, and NLH rats were given IS, and both TC and NLH rats were tested for escape behavior.

The observed modulation of PLC in the brain of LH rats is quite intriguing. Very recently, we examined the effects of psychoactive drugs on PI-PLC and observed that chronic administration of antidepressants, such as fluoxetine, desipramine, phenelzine, and the anti-anxiety drug buspirone, to normal rats decreased, whereas administration of

the anxiogenic drug metachlorophenylpiperazine increased, PI-PLC activity as well as expression of the PLC β_1 isozyme in rat cortex and hippocampus, without any change in the expression of PLC δ_1 or γ_1 (Dwivedi *et al*, 2002). Interestingly, LH rats exhibit anxious behavior, and buspirone was found to attenuate LH behavior in rats (Drugan *et al*, 1987). Given that antidepressants and buspirone as well decrease PI-PLC activity and expression of the PLC β_1 isozyme, one might expect the opposite effects on PI-PLC to be associated with LH behavior. However, this was not the case. We found that both antidepressants and LH cause a decrease in PI-PLC. Such findings are not surprising. It has been reported that 5HT_{2A} receptors are upregulated in depression (Biegon *et al*, 1987; Arora and Meltzer, 1989a; Hrdina *et al*, 1995) and that antidepressant administration to normal human controls (Lowther *et al*, 1994) and electroconvulsive shock (Kellar *et al*, 1981) to rats also increase 5HT_{2A} receptors. Similarly, β -adrenergic receptors are upregulated in the leukocytes of depressed patients (Extein *et al*, 1979; Carstens *et al*, 1987; Pandey *et al*, 1987; Magliozzi *et al*, 1989) and after antidepressant treatment (Sulser, 1979; Peroutka and Snyder, 1980; Pandey *et al*, 1985). Although the idea is speculative in nature, it is quite possible that PI-PLC may be regulated differently under the physiological conditions induced by learned helplessness/depression. Whereas PI-PLC may be down-regulated after antidepressant administration to wild-type animals, antidepressants may reverse this decrease when given to LH rats. Future studies are needed to clarify this issue.

Interestingly, as mentioned earlier, the single-stress paradigm decreased only PI-PLC activity, whereas repeated stress decreased not only PI-PLC activity but also the expression of PLC β and γ isozymes. It appears that a decrease in PI-PLC activity after repeated stress may be due to decreased synthesis of PLC β and γ isozymes. This is further supported by the notion that upon activation PLC is translocated from the membrane to cytosol. Since we observed that PI-PLC activity as well as expression of β and γ isozymes was decreased in both membrane and cytosol fractions, this supports the possibility that the decrease in PI-PLC activity could be due to the decreased synthesis of these specific PLC isozymes. As far as decrease in PI-PLC activity after single stress is concerned, no

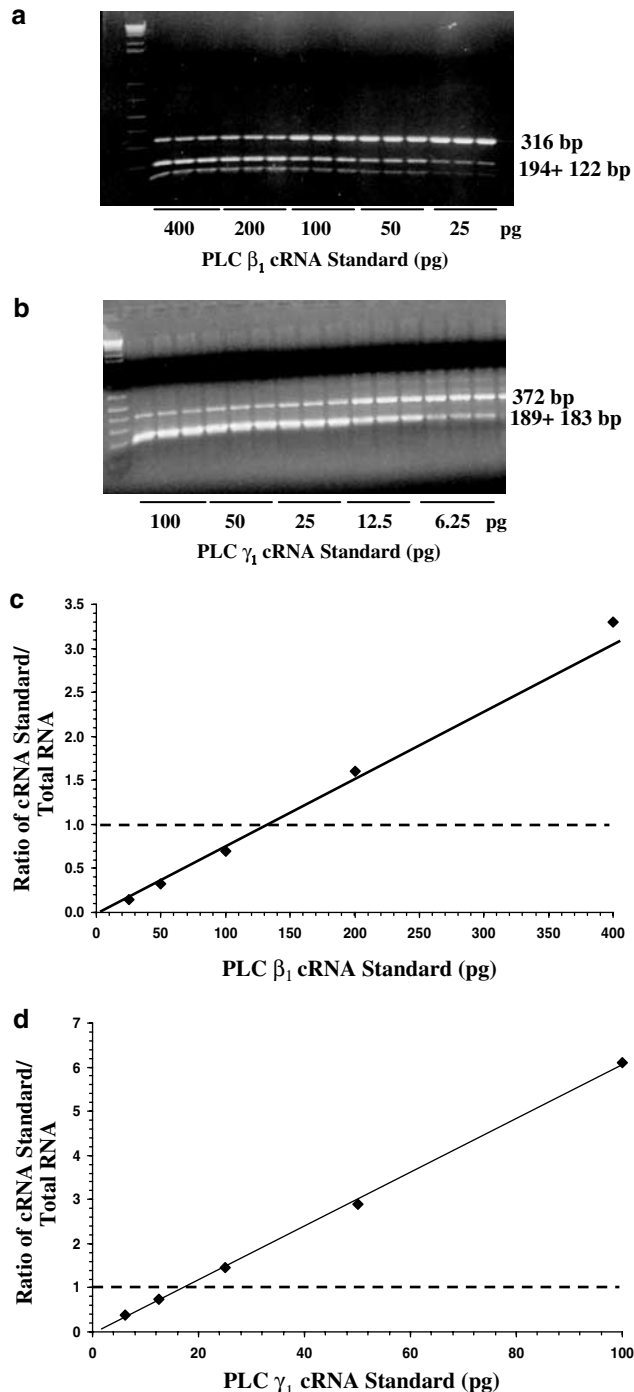


Figure 4 Representative experiments showing competitive PCR analyses for PLC β_1 (a) and γ_1 (b) mRNA content in the frontal cortex of a normal rat brain. Decreasing concentrations of PLC β_1 (400–25 pg) or PLC γ_1 (100–12.5 pg) standard cRNA were added to a constant amount (1 μ g) of total RNA isolated from the frontal cortex. The mixtures were reverse-transcribed and PCR-amplified in the presence of trace amounts of [³²P]dCPT; aliquots were digested by BglII and electrophoresed on 1.5% agarose gel. The higher molecular size band (316 bp for PLC β_1 and 372 bp for PLC γ_1) corresponds to the amplification products arising from the mRNA, whereas the lower bands (194 + 122 bp for PLC β_1 and 189 + 183 bp for PLC γ_1) arise from cRNA generated from the internal standard digested by BglII. Data derived from the agarose gel are plotted as the counts incorporated into the amplified cRNA standard divided by the counts incorporated into the corresponding isozyme mRNA amplification product vs the known amount of internal standard cRNA added to the test sample. The point of equivalence represents the amount of PLC β_1 (c) or PLC γ_1 (d) mRNA.

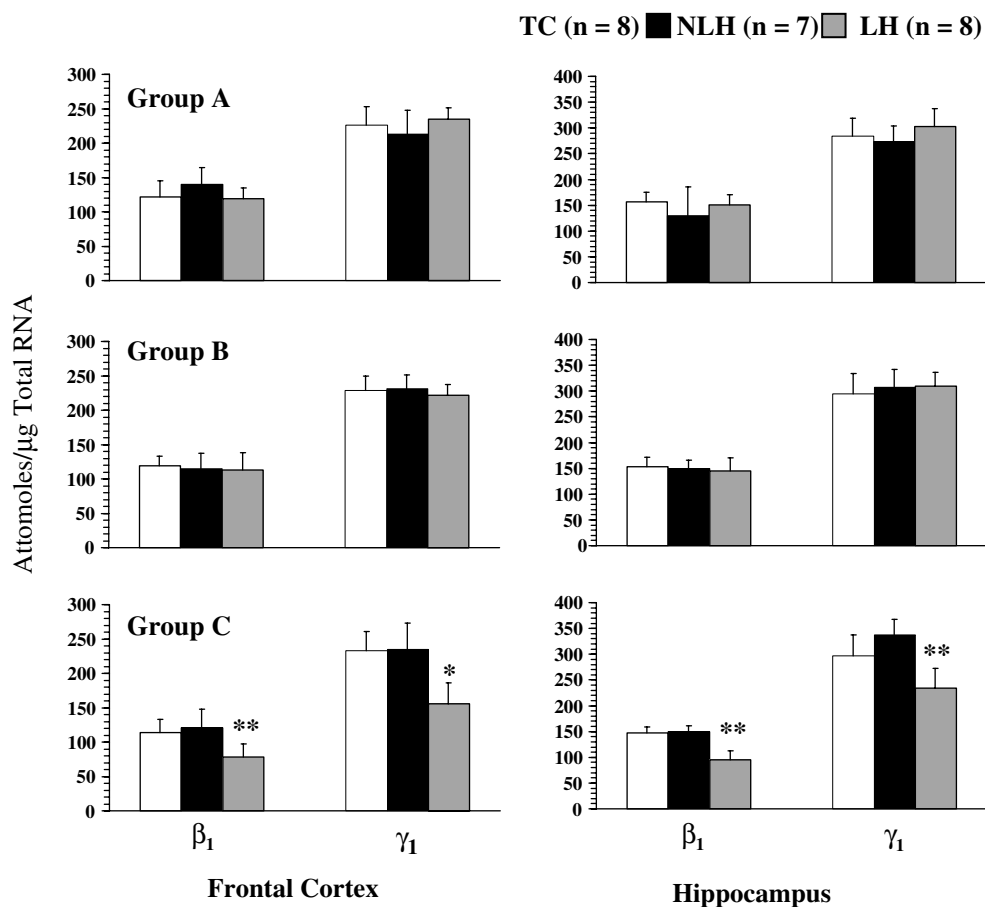


Figure 5 mRNA levels of PLC isozymes in the frontal cortex and hippocampus of groups A (given inescapable shock on day 1 and tested for escape latency on day 2), B (given inescapable shock on day 1 and tested for escape latency on day 4), and C (given inescapable shock on day 1 and tested for escape latency on day 2; rats were again given shock on day 7 and tested for escape latency on day 8, and re-tested on day 14), comprised of TC, NLH, and LH rats. Rats were decapitated 24 h after the last testing. Values are means \pm SD. Overall group differences in different groups were as follows: Group A (frontal cortex: PLC β , $df = 2, 20$, $F = 1.9$, $P = 0.17$; PLC γ , $df = 2, 20$, $F = 1.21$, $P = 0.32$; hippocampus: PLC β , $df = 2, 20$, $F = 0.37$, $P = 0.69$; PLC γ , $df = 2, 20$, $F = 1.3$, $P = 0.26$); Group B (frontal cortex: PLC β , $df = 2, 20$, $F = 0.18$, $P = 0.83$; PLC γ , $df = 2, 20$, $F = 0.48$, $P = 0.62$; hippocampus: PLC β , $df = 2, 20$, $F = 0.31$, $P = 0.74$; PLC γ , $df = 2, 20$, $F = 0.39$, $P = 0.68$); and Group C (frontal cortex: PLC β , $df = 2, 20$, $F = 8.34$, $P = 0.002$; PLC γ , $df = 2, 20$, $F = 15.17$, $P < 0.001$; hippocampus: PLC β , $df = 2, 20$, $F = 29.01$, $P < 0.001$; PLC γ , $df = 2, 20$, $F = 14.61$, $P < 0.001$). * $P = 0.002$; ** $P < 0.001$ as compared with TC.

specific explanation is currently available; however, the possibility of a transient effect of acute stress on PI-PLC activity cannot be ruled out.

How PLC is regulated in brain of LH rats is not clear at the present time. In an earlier study, we demonstrated that PLC is regulated in response to glucocorticoids. For example, we found that, whereas the synthetic glucocorticoid dexamethasone increased, corticosterone administration decreased PI-PLC activity and expression of the PLC β_1 isozyme in rat brain (Dwivedi and Pandey, 1999; Dwivedi et al, 2000). In the present study, although we did not find significant differences in the levels of corticosterone among TC, NLH, and LH rats, a much higher level of corticosterone was noted in LH rats; however, due to the large variation, this increase was not significantly different. It is pertinent that a hyperactive hypothalamic-pituitary-adrenal axis has been reported during stress, anxiety, and depressive behavior (Plotsky et al, 1998; Strohle and Holsboer, 2003), which is associated with the release of cortisol. Many studies have shown that the induction of LH behavior is sensitive to the effects of corticosterone (De Kloet et al, 1988; Haracz

et al, 1988; Greenberg et al, 1989). Edwards et al (1990) has reported that adrenalectomy increases the number of animals who become helpless, and that this is reversed by corticosterone administration. There is a possibility that PLC might be regulated in response to elevated stress hormones.

Another possibility could be the desensitization of PLC in response to upstream events at the level of neurotransmitters. It is pertinent to mention that a number of studies demonstrate upregulated serotonin (5HT_{2A}) receptors, linked to the phosphoinositide signaling pathway, in postmortem brain of depressed patients and suicide victims (Mann et al, 1986; Arora and Meltzer, 1989b; Arango et al, 1990; Pandey et al, 2002) and in platelets of depressed patients (Pandey et al, 1995; see Mendelson, 2000). It is quite possible that the decrease in PLC may be in response to the activation of 5HT_{2A} receptors. Although a functional deficit of 5HT in LH rats has been reported (Hellhammer et al, 1984; Petty et al, 1992), the results pertaining to changes in 5HT_{2A} receptor number in LH behavior and stress have been inconsistent (Ferretti et al, 1995; Wu et al, 1999).

Regardless of the mechanisms regulating PLC in brain of LH rats, it is interesting to note that only PI-PLC activity was decreased after the single-stress paradigm, whereas repeated stress decreased not only PI-PLC activity but also the gene expression of specific PLC isozymes. These results suggest that the induction of LH could be a two-step process, an acute followed by long-term stabilization. It is quite possible that acute stress could be causing transient effects, whereas repeated stress might act at the level of gene expression, whose effects would be longer lasting.

The functional significance of the decrease in PLC associated with LH behavior is yet to be determined, but it is well established that a wide variety of extracellular signals produce many biological responses by regulating the state of phosphorylation and dephosphorylation of specific proteins, which are then involved in the regulation of cellular functions. It has been shown that PLC is involved in various neuronal functions, such as synthesis and release of neurotransmitters, regulation of receptors and ion channels, growth, differentiation, and gene expression. Given that depression may be associated with cellular adaptation and neuronal plasticity (Malberg and Duman, 2003), a decrease in PLC is quite relevant in depressive behavior. That there is a decrease in PLC γ_1 expression in the brain of LH rats is quite striking. Whereas PLC β_1 is regulated by G-protein-coupled receptors, PLC γ is regulated by several tyrosine kinase receptors (Rhee, 2001), including those with trophic activity in brain, such as nerve growth factor and brain-derived neurotrophic factor, thus regulating cell division, differentiation, cell survival, and apoptosis. In addition, it has been shown that inhibition of PLC γ strongly inhibits the activation of extracellular signal-regulated kinase and Akt in fibroblasts (Amin et al, 2003), which are critical in the regulation of cell survival and apoptosis. As is true for both PLC β and γ of their role in releasing Ca^{2+} from intracellular sources, a new role of PLC γ has emerged. Recently, Patterson et al (2002) have found that PLC γ is essential for the activation of calcium entry into cells after stimulation of cell surface receptors. It is pertinent to mention that disturbances in Ca^{2+} regulation in depression (Mikuni et al, 1991; Kusumi et al, 1994; Dwivedi et al, 1998; Kusumi et al, 2000; Plein and Berk, 2000; Berk et al, 2001), and recently in lymphocytes of LH rats (Velbinger et al, 2000), have been reported. Also, Turecki et al (1998) have reported evidence that PLC γ_1 may be involved in the predisposition to bipolar disorder of the lithium-responsive patients, suggesting a role of PLC γ_1 in the pathogenesis of affective disorder.

In conclusion, to our knowledge, this is the first study that examines the crucial components of the PI signaling system, that is, PI-PLC, in LH behavior. Our study demonstrates diminished activation of PLC in the brain of LH rats, which apparently has different responsive mechanisms at the level of PLC isozyme expression as a consequence of single vs repeated stress. Whereas single stress causes a decrease in PI-PLC activity, repeated stress causes lower expression of PLC β_1 and γ_1 isozymes as well. Our study not only suggests that alterations in PLC may be important in the pathophysiology of depression and other stress-related disorders but also raises the interesting possibility that PLC may be playing different roles in acute vs recurrent depression.

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