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Fear conditioning is associated with altered integration of PLC and ERK signaling in the hippocampus

Colin T. Buckley*, Kevin K. Caldwell*

Department of Neurosciences, MSC08 4740, University of New Mexico School of Medicine, 1 University of New Mexico, Albuquerque, NM 87131-0001, United States

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Abstract

The extracellular signal-regulated protein kinases (ERKs) are proline-directed, serine/threonine kinases that regulate a variety of cellular functions, including proliferation, differentiation, and plasticity. In the present report, we provide evidence that ERK2 and phosphatidylinositol-specific phospholipase C (PLC)-β and -γ isozymes interact in the rat hippocampal formation. We found that anti-PLC-β1a, -β2, -β4, -γ1 and -γ2, but not -β3, immune complexes isolated from rat hippocampal formation postnuclear fractions contain anti-ERK2 immunoreactivity. Further, we show that PLC catalytic activity is associated with anti-ERK2 immunoprecipitates isolated from the hippocampal formation, and that the amount of enzyme activity is significantly increased following fear-conditioned learning. The observed interactions may be mediated by consensus sequences conforming to an ERK2 docking site, termed a D-domain, that we identified in PLC-β1a, -β2, -β4 -γ1 and -γ2. Based on these results, we propose that PLC-β and PLC-γ isozymes form signaling complexes with ERK2 in rat brain, and these complexes play critical roles in learning and memory, as well as a variety of other neuronal functions.

Keywords: Docking; Hippocampal formation; PLC; MAPK; Fear conditioning

1. Introduction

Cells possess a variety of means to integrate diverse extracellular stimuli into coordinated cellular responses. Both direct and indirect interactions between the components of signal transduction pathways are important means of signal integration (Pawson and Nash, 2000). Several published studies reveal that there is substantial crosstalk between phosphatidylinositol-specific phospholipase C (PLC)-dependent and mitogen-activated protein kinase (MAPK)-dependent signaling systems in cells. Three of these studies demonstrate that direct, protein–protein interactions between components of these pathways can occur: Xu et al. (2001) found that PLC-β1 associates with both extracellular signal-regulated protein kinase 1 (ERK1) and ERK2 in cells over-expressing PLC-β1, Barr et al. (2002) demonstrated that PLC-β2 binds to p38 protein kinase, and we have shown that PLC-γ1 directly binds to ERK2 (Buckley et al., 2004). In the present studies we tested the hypothesis that PLC-β and PLC-γ isozymes interact with ERK2 in rat brain.

ERKs comprise one of the major families of MAPKs; c-Jun amino-terminal kinases (JNKs, also called stress-activated protein kinases) and p38 stress-activated protein kinases are the other major families of MAPKs. Multiple members of the ERK family have been cloned (Pearson et al., 2001, Johnson and Lapadat, 2002); of these, ERK1 and ERK2 are the most abundant in brain. Although ERK1 and ERK2 possess high sequence homology (90% identical; Boulton et al., 1991), they display similar, but distinct, subcellular distributions in the rat forebrain (Suzuki et al., 1995), they perform unique roles in synaptic functioning (Mazzucchelli et al., 2002), and disruption of the mouse ERK2 gene produces embryonic lethality (Hatano et al.,

* Corresponding authors. Tel.: +1 505 272 0033; fax: +1 505 272 8082.
E-mail address: kcaldwell@salud.unm.edu (K.K. Caldwell).

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2003), whereas ERK1 knockout mice are viable (Mazzucchelli et al., 2002). We chose to focus our studies on hippocampal formation ERK2, which has shown to play a prominent role in synaptic plasticity (English and Sweatt, 1997, Davis et al., 2000, Watabe et al., 2000, Selcher et al., 2003 Ying et al., 2002) and processes important for spatial and associative learning (Selcher et al., 1999, Atkins et al., 1998, Sweatt, 2001).

PLC isozymes catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), into the important intracellular messengers, inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3, "IP_3") and 1,2-diacylglycerol (1,2-DAG) (Majerus et al., 1990; Williams, 1999). Eleven distinct mammalian PLC isozymes, with multiple subtypes, and in some instances splice variants of these subtypes, have been identified (Rhee, 2001). These 11 PLC isozymes have been grouped into four types: PLC-β, PLC-δ, PLC-γ, and PLC-ϵ. Each of the known PLC-β (Watanabe et al., 1998) and PLC-γ (Homma et al., 1989) isozymes has been reported to be present in brain. Growing evidence implicates PLC isozymes as important components of signal transduction processes involved in brain functioning. Several lines of research indicate that PLC-β1 may participate in various forms of synaptic plasticity, including fear-conditioned learning and memory (see Weeber et al., 2001 and references therein). To our knowledge, there are no published studies that have implicated PLC-β2 in brain functioning. PLC-β3 has been reported to play a role in μ-opioid receptor-dependent responses (Xie et al., 1999). PLC-β4 coupling to metabotropic glutamate receptors in the cerebellum (Kim et al., 1997) may account for its role in delay eyeblink conditioning and cerebellar synaptic plasticity (Kishimoto et al., 2001; Miyata et al., 2001). PLC-γ isozymes have been implicated in the control of various neuronal responses, including neuronal plasticity (Minichiello et al., 2002) and nerve growth cone guidance (Ming et al., 1999).

In the present studies, we found that PLC-β and PLC-γ isozymes interact with ERK2 in the rat hippocampal formation. This interaction is a mechanism through which PLC- and ERK2-dependent signaling may be integrated. Further, we provide evidence that the interaction of PLC and ERK2 may play an important role in hippocampal-dependent learning and memory.

2. Materials and methods

2.1. Animals

Female Sprague-Dawley rats (150–200 days old) were maintained as described in Weeber et al. (2001). The rats were given unlimited access to standard rat chow and tap water. All procedures employed for the housing, handling, fear conditioning and sacrificing of rats were approved by the University of New Mexico Health Sciences Center Laboratory Animal Care and Use Committee.

2.2. Tissue preparation and subcellular fractionation

Rats were sacrificed by decapitation. The hippocampal formations were rapidly removed and were homogenized in 1.5 mL of ice-cold homegenization buffer (HB): 20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 320 mM sucrose, 40 μg leupeptin/mL, 20 μg aprotonin/mL, 30 μM calpain inhibitor III, 0.5 mM AEBSF, and 200 μM sodium orthovanadate, as described in Weeber et al. (2001). Hippocampal formation homogenates were frozen in liquid nitrogen, and stored at −80 °C for further fractionation. The homogenates were thawed and then centrifuged (1000×g, 7 min, 4 °C). The supernatant was decanted; the pellet was resuspended in 0.5 mL HB, homogenized and centrifuged as before. The supernatant was combined with the supernatant from the first centrifugation to form the S1 fraction, and centrifuged (200,000×g, 30 min, 4 °C.). The soluble (S2) fraction was decanted and the pellet (P2) fraction was resuspended in 500 μL of extraction buffer [HB supplemented with 75 mM NaCl, 75 mM KCl, and 1% (v/v) Triton X-100], homogenized as described above, and stored on ice. After 20 minutes, the mixture was spun (200,000×g, 20 min, 4 °C). The Triton X-100 soluble material (herein referred to as the "P2" fraction) was decanted, aliquoted into storage tubes, frozen in liquid nitrogen, and stored at −80°C until further use. Protein determinations were performed as described in Weeber et al. (2001).

2.3. Immunoprecipitation

Anti-PLC isozyme immune complexes were isolated as follows. Hippocampal formation P2 preparations (100 μg protein) were incubated with 6 μg of rabbit anti-PLC isozyme antibody overnight with mixing at 4 °C. The immune complexes were recovered with protein A-Sepharose beads as described in Weeber et al. (2001) and washed twice with 1 mL of extraction buffer. Anti-ERK2 immune complexes were isolated as follows. Fifteen micrograms of rat hippocampal formation P2 proteins were incubated with 4 μg of mouse anti-ERK2 antibody. Immune complexes were recovered using 100 μL protein A-Sepharose beads and washed twice with 1.25× PLC activity assay buffer.

2.4. Immunoblotting

Anti-ERK2 immunoreactivity associated with anti-PLC immune complexes was measured as follows. Immune complexes were released from the beads by adding 50 μL of 2X SDS-PAGE sample buffer (125 mM Tris, pH 6.8, 0.005% bromophenol blue, 100 mM DTT, 17.5 % glycerol, 4.0 % SDS) and boiling for 5 min. Eluted proteins were separated using 7.5% SDS PAGE gels prior to transfer to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in 5% (w/v) fat-free milk in Tris buffered saline (TBS), and blotted with mouse anti-ERK2 (1:1000) antibody, or mouse anti-phospho-ERK1/2 (1:2000) antibody

followed by anti-mouse horseradish peroxidase-conjugated (1:20,000) antibody. The immunoreactive proteins were detected using enhanced chemiluminescene from Amersham Pharmacia Biotech (Piscataway, NJ).

2.5. In vitro treatment of anti-PLC immune complexes with phospho-ERK2

Biotinylated goat anti-rabbit IgG (100 μl of 10.0 μg IgG/mL PBS) was coated onto a streptavidin coated well, using 8-well strips. Each strip was incubated overnight at 4°C, and then washed three times with PBS at room temperature (5 min each). Rabbit anti-PLC-β1a, PLC-β4, and PLC-γ2 were diluted in PBS to a concentration of 2 μg/mL and incubated (100 μL per well) with the biotinylated goat anti-rabbit IgG-coated streptavidin strips overnight at 4 °C. Control wells were processed in a similar fashion except normal rabbit IgG (2 μg/mL) was substituted for anti-PLC antibody. The next day, the strips were washed with PBS three times (for 5 min) at room temperature. Hippocampal P2 fraction extracts were diluted with PBS to a final concentration of 30 μg/100 μL, added to each well, and incubated overnight at 4 °C. Unbound proteins were removed with three washes with PBS (5 min per wash). Captured PLC was treated (20 min, 35 °C) with one of the following: (1) Assay Dilution Buffer I (ADBI): 20 mM [3-(*N*-Morpholino)propanesulfonic acid], pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 20 μM PKC inhibitor peptide, 2 μM PKA inhibitor peptide, and 20 μM Compound R24571; (2) ADBI with 90 μM ATP, 13.5 mM $MgCl_2$; (3) ADBI with 0.4 U of recombinant phospho-ERK2 (Upstate Biotechnology); (4) ADBI containing 0.4 U of recombinant phospho-ERK2 and 90 μM ATP, 13.5 mM $MgCl_2$. Next, the wells were washed three times (5 min each at room temperature) with 1.25× PLC assay buffer (43.75 mM sodium phosphate, pH 6.8, 87.5 mM KCl, 1.0 mM EGTA, 1.0 mM $CaCl_2$) prior to quantification of PLC activity (see below).

2.6. Immune complex PLC activity measurement

Immune complex-associated PLC activities were quantified as described in Buckley and Caldwell (2003). Activity was calculated as nmol (or pmol) $Ins(1,4,5)P_3$ product formed/min/mg protein present within the well from which the enzyme was affinity captured.

2.7. Fear conditioning

One trial, delay fear conditioning was performed essentially as described by Weeber et al. (2001) except that the conditioning apparatus was a Habitest System equipped with a Precision Regulated Shocker (Coulbourn Instruments, Allentown, PA). Rats were randomly assigned to either the one-trial fear-conditioned (paired tone-shock, PTS) group or the unpaired control (UPC) group. Thirty minutes following shock delivery, rats were sacrificed, their hippocampal formation was removed, and the postnuclear particulate (P2) fraction was isolated as described above.

2.8. Materials

Rabbit polyclonal antibodies against PLC-β1a, -β2, -β3, -β4a, -γ1, and -γ2, mouse monoclonal antibody to ERK2, goat polyclonal antibodies against ERK1, and biotinylated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA). HRP labeled goat anti-mouse IgG secondary antibody and [^{3}H] $PtdIns(4,5)P_2$ were purchased from PerkinElmer Life Sciences (Boston, MA). Mouse monoclonal anti-phospho-ERK1/2 antibody was purchased from Cell Signaling Technology (Beverly, MA). Streptawells, streptavidin coated microtiter plates and Triton X-100 were purchased from Roche Applied Science (Indianapolis, IN). SDS-PAGE gels and buffers and PVDF membranes were from BioRad Laboratories (Hercules, CA). Protein A-Sepharose CL-4B and ECL Plus Western Blotting Detection System were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Leupeptin, AEBSF, aprotinin, calpain inhibitor III, and recombinant, activated and non-activated ERK2 from rat, were purchased from Calbiochem (San Diego, CA). All other chemicals and supplies were purchased from commercial sources.

3. Results

3.1. ERK2 co-immunoprecipitates with PLCs

In order to test the hypothesis that PLC-β and -γ isozymes associate with ERK2 in rat brain, anti-PLC-β and anti-PLC-γ immune complexes were isolated from rat hippocampal formation postnuclear membrane (P2) extracts and probed for anti-ERK2 immunoreactivity. We found that ERK2 co-immunoprecipitated with PLC-β1a, -β2, -β4 (Fig. 1A), -γ1 and -γ2 (Fig. 1B). In contrast, the amount of anti-ERK2 immunoreactivity associated with anti-PLC-β3 immunoprecipitates (Fig. 1A) was not different from the background (i.e., IgG control). When anti-PLC-β and PLC-γ immunoprecipitates were probed for anti-ERK1 immunoreactivity, no signals were detected, whereas anti-phospho-ERK1/2 immunoreactivities were minimal, or no signals were detected (data not shown).

3.2. Fear conditioning increases the amount of PLC activity associated with anti-ERK2 immunoprecipitates

We questioned whether associations between ERK2 and PLC isozymes are changed by physiologic stimuli that signal through ERK-dependent pathways. ERK2 has been implicated as important for learning and memory in several paradigms, including contextual fear conditioning (Atkins et al., 1998); thus, we sought to determine whether one trial,

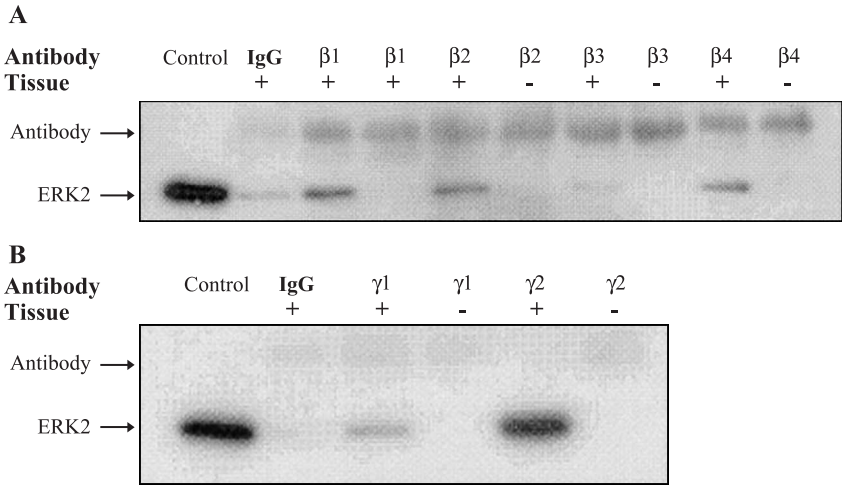

Fig. 1. ERK2 co-immunoprecipitates with (A) PLC-β1, -β2 and -β4, but not -β3, and (B) PLC-γ1 and -γ2. Hippocampal membrane proteins were incubated with rabbit polyclonal antibody specific for each of the designated PLC isozymes or with normal rabbit IgG. Immune complexes were recovered using protein A-Sepharose beads, washed to remove nonspecific proteins, and then separated on SDS-PAGE gels. Gels were also loaded with rat hippocampal membrane protein to serve as internal blotting control (Control). Samples were then transferred to PVDF membranes and were blotted with mouse monoclonal antibody specific for ERK2. The blots shown are representative of at least three blots for each isozyme.

delay fear conditioning exerted an effect on PLC-ERK2 interactions. Anti-ERK2 immune complexes were isolated from the hippocampal formation of rats that had either undergone one-trial fear conditioning (paired tone-shock, PTS) or an "unpaired" control (UPC) paradigm, and assayed for associated PLC activity (Fig. 2A). First, it should be noted that these results demonstrate that one or more of the PLCs associated with anti-ERK2 immunoprecipitates were catalytically active. Further, 30 min following fear conditioning there was a significant increase in the amount of PLC activity associated with ERK2 (Fig. 2B). We have obtained similar results in studies employing anti-ERK2 immune complexes isolated from mouse hippocampal formation P2 fraction 30 min following fear conditioning or a behavioral control (immediate shock) procedure (data not shown).

3.3. In vitro treatment of affinity captured phospholipase C -β1a, -β4, and -γ2 with phospho-ERK2

We sought to determine whether phospho-ERK2 treatment exerted an effect on PLC lipase activity. Affinity purified phospholipase C-β1a, -β4, and -γ2 were incubated under conditions that allowed for substrate phosphorylation (i.e., in the presence of Mg^{2+} and ATP), or not, prior to measuring the catalytic activity of each isozyme. In vitro treatment of anti-PLC-β1 (Fig. 3A) and anti-PLC-β4 (Fig. 3B) immune complexes with phospho-ERK2 in the absence of Mg^{2+}-ATP did not alter phospholipase activity, whereas the same treatment of anti-PLC-γ2 (Fig. 3C) immune complexes reduced PLC activity. Incubation of both anti-PLC-γ2 and anti-PLC-β4 immune complexes with Mg^{2+}-ATP stimulated lipase activity. This is similar to results that we reported for anti-PLC-γ1 immune complexes (Buckley and Caldwell, 2003). Treatment of captured PLC-γ2 and PLC-β4 with phospho-ERK2 and Mg^{2+}-ATP opposed this ATP-dependent stimulation. PLC-β2 demonstrated only negligible lipase activity under the conditions used in these assays; therefore, the effect of phospho-ERK2 could not be reliably assayed. Phospho-ERK2-dependent regulation of PLC-γ1 enzyme activity is addressed in a separate report (Buckley et al., 2004).

3.4. Identification of MAPK docking motifs in PLC isozymes

The identification of sequence motifs that mediate protein-protein interactions, when combined with available protein amino acid sequence data, allows for the identification of putative protein binding pairs. Two ERK-binding motifs have been identified by Kornfeld et al. (Jacobs et al., 1999; Fantz et al., 2001): (1) the FXFP motif, having the consensus sequence of F-X-F-P, and (2) the D-domain motif, having two possible consensus sequences: either (K/R)-X-(X/K/R)-(K/R)-$X_{(1-4)}$-(L/I)-X-(L/I) or (K/R)-(K/R)-(K/R)-$X_{(1-5)}$-(L/I)-X-(L/I). We searched PLC-β, PLC-γ, PLC-δ and PLC-ϵ isozymes for amino acid sequences that fit these consensus sequences and found that PLC-β1, -β2, -β4, -γ1, -γ2, -δ1, -δ3, and -ϵ each contain at least one putative D-domain, while PLC-β3 is the only isozyme in which we identified an FXFP motif (Table 1).

4. Discussion

We have made several important observations in these studies. First, we demonstrated that ERK2 associates with PLC-β1a, -β2, -β4, -γ1 and -γ2 isozymes in the rat hippocampal formation. Previously, Xu et al. (2001) demonstrated that, in 3T3 cells overexpressing PLC-β1, an association between ERK2 and PLC-β1 could be detected.

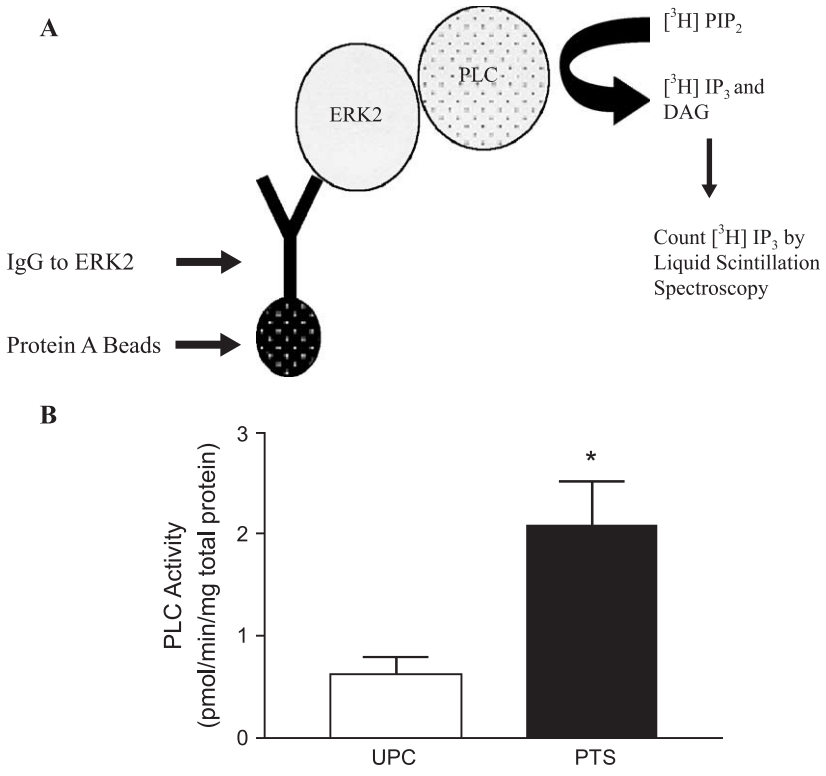

Fig. 2. PLC activity present in anti-ERK2 immunoprecipitates isolated from rat hippocampal formation postnuclear membrane fractions. (A) Schematic representation of Enzyme Linked Immunosorbent Assay (ELISA) of PLC co-immunoprecipitating with ERK2. ERK2 was affinity captured with an anti-ERK2 monoclonal antibody bound to protein A-Sepharose beads. The beads were washed, then incubated in the presence of substrate, $[^3H]PtdIns(4,5)_2$ ($[^3H]PIP_2$). $[^3H]Ins(1,4,5)P_3$, $[^3H]IP_3$, product formation was measured by liquid scintillation spectroscopy. Background measurements were taken using a matching non-immune mouse IgG and treated similarly to anti-ERK2 immune complexes. Activity specific to the anti-ERK2 immune complex was measured as lipase activity present in the anti- ERK2 immune complexes minus lipase activity within the non-immune IgG sample. (B) The hippocampal formation was isolated from adult rats 30 min following completion of the fear conditioning (paired tone-shock, PTS) or behavioral control (unpaired control, UPC) paradigm. Postnuclear membrane fractions were prepared and ERK2 was immunoprecipitated as described in Materials and methods. Immune complex PLC activity was measured and expressed as pmol $Ins(1,4,5)P_3$ product formed/min/mg tissue. n=6 for UPC and n=9 for PTS; asterisk designates significance determined by Student's t-test ($p<0.02$).

We found that the association is not specific for PLC-β1, but also occurs with other PLC-β, as well as PLC-γ isozymes. Further, this interaction is not unique to PLC-β1 within the nucleus, as reported by Xu et al. (2001). Second, we were unable to detect an association between any of the PLC-β or -γ isozymes and ERK1. This result is in contrast to results of Xu et al. (2001), who reported that PLC-β1 was associated with phospho-ERK1. Our studies indicate that either ERK2 and ERK1 differ in their affinities for PLC isozymes or ERK1 and PLC isozymes are differentially compartmentalized in the rat hippocampal formation. Third, the finding that there was an increase in the amount of PLC activity associated with anti-ERK2 immunoprecipitates following fear-conditioned learning indicates that the association of ERK2 and PLC isozymes is of physiologic significance. Fourth, ATP-stimulated PLC-γ2 lipase activity is completely reversed in the presence of phospho-ERK2. The simplest explanation for this observation is that it is an effect that is secondary to phospho-ERK2-catalyzed phosphorylation of PLC-γ2. However, we have found that PLC-γ2 is a relatively poor in vitro substrate for phospho-ERK2, at least under conditions that allow for the robust phosphorylation of PLC-γ1 (Buckley et al., 2004). These results support, instead, a mechanism in which phospho-ERK2 either indirectly regulates PLC-γ2 enzyme activity (e.g., secondary to effects on one or more regulatory proteins, such as a protein kinase, that are associated with the anti-PLC-γ2 immune complex) or directly regulates PLC-γ2 catalytic activity by a phosphorylation-independent mechanism. This latter proposal is supported by the observation that the effect of phospho-ERK2 was observed in the absence of Mg^{2+}-ATP (Fig. 3C) and by reports showing that ERKs regulate the catalytic activity of other enzymes (e.g., MAPK phosphatase-3, Camps et al., 1998; phosphotyrosine-specific phosphatase PTP-SL, Buschbeck et al., 2002) by a mechanism that is dependent on direct interaction between ERK and the phosphatase, but is independent of ERK catalytic activity. Finally, employing consensus sequences reported by Kornfeld et al. (Jacobs et al., 1999; Fantz et al., 2001), we identified putative ERK2 binding sites in nine of the 11 known types of PLC. These identifications have not previously been reported.

We have not yet identified which PLC isozyme(s) underlies the observed increase in ERK2-associated PLC

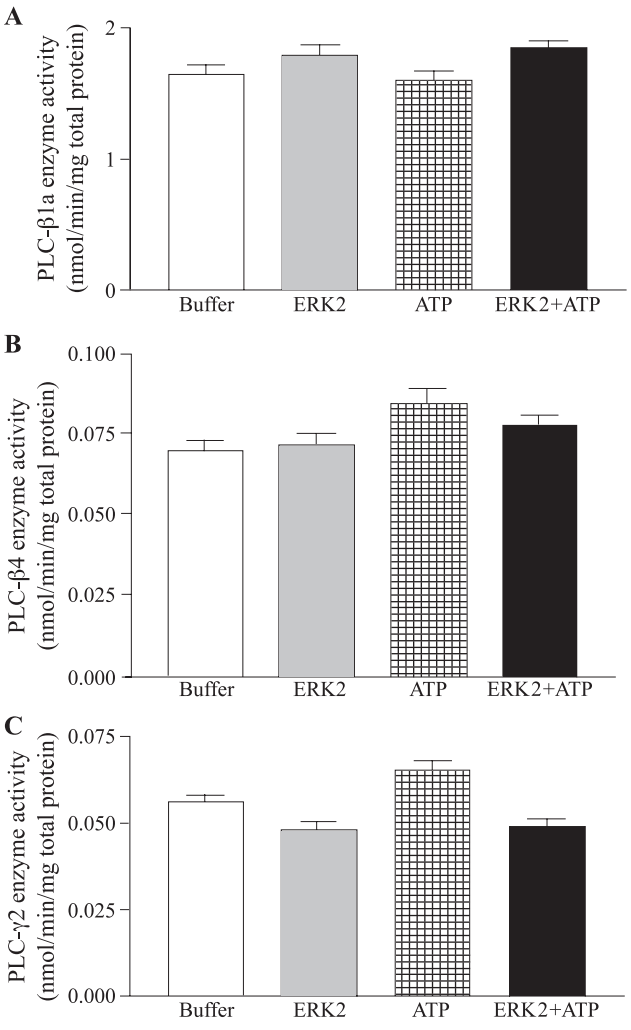

Fig. 3. Effects of phospho-ERK2 treatment on anti-PLC-β1a (top), -β4 (middle), and -γ2 (bottom) immune complex PLC activity. Affinity captured PLC isozymes were treated with buffer, buffer with phospho-ERK2, buffer plus ATP, or buffer with phospho-ERK2 and ATP; subsequently, PLC activity was determined as described in Materials and methods. Data are from six replicates and are representative of two experiments for PLC-β1a and PLC-β4 and three experiments for PLC-γ2.

activity in fear-conditioned animals. Preliminary experiments have shown that the amount of particulate-associated ERK2-PLC-γ2 complex is significantly increased 30 min following fear conditioning, implicating PLC-γ2 as an important contributor to the observed increase in ERK2-associated PLC activity (data not shown).

We identified D-domain sequences in PLC isozymes employing consensus sequences defined by Kornfeld et al. (Jacobs et al., 1999; Fantz et al., 2001). Other investigators (e.g., Pearson et al., 2001) have identified different, but similar, docking motifs for MAPKs. In general, binding motifs for MAPKs are composed of approximately 20 amino acids. In contrast to specifying consensus sequences, Sharrocks et al. (Barsyte-Lovejoy et al., 2002) have proposed models for MAPK docking domains that define regions of sequence similarity: basic, LXL, hydrophobic. These regions play differing roles in determining specificity for interactions with ERK, JNK and p38 family members. Each of the D-domains that we identified consists of a basic region and an L-X-L motif. Further, an adjacent hydrophobic region is readily identifiable in several PLC isozymes (e.g., PLC-γ1, ^{953}LVV; PLC-γ2, ^{187}VSGI). Thus, it is possible that, in addition to binding ERK2, the D-domains of PLC isozymes bind MAPKs belonging to the p38 and/or JNK families. We have begun studies aimed at addressing this possibility. In support of this proposal is the demonstration by Barr et al. (2002) that p38 binds to PLC-β2. The PLC-β2 D-domain that we identified is within the portion of PLC-β2 that they identified (amino acids 774–1181) as mediating the interaction. Differential coupling of PLC-β and PLC-γ isozymes to members of the MAPK family could explain how different cellular responses (e.g., growth, differentiation, apoptosis, neurite outgrowth) are elicited by enzymes (i.e., PLC isozymes) that perform the same catalytic function (i.e., Ins(1,4,5)P_3 and DAG production). The formation of PLC-MAPK signaling complexes is likely to facilitate, and restrict, the transduction of PLC-dependent signals to specific MAPK signaling pathways. Peptide versions of these docking sites may prove to be important pharmacologic tools in the treatment of a variety of diseases.

In conclusion, we provide evidence that PLC-β and PLC-γ isozymes interact with ERK2 in rat hippocampal formation. These interactions may play an important role in hippocampal-dependent learning and memory. These

Table 1
Identification of peptide sequences within PLC isozymes that conform to consensus sequences of the D-domain and the FXFP motif

PLC isozyme	FXFP motif	D-domain motif
-β1	none	^{777}R-N-E-R-N-Q-P-L-M-L
		^{1028}K-Y-Q-K-R-E-H-I-K-L
		^{1153}K-F-K-R-L-P-L-E-I
-β2	none	^{977}R-E-L-K-D-R-L-E-L(-E-L)
-β3	^{782}F-D-F-P	none
-β4	none	^{985}K-K-K-G-G-S-N-C-L-E-I
-γ1	none	^{945}R-R-K-K-I-A-L-(E-L)
-γ2	none	^{164}R-E-L-K-T-I-L-P-L
-δ1	none	^{102}K-D-Q-R-N-T-L-D-L
		^{335}K-G-C-R-C-L-E-L
-δ2	none	none
-δ3	none	^{93}R-R-K-N-L-D-L
-δ4	none	none
-ε	none	^{1369}R-E-N-K-K-D-L-Q-L-(P-L)
		^{1941}R-G-Y-R-H-L-Q-L

D-domain and FXFP motifs were identified employing consensus sequences identified by Kornfeld et al. (Jacobs et al., 1999; Fantz et al., 2001). Brackets indicate that two L/I-X-L/I motifs were identified at the C-terminal of the peptide. The position of the amino-terminal residue in the protein is identified as a superscript numeral. Sequences employed for these determinations were accession numbers P10687 (PLC-β1a), NP004564 (PLC-β2), NP032900 (PLC-β3), AAB28484 (PLC-β4a), P10686 (PLC-γ1), P24135 (PLC-γ2), P10688 (PLC-δ1), S14113 (PLC-δ2), NP542419 (PLC-δ4), and BAC00906 (PLC-ε); the sequence for PLC-δ3 was taken from Lee and Rhee (1996).

studies provide the basis to hypothesize that behavioral challenge elicits spatial and temporal alterations in the integration of PLC- and ERK-dependent signaling in the rat brain, and that errors in the coordination of these signals may lead to cognitive and behavioral impairments.

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