A Cell-Permeable Phospholipase Cγ1-Binding Peptide Transduces Neurons and Impairs Long-Term Spatial Memory

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Growth factor-mediated signaling has emerged as an essential component of memory formation. In this study, we used a phospholipase C gamma 1 (PLCγ1) binding, cell-penetrating peptide to sequester PLCγ1 away from its target, the phosphotyrosine residues within the activated growth factor receptor. Peptides appear to transduce neurons but not astrocytes or oligodendrocytes. The presence of the peptides in the hippocampus during training in the Morris water maze significantly impaired long-term memory, but not memory acquisition. These results, along with previous studies on extracellular signal-regulated kinase (ERK) and phosphoinositide-3 kinase (PI3K), implicate all three key growth factor receptor-activated intracellular signaling pathways in memory storage.

One of the basic goals in the study of memory is the identification of the molecular cascades that contribute to its formation and storage. The brain contains several trophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), whose traditionally accepted roles are in neuronal survival and differentiation/development. In the last decade, it has become clear that some of these factors play a role in regulation of synaptic transmission and plasticity (Kang and Schuman 1995). Trophic factors stimulate intracellular signaling cascades via membrane tyrosine kinase receptors, which activate three key transducer molecules, phospholipase Cγ1 (PLCγ1), phosphoinositide-3 kinase (PI3K), and extracellular signal-regulated kinase (ERK) via growth factor receptor binding protein 2 (Grb2) and/or fibroblast growth factor receptor substrate 2 (Frs2). These molecular cascades are initiated by protein–protein interactions between src homology 2 (SH2) domains and short sequences that flank phosphotyrosine residues within the cytoplasmic domain of the activated growth factor receptor. A few studies have implicated ERK and PI3K in mammalian learning and memory (Atkins et al. 1998; Blum et al. 1999; Lin et al. 2001; Dash et al. 2002). However, the role of PLCγ1-mediated signaling in memory formation is not known. Several recent studies implicate PLCs in long-term potentiation (LTP), a proposed mechanism for learning and memory. For example, it has been shown that BDNF as well as NT-3-mediated synaptic potentiation is blocked by U73122, a pharmacological inhibitor of the PLC family of enzymes (Kleiman et al. 2000, Yang et al. 2001). Unfortunately, isoform-specific PLC inhibitors are not available, making it difficult to evaluate the role of the trophic factor-activated isoform of PLC, PLCγ1, in memory. However, a study by Minichiello et al. (2002) showed that a targeted mutation of the PLCγ1 binding site within the BDNF receptor TrkB significantly impaired hippocampal LTP.

The aim of this study was to examine whether PLCγ1 is necessary for acquisition and/or retention of spatial memory. For this purpose, we used cell-penetrating peptides designed to bind to the SH2 domain of PLCγ1, making the receptor binding site of PLCγ1 unavailable to interact with the phosphotyrosine residues within the activated growth factor receptor. It has been shown previously that such peptides can effectively compete for interaction with target molecules and in this way inhibit function of PLCγ1 (Hall et al. 1996) or other molecules, such as c-Jun N-terminal kinase (Borsello et al., 2003). Hall and colleagues (1996) demonstrated that when cell transduction domain-containing PLCγ1 monophosphoryl tyrosine peptides are applied to neurons in culture, growth factor-induced IP3 production is completely abolished. In vitro binding experiments have shown that bisphosphorylated peptides bind PLCγ1 with significantly higher affinity and specificity than monophosphorylated peptides (Ottenger et al. 1998). For these studies, we used a bisphosphoryl tyrosine peptide (PLCγ1 binding sequence from the rat platelet-derived growth factor (PDGF) receptor N‘-SVLY(P)TAVQPNEGDN DY(P)HPLDPK-C‘) and the monophosphoryl tyrosine peptide (PLCγ1 binding sequence from the rat FGF receptor N‘-SNQEYPILDLS-C‘) (Hall et al. 1996). All peptides were linked to a 10-amino-acid human immunodeficiency virus (HIV) TAT (48–57) cell transduction domain N‘-YGRKKRRQRPR-C‘ (Vives et al. 1997) and to a biotin molecule to allow detection. For controls, we used a TAT “ghost” peptide (containing only the TAT transduction sequence and the biotin modification) and a nonphosphorylated PLCγ1 peptide identical in sequence to the bisphosphoryl peptide. For all experiments, Long-Evans rats (285–325 g) were used. They were housed in a 12-h light/dark cycle, with ad libitum access to food and water. All of the training and testing was done during the light cycle by an experimenter who was kept blind with respect to the treatment schedule. All protocols involving the use of animals were in compliance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

The binding specificity of the cell-penetrating PLCγ1 phosphopeptides, as well as that of control peptides, to PLCγ1 was assessed in a peptide precipitation experiment using hippocampal extracts (Derossi et al. 1998). The extracts were prepared as described previously (Blum et al. 1999). Extracts were allowed to interact with streptavidin-bound peptides, in a binding buffer containing 150 mM NaCl, 20 mM Tris-HCl, 1% Nonidet-40, 1
mM pyrophosphate, 1 mM NaF, and 1 mM Na3VO4. The streptavidin-coated agarose beads, along with the peptide and any bound proteins from the hippocampal extract, were precipitated out by centrifugation and washed briefly in the binding buffer, and the pelletized material was boiled in 1× SDS PAGE gel load buffer. Samples were run on a 10% Tris/Tricine SDS-PAGE gel. For Western blots, resolved proteins were transferred to Immobilon-P (Millipore) membrane and blocked overnight in 5% BSA in TBST (10 mM Tris/HCl at pH 7.9, 150 mM NaCl, 0.05% Tween-20), followed by a 3-h incubation with 0.2 µg/mL primary antibody at room temperature. Immunoreactivity was visualized by an alkaline-phosphatase-conjugated secondary antibody and a CDP-Star chemiluminescence substrate. Weak immunoreactivity for PLCγ1 was detected in the control TAT and nonphosphorylated peptide precipitates, which did not change with increasing peptide amounts, suggesting nonspecific protein trapping in the precipitated material (Fig. 1A). Consistent with the published studies in which bisphosphoryl peptides were found to have 100- to 1000-fold higher affinity for PLCγ1 (Ottinger et al. 1998), we found that the dually tyrosine-phosphorylated peptide bound PLCγ1 more effectively than the singly tyrosine-phosphorylated peptide used by Hall et al. (1996). Thus, the monophosphoryl peptides were not used for any further experiments. The binding of the peptides appeared to be specific to PLCγ1, and not other SH2 domain-containing proteins, such as Grb2 and PI3K p85 (Fig. 1B and C). However, this study cannot rule out the possibility that the peptide may be nonspecifically binding to other signal transduction molecules that were not directly examined.

Next, we investigated the ability of the phosphopeptides to enter hippocampal cells. The phosphopeptides were delivered into the dorsal hippocampus via bilateral guide cannulae (AP = 3.3 mm, L = 2.0 mm from bregma and V = −2.0 mm from the dura). During infusion, the injection cannulae extended 1.75 mm beyond the tips of the guides, yielding a total depth of 3.75 mm. Two concentrations of peptide, 1 µg and 10 µg per hippocampus, were used. Stock solutions of peptides were prepared in water and diluted to working concentrations in sterile saline. All injections were performed in freely moving animals at a rate of 0.25 µL/min, using a dual syringe infusion pump (Stoelting), to
deliver 1 µL total volume of peptide solution. Animals were perfused with 4% paraformaldehyde at either 30 min, 1.5 h, or 3 h following the infusion. Biotinylated peptides were detected using a streptavidin-Alexa488 conjugate. At 1 µg/hippocampus, the peptides were detected only diffusely in the granule neurons of the dentate gyrus (Fig. 2A). The hippocampal cell layers appeared intact when visualized using the neuron-specific NeuN (neuron-specific nuclear protein) antibody (Fig. 2B). At 10 µg/hippocampus, the peptides were easily detectable in all hippocampal cell layers (Fig. 2C). The peptides were detectable at maximum level at 30 min, detectable at intermediate level at 1.5 h, and no longer detectable by 3 h post infusion (data not shown).

To determine what types of cells were taking up the peptide, double labeling was performed using antibodies against NeuN, GFAP (glial fibrillary acid protein), and R îp to identify neurons, astrocytes, and oligodendrocytes, respectively. The hippocampal neuronal layers were visualized using the NeuN antibody, and the peptide was detected in NeuN positive cells (Fig. 2D). At higher magnifications of 100 × (Fig. 2E) and 300 × (Fig. 2F), the peptide was seen throughout the cytoplasm, as well as in the nucleoli of the neurons. In contrast, the peptides were not detected in astrocytes (Fig. 2G) or oligodendrocytes (Fig. 2H). At this time, we are not aware of the mechanism for this neuron-selective uptake and/or stability for the peptide. Because TAT is thought to mediate the transduction of small peptides in a non-temperature-sensitive, nonendocytotic mechanism (Zaro et al. 2003), it is not clear at present why neurons are preferentially transduced.

There is evidence that the full-length TAT protein can cause neurotoxicity in the rat brain (Bruce-Keller et al. 2003); however, there have been no published reports that the cell-transduction domain of TAT has such properties. To examine whether intrahippocampal infusion of TAT cell-transduction domain-containing peptides causes hippocampal neuronal death, 10 µg of the peptide was infused into the dorsal hippocampus and cell and nuclear morphology were examined visually using the non-specific DNA/RNA stain Hoechst 33258 (bis-benzimide) in tissue taken at 30 min, 1.5 h, or 48 h following the infusion. The cells appeared uniform in shape and size and there were no apparent differences in cell density or cell layer continuity at any of the times, when compared with uninjected controls (Fig. 3).

To test whether PLCγ1 plays a role in memory formation and storage, the PLCγ1 peptides were bilaterally infused into the dorsal hippocampi. The effects on memory acquisition and retention were tested using a single-block design paradigm of the Morris water maze task essentially as described previously (Guzowski and McGaugh 1997; Teather et al. 2002). In two separate experiments, the bisphosphoryl PLCγ1- or the nonphosphorylated PLCγ1 peptide-infused animals were each compared with TAT ghost peptide-infused animals. Following the first four training trials in the Morris water maze, animals were infused with either 10 µg of the PLCγ1 or an equal molar amount of the TAT ghost peptide (3 µg). After a 30-min rest period, animals were given an additional nine training trials. This delay time was chosen because the peptides were maximally detected in the hippocampus starting at 30 min following infusion. No difference in acquisition of the task was observed between either the bisphosphoryl or nonphosphorylated PLCγ1 peptide and the TAT ghost peptide (Fig. 4A, data not shown). This indicates that PLCγ1 may not play a role in spatial learning.

Forty-eight hours after training, animals were tested for retention by a transfer test in which the hidden platform was removed from the maze and animals were allowed to search for a period of 60 sec (probe trial; Fig. 4A). The performance of TAT peptide-infused animals across all parameters assessed was consistent with saline- and vehicle-infused controls from previous studies performed in our laboratory. In contrast, animals infused with the bisphosphoryl PLCγ1 peptide took a significantly longer time than the TAT-infused animals to cross the previous location of the platform (latency: TAT (n = 8) 15.49 ± 5.7 sec vs. pp-PLCγ1 (n = 7) 47.35 ± 6.01 sec, P = 0.015), suggesting long-term memory impairment. Consistent with our binding study, which indicated no specific interaction of the nonphosphorylated peptide and PLCγ1, the nonphosphorylated peptide-infused animals performed comparable with TAT control peptide-infused animals (latency: TAT (n = 8) 15.72 ± 3.18 sec vs. nonphospho-PLCγ1 (n = 8) 14.20 ± 5.39 sec, P = 0.832). This suggests that the effect on memory retention is specific to the phosphorylated version of the peptide. This effect was not due to differences in swimming speed (TAT (n = 8) 1571.54 ± 94.14 cm/sec vs. pp-PLCγ1 (n = 7) 1587.36 ± 118.30 cm/sec, P = 0.92). To further analyze the probe trial performance, latency and dwell time in defined concentric areas around the platform were compared. As the blocked training used in these studies does not give rise to strong quadrant preferences, concentric areas surrounding the platform location were used to determine localization effects (Gallagher et al. 1993). In all of the parameters tested, the nonphosphorylated peptide infused animals performed equally well as their TAT ghost peptide-infused controls (data not shown). In contrast, the bisphosphoryl PLCγ1 peptide-infused animals took a significantly longer time to get to the area near the platform (if at all) and spent significantly less time searching in the target area.
when compared with the TAT control peptide-infused animals (Fig. 4, B and C). The representative probe trial traces (Fig. 4B) illustrate that the bisphosphorylated peptide-infused animals appeared to be searching for the platform but in an incorrect location. This suggests that these animals have retained the memory for the goal of the task, as well as the strategy, but may not have the memory for the precise platform location. To ensure that the retention deficit observed in the bisphosphorylated PLCγ1-infused animals was due to a lack of memory for the platform location rather than a general hippocampal dysfunction, animals were briefly retrained (placed on platform for 30 sec, and then given three retraining trials). Animals from the bisphosphorylated PLCγ1 peptide, which had performed poorly on retention testing, were able to locate the hidden platform just as quickly as the TAT control group once they were shown the location of the platform, and in a second retention test 48 h following retraining, both groups performed equally well (latency: TAT 15.26 ± 6.43 sec vs. pp-PLCγ1 16.28 ± 4.73 sec, p = 0.91). This is consistent with our suggestion that intrahippocampal administration of the peptide did not result in a complete amnesia for the task rather than a lack of information specifically related to platform location.

There are several recent studies implicating PLC in BDNF- and NT-3-mediated synaptic potentiation (Kleiman et al. 2000, Yang et al. 2001; Minichiello et al. 2002). The present study demonstrates that intrahippocampal infusion of a PLCγ1 binding peptide impairs spatial memory, suggesting a role for PLCγ1-mediated signaling in this process. Several growth factor receptors, including the TrkB (receptor for BDNF), TrkC (receptor for NT-3), and insulin-like growth factor-1 receptor, can activate PLCγ1 when engaged with the appropriate ligand. The present study implicates PLCγ1-mediated signaling, initiated by one or several of these growth factor receptor types, in long-term memory formation, a process that requires gene expression. At present, it is not known whether PLCγ1-mediated signaling directly activates genes and/or mediates its effect via cross talks with ERK and PI3K cascades to cause gene activation. Future experiments will examine these questions.

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