Cellular Pharmacology of Protein Kinase Mζ (PKMζ) Contrasts with Its in Vitro Profile

**IMPLICATIONS FOR PKMζ AS A MEDIATOR OF MEMORY**

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**Background:** Based on their in vitro effects, ZIP and chelerythrine have been used as PKMζ inhibitors and staurosporine as a negative control to implicate PKMζ in memory.

**Results:** ZIP and chelerythrine do not and staurosporine does inhibit PKMζ in cells and brain slices.

**Conclusion:** Cellular pharmacology of PKMζ contrasts with its in vitro profile.

**Significance:** Contrary to current dogma, PKMζ may not mediate memory.

A number of recent studies have used pharmacological inhibitors to establish a role for protein kinase Mζ (PKMζ) in synaptic plasticity and memory. These studies use zeta inhibitory peptide (ZIP) and chelerythrine as inhibitors of PKMζ to block long term potentiation and memory; staurosporine is used as a negative control to show that a nonspecific kinase inhibitor does not block long term potentiation and memory. Here, we show that neither ZIP nor chelerythrine inhibits PKMζ in cultured cells or brain slices. In contrast, staurosporine does block PKMζ activity in cells and brain slices by inhibiting its upstream phosphoinositide-dependent kinase-1. These studies demonstrate that the effectiveness of drugs against purified PKMζ may not be indicative of their specificity in the more complex environment of the cell and suggest that PKMζ is unlikely to be the mediator of synaptic plasticity or memory.

Synaptic plasticity is generally thought to be the cellular correlate of memory (1). In particular, long term potentiation (LTP) of synaptic transmission, which is triggered by a brief high frequency activation of synapses, is commonly thought to account for the maintenance of memory. Research directed toward understanding the molecular basis of LTP and memory has firmly established the role of such molecules as synaptic NMDA receptors and such processes as a rise in intracellular calcium in triggering synaptic plasticity (1). Subsequent biochemical events, especially those that maintain LTP and memory, however, have not been well established. One proposal has been that the transient rise in intracellular calcium leads to production of a brain-specific alternative transcript of protein kinase Cζ (PKCζ) that encodes only the catalytic domain (PKMζ) (2). This persistently active enzyme could phosphorylate downstream activators and maintain plasticity and memory beyond the initial triggering events (3-7).

Evidence that PKMζ is required for LTP and for the maintenance of several forms of memory has depended almost exclusively on the use of pharmacological approaches (3-7). These studies have relied on the use of zeta inhibitory peptide (ZIP), a myristoylated putative PKCζ-inhibiting peptide derived from the autoinhibitory pseudosubstrate peptide sequence within PKCζ, chelerythrine, an apoptosis-inducing compound that is marketed, and extensively used, as a PKC inhibitor (8, 9), and staurosporine, a general protein kinase inhibitor (10). ZIP and chelerythrine have been found to block LTP and memory, theoretically by inhibiting PKMζ, whereas staurosporine, which does not inhibit purified PKMζ in vitro, fails to do so. Beyond their in vitro testing against pure protein, however, the effectiveness of ZIP or chelerythrine and the ineffectiveness of staurosporine in inhibiting PKMζ within the complex milieu of mammalian cells and tissues have never been established. Indeed, studies show that the cellular effects of chelerythrine are not mediated by PKCζ (11), nor does the compound inhibit PKCζ isoforms (or any other kinase in a screen of 34 structurally diverse kinases) in vitro (12, 13) or in cells (14). Furthermore, the effectiveness of pseudosubstrate peptides in binding and inhibiting an enzyme depends largely on their intramolecular nature, an advantage not possessed by ZIP. And lastly, staurosporine, though it may not inhibit PKMζ directly in vitro (3, 15), does inhibit phosphoinositide-dependent kinase-1 (PDK1) (10, 16), the upstream kinase whose constitutive phosphorylation of all PKCζ isoforms is required for their kinase activity (17-19). The discrepancies between the potential effectiveness of these compounds in inhibiting PKMζ activity in cells and their
reported effects on learning and memory suggest that PKMζ is not mediating the effects of these drugs in cells.

Here, we examine whether the inhibitors used to implicate PKMζ in learning and memory block PKMζ activity in the context of heterologous cells and brain slices. We demonstrate that those inhibitors that have been reported to impact learning and memory, ZIP and chelerythrine, do not inhibit PKMζ in cells, whereas an inhibitor reported not to impact learning and memory, staurosporine, does inhibit PKMζ in cells. These data indicate that PKMζ is not the cellular target of ZIP or chelerythrine, that PKMζ is a cellular target of staurosporine, and that PKMζ likely does not mediate learning or memory.

EXPERIMENTAL PROCEDURES

Materials—ZIP and scrambled ZIP were obtained from AnaSpec and dissolved in PBS obtained from Cellgro. Chelerythrine, staurosporine, and bisindolylmaleimide IV (bisIV) were obtained from Calbiochem and dissolved in DMSO obtained from Sigma. Ser(P) PKC antibody was obtained from Cell Signaling. Phospho-MARK2 antibody was obtained from Abcam. DsRed antibody, which recognizes DsRed variants including mRFP and tdTomato, was obtained from Clontech. β-Actin antibody was obtained from Sigma. An antibody that specifically recognizes the phosphorylated activated loop of PKC isozymes (pAL) was characterized previously (17).

Plasmids—The C kinase activity reporter (CKAR) construct was described previously (20). PKMζ constructs consist of monomeric RFP fused to the C terminus of the last 409 amino acids of rat PKCζ. Mammalian PKMζ-RFP and mRFP vector control constructs were cloned into pcDNA3. Sindbis viral PKMζ-RFP and tdTomato vector control constructs were cloned into pSinRep5, and Sindbis virus was prepared as described previously (21).

Cell Culture and Transfection—293T, HeLa, and COS-7 cells were maintained in DMEM (Cellgro) containing 5% FBS (HyClone or Invitrogen) and 1% penicillin/streptomycin (HyClone or Invitrogen) and 1% penicillin/streptomycin (HyClone or Invitrogen). Transient transfections were carried out using FuGENE 6 (Roche Applied Science) or lipofectamine (HyClone or Invitrogen) and 1% penicillin/streptomycin (HyClone or Invitrogen). Cells were maintained in DMEM (Cellgro) containing 5% FBS and 1 mM CaCl2 in the dark at room temperature.

Immunoprecipitation—293T cells were transfected with mRFP or PKMζ-RFP and treated with inhibitors for 30 min in cell culture medium at 37 °C. Cells were then rinsed with ice-cold PBS and lysed on ice in a buffer containing 1% Triton X-100, 50 mM Tris (pH 7.5), 10 mM Na3PO4, 50 mM NaF, 150 mM NaCl, 5 mM EDTA, 2 mM benzamidine, 50 μg/ml leupeptin, 1 mM PMSF, and 1 mM sodium vanadate, and centrifuged at 16,000 × g for 2.5 min. Detergent-solubilized lysates were separated on SDS-polyacrylamide gels, transferred onto PVDF membranes, and probed using the indicated antibody. Blots were visualized via chemiluminescence on a FluorChem imaging system (Alpha Innotech). Densitometric analyses were performed on AlphaView software (Alpha Innotech).

Immunoblotting—293T cells were plated onto sterilized glass coverslips in 35-mm imaging dishes and co-transfected with CKAR and either mRFP or PKMζ-RFP. Approximately 48 h post-transfection, the cells were washed and subsequently imaged in Hanks’ balanced salt solution (Cellgro) containing 1 mM CaCl2, in the dark at room temperature, and the specified drugs were introduced during live cell imaging. Images were acquired via a 40× objective on a Zeiss Axiovert microscope (Carl Zeiss Microimaging) using a MicroMax digital camera (Roper-Princeton Instruments) controlled by MetaFluor software version 3.0 (Universal Imaging Corp.).

Optical filters were obtained from Chroma Technologies. Time-lapse images of cyan fluorescent protein (CFP), fluorescence resonance energy transfer (FRET), and yellow fluorescent protein (YFP) were collected every 15 s through a 10% neutral density filter. CFP and FRET images were obtained through a 420/20 nm excitation filter, a 450 nm dichroic mirror, and either a 475/40 nm emission filter (CFP) or a 535/25 nm emission filter (FRET). YFP images monitored as a control for photobleaching were obtained through a 495/10 nm excitation filter, a 505 nm dichroic mirror, and a 535/25 nm emission filter. Excitation and emission filters were switched in filter wheels (Lambda 10-2, Sutter). Integration times were 200 ms for CFP and FRET and 100 ms for YFP.

For each cell imaged, MetaFluor calculated a FRET ratio consisting of the average CFP/FRET for a manually selected cellular region. Base-line FRET ratios were acquired for 5 min before introduction of inhibitors, and the trace for each cell was normalized either to its average base-line value or to its minimum

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point. Normalized FRET ratios were combined from \( n \geq 11 \) cells/group over at least three independent experiments and plotted as mean ± S.E.

**In Vitro Kinase Activity Assay**—The effect of 1 \( \mu M \) ZIP or scrambled ZIP on PKC\( \zeta \) activity in vitro was assayed by monitoring \( ^{32} \text{P} \) incorporation from \( [\gamma^{32} \text{P}] \text{ATP} \) (3000 Ci/mmol; PerkinElmer Life Sciences) into a synthetic PKC-selective substrate peptide (Ac-FKKSFKL-NH\(_2\), AnaSpec) by purified PKC\( \zeta \) (Millipore) essentially as described previously (23). Briefly, purified PKC\( \zeta \) (7 nm) was incubated with substrate peptide (100 \( \mu M \)) in the presence of PBS vehicle control, 1 \( \mu M \) ZIP, or 1 \( \mu M \) scrambled ZIP in a total reaction volume of 80 \( \mu L \) in buffer containing 16 \( \text{mM} \) HEPES (pH 7.5), 1.2 \( \text{mM} \) DTT, 1 \( \text{mM} \) EGTA (pH 7.9), 5 \( \text{mM} \) MgCl\(_2\), and [\( ^{32} \text{P} \)]ATP (100 \( \mu M \); 0.1 Ci/mmol) for 11 min at 30 °C. Reactions were quenched by the addition of 25 \( \mu L \) of a solution containing 0.1 \( \text{m} \) ATP and 0.1 \( \text{m} \) EDTA (pH 8). Aliquots (85 \( \mu L \)) were spotted onto P81 cation-exchange chromatography paper (Whatman), washed four times with 0.4% (v/v) phosphoric acid and once with 95% ethanol, and radioactivity was determined by liquid scintillation counting. One unit is defined as 1 nmol of phosphate incorporated/min at 30 °C; data are expressed as the specific activity of PKC\( \zeta \) (milli-units \( \mu g^{-1} \)) and plotted as the mean ± S.E. of triplicate assays.

**RESULTS**

**ZIP and Chelerythrine Do Not Inhibit PKM\( \zeta \) in Mammalian Cells**—We examined the effects of ZIP and chelerythrine on PKM\( \zeta \) activity in mammalian cell lines using two approaches. First, we used a Ser(P) PKC substrate antibody to detect any substrates whose phosphorylation was significantly enhanced in cells overexpressing PKM\( \zeta \)-RFP compared with cells expressing RFP alone; this antibody effectively probes PKC substrates in cells (14). Overexpression of PKM\( \zeta \) in 293T cells robustly enhanced the phosphorylation of multiple PKC substrates, in particular of a protein with an apparent molecular mass of \( \sim 180 \text{kDa} \) (Fig. 1A; compare lane 1 with 4 and lane 7 with 9). Cells were mock treated or treated for 1 h with 1 \( \mu M \) either ZIP, scrambled ZIP, or chelerythrine, the inhibitor concentrations used in previous studies on LTP and the treatment duration selected based on the fact that 1 \( \mu M \) chelerythrine had been observed to achieve full inhibition of potentiated excitatory postsynaptic currents within 10 min during whole cell
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**FIGURE 2. Staurosporine inhibits PKMζ activity in mammalian cells by inhibiting its constitutive activation loop phosphorylation.** A and B, COS-7 cells co-transfected with CKAR and either RFP or PKMζ-RFP were monitored using live cell fluorescence imaging for changes in FRET ratio in response to addition of (A) 100 nM staurosporine followed by a further 900 nM staurosporine or (B) 6 μM bisIV followed by 100 nM staurosporine and a further 900 nM staurosporine. C, COS-7 cells co-transfected with the phosphoacceptor Thr/Ala mutant of CKAR (CKART/A) and either RFP or PKMζ-RFP were monitored for changes in FRET ratio in response to addition of 1 μM staurosporine. For all imaging experiments in this figure, the trace for each cell imaged was normalized to its minimum value, and normalized FRET ratios were combined from ≥13 cells/group over at least three independent experiments and plotted as mean ± S.E. D, 293T cells transfected with RFP (immunoprecipitation control) or PKMζ-RFP were treated with vehicle, 100 nM or 1 μM staurosporine, or 1 μM ZIP, then lysed. DsRed antibody was used to immunoprecipitate the transfected proteins, and samples were Western blotted with an antibody specific to the phosphorylated activation loop of PKC isozymes (pAL) or with DsRed antibody. The graph quantifies activation loop phosphorylation of immunoprecipitated PKMζ-RFP normalized for PKMζ-RFP expression and relative to the vehicle-treated group (mean ± S.E. error bars) for n ≥ 8/group. **, p < 0.01 and ***, p < 0.001 compared with DMSO- and 1 μM ZIP-treated by Bonferroni post hoc test after one-way ANOVA.

recordings (3). Phosphorylation of the Ser(P) substrate by recombinant PKMζ-RFP was not affected by treatment of cells with ZIP or scrambled ZIP (compare lanes 4-6); nor was it affected by treatment with chelerythrine (compare lane 9 with 10). Basal phosphorylation of this substrate was also unaffected by these compounds (compare lanes 1-3, 7 with 8). Quantitation of data from six independent experiments confirmed no significant effects of any of these compounds on the phosphorylation of this PKMζ substrate (Fig. 1A, graph) or any of the other substrates recognized by this antibody (data not shown). Similar results were obtained in HeLa cells (data not shown). In a second and independent approach, we took advantage of CKAR, a genetically encoded FRET-based reporter of PKC activity (20), to measure the activities of endogenous PKC and overexpressed PKMζ-RFP in real time in live cells in response to addition of inhibitors. COS-7 cells were co-transfected with CKAR and either RFP vector control or PKMζ-RFP. Addition of 1 μM ZIP had no effect on the base-line-normalized FRET ratio in either case and therefore inhibited neither endogenous PKCs nor overexpressed PKMζ (Fig. 1B). As a positive control, staurosporine (a potent inhibitor of PKCs, see Fig. 2) robustly inhibited endogenous PKC activity and caused an even greater decrease in FRET ratio in the presence of overexpressed PKMζ (Fig. 1B). An analogous CKAR experiment also showed that 1 μM chelerythrine did not inhibit overexpressed PKMζ (data not shown).

The effectiveness of the ZIP and scrambled ZIP used in this study was confirmed by their inhibition of purified PKCζ in vitro (Fig. 1C; similar results were obtained in the presence of phosphatidylserine except that activity was ~2-fold higher). The chelerythrine used in this study was also confirmed to be biologically active, as evidenced by its ability to trigger apoptosis in HeLa (Fig. 1D) and 293T cells (data not shown).

**Staurosporine Inhibits PKMζ Activity in Mammalian Cells by Inhibiting Activation Loop Phosphorylation—**We next tested the effects of staurosporine, a general kinase inhibitor reported not to affect LTP (3) or memory (4), on PKMζ activity in cells. COS-7 cells co-transfected with CKAR and either RFP or PKMζ-RFP were monitored for changes in FRET ratio in response to addition of staurosporine. 100 nM staurosporine, the concentration used in the studies on LTP and memory, resulted in a significant drop in CKAR phosphorylation both in control cells and those overexpressing PKMζ (Fig. 2A). 100 nM staurosporine was enough to inhibit endogenous PKC activity...
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fully, as addition of more staurosporine did not further decrease the FRET ratio of CKAR+RFP (Fig. 2A). In contrast, this addition of another 900 nM staurosporine did cause an additional drop in the FRET ratio of CKAR+PKMζ-RFP, revealing inhibition of the overexpressed PKMζ (Fig. 2A). Thus, the basal activity of cellular PKMζ is effectively inhibited by 1 μM staurosporine.

To discriminate whether 100 nM staurosporine inhibits only endogenous PKC activity or whether it also inhibits PKMζ activity, cells were pretreated with 6 μM general PKC inhibitor bisIV to abolish endogenous PKC activity before the addition of staurosporine. 6 μM bisIV completely inhibited endogenous PKC activity, as subsequent addition of 100 nM or up to 1 μM staurosporine did not further decrease the FRET ratio of CKAR+RFP (Fig. 2B). The smaller response of CKAR+PKMζ-RFP to bisIV compared with CKAR+RFP can be attributed to the antagonizing action of uninhibited PKMζ-RFP replacing phosphates on CKAR lost due to bisIV inhibition of endogenous PKCs. Importantly, the PKC activity in cells overexpressing PKMζ and pretreated with bisIV was additionally inhibited by 100 nM staurosporine (Fig. 2B), revealing that this concentration of staurosporine is sufficient to inhibit PKMζ. Thus, the bisIV-induced drop in the PKC FRET ratio in Fig. 2B reflects the contribution of endogenous PKC (~20% of the maximal drop), and the subsequent staurosporine-induced drops reflect inhibition of overexpressed PKMζ. These FRET ratio changes arise from bona fide phosphorylation of CKAR at its phosphoacceptor site, as a mutant construct of CKAR with Ala at this position, have no significant catalytic activity.

Staurosporine is relatively ineffective at inhibiting PKCζ itself in vitro (15). However, it binds with nanomolar affinity to and potently inhibits the upstream kinase PDK1 (10, 16), which is required for the activation loop phosphorylation and catalytic competence of all PKC isoforms (17, 19), including PKCζ (18). Of particular relevance to this study, phosphorylation at the PKCζ site on PKC (Thr-410) is required for catalytic activity: enzyme that is not phosphorylated on Thr-410, or constructs with an Ala at this position, have no significant catalytic activity (24). Staurosporine treatment of 293T cells expressing PKMζ caused a marked reduction (54 ± 6% (100 nM), 59 ± 6% (1 μM); p < 0.0001, one-way ANOVA) in the activation loop phosphorylation of immunoprecipitated PKMζ (Fig. 2D). Thus, staurosporine inhibits PKMζ activity in cells by inhibiting its activation loop phosphorylation by PDK1.

ZIP and Chelerythrine Do Not, But Staurosporine Does, Inhibit PKMζ in Rat Hippocampal Brain Slices—We then examined the effects of these inhibitors on PKMζ activity when administered in situ to organotypic hippocampal brain slices. Here, we took advantage of the high expression of the PKCζ-specific substrate MARK2/Par-1b (25), whose phosphorylation in brain slices was assessed using a phosphoantibody specific to its PKCζ phosphorylation site (Fig. 3A). Overexpression of PKMζ-RFP using Sindbis virus (21) increased the phosphorylation of MARK2 2.2 ± 0.4-fold (n = 15; compare lane 1 with 6). Brain slices were bath-treated with the concentrations of ZIP and chelerythrine used in previous studies on synaptic plasticity (1 μM) (3) or with a 10-fold higher concentration for 4 h, long enough for ZIP and chelerythrine to have exerted their full effects on LTP when applied in bath to hippocampal slices in previous studies (3). Indeed, ZIP and chelerythrine can disrupt hippocampal LTP maintenance in rat brain slices and in vivo in rat and mouse in as little as 2 h (26). Quantitative analysis of multiple independent experiments revealed no inhibition by ZIP, scrambled ZIP, or chelerythrine of either endogenous kinase or overexpressed PKMζ (Fig. 3A, bar graph), even at concentrations of 10 μM (overexpression p < 0.001, drug p = 0.90, interaction p = 0.89, two-way ANOVA). In fact, peptides had a modest stimulatory effect on phosphorylation by both endogenous kinase and overexpressed PKMζ-RFP, indicating biological activity of the compounds in this preparation. Similar results were observed in brain slices probed with the Ser(P) PKC substrate antibody used in Fig. 1A (data not shown).

In contrast to ZIP and chelerythrine, staurosporine inhibited the phosphorylation of MARK2 at the PKMζ site. Quantitative analysis of eight independent experiments revealed that 100 nM staurosporine resulted in a significant decrease in the phosphorylation of MARK2 in brain slices overexpressing PKMζ to 50 ± 20% that observed in mock-treated samples (Fig. 3B). Taken together, these data establish in hippocampal brain slices that ZIP and chelerythrine do not inhibit PKMζ but that staurosporine does via its potent inhibition of the upstream kinase PDK1.

DISCUSSION

Identification of the molecules responsible for the long lasting maintenance of memory continues to be an important biological question. A number of recent studies have used pharmacological approaches to implicate the action of PKMζ as a potential mechanism (3–7). To our knowledge, the effectiveness of ZIP, chelerythrine, or staurosporine in inhibiting PKMζ within the complex milieu of mammalian cells and tissues has not been previously demonstrated. Here, we find that ZIP and chelerythrine fail to inhibit PKMζ activity in heterologous cells or brain slices. Based on the oriented peptide library work of Cantley and co-workers (27), the sequences of ZIP (SIYR- GARRWRKL), which is identical to that of the PKCζ pseudo-substrate, and scrambled ZIP (RLYRKRIWRSAGR), when substituted with Ser at the phosphoacceptor position (underlined), are predicted to be equally good substrates for PKCζ, which may explain the partial inhibition of PKCζ in vitro even by scrambled ZIP (Fig. 1C), a negative control peptide. Furthermore, the pseudo-substrate sequence of PKCζ, when substituted with Ser at the phosphoacceptor site, is actually phosphorylated with an order of magnitude lower Km and an order of magnitude higher Vmax/Km by a different PKC isoform, PKCδ (27). Thus, the lack of a defined substrate consensus sequence for PKCζ, including PKCζ, precludes the use of pseudo-substrate peptides as specific pharmacological tools. Because ZIP clearly affects LTP, we conclude that this basic peptide likely disrupts a macromolecular interaction in cells via a target unrelated to PKMζ. Finally, we find that staurosporine actually does inhibit PKMζ in cells and brain slices by inhibiting its constitutive phosphorylation by the upstream kinase PDK1. This effect of staurosporine would be overlooked in in vitro
assays, from which PDK1 would be absent, because the purified PKM/H9256 used would already be phosphorylated at the PDK1 site and thus catalytically competent.

Measurement of the phosphorylation of multiple distinct endogenous PKM/H9256 substrates in cell lines and hippocampal brain slices as well as an overexpressed PKM/H9256 substrate in the form of CKAR all show that, in contrast to their effects in vitro, ZIP and chelerythrine do not, but staurosporine actually does inhibit PKM/H9256 in the complex milieu of mammalian cells and tissues. The inconsistencies between the effectiveness of ZIP, chelerythrine, and staurosporine in inhibiting PKM/H9256 activity in cells and tissues and their reported effects on learning and memory provide a double dissociation between PKM/H9256 activity and synaptic plasticity. We note that genetic studies in which PKM/H9256 has been overexpressed have been used to support a role for PKM/H9256 in memory (3, 28). Given that overexpressed kinases can mislocalize and increase the phosphorylation of both specific and nonspecific substrates in cells and given that these reports have not included specificity controls, their results are inconclusive. Because the cellular pharmacology of PKM/H9256 contrasts with its in vitro profile, we conclude that PKM/H9256 cannot be implicated as the molecular substrate of long term plasticity or memory based on the prevalent studies using the pharmacological tools of ZIP, chelerythrine, and staurosporine.

Acknowledgments—We thank Matt Niederst and Maya Kunkel for performing the apoptosis assay in Fig. 1D.

REFERENCES


Signal Transduction:
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doi: 10.1074/jbc.M112.357244 originally published online February 29, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.357244

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