Substrate Phosphorylation in the Protein Kinase Cγ Knockout Mouse*

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The phosphorylation state of three identified neural-specific protein kinase C substrates (RC3, GAP-43/B-50, and MARCKS) was monitored in hippocampal slices of mice lacking the γ-subtype of protein kinase C (PKCγ). Wild-type controls by quantitative immunoprecipitation following 32P, labeling. Depolarization with potassium, activation of glutamate receptors with glutamate, or direct stimulation of protein kinase C with the phorbol ester 12,13-dibutyrate (PDB). We show that stimuli that readily increase RC3 phosphorylation in wild-type mice fail to affect RC3 phosphorylation in PKCγ-deficient mice.

Mice lacking the γ-subtype of protein kinase C (PKCγ) show mild spatial learning deficits and impaired hippocampal LTP observed in these mice.

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EXPERIMENTAL PROCEDURES
The effects of depolarization, glutamate receptor stimulation, and direct PKC activation were determined in PKCγ knockout and littermate control mice (bred into a C57/B1 background for six generations and kindly provided to us by Dr. J. M. Wehner).

For Western blotting, the forebrain of each mouse was homogenized in 10 ml of buffer containing 50 mM Tris (pH 7.5), 100 mM NaCl, 2 mM EDTA, 1 mM EGTA, 50 mM dithiothreitol, 0.6 mM phenylmethylsulfonyl fluoride, and 1% SDS. We equalized the protein concentrations of the homogenates based on three independent protein assays performed in triplicate prior to adding SDS and dithiothreitol using the BCA method and then immuno-blotted serial dilutions of each homogenate. The blots were probed with polyclonal rabbit anti-RC3 (Affinity Research Products Ltd., Exeter, UK) at a dilution of 1:1000 and then developed with a horseradish peroxidase-based, enhanced chemiluminescence protocol.

Hippocampal slices of wild-type (n = 8) and knockout (n = 8) mice were prepared as described (6) and incubated in cobenagated phosphate-free ACSF containing 124 mM NaCl, 4.5 mM KCl, 1.3 mM MgSO4, 2.5 mM CaCl2, 10 mM glucose, and 20 mM NaHCO3 at room temperature. After 30 min slices were transferred to reaction tubes containing 900 μl of carbogenated phosphate-free ACSF at 30 °C, and 45 min later 100 μCi of 32P (specific activity, 40 mCi/ml; ICN Pharmaceuticals) was added. Slices were labeled for 90 min, and the medium was changed to phosphate-free ACSF containing 30 mM K+, 1 mM glutamate (Fluka), or 0.1 μM PDB (Sigma). 32P incorporation into RC3, GAP-43/B-50, and MARCKS was determined using a quantitative immunoprecipitation as described (8). Briefly, protein homogenate was incubated overnight at 4 °C with polyclonal rabbit antibodies 8420 (final dilution for RC3, 1:100), 9727 (final dilution for GAP-43/B-50, 1:200), or 0.1 μM PDB (Sigma). 32PO4 incorporation into proteins was detected using a Fuji BAS1000 imaging system (Raytest, Germany) and quantified using TINA analysis software. The total 32PO4 incorporation into proteins was determined by trichloroacetic acid precipitation as described previously, and 32PO4 incorporation into RC3, GAP-43/B-50, and MARCKS was normalized accordingly.

RESULTS
Protein levels of RC3 were not different between wild-type, heterozygous, and PKCγ knockout mice (Fig. 1A), showing that there was no up- or down-regulation of RC3 levels induced by the PKCγ knockout. Basal in situ phosphorylation of RC3 and MARCKS in hippocampal slices from mice lacking PKCγ did not differ significantly from those observed in wild-type littermate controls (102.3 ± 8.1% (mean ± S.E.) and 99.3 ± 9.2% of basal phosphorylation in controls for RC3 and MARCKS respectively, p > 0.1, n = 8, and n = 4). However, increased basal

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§ The abbreviations used are: PKC, protein kinase C; ACSF, artificial cerebrospinal fluid; LTP, long term potentiation; PDB, 4α-phorbol 12,13-dibutyrate.

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increase in RC3 phosphorylation in slices from PKCγ-deficient mice. Thus, RC3 is a highly specific substrate for PKCγ during excitation. PDB increased the phosphorylation of MARCKS and GAP-43/B-50 in slices derived from knockout (147.4 ± 6.6% (n = 4) and 162.7 ± 14.2% (n = 8)), as well as the wild-type (173.7 ± 14.5% (n = 4) and 288.2 ± 35.2% (n = 8)) mice (Fig. 1B). However, GAP-43/B-50 phosphorylation was significantly attenuated in the former compared with the latter (43.5 ± 4.4% of wild type, p < 0.01, n = 8). Decreased incorporation of $^{32}$PO$_4$ into GAP-43/B-50 in knockout mice upon direct stimulation of PKC was probably due to higher initial levels of phospho-GAP-43/B-50 because the two appear to offset each other, although the possibility that GAP-43/B-50 could be a substrate for PKCγ cannot be ruled out.

**DISCUSSION**

The experiments described here demonstrate that depolarization with potassium, activation with glutamate, or direct stimulation of PKC with a phorbol ester leads to phosphorylation of RC3 solely by PKCγ. Thus, the results unequivocally delineate the following biochemical pathway: activation of a postsynaptic (metabotropic) glutamate receptor stimulates PKCγ, which in turn phosphorylates RC3. Basal levels of phospho-RC3 appear to be dictated by a calcium and diacylglycerol-independent atypical isoform of PKC, possibly $\lambda$ or $\zeta$. Basal levels of phospho-GAP-43/B-50 are higher in the PKCγ knockout mouse, and this might be a presynaptic mechanism to compensate for the inability to phosphorylate RC3 in dendrites, perhaps by increasing neurotransmitter release in response to decreased postsynaptic gain (6, 10–13). Inability to phosphorylate RC3 in the PKCγ knockout mouse by either membrane depolarization or by activation of postsynaptic glutamate receptors may contribute to the electrophysiological and behavioral phenotypes of the PKCγ knockout mouse.

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