Depolarization Activates ERK and Proline-rich Tyrosine Kinase 2 (PYK2) Independently in Different Cellular Compartments in Hippocampal Slices

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In the hippocampus, extracellular signal-regulated kinase (ERK) and the non-receptor protein proline-rich tyrosine kinase 2 (PYK2) are activated by depolarization and involved in synaptic plasticity. Both are also activated under pathological conditions following ischemia, convulsions, or electroconvulsive shock. Although in non-neuronal cells PYK2 activates ERK through the recruitment of Src-family kinases (SFKs), the link between these pathways in the hippocampus is not known. We addressed this question using K⁺-depolarized rat hippocampal slices. Depolarization increased the phosphorylation of PYK2, SFKs, and ERK. These effects resulted from Ca²⁺ influx through voltage-gated Ca²⁺ channels and were diminished by GF109203X, a protein kinase C inhibitor. Inhibition of SFKs with PP2 decreased PYK2 tyrosine phosphorylation dramatically, but not its autophosphorylation on Tyr-402. Moreover, PYK2 autophosphorylation and total tyrosine phosphorylation were profoundly altered in fyn−/− mice, revealing an important functional relationship between Fyn and PYK2 in the hippocampus. In contrast, ERK activation was unaltered by PP2, Fyn knock-out, or LY294002, a phosphatidylinositol-3-kinase inhibitor. ERK activation was prevented by MEK inhibitors that had no effect on PYK2. Immunofluorescence of hippocampal slices showed that PYK2 and ERK were activated in distinct cellular compartments in somatodendritic regions and nerve terminals, respectively, with virtually no overlap. Activation of ERK was critical for the rephosphorylation of a synaptic vesicle protein, synapsin I, following depolarization, underlining its functional importance in nerve terminals. Thus, in hippocampal slices, in contrast to cell lines, depolarization-induced activation of non-receptor tyrosine kinases and ERK occurs independently in distinct cellular compartments in which they appear to have different functional roles.

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1 The abbreviations used are: ERK, extracellular signal-regulated kinase; ACSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; MAP-2, microtubule-associated protein 2; MEK, mitogen-activated protein kinase/ERK kinase (MEKK) inhibitor PD98059 (12), demonstrating the important functional role of these signaling pathways. In addition, both ERK and PYK2 are activated in the hippocampus following brain ischemia (13–16), and convulsions (15, 17, 18), suggesting their possible role in pathology. However, the links between these two pathways in neurons remain unclear.

ERK activation results from the phosphorylation of a threonine residue and a tyrosine residue in its activation loop by MEK downstream from signaling pathways involving small GTPases and protein kinases of the Raf family (19). In the nervous system, the ERK pathway can be activated in response to many stimuli acting through receptor tyrosine kinases (20), G protein-coupled receptors (6), NMDA glutamate receptors (21–23), and membrane depolarization (10, 24). ERK activation can regulate synaptic transmission indirectly through the control of protein synthesis and gene expression in the nucleus (25, 26) or directly by phosphorylating ion channels or synaptic vesicle proteins important for neuronal excitability or neurotransmitter release (27–30).

PYK2 is a non-receptor tyrosine kinase member of the focal adhesion kinase family, highly enriched in the central nervous system (5). PYK2 has been implicated in the signaling of G protein-coupled receptors, nicotinic acetylcholine receptors, stress stimuli, and membrane depolarization (31–35). In most cells, including neurons, activation of PYK2 appears to be a Ca²⁺/protein kinase C (PKC)-dependent mechanism (5, 31). PYK2 activation results from its autophosphorylation on Tyr-402, which creates an Src homology 2-binding site that recruits...
Src family kinases (SFKs), which phosphorylate several other tyrosine residues of PYK2 (34, 36). Interactions between the activated PYK2/Src module and other proteins such as the Grb2-Sos complex, p130Cas, paxillin, and Graf regulate intracellular signaling (37). In addition, in hippocampal neurons PYK2 is associated with NMDA receptors and regulates their function in combination with SFKs (11, 38).

PYK2 acts as an upstream regulator of ERK via activation of SFKs in several cell types in culture (31, 34, 35, 39). For example, in PC12 cells, a chromaffin cell line often used as a neuronal model, PYK2 appears to link bradykinin and lysophosphatidic acid receptors with ERK after recruitment of the Grb2-Sos complex and the subsequent activation of the Ras/ERK signaling pathway (34). Moreover, in these cells ERK activation in response to increases in intracellular free Ca2+ requires PYK2 (40). Hence, it has been postulated that PYK2 and ERK are components of the same important pathway controlling neuronal plasticity (7). However, the role of PYK2 in the activation of ERK in the nervous system is not known. To investigate this issue we examined PYK2, SFK, and ERK phosphorylation states, reflecting their activation, in KCl-depolarized hippocampal slices. We demonstrate that KCl depolarization activates the PYK2/SFK module and ERK independently from each other in distinct cellular compartments where they may have different functions.

MATERIALS AND METHODS

Reagents—4-Amino-5-[(4-chlorophenyl)-7-[(butyl)pyrazol](3,4-d)pyrimidine (PP2) and 4-amino-7-phenylpyrazol (3,4-d)pyrimidine (PP3) were from Calbiochem, LY290004 was from Biomol, and PD98059 was from New England Biolabs. U0126 was kindly provided by Dr. James Trzaskos (DuPont Merck). Mouse monoclonal anti-phospho-tyrosine (4G10) (diluted 1:4000 for immunoblotting) was from Upstate Biotechnology. Mouse monoclonal anti-phospho-ERK, reacting with active ERK1/2 and phospho-Threonine and threonine residues of the activation loop (clone MAPK-YT), was from Sigma (1:4000; fluorescence, 1:200). Rabbit polyclonal anti-ERK1/2 (1:2,000) and anti-phospho-SFK (1:25,000) were from Biosource and Upstate Biotechnology, respectively. Polyclonal affinity-purified anti-PYK2 antibody (1:2,000) was obtained by immunizing a rabbit against a 17-amino acid peptide encompassing residues 2–18 of rat PYK2 (32). Polyclonal affinity-purified anti-phospho-Tyr-402-PYK2 antibodies were either from Biosource (1:2,000) or raised in rabbits immunized with a keyhole limpet hemocyanin-conjugated phosphopeptide (Cys-Ser-Ile-Glu-Ser-Asp-Ile-Tyr-P)(Ala-Glu-Pro-Asp-Glu-Thr) corresponding to amino acids 395–409 of PYK2 (fluorescence, 1:200) (15). For immunoprecipitation of antibody G-526, reacting specifically with synthetic 1 phosphorilated on Ser-62 and Ser-67 (sites 4 and 5; blot, 1:4000), was a gift of Prof. Benfenati, Genoa, Italy (30). Microtubule-associated protein 2 (MAP-2; fluorescence, 1:2000) and synaptotherin (1:1000) mouse monoclonal antibodies were from Chemicon and Synaptic System, respectively. Alexa 488- or Cy3-coupled secondary antibodies (1:400) were from Molecular Probes.

Rat Hippocampal Slices—Rat hippocampal slices (300 μm) were prepared from young male Sprague-Dawley rats (100–150 g) with a McIlwain tissue chopper and incubated as described previously (32). Briefly, slices were dissected in ice-cold Ca2+-free artificial cerebrospinal fluid (ACSF) and placed for 10 min in polypyrrolene tubes (three slices per tube) containing 1 ml of Ca2+-free ACSF at 34 °C and equilibrated at pH 7.4 in O2/CO2 (95:5, v/v). The slices were then incubated at 34 °C in 900 μl of ACSF containing 1.1 mM Ca2+ and 1 μM tetrodotoxin for 45 min before depolarization by 40 mM (final concentration) KCl or vehicle. The addition of 40 mM NaCl as a control for possible osmotic effects did not alter protein phosphorylation (data not shown). Tetrodotoxin was added to prevent indirect effects due to neuronal firing and had no effect on tyrosine phosphorylation by itself (data not shown) and was added before depolarization by KCl. At the end of the experiment, ACSF was aspirated, and the slices were immediately frozen on dry ice and kept at −80 °C. Slices were either used for immunoprecipitation (see below) or solubilized by sonication in 200 μl of a 100 °C solution of 1% (w/v) SDS and 1 mM sodium orthovanadate and placed on a boiling water bath for 5 min. Mice hippocampal slices from wild type and $f$yn knock-out mice (obtained from The Jackson Laboratory, Bar Harbor, ME) were prepared as rat slices, except that the hippocampus was dissected in ice-cold ACSF from coronal sections (300 μm) obtained with a vibratome.

Western Blot Analysis—Equal amounts of SDS slice lysates (100 μg) were separated by SDS-polyacrylamide gel electrophoresis prior to electrophoretic transfer onto nitrocellulose membrane (Hybond Pure, Amersham Biosciences). Membrane were blocked for 1 h at room temperature in Tris-buffered saline (100 mM NaCl, 10 mM Tris, pH 7.5) with 0.1% Tween 20 (Tris-buffered saline-Tween) or 5% nonfat milk (Tris-buffered saline-milk) for the detection of phosphorylated or non-phosphorylated proteins, respectively. The membranes were then incubated overnight at 4 °C with the primary antibody, washed three times, and incubated for 2 h at room temperature with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (diluted 1:4000; Amersham Biosciences). Bound antibodies were visualized by enhanced chemiluminescence detection (ECL, Amersham Biosciences). When necessary, membranes were stripped in buffer containing 100 mM glycine, pH 2.5, 200 mM NaCl, 0.1% Tween 20 (w/v), and 0.1% β-mercaptoethanol (w/v) for 45 min at room temperature, followed by extensive washing in Tris-buffered saline before reblocking and reblotting. Quantifications were carried out by scanning the autoradiograms and measuring relative optical density with Scion Image software. Data were normalized to the mean value of untreated controls in the same autoradiograms. Statistical analysis was done with ANOVA followed by Bonferroni’s test using Prism 3.02 software.

Depolarization Induces a Rapid and Reversible Phosphorylation of PYK2, SFKs, and ERK in Hippocampal Slices—Treatment of rat hippocampal slices with 40 mM KCl for 2 min induced the appearance of two major phosphorytrosine immunoreactive bands at 115 and 42 kDa (Fig. 1A). These bands comigrated with PYK2 and P-ERK2, respectively (data not shown). Depolarization-induced tyrosine phosphorylation of PYK2 was directly demonstrated by immunoprecipitation followed by phosphotyrosine immunoblotting (Fig. 1B). Phosphorylation of ERK was demonstrated by immunoblotting with antibodies specifically reacting with ERK doubly phosphorylated on threonine and tyrosine in its activation loop (Fig. 1C). Although the amounts of ERK1 and ERK2 were similar, phosphorylation predominated on ERK2 as reported previously in the hippocampus after high frequency electrical stimulation or other stimuli (10, 43). This difference may indicate a preferential activation of ERK2 by depolarization, although we cannot exclude a difference in the affinity of antibodies for the two isozymes. These results confirm previous reports of dephosphorylation-induced activation of ERK and PYK2 in hippocampal slices (24, 32, 44). By contrast, no change in the phosphorylation of c-Jun N-terminal kinase and p38 mitogen-activated protein kinase was observed in the same experiments (data not shown).

We examined the time course of PYK2 and ERK activation and compared it with that of SFK phosphorylation using an
antibody specific for SFKs phosphorylated on tyrosine in the activation loop (Fig. 1D). Kinetics of PYK2, SFK, and ERK activation were slightly different after a 2-min KCl depolarization. PYK2 and SFK phosphorylation states followed the same time course with a peak at 1 min and a return to baseline after 10 min, suggesting a common mechanism in PYK2 and SFK activation. By contrast, ERK2 activation was slower (peak at 3 min) but more sustained (Fig. 1D).

Depolarization-induced Phosphorylation of PYK2 and ERK Involves Voltage-gated Ca²⁺ Channels and PKC—PYK2 and ERK activation were both prevented in the absence (Control) or presence (KCl) of 40 mM KCl. Arrows indicate the major immunoreactive bands comigrating with PYK2 (upper) and ERK1 (lower) (data not shown). B, PYK2 immunoprecipitation (IP) from slices treated as described for panel A followed by immunoblotting for phosphotyrosine and total PYK2. C, homogenates of slices prepared as described for panel A and analyzed by immunoblotting for P-ERK or total ERK. D, time course of the activation of kinases following depolarization by 40 mM KCl (duration of treatment is indicated by the horizontal bar, representing 2 min). PYK2 tyrosine phosphorylation was measured by immunoblotting with phosphotyrosine antibodies after PYK2 immunoprecipitation (PYK2/P-Tyr). Phosphorylated Src-family kinases (P-SFK) and active ERK (P-ERK) were measured by immunoblotting with specific antibodies. Changes in phosphorylation were studied in different experiments from 0 to 60 min (left, main graph) or from 0 to 5 min (right, inset).

FIG. 1. Depolarization activates PYK2 and ERK in hippocampal slices. A, phosphotyrosine (P-Tyr) immunoblot of homogenates from slices incubated for 2 min in the absence (Control) or presence (KCl) of 40 mM KCl. Arrows indicate the major immunoreactive bands comigrating with PYK2 (upper) and ERK1 (lower) (data not shown). B, PYK2 immunoprecipitation (IP) from slices treated as described for panel A followed by immunoblotting for phosphotyrosine and total PYK2. C, homogenates of slices prepared as described for panel A and analyzed by immunoblotting for P-ERK or total ERK. D, time course of the activation of kinases following depolarization by 40 mM KCl (duration of treatment is indicated by the horizontal bar, representing 2 min). PYK2 tyrosine phosphorylation was measured by immunoblotting with phosphotyrosine antibodies after PYK2 immunoprecipitation (PYK2/P-Tyr). Phosphorylated Src-family kinases (P-SFK) and active ERK (P-ERK) were measured by immunoblotting with specific antibodies. Changes in phosphorylation were studied in different experiments from 0 to 60 min (left, main graph) or from 0 to 5 min (right, inset).

FIG. 2. Role of Ca²⁺ influx and PKC in the activation of PYK2 and ERK by depolarization in hippocampal slices. A, hippocampal slices were incubated for 2 min in the absence (Control) or the presence of 40 mM KCl in standard ACSF, ACSF without Ca²⁺, or ACSF with 5 mM Mn²⁺, as indicated. PYK2 phosphorylated on tyrosine and active ERK were detected by immunoblotting. B, slices were incubated for 2 min in the absence (0) or presence (+) of 40 mM KCl and the indicated concentrations of a PKC inhibitor, GF109203X, applied 30 min before KCl. C, quantification of results obtained from the data presented in panel B in three independent experiments. Data are means ± S.E. Statistical analysis was by ANOVA followed by Bonferroni’s test. Control versus KCl: **, p < 0.01; ***, p < 0.001. Absence versus presence of GF109203X: °, p < 0.05.

antibody specific for SFKs phosphorylated on tyrosine in the activation loop (Fig. 1D). Kinetics of PYK2, SFK, and ERK activation were slightly different after a 2-min KCl depolarization. PYK2 and SFK phosphorylation states followed the same time course with a peak at 1 min and a return to baseline after 10 min, suggesting a common mechanism in PYK2 and SFK activation. By contrast, ERK2 activation was slower (peak at 3 min) but more sustained (Fig. 1D).

Depolarization-induced Phosphorylation of PYK2 and ERK Involves Voltage-gated Ca²⁺ Channels and PKC—PYK2 and ERK activation were both prevented in the absence of extracellular Ca²⁺ or in the presence of Co²⁺, a nonspecific voltage-gated Ca²⁺ channel blocker (Fig. 2A). In contrast, NMDA or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor inhibitors (MK801 and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), respectively) did not alter the depolarization-induced phosphorylation of PYK2 and ERK (Table I), ruling out a major contribution of endogenously released glutamate. Blockade of a single class of voltage-gated Ca²⁺ channels did not suffice to prevent depolarization-induced phosphorylation of PYK2 and ERK, which persisted in the presence of nifedipine, a L- and T-type channel blocker, Ni²⁺, a R-type blocker, or ω-conotoxin, a N/P/Q-type inhibitor (Table I). These results indicate that depolarization induces the phosphorylation of PYK2 and ERK by triggering Ca²⁺ influx through the opening of multiple types of voltage-gated Ca²⁺ channels in hippocampal slices and that the blockade of a single type of channel is not sufficient to prevent this effect.

In hippocampal slices the activation of PYK2 by depolarization is impaired by inhibitors of PKC (32), and ERK phosphorylation is stimulated by activators of PKC (10). We examined the contribution of PKC in the activation of ERK by depolarization using a specific inhibitor of this enzyme, GF109203X (45). In the presence of GF109203X, the depolarization-induced
phosphorylation of PYK2 and ERK was markedly diminished (Fig. 2, B and C), suggesting that an active PKC is involved in both responses.

Role of SFKs and Fyn in Depolarization-induced PYK2 Tyrosine Phosphorylation—The first step of PYK2 activation is thought to be its autophosphorylation on Tyr-402, followed by the recruitment of SFK and the phosphorylation of other tyrosine residues (35). We examined the effects of depolarization on PYK2 autophosphorylation using antibodies specific for the phosphorylated form of Tyr-402 and found that it was dramatically increased (Fig. 3, A and B). We then tested the role of Src-family kinases using PP2, an inhibitor of this family of enzymes (46). Total tyrosine phosphorylation of PYK2 after immunoprecipitation was dramatically reduced in the presence of PP2, whereas its inactive analog, PP3, had no effect (Fig. 3, A and B). In contrast, phosphorylation of PYK2 on Tyr-402 was not altered in the presence of PP2 (Fig. 3, A and B). These results support the model of an autophosphorylation of PYK2 followed by a recruitment of SFKs. They also demonstrate that depolarization-induced Ca^{2+} influx triggers PYK2 phosphorylation on Tyr-402 independently of SFKs.

SFKs expressed in the hippocampus include Src, Fyn, Yes, and Lck (47–49). We have previously shown that the focal adhesion kinase, a tyrosine kinase closely related to PYK2, is associated with Fyn in response to endocannabinoids in hippocampal slices (41). We examined the contribution of Fyn in the phosphorylation of PYK2 using hippocampal slices from fyn−/− mice. The levels of PYK2 were not altered in the hippocampus from mutant mice (Fig. 3C). However, the basal total tyrosine phosphorylation of PYK2, as well as its phosphorylation on Tyr-402, was dramatically reduced in hippocampal slices from fyn−/− mice (Fig. 3, C and D). Following the depolarization of fyn−/− slices, total tyrosine phosphorylation of PYK2 and Tyr-402 phosphorylation increased significantly, although they did not reach the levels observed in wild type hippocampus (Fig. 3, C and D). These results demonstrate that Fyn plays a critical role in the control of PYK2 tyrosine phosphorylation and its activation by depolarization. The results also show that other kinases presumably contribute to the phosphorylation of PYK2 in the hippocampus.

TABLE I
Study of Ca^{2+} channels and glutamate receptors antagonists on PYK2 and ERK2 phosphorylation

| Ca^{2+} channel blockers | P-PYK2 | P-ERK2 |
|--------------------------|--------|--------|
| Control                  | 100 ± 1| 100 ± 3|
| KCl                      | 446 ± 68a| 382 ± 62a|
| KCl + Ni^{2+} (50 μM)    | 512 ± 21a| 367 ± 18a|
| KCl + nifedipine (100 μM)| 396 ± 46a| 380 ± 48a|
| KCl + α-conotoxin (1 μM) | 406 ± 14a| 342 ± 14a|

| Glutamate receptor antagonists | P-PYK2 | P-ERK2 |
|-------------------------------|--------|--------|
| Control                       | 100 ± 9| 100 ± 6|
| KCI                           | 337 ± 30a| 320 ± 22a|
| KCI + MK801 (100 μM)          | 357 ± 23a| 325 ± 9a|
| KCI + CNQX (10 μM)            | 366 ± 11a| 315 ± 6a|

*p < 0.001.  
*p < 0.05.  
5-Cyano-7-nitroquinoxaline-2,3-dione.

**Fig. 3. Role of SFKs and Fyn in the activation of PYK2 by depolarization in hippocampal slices.** A, hippocampal slices were incubated for 2 min in the absence (Control) or presence of KCl with or without a 30-min pretreatment with the indicated drugs. P-PYK2 was measured by immunoprecipitation followed by phosphotyrosine immunoblotting and P-ERK2 by immunoblotting. Results are means of controls ± S.E. (n = 3–7). Statistics were by ANOVA followed by Bonferroni’s multiple comparison test.

A. Biotinylated phosphotyrosine antibodies reveal a dramatic increase of PYK2 phosphorylated on Tyr-402 and found that it was dramatically increased (Fig. 3, A and B). The results support the model of an autophosphorylation of PYK2 followed by a recruitment of SFKs. They also demonstrate that depolarization-induced Ca^{2+} influx triggers PYK2 phosphorylation on Tyr-402 independently of SFKs.

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Effects of MEK inhibitors on the activation of ERK and PYK2 by depolarization in hippocampal slices. Hippocampal slices were incubated for 2 min in the absence (Control) or the presence of 40 mM KCl following a 30-min pretreatment with U0126 (30 μM) or PD98059 (50 μM), two inhibitors of MEK, where indicated. A, immunoblotting for P-ERK and ERK. B, PYK2 was immunoprecipitated (IP) and analyzed by immunoblotting for phosphorytosine (P-Tyr) or total PYK2 (PYK2). Results were quantified as described in the Fig. 1 legend. Data are means ± S.E. (n = 5), and statistical analysis was done with ANOVA followed by Bonferroni’s test. Control versus KCl: *, p < 0.05; ***, p < 0.001. Absence versus presence of U0126 or PD98059: ++*, p < 0.001.

**Role of MEK in Depolarization-induced Activation of ERK**—ERK is activated by the phosphorylation of its activation loop by a dual specificity protein kinase, MEK. We examined the effects of two inhibitors of MEK, PD98059 (50) and U0126 (51), on the phosphorylation of ERK in response to KCl depolarization of hippocampal slices (Fig. 4A). Pretreatment with U0126 completely prevented ERK phosphorylation, whereas PD98059 had only a partial effect. A similar difference was already observed in the case of ERK activation by endocannabinoids (43) and may reflect a preferential involvement of MEK2 in the regulation of ERK in hippocampal neurons, because PD98059 is 10-fold less potent on MEK2 than on MEK1 (52). The study of the time course of activation of PYK2 and ERK in response to depolarization (Fig. 1D) indicates it is unlikely that PYK2 activation is a consequence of ERK activation. This was further ruled out by the observation that PYK2 phosphorylation was not altered in slices pre-treated with the MEK inhibitors (Fig. 4B).

**Depolarization-induced ERK Activation Is Independent of SFKs and Phosphatidylinositol-3-kinase**—We next examined the role of SFKs in the activation of ERK by depolarization in hippocampal slices. In slices from fyn−/− mice, the phosphorylation of ERK induced by depolarization was the same as in wild type mice (Fig. 5A). Moreover, the phosphorylation of ERK was not altered when slices were exposed to KCl depolarization in the presence of PP2, ruling out the role of other SFKs (Fig. 5B). These results demonstrate that tyrosine phosphorylation by SFKs of PYK2 or of other proteins is not necessary for depolarization-induced ERK phosphorylation. Because most previously reported pathways activated by PYK2 and leading to ERK activation involve the phosphorylation of PYK2 by SFKs (37), these results argue against a role of PYK2 in the depolarization-induced activation of ERK.

PYK2 can also activate phosphatidylinositol-3-kinase, which might, in turn, activate the ERK pathway (53, 54). We tested the role of this pathway in depolarization-induced ERK phosphorylation using a phosphatidylinositol-3-kinase inhibitor, LY294002 (55). Pretreatment of hippocampal slices did not alter ERK activation after KCl depolarization (with no pretreatment, control was 100 ± 23% and KCl was 398 ± 25%; with pretreatment using 50 μM LY294002, control was 54 ± 16% and KCl was 393 ± 8%; n = 3 in each group). These results indicate that phosphatidylinositol-3-kinase is not involved in ERK activation by depolarization.

**PYK2 and ERK Are Activated in Different Compartments after KCl-depolarization of Hippocampal Slices**—Altogether, our biochemical results showed that the conditions that impaired signaling by SFKs or phosphatidylinositol-3-kinase did not alter the activation of ERK by depolarization. Because these enzymes are responsible for the activation of ERK downstream from PYK2 in other cell types, it is unlikely that the activation of ERK results from the activation of PYK2 in depolarized hippocampal slices. Thus, hippocampal slices appear to differ from many cell lines, including PC12 cells. One major difference between mature brain tissue and cell lines is the degree of morphological differentiation of cells and their...
marked compartmentalization. Therefore, we examined whether activated PYK2 and ERK were located in different or similar cellular compartments. To address this issue, we performed immunofluorescence studies in control and 2-min KCl-depolarized hippocampal slices using antibodies specific for P-Tyr-402-PYK2 and P-ERK (Fig. 6). No or barely visible immunostaining with either antibody was observed in control slices, whereas a strong immunoreactivity appeared after KCl-depolarization, confirming the results of biochemical experiments. However, localization of P-PYK2 and P-ERK immunostaining after depolarization was dramatically different (Fig. 6). P-PYK2 labeling appeared concentrated in the neuronal cell bodies of the pyramidal cell layer, including nuclei and perikarya, and in the proximal dendrites. On the other hand, P-ERK immunoreactivity appeared essentially in the neuropil, whereas the principal cells were devoid of labeling. In addition to neurons, some P-PYK2 immunoreactivity was also observed in glial cells, including astrocytes identified by glial fibrillary acidic protein immunoreactivity (data not shown). P-ERK was not observed in glial cells.

To determine more precisely the localization of P-PYK2 and P-ERK we used double immunolabeling and laser-scanning confocal microscopy (Fig. 7). We observed virtually no colocalization of P-ERK and P-PYK2 (Fig. 7A). We determined the localization of P-ERK and P-PYK2 in neurons using double labeling with antibodies for MAP-2, a protein enriched in dendrites, and for synaptolectin, a synaptic vesicle-associated marker of nerve terminals. P-ERK immunostaining was colocalized with synaptolectin (Fig. 7B) but not with MAP-2 (Fig. 7C). Conversely, P-PYK2 immunostaining was colocalized with MAP-2 (Fig. 7D) but not with synaptolectin (Fig. 7E). Although maximal changes after KCl depolarization occurred in CA1, the localization of P-ERK and P-PYK2 was similar throughout the hippocampus, including CA1, CA3, and dentate gyrus (data not shown). Altogether, these experiments revealed that PYK2 and ERK were not activated in the same cellular compartments after KCl-depolarization; P-PYK2 was mostly found in the cell bodies and dendrites of pyramidal cells, whereas P-ERK was in the neuropil, where it colocalized with a presynaptic marker.

Depolarization-induced Activation of ERK Is Critical for the Rephosphorylation of the Synaptic Vesicle-associated Protein Synapsin I—Because P-ERK immunoreactivity following depolarization of hippocampal slices was enriched in nerve terminals, we examined its possible role at this level following depolarization. A major substrate for ERK in nerve terminals is the synaptic vesicle-associated protein synapsin I (30, 56). We measured the phosphorylation of synapsin I by immunoblotting with antibodies specific for the ERK-phosphorylated form of synapsin I at Ser-62 and Ser-67 (sites 4/5) (30). Depolarization induced a complete dephosphorylation of synapsin I at this site, followed by a rapid rephosphorylation (Fig. 8, A and B). The initial dephosphorylation is in agreement with the reported dephosphorylation of synapsin I sites 4/5 by calcineurin (56). In hippocampal slices, the rephosphorylation of synapsin I paralleled the activation of ERK, as indicated by P-ERK immunoreactivity (Fig. 8, A and B). The rephosphorylation of synapsin I was dramatically reduced in the presence of U0126, confirming the role of an active MEK/ERK module in the regulation of this site (Fig. 8, A and C). In contrast, PP2 had no effect on synapsin I rephosphorylation, showing the lack of involvement of SFKs in these regulations (Fig. 8, A and C).

DISCUSSION

Using a combination of biochemical and immunofluorescence approaches, we have characterized the effects of KCl-induced depolarization on the activation of ERK and PYK2, two kinases important for synaptic plasticity. In contrast with previous reports that dealt mostly with cell lines, we found that ERK and PYK2 phosphorylation induced by the depolarization of hippocampal slices occurred independently in different cellular compartments. Stimulation of PYK2 phosphorylation was reported following the depolarization of PC12 cells (31) and the depolarization or high frequency electrical stimulation of hippocampal slices (11, 32, 57). ERK activation has also been reported independently in similar conditions (10, 24, 39). The aim of our study was to address the possible relationship between the two pathways. Under our experimental conditions, ERK2 and PYK2 were the two major proteins phosphorylated on tyrosine after KCl-depolarization. The time course of phosphorylation was slightly slower for ERK than for PYK2, which would be compatible with an activation of ERK by PYK2. The blockade of both effects in the presence of Co2+, but not of glutamate receptor inhibitors, indicates that depolarization-induced activation of ERK and PYK2 was mediated by the Ca2+ influx through voltage-gated Ca2+ channels and not by endogenously released glutamate. Several types of Ca2+ channels appeared to be involved in this activation, because it was not blocked by any of the specific inhibitors tested. An active PKC was necessary for these effects, because they were markedly diminished in the presence of GF109203X, a PKC inhibitor. This supports the apparent role of PKC upstream from PYK2 that has been reported in numerous models since the initial demonstration of PYK2 activation by phorbol esters (31) and the blockade of its activation by PKC inhibitors (32). However, it should be emphasized that the precise mechanism of action of PKC in the regulation of PYK2 remains to be established. The effect of PKC inhibition is also compatible with the role of this enzyme in ERK activation in various cell types, including the hippocampus (28).

The use of an SFK inhibitor, PP2, allowed us to better characterize the mechanisms of PYK2 activation following depolarization. We could distinguish between the phosphorylation of the Tyr-402 of PYK2 that was unaltered by PP2 and the total tyrosine phosphorylation that was dramatically reduced by this inhibitor. These results support the model proposed for PYK2 activation (36), starting with an SFK-independent mechanism involving the trans-autophosphorylation of Tyr-402 and followed by the recruitment of SFKs, which is similar to the model suggested for the focal adhesion kinase (58). Among the SFKs capable of interacting with PYK2 after its autophosphorylation,
tion, our results provide evidence for an important functional role of Fyn. The activation of PYK2 was profoundly altered in hippocampal slices of Fyn-deficient mice, both in unstimulated slices and following depolarization. Interestingly, Tyr-402 phosphorylation was dramatically decreased in fyn−/− mice, whereas it was unaltered by an SFK inhibitor. This surprising result, similar to that obtained for the focal adhesion kinase in the hippocampus (41), suggests that Fyn plays a role in the control of PYK2 that is not limited to its catalytic activity. It should be noted, however, that a small but significant increase

**Fig. 7.** Colocalization of P-ERK, P-PYK2, MAP-2, and synaptobrevin in depolarized hippocampal slices. Rat hippocampal slices were incubated in the presence of 40 mM KCl for 2 min, fixed immediately, and resectioned before processing for immunofluorescence with antibodies against active ERK (P-ERK; green) and phospho-Tyr-402-PYK2 (P-PYK2; red) (A), MAP-2 (green) and active ERK (red) (B), synaptobrevin (green) and active-ERK (red) (C), MAP-2 (green) and phospho-Tyr-402-PYK2 (red) (D), and synaptobrevin (green) and phospho-Tyr-402-PYK2 (red) (E). Images were obtained with a laser-scanning confocal microscope (single sections shown). Colocalization was determined and indicated by white dots (right). The graph for each double labeling shows the distribution of green and red fluorophores across a line indicated by a white bar in the higher magnification inset. Scale bar, 2 μm.

**Fig. 8.** Time course of synapsin I and ERK phosphorylation during the depolarization of hippocampal slices. A, hippocampal slices were incubated for 2 min in the presence of 40 mM KCl (black bar), and P-ERK and synapsin I, phosphorylated on sites 4/5 (P-Synapsin 4/5), were studied at various time points by immunoblotting (left). The same experiment was repeated in the presence of U0126 (a MEK inhibitor, 30 μM; middle) or PP2 (an SFK inhibitor, 20 μM; right). B, quantification of P-ERK and P-synapsin 4/5 immunoreactivity (data are means ± S.E., n = 3). C, quantification of P-synapsin 4/5 immunoreactivity in the absence or presence of kinase inhibitors (data are means ± S.E., n = 3). The repolysynthesis of synapsin I, following its depolarization-induced dephosphorylation, was severely impaired in the presence of U0126 (***, p < 0.001).
in the tyrosine phosphorylation of PYK2 was observed in *fyn*−/− mice following depolarization, in agreement with the reported association of PYK2 with other SFKs, including Src (57). Because of the role of PYK2 in long term potentiation (11), its functional alterations are likely to contribute to the impaired long term potentiation induction and spatial learning observed in *fyn*−/− mice (48). Such alterations may result in part from dysregulation of the NMDA receptor, which is a substrate of Fyn (59, 60) functionally altered in *fyn*−/− mice (61). Thus, the functional association between PYK2 and Fyn reported here supports a major role of this module in the regulation of NMDA receptors. It is interesting to emphasize that these regulations may play an important role in pathological conditions, because several reports suggest that the PYK2/NMDA receptor pathway is also activated *in vivo* following brain ischemia (14, 15, 62) as a consequence of Ca$^{2+}$/H$^{+}$ influx through voltage-gated Ca$^{2+}$ channels (63).

In contrast to the role of SFKs and Fyn in the regulation of PYK2, these enzymes did not appear to be involved in the depolarization-induced activation of ERK. ERK activation by depolarization was unchanged under conditions in which PYK2 tyrosine phosphorylation was severely altered. Because the reported pathways linking ERK activation to PYK2 involve the tyrosine phosphorylation of the latter, it appears extremely unlikely that PYK2 is a major component in ERK activation in depolarized hippocampal slices. Moreover, phosphatidylinositol 3-kinase, which can be recruited by PYK2 and has the capacity to activate ERK (53, 54), also appeared not to be involved in ERK activation in these experimental conditions. Thus, our biochemical data provide strong evidence that the PYK2/SFK module is not involved in ERK activation by KCl depolarization in hippocampal slices. This conclusion differs from that drawn from studies in PC12 cells in which PYK2 acts as an upstream regulator of ERK, recruiting the Grb2-Sos complex after tyrosine phosphorylation induced by lysophosphatidic acid and bradykinin (31, 34). Moreover, in PC12 cells the absence of PYK2 prevents the activation of ERK by Ca$^{2+}$/H$^{+}$ influx induced by a Ca$^{2+}$/H$^{+}$ ionophore (40) or by KCl-induced depolarization.\(^2\) These differences between PC12 cells and hippocampal slices underline the cell specificity of signaling pathways and the importance of studying these pathways in physiologically relevant preparations. Several alternative mechanisms may account for the regulation of the MEK/ERK module by Ca$^{2+}$/H$^{+}$ influx in the hippocampus (9), including the inhibition of a GTPase-activating protein such as SynGAP (64, 65) or the activation of a guanine nucleotide exchange factor such as Ras-GRF (66). The contribution of PKC in the activation of ERK is dependent on cell types and can play a modulatory role (for reviews, see Refs. 6 and 67), which would be consistent with the partial impairment of ERK activation by PKC inhibition in hippocampal slices. Finally, it should be emphasized that two other mitogen-activated protein kinase modules that have been reported to be activated downstream from PYK2, the c-Jun N-terminal kinase module (3, 33), and the p38 mitogen-activated protein kinase module (68), were not activated in depolarized hippocampal slices.

The independence between the activation of PYK2 and ERK was further supported by our immunofluorescence studies. Indeed, P-PYK2 appeared essentially located in the cell bodies and dendrites of pyramidal neurons, whereas P-ERK immunoreactivity was observed in the neuropil, mostly associated with nerve terminals. In addition, some P-PYK2 immunoreactivity was observed in glial cells in agreement with the previously reported regulation of this kinase in astrocytes (69) and microglia (15, 70). It is unclear whether the activation of glial PYK2 resulted from a direct effect of the KCl pulse on these cells or from the action of mediators released by depolarized neurons. We did not observe any P-ERK immunoreactivity in glial cells, further supporting the independence between the two pathways.

The presence of P-PYK2 in neuronal cell bodies and dendrites, colocalized with MAP-2, corresponded with the previously reported distribution of PYK2 in the hippocampus (71). This localization is also compatible with the association of PYK2 with post-synaptic proteins (11, 38). However, it should be noted that depolarization-induced PYK2 activation was mostly detected in dendritic shafts and not in spines where most excitatory synapses are located, an observation supported by the small proportion of PYK2 associated with synaptic membranes (72).

P-ERK immunostaining did not colocalize with P-PYK2 but was found in the neuropil, where it was colocalized with synaptobrevin, a synaptic vesicle-associated protein. These immunofluorescence experiments do not rule out the presence of P-ERK at the postsynaptic level, which is closely associated with nerve terminals and could not be separated by light microscopy. Interestingly, however, even when studied at a later time after depolarization (10 min) P-ERK was confined to the neuropil and did not accumulate in dendritic shafts, soma, or nuclei.\(^3\) These results are consistent with a restriction of ERK activation to nerve terminals. Thus, the localization of P-ERK following depolarization contrasts with that observed with other stimuli, including the direct activation of protein kinases A or C (28) or the stimulation of CB1 cannabinoid receptors (43), all of which lead to a somatodendritic and nuclear localization of P-ERK. These observations are important, because they demonstrate that although ERK is widely distributed in hippocampal neurons (73), it is specifically activated in distinct cellular compartments in response to different stimuli. This selective spatial pattern of activation may have important functional consequences.

Because of its predominant localization, the activation of ERK by depolarization is likely to be important in the regulation of cytoplasmic or membrane proteins present in the neuropil rather than in the control of nuclear or somatodendritic targets. A major substrate of ERK in nerve terminals is synapsin I, and we have demonstrated that in hippocampal slices depolarization induced a sequence of dephosphorylation-re-phosphorylation of synapsin I sites 4/5, similar to that reported in isolated nerve terminals (56). We found that activated ERK mediates the rephosphorylation of synapsin I in hippocampal slices as reported *in vivo* (74), a regulation that appears to be critical for controlling the availability of synaptic vesicles (75). Altogether, these results confirm that ERK is activated in nerve terminals following depolarization and has an important functional role.

In conclusion, our study demonstrates that, in contrast to cell lines, depolarization activates PYK2 and ERK independently in two distinct cellular compartments in the adult hippocampus. The somatodendritic activation of PYK2 is severely altered in *fyn*-deficient mice, suggesting a strong functional association between these two kinases in the hippocampus. In contrast, ERK activation predominates in nerve terminals, where it controls rephosphorylation of synapsin independently of Src-family kinases and may thus regulate neurotransmitter release.

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\(^2\) M. Toutant and J.-A. Girault, unpublished observations.

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Depolarization Activates ERK and Proline-rich Tyrosine Kinase 2 (PYK2) Independently in Different Cellular Compartments in Hippocampal Slices
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