Differential Regulation of Proline-rich Tyrosine Kinase 2/Cell Adhesion Kinase β (PYK2/CAKβ) and pp125FAK by Glutamate and Depolarization in Rat Hippocampus*

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The mechanisms by which stimuli that raise cytosolic free Ca2+ concentrations in neurons can increase protein tyrosine phosphorylation are not known. Using rat hippocampal slices and cortical synaptosomes, we have examined the regulation of two highly related cytoplasmic tyrosine kinases, pp125 focal adhesion kinase (pp125FAK) and proline-rich tyrosine kinase 2/cell adhesion kinase β (PYK2/CAKβ). Membrane depolarization increased tyrosine phosphorylation of PYK2/CAKβ and pp125FAK. These effects were blocked by EGTA or by protein kinase C inhibitors (RO31-8220; GF109203X) and mimicked by ionomycin or phorbol 12-myristate 13-acetate, in the case of pp125FAK, or their combination in the case of PYK2/CAKβ. Glutamate and specific agonists of ionotropic (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate and N-methyl-D-aspartate) or metabotropic (trans-1-amino cyclopentane-1,3,5-dicarboxylate) glutamate receptors stimulated the phosphorylation of pp125FAK but not of PYK2/CAKβ. Glutamate effects were prevented by GF109203X. Thus, in hippocampal slices, tyrosine phosphorylation of pp125FAK and PYK2/CAKβ are regulated differentially by pathways involving Ca2+ and protein kinase C. pp125FAK and PYK2/CAKβ may provide specific links between neuronal activity, increases in cytosolic Ca2+ and protein tyrosine phosphorylation, which may be important for neuronal survival, and synaptic plasticity.

Increases in cytosolic free Ca2+ are critical for many neuronal functions including neurotransmitter synthesis and release, and neuronal survival, while excess in Ca2+ can lead to neuronal death. The induction of two well-studied types of synaptic plasticity, long term potentiation (LTP) and long term depression (LTD) requires also a marked increase in cytosolic Ca2+, which results usually from the stimulation of glutamate NMDA receptors (see Ref. 1 for a review). Many of the effects of Ca2+ are mediated by the activation of serine/threonine kinases, including classical PKCs (2) and Ca2+/calmodulin kinases (3), and of a serine/threonine phosphatase, calcineurin (4). In addition, cytosolic Ca2+ is able to activate protein tyrosine phosphorylation and signaling cascades involving Ras and MAP kinase which result in alterations in gene transcription (see Ref. 5 for a review). Depolarization and neurotransmitter agonists increase tyrosine phosphorylation of several proteins in synaptosomes (6), hippocampal slices (7), neurons in culture (7), and cell lines with neuronal characteristics (8, 9). This link between tyrosine phosphorylation and Ca2+ in neurons opens exciting perspectives since tyrosine phosphorylation appears necessary for the establishment of LTP (10) and LTD (11) and plays an important role in the control of neuronal differentiation and survival (5). However, the molecular basis for the stimulation of tyrosine phosphorylation in response to Ca2+ is not known. Evidences obtained in PC12 cells indicate that Src (8) and PYK2 (9) are involved in the Ca2+-induced activation of tyrosine phosphorylation and downstream signaling cascades. Yet, the mechanism by which Ca2+ activates Src and PYK2 in PC12 cells is not known, and it is not clear which tyrosine kinase is activated first. In addition, the regulation of these, or related, kinases has not been demonstrated in normal neuronal tissue. Here, we have investigated, in rat hippocampus, the possible regulation by Ca2+ of two structurally related cytoplasmic tyrosine kinases, pp125FAK and PYK2/CAKβ, which could be associated with Src family kinases in neurons.

pp125FAK is a 125-kDa cytosolic tyrosine kinase devoid of SH2 or SH3 domains, which is associated with focal adhesions (12, 13). pp125FAK is phosphorylated on tyrosine in response to integrin engagement and to stimulation of various G protein-coupled receptors (14). Autophosphorylated pp125FAK binds to the SH2 domain of Src or Fyn (15). Phosphorylation of pp125FAK by Src on multiple residues (16) allows the recruitment and activation of phosphatidylinositol-3-kinase (17, 18) and the binding of Grb2, leading to the activation of MAP kinase cascade (19). pp125FAK is highly expressed in nervous tissue during development, a period at which it is enriched in neuronal growth cones (20). In the brain of adult rats, pp125FAK is expressed at higher levels than in most other tissues, especially in the hippocampus, and the cerebral cortex (20). However, pp125FAK immunoreactivity is rather diffuse in adult neurons (20), and its precise localization and function are not known. Interestingly, pp125FAK appears to be a major phosphoprotein altered in Fyn knock-out mice (21), which dis-
play anomalous hippocampal development and LTP (22). This observation, together with the ability of pp125FAK to trigger multiple signaling cascades and to regulate interactions between actin-based cytoskeleton and extracellular matrix receptors, suggests a role for pp125FAK in synaptic plasticity.

Recently, a 110-kDa tyrosine kinase which shares a high degree of sequence homology with pp125FAK has been cloned independently by several groups and named proline-rich tyrosine kinase 2 (PYK2) (9), cell adhesion kinase β (CAKβ) (23), related adhesion focal tyrosine kinase (24), or pp125FAK2 (25). In transfected COS-7 cells, PYK2/CAKβ is not localized in focal adhesions, but in regions of cell-cell contacts (23). In PC12 cells, PYK2 is phosphorylated on tyrosine and activated in response to various stimuli which raise intracellular Ca2+ and stimulate protein kinase C (9), although the precise mechanism of this activation is not known. In addition, PYK2/CAKβ induces the phosphorylation on tyrosine of several signal-transducing proteins and ion channels and activates the MAP kinase pathway (9). Since PYK2/CAKβ is highly expressed in adult brain (23, 24), these observations make it an ideal candidate for coupling depolarization and/or activation of neurotransmitter receptors to tyrosine phosphorylation pathways in neurons.

To assess the potential role of PYK2/CAKβ and pp125FAK in the nervous system, we have investigated the ability of glutamate agonists and membrane depolarization to regulate tyrosine phosphorylation of these two kinases in rat hippocampal slices, one of the most widely used model for studying synaptic plasticity. Our results show that membrane depolarization stimulates dramatically the phosphorylation of PYK2/CAKβ, whereas agonists of three different types of glutamate receptors increase specifically phosphorylation of pp125FAK. In addition, we show that phosphorylation of PYK2/CAKβ and pp125FAK requires an active PKC. These results demonstrate a high degree of selectivity in the activation of PYK2/CAKβ and pp125FAK in mature nervous systems where these kinases may be important to connect neuronal activity and tyrosine phosphorylation pathways.

**EXPERIMENTAL PROCEDURES**

**Rat Hippocampal Slices**—Rat hippocampal slices (300 μm) were prepared from male Sprague-Dawley rats (100–150 g) with a McIlwain tissue chopper and incubated (3 slices per tube) in 1 ml of artificial cerebrospinal fluid (ACSF), at 35 °C for 50 min before pharmacological treatments, as described previously (7). To avoid indirect effects due to neuronal firing, experiments were all carried out in the presence of 1 mM TTX, which was added at the beginning of slice incubation. Treatments with NMDA were carried out in Mg2+-free ACSF. Neither TTX, nor the absence of Mg2+, had any effect on protein tyrosine phosphorylation by themselves (data not shown). At the end of the experiment, ACSF was aspirated, and the slices were frozen and kept at −80 °C. Slices were either used for immunoprecipitation (see below) or sonicated in 200 μl of 1% SDS, 5% β-mercaptoethanol.

**Subcellular Fractionation**—Subcellular fractionation was carried out as described (26). Synaptosomes were prepared from P2 over a Percoll density gradient (27) and incubated in ACSF. After treatment, they were collected by centrifugation and homogenized by sonication as described above for slices.

**Immunoprecipitation**—For each immunoprecipitation, 6 hippocampal slices (total protein = 500–600 μg) from two independent samples were pooled and homogenized in ice-cold buffer (100 mM NaCl, 50 mM TrisCl pH 7.4, 5 mM EDTA, 50 mM NaF) containing 1 mM Na+ orthovanadate, 1% (v/v) Nonidet P-40, and protease inhibitors (Complete, Boehringer Mannheim). Immunoprecipitation was carried out with 20 μl of preimmune or immune rabbit antisera predetermined and washed on protein A-Sepharose beads, as described previously (28). The amount of PYK2/CAKβ or pp125FAK in the immunoprecipitate was estimated using one-third of each immune precipitate for an in vitro kinase assay (incubation for 10 min at 30 °C in 50 μl of buffer containing 50 mM HEPES, pH 7.4, 10 mM MnCl2, and 5 μCi of [γ-32P]ATP, 3000 Ci/mmol).

**Immunoblotting**—Slice lysates (40 μg of protein measured with a bicinchoninic acid-based method, Ref. 29) or pellets from immunoprecipitation, or pp125FAK in the immunoprecipitate was estimated and quantified were carried out as described previously (30). Stripping of antibodies was achieved by incubating the membrane for 1 h in a buffer containing 100 mM glycine, pH 2.5, and 100 mM β-mercaptoethanol.

**Antibodies**—Antiphosphotyrosine mouse monoclonal antibody 4G10 was from UBI. Serum SL38 was prepared by immunizing a rabbit against the amino-terminal fragment of rat pp125FAK (31) (residues 1–376) expressed in Escherichia coli as a hexahistidine fusion protein. Serum 539754 was obtained by immunizing a rabbit against a 17-amino acid peptide encompassing residues 2–18 of rat PYK2/CAKβ. Serum 670–716 was obtained by immunizing a rabbit against a glutatione S-transferase-fusion protein including residues 670–716 of PYK2/CAKβ and affinity-purified (23).

**RESULTS AND DISCUSSION**

Membrane depolarization results in Ca2+ influx by opening voltage-sensitive Ca2+ channels in neurons, while stimulation of specific glutamate receptors may lead to increases in cytosolic Ca2+ by several mechanisms (see Ref. 32). NMDA receptors, in the absence of extracellular Mg2+, are highly permeant to Ca2+, and stimulation of AMPA receptors may result in Ca2+ influx by opening neighboring voltage-sensitive Ca2+ channels. In addition, AMPA receptors of the GluR2 subtype, which are highly expressed in hippocampus, are permeable to Ca2+ (32). Finally, metabotropic glutamate receptors are coupled to phospholipase C and can release Ca2+ from intracellular stores (33).

Treatment of rat hippocampal slices for 5 min with glutamate (Ref. 7 and see below) or specific agonists of glutamate receptors (100 μM AMPA, NMDA in the absence of Mg2+), or t-ACPD)
increased tyrosine phosphorylation of several proteins, including major components of 120–130 kDa (Fig. 1A). Membrane depolarization induced by a 2-min increase in the extracellular concentration of K⁺ ions (40 mM KCl) resulted also in a marked stimulation of tyrosine phosphorylation of several proteins (Fig. 1A), confirming and extending our previous results (7). As already reported, the effects of KCl were more pronounced than those of glutamate agonists on a 110-kDa protein (Fig. 1A).

Following stripping of the membrane from antiphosphotyrosine antibodies, immunoblotting with specific antibodies revealed that pp125FAK comigrated with a major 125-kDa band phosphorylated in response to glutamate agonists, whereas PYK2/CAKβ comigrated with a 110-kDa band phosphorylated in response to KCl-induced depolarization (Fig. 1A).

PYK2/CAKβ and pp125FAK were immunoprecipitated from slice homogenates with specific antibodies, and their state of phosphorylation was studied by antiphosphotyrosine immunoblotting. A marked increase in tyrosine phosphorylation of pp125FAK, but not of PYK2/CAKβ, was observed in response to glutamate agonists (Fig. 1B). It should be noted that a 110-kDa protein phosphorylated in response to glutamate agonists was visible on immunoblots of total homogenates (Fig. 1A). Since it was not immunoprecipitated by two different antibodies against PYK2/CAKβ (Fig. 1B and data not shown), it is likely to be a different protein. However, we cannot rule out formally the existence of a form of PYK2/CAKβ not recognized by our antibodies because of strong interaction with other proteins or of post-translational modifications. In contrast, KCl-induced depolarization increased dramatically tyrosine phosphorylation of PYK2/CAKβ (Fig. 1B, lane 6). The effects of depolarization on pp125FAK phosphorylation were less pronounced than on PYK2/CAKβ (Fig. 2B) and failed to be observed in about one-fourth of the slice preparations (compare Figs. 1B and 2). Neither glutamate agonists nor depolarization altered significantly the amounts of pp125FAK or PYK2/CAKβ recovered in the immune precipitates, as estimated by a phosphorylation assay (data not shown).

The marked difference observed between the phosphorylation of pp125FAK and PYK2/CAKβ in response to glutamate agonists and depolarization raised the possibility that they may have different locations. For instance, PYK2/CAKβ could be selectively enriched at the presynaptic level and pp125FAK at the postsynaptic level, where most glutamate receptors are located. However, fractionation experiments demonstrated that only low amounts of the two kinases were present in the crude synaptosomal fraction (P2), in cerebral cortex (Fig. 3A), and in hippocampus (data not shown). Moreover, when purified synaptosomes were incubated in vitro and depolarized by the application of KCl, tyrosine phosphorylation of pp125FAK was increased (Fig. 3B), whereas no change in PYK2/CAKβ phosphorylation was detected (data not shown). These results suggest that the selective sensitivity of PYK2/CAKβ phosphorylation to KCl-induced depolarization is not due to its enrichment in nerve terminals.

The role of Ca²⁺ in the effects of depolarization on pp125FAK and PYK2/CAKβ tyrosine phosphorylation were examined. External Ca²⁺ was absolutely required for the tyrosine phosphorylation of PYK2/CAKβ in response to depolarization, which was completely blocked in the presence of 3 mM EGTA applied 3 min before KCl (Fig. 2). The application of EGTA by itself enhanced tyrosine phosphorylation of pp125FAK in hippocampal slices (Fig. 2) and in cortical synaptosomes (Fig. 3B), but prevented any further increase upon depolarization in both preparations. The basis for the effect of EGTA on pp125FAK basal phosphorylation is not known and may be related, in part, to nonexocytotic release of endogenous neurotransmitters (34). To examine further the action of Ca²⁺, we studied the ability of a Ca²⁺ ionophore and a phorbol ester, alone or in
combination, to reproduce the effects of depolarization in hippocampal slices. A 10-min application of 0.1 μM PMA or a 2-min application of 1 μM ionomycin was sufficient to increase the phosphorylation of pp125FAK, but had no consistent effect on PYK2/CAKβ phosphorylation (Fig. 4). However, the combination of PMA and ionomycin treatments resulted in a synergistic effect on PYK2/CAKβ phosphorylation (Fig. 4). These results suggest strongly that activation of PKC is sufficient to induce tyrosine phosphorylation of pp125FAK. In contrast, PYK2/CAKβ phosphorylation required the simultaneous addition of Ca2+ ionophore and phorbol ester, an observation which emphasizes the difference in the regulation of these two tyrosine kinases.

To test the potential role of PKC or other Ca2+-activated enzymes in mediating the effects of depolarization on tyrosine phosphorylation of pp125FAK and PYK2/CAKβ, various pharmacological inhibitors were used. Pretreatment of slices with antagonists of calmodulin (calmidazolium, trifluoperazine) or inhibitors of Ca2+/calmodulin-dependent kinases (KN62) or of calcineurin (FK506, cyclosporin A, alone or in combination) did not prevent the stimulation of protein tyrosine phosphorylation in response to depolarization (data not shown). In contrast, two inhibitors of PKC, RO31-8220 (35) (Fig. 5) or GF109203X (36) (data not shown) inhibited markedly the effects of depolarization in hippocampal slices. A 10-min application of 0.1 mM PMA, or 2 min to 1 mM ionomycin, or the combination of the two treatments, A, PYK2/CAKβ and pp125FAK were immunoprecipitated with specific antibodies (539 754 and SL38, respectively), and their tyrosine phosphorylation was assessed by antiphosphotyrosine immunoblotting. B, quantification of phosphorylation and statistical analysis were done as indicated in the legend to Fig. 2. Data correspond to the mean ± S.E. of 4–15 samples per condition.

Our results show that tyrosine phosphorylation of PYK2/CAKβ and pp125FAK is increased in response to extracellular signals in rat hippocampal slices. However, we found a marked preferential response of PYK2/CAKβ to depolarization. Although we cannot rule out that PYK2/CAKβ is activated by neurotransmitters in some circumstances, the situation in hippocampal slices contrasts with that reported in PC12 cells, in which an increase in PYK2 phosphorylation occurred in response to various stimuli (9). Thus, our results suggest the existence of a more specific mechanism of activation of PYK2/CAKβ in mature nervous system than in cell lines. This specific activation cannot be attributed to an enrichment of PYK2/CAKβ in nerve terminals, but may be related to a preferential localization of PYK2/CAKβ in the vicinity of voltage-activated Ca2+ channels. In contrast, pp125FAK phosphorylation was stimulated not only following depolarization, but also in response to stimulation of glutamate receptors. These results argue for the presence of pp125FAK at the postsynaptic membrane, where these receptors are mostly found, in agreement with our previous immunocytochemical observations (20). Nevertheless, we found that pp125FAK is also present in nerve terminals where it is phosphorylated in response to depolarization. It should be noted that, since phosphorylation of pp125FAK is enhanced in brain in response to several neurotransmitters including glutamate (this study), acetylcholine,2 and anandamide (37), it is possible that the increase in pp125FAK phospho-

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Phosphorylation in response to depolarization results in part from the release of endogenous neurotransmitters.

PYK2/CAKβ and pp125FAK tyrosine phosphorylation in response to depolarization required the presence of extracellular Ca\(^{2+}\) and is likely to result from Ca\(^{2+}\) influx. In addition, several observations support a critical role for PKC. First, PKC activation, by a phorbol ester alone in the case of pp125FAK, or in combination with a Ca\(^{2+}\) ionophore in the case of PYK2/CAKβ, increased tyrosine phosphorylation of these proteins. Second, among all the various inhibitors of Ca\(^{2+}\)-activated enzymes that we have tried, only PKC inhibitors were able to decrease significantly the phosphorylation of PYK2/CAKβ and pp125FAK resulting from depolarization and the phosphorylation of pp125FAK induced by glutamate. However, we cannot exclude that PKC plays only a permissive role and that direct stimulation of another Ca\(^{2+}\)-activated protein is responsible for the activation of pp125FAK and/or PYK2/CAKβ phosphorylation.

Phosphorylation of pp125FAK and PYK2/CAKβ provides a link between increases in Ca\(^{2+}\) and tyrosine phosphorylation pathways, which may be involved in neuronal survival and synaptic plasticity (5). In light of the known importance of Ca\(^{2+}\)-activated processes, PKC and tyrosine phosphorylation in LTP and LTD (10, 11, 38), it is tempting to speculate that phosphorylation of pp125FAK induced by glutamate. However, we can clearly sensitive to stimulation of NMDA receptors which appears unable to induce LTP or LTD in response to stimulation of AMPA and metabotropic glutamate receptors, which plays a critical role in synaptic plasticity (1, 38). On the other hand, pp125FAK phosphorylation was also increased in response to stimulation of AMPA and metabotropic glutamate receptors, which appears unable to induce LTP or LTD in hippocampus (1, 38), indicating that phosphorylation of pp125FAK is not sufficient for generating these long term changes in synaptic efficacy. Thus, both pp125FAK and PYK2/CAKβ appear to be involved in the Ca\(^{2+}\)-induced activation of tyrosine phosphorylation in nervous tissue. However, in spite of the similarities between these two tyrosine kinases, their regulation and, probably, their role appear different.

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