Protein tyrosine kinase PYK2 involved in Ca^{2+}-induced regulation of ion channel and MAP kinase functions

S. Lev*, H. Moreno†, R. Martinez‡, P. Canoll*, E. Peles‡, J. M. Musacchio*, G. D. Plowman‡, B. Rudy† & J. Schlessinger*§

Departments of Pharmacology* and Physiology†, New York University Medical Center, 550 First Avenue, New York, New York 10016, USA
‡ SUGEN, Inc., 515 Galveston Drive, Redwood City, California 94063-4720, USA

The protein tyrosine kinase PYK2, which is highly expressed in the central nervous system, is rapidly phosphorylated on tyrosine residues in response to various stimuli that elevate the intracellular calcium concentration, as well as by protein kinase C activation. Activation of PYK2 leads to modulation of ion channel function and activation of the MAP kinase signalling pathway. PYK2 activation may provide a mechanism for a variety of short- and long-term calcium-dependent signalling events in the nervous system.

PROTEIN tyrosine kinases are important for the control of cell growth and differentiation[1,2]. Several protein tyrosine kinases are highly expressed in the central nervous system[3], and there is evidence that protein phosphorylation is important for regulation in the nervous system. Neurotrophic factors that control the differentiation and survival of different types of neuronal cells mediate their biological effects by activation of cell surface receptors with intrinsic protein tyrosine kinase activity[4]. Furthermore, protein phosphorylation is a key regulatory mechanism for membrane excitability and ion channel function. It has been shown that ion channels are subjected to phosphorylation that alters their functional properties[5,6]. Most of the currently available information is restricted to modulation of ion channel functions by serine/threonine kinases such as protein kinase C (PKC), cyclic AMP-dependent protein kinase and calmodulin-dependent kinase[7-10]. It has been shown that PKC can regulate the action of a variety of ion channels, including voltage-gated potassium channels[10], voltage-dependent sodium channels[7] and the nicotinic acetylcholine receptor[3]. Tyrosine phosphorylation also has a regulatory role in the action of specific ion channels, such as the *N*-methyl-D-aspartate (NMDA) receptor[11] and a delayed rectifier-type potassium channel[12]. In the latter example, inhibition of potassium currents is mediated in part by tyrosine phosphorylation of the channel protein by an unknown protein tyrosine kinase[12]. Thus it is clear that the action of protein kinases and phosphatases is crucial for the control of proliferation, differentiation and survival of neuronal cells, as well as for the regulation of neuronal excitability, plasticity and excitotoxicity.

Cloning of PYK2

To isolate Grb2-binding proteins, we have used the Grb2 adaptor protein as a specific probe for screening expression libraries. One of the cloned proteins encoded a protein tyrosine kinase with a proline-rich region that can bind *in vitro* to the SH3 domains of Grb2 (J. Ureña and J.S., unpublished results). This protein was termed proline-rich tyrosine kinase 1 (PYK1). Comparison of the amino-acid sequence of PYK1 to other tyrosine kinases indicated that PYK1 is related to the Ack protein tyrosine kinase[13], and that it represents a member of a new class of cytoplasmic protein tyrosine kinases.

To isolate kinases related to PYK1, we applied the polymerase chain reaction (PCR), using degenerate oligonucleotide primers derived from the PYK1 sequences in conserved motifs of catalytic domains of PTKs[14]. Figure 1*a* shows the complete amino-acid sequence of a protein tyrosine kinase that was isolated from a human brain complementary DNA library and termed PYK2. The open reading frame of PYK2 encodes a protein of 1,009 amino acids, containing a long amino-terminal sequence of 425 amino acids followed by a protein tyrosine kinase domain, two proline-rich domains (29 and 23.3% proline, respectively), and a large carboxy-terminal region. The kinase domain of PYK2 (Fig. 1*a*, box) contains several sequence motifs conserved among protein tyrosine kinases, including the tripeptide motif DFG that is found in most kinases, and a consensus ATP-binding motif GXGXXG followed by an AXK sequence 17 amino-acid residues downstream[14].

Comparison of the amino-acid sequence of the kinase domain of PYK2 with other protein tyrosine kinases showed that the kinase core of PYK2 is most similar to the protein tyrosine kinase domains of Fak, Fer, Her4 and Abl (Fig. 1*b*). In addition to the sequence homology in the kinase domain, the flanking sequences and the overall structural organization of the PYK2 protein are most similar to those of Fak[15]. This suggests that PYK2 and Fak belong to the same family of non-receptor protein tyrosine kinases.

We next examined the expression pattern and tissue distribution of PYK2 by northern blot and *in situ* hybridization analyses. Northern blot analysis of multiple human tissues demonstrated that a 4.5-kilobase (kb) PYK2 transcript is most abundant in the human brain, with a low level of expression detected in the kidney (data not shown). *In situ* hybridization analysis showed that PYK2 was expressed in discrete populations of neurons within the rat brain, with the highest levels of mRNA seen in the hippocampus, dentate gyrus and olfactory bulb (data not shown). These results are consistent with northern blot analysis of messenger RNA isolated from various human brain sections (data now shown).

To characterize the PYK2 protein, specific antibodies were raised in rabbits against a PYK2 fusion protein. These antibodies precipitated a protein from PC12 cells, as well as from transfected NIH 3T3 cells, that migrated in SDS gels with an apparent relative molecular mass (M_r) of 112K (data not shown).

Activation of PYK2

Because PYK2 is highly expressed in the central nervous system and PC12 cells, we examined the effect of a variety of neuronal stimuli on the phosphorylation state of PYK2. In these experiments, PC12 cells were treated with agonist, lysed, and immuno-

§ To whom correspondence should be addressed.

FIG. 1 Primary structure of human PYK2 protein tyrosine kinase. *a*, Deduced amino-acid sequence of human PYK2 from cDNA clones. The tyrosine kinase domain is highlighted by a dark shaded box. Two proline-rich domains in the C-terminal region are boxed with light shading. Amino-acid residues are numbered on the left. *b*, Comparison of the amino-acid sequence of the catalytic domain of PYK2 with the four most related human protein tyrosine kinases demonstrated 61%, 43%, 40% and 41% sequence identity between PYK2 and Fak, Fer, HER4 and Abl, respectively. The homology between PYK2 and Fak extends beyond the catalytic domain with 42% and 36% amino-acid identity in the N-terminal and C-terminal regions, respectively.

METHODS. Total RNA from rat spinal cord was used to prepare cDNA using the reverse transcriptase of Molony murine leukaemia virus ('Superscript', BRL) according to the manufacturer's protocol. The cDNA was amplified by PCR utilizing degenerate oligonucleotide primers corresponding to conserved tyrosine kinase motifs from subdomains TK6 and TK9 of PYK1 (J. Ureña and J.S., unpublished results); (the sense and antisense primers correspond to amino-acid sequences IHRDLAARN and WMFGVTLW, respectively). The PCR was performed under the following conditions: 1 min at 94 °C; 1 min at 50 °C and 1 min at 68 °C for 35 cycles. Amplified DNA was subcloned and sequenced, resulting in identification of a novel tyrosine kinase termed PYK2. A λgt10 human fetal brain cDNA library (Clontech) was screened with a ^{32}P-labelled probe derived from the PCR clone corresponding to rat PYK2. Four overlapping clones were isolated and their DNA sequence was determined on both strands using series of oligonucleotide primers. The 3,414-bp consensus sequence contains a single open reading frame of 3,027 nucleotides preceded by a 105-nucleotide 5′-untranslated region. The complete nucleotide sequence was deposited in GenBank (accession no. 33289). Amino-acid sequence comparisons were performed using the Smith–Waterman algorithm of MPSRCH (IntelliGenetics) on a MasPar computer.

precipitated with anti-PYK2 antibodies, followed by SDS-PAGE (polyacrylamide gel electrophoresis) analysis and immunoblotting with phosphotyrosine-specific antibodies.

Stimulation of PC12 cells with carbachol induces strong tyrosine phosphorylation of PYK2 (Fig. 2*a*). Because carbachol can activate both the nicotinic and muscarinic receptors[16], we explored whether activation of both cholinergic receptor subtypes leads to tyrosine phosphorylation of PYK2. Pharmacological analysis with either subtype-specific agonists, muscarine and dimethylphenylpiperazinium (DMPP), or subtype-specific antagonists, atropine and mecamylamine (Fig. 2*a*) indicated that activation of PYK2 by carbachol is mediated by means of the nicotinic acetylcholine receptor. The phosphorylation of PYK2 in response to carbachol is very rapid; 5 s after applying carbachol to the cells, PYK2 became phosphorylated on tyrosine residues. Elimination of extracellular calcium by EGTA completely blocked this agonist-induced tyrosine phosphorylation of PYK2 (Fig. 2*a*, lower panels), indicating that calcium influx is required for carbachol-induced PYK2 phosphorylation.

Stimulation of the nicotinic acetylcholine receptor induces membrane depolarization by cation influx through the ion channel pore. This depolarization opens voltage-gated calcium channels, resulting in a large influx of calcium into the cell. We therefore checked whether membrane depolarization induced by increasing the extracellular concentration of potassium ions would give the same effect on PYK2 tyrosine phosphorylation. Depolarization of PC12 cells with 75 mM KCl induced rapid tyrosine phosphorylation of PYK2 (Fig. 2*b*). The omission of calcium from the extracellular medium completely abolished this PYK2 tyrosine phosphorylation, indicating that activation of PYK2 is due to calcium influx rather than membrane depolarization *per se*. To explore this possibility further, we examined the effect of a calcium ionophore (A23187) on PYK2 activation: PYK2 is phosphorylated on tyrosine residues after incubation with A23187 (Fig. 2*b*, right). Elevation of intracellular calcium in response to a variety of stimuli therefore causes tyrosine phosphorylation of PYK2. Moreover, the intrinsic protein tyrosine kinase activity of PYK2 was stimulated by carbachol or KCl, leading to strong tyrosine phosphorylation of the exogenous substrate poly(Glu–Tyr) (4:1) (Fig. 2*c*).

Role of G-protein-coupled receptors

The neuropeptide bradykinin elicits a wide range of biological responses in various cell types and tissues[17]. In PC12 cells, bradykinin activates a G-protein-coupled receptor that stimulates the production of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) by activation of phospholipase C[18]. DAG activates the Ser/Thr protein kinase C (PKC), whereas Ins(1,4,5)P_3 increases cytosolic calcium levels by triggering the release of calcium from intracellular stores[19]. Because bradykinin increases intracellular calcium concentration, we analysed its effect on the phosphorylation state of PYK2 (Fig. 2*d*, *e*). These experiments show that bradykinin induces rapid tyrosine phosphorylation of PYK2 in PC12 cells. In contrast with stimulation of PYK2 phosphorylation in response to carbachol treatment (Fig. 2*a*) or membrane depolarization (Fig. 2*b*), the effect of bradykinin was not influenced by the omission of extracellular calcium (Fig. 2*e*): bradykinin induced PYK2 phosphorylation in the absence of extracellular calcium and in the presence of EGTA. PYK2 activation therefore appears to be a direct response to elevation of cytsolic calcium itself.

Incubation of PC12 cells with phorbol myristyl acetate (PMA) induced tyrosine phosphorylation of PYK2 (Fig. 2*e*), suggesting that tyrosine phosphorylation of PYK2 could also be mediated

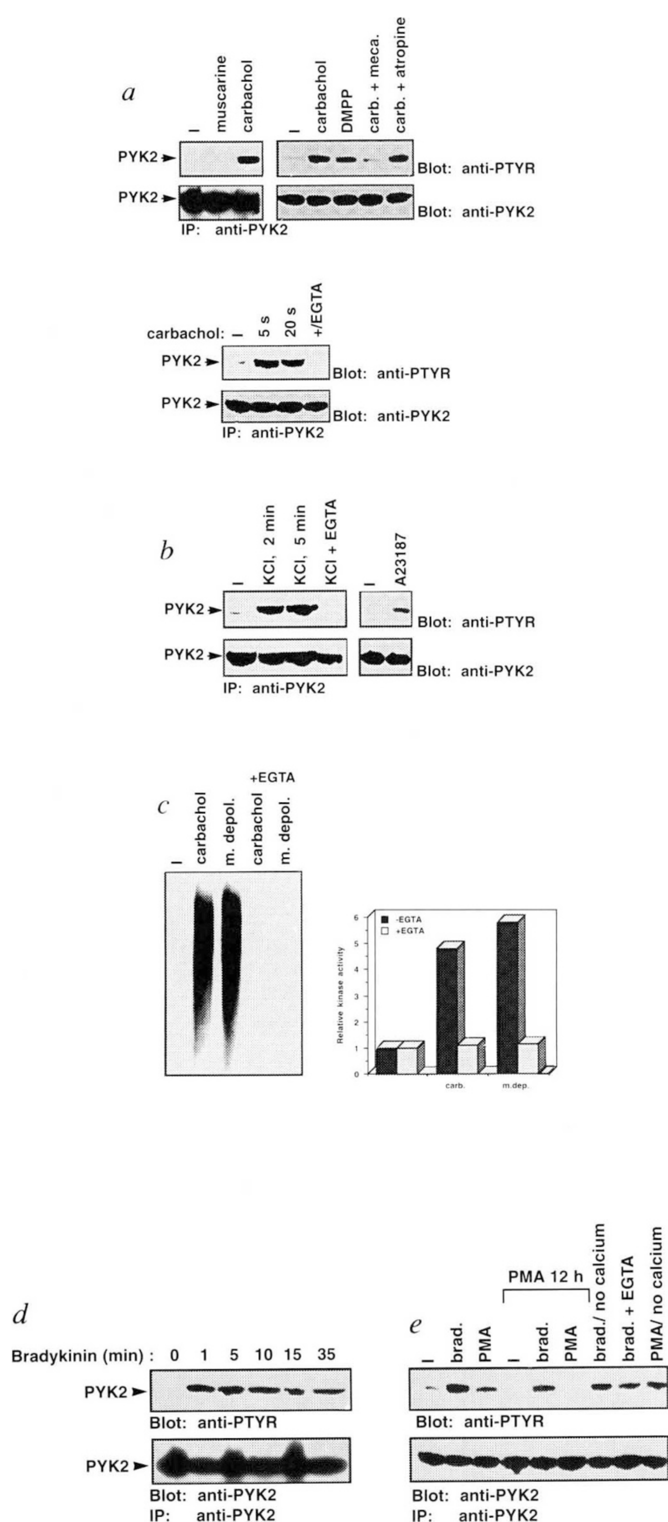

FIG. 2 Tyrosine phosphorylation of PYK2 in response to carbachol (carb.), bradykinin, membrane depolarization (m. depol.) and Ca^{2+} influx. *a*, Carbachol induces tyrosine phosphorylation of PYK2 by activation of the nicotinic acetylcholine receptor. Immunoprecipitates of PYK2 from PC12 cells that were subjected to the following treatments: muscarine (1 mM) or carbachol (carb.) (1 mM) for 20 s at 37 °C (left); carbachol (1 mM), DMPP (100 μM), or carbachol after pretreatment with the muscarinic antagonist atropine (100 nM) or the nicotinic antagonist mecamylamine (meca.) (10 μM) for 5 min at 37 °C (right). Incubation with carbachol in the presence or absence of EGTA (3 mM) as indicated (lower panels). The immunocomplexes were resolved by SDS–PAGE, transferred to nitrocellulose, and probed with either anti-phosphotyrosine antibodies or with anti-PYK2 antibodies as indicated. *b*, Membrane depolarization and calcium ionophore induce tyrosine phosphorylation of PYK2. Immunoprecipitates of PYK2 from quiescent PC12 cells that were subjected to the following treatments: incubation with 75 mM KCl in the presence or absence of EGTA (3 mM) (left), incubation with 6 μM of the calcium ionophore A23187 for 15 min at 37 °C (right). The immunoprecipitates were washed, resolved by 7.5% SDS–PAGE and immunoblotted with either anti-phosphotyrosine antibodies or with anti-PYK2 antibodies. *c*, Tyrosine phosphorylation of exogenous substrate by PYK2. PYK2 was immunoprecipitated from quiescent PC12 cells or following stimulation with carbachol or KCl in the absence or presence of EGTA. The immunoprecipitates were subjected to an *in vitro* kinase assay in the presence of [γ^{32}-P]ATP and the exogenous substrate poly(Glu-Tyr) (4:1). The phosphorylated products were resolved by SDS–PAGE, analysed by autoradiography (left) and quantified by a phosphoimager and Image Quant software (Molecular Dynamics, Inc.). The data are presented as kinase activity relative to unstimulated cells (right). *d*, Time course of bradykinin-induced tyrosine phosphorylation of PYK2. Quiescent PC12 cells were incubated at 37 °C with 1 μM bradykinin (brad.) for the indicated periods of time. PYK2 was immunoprecipitated from untreated (−) or treated cells. The immunocomplexes were washed, resolved by SDS–PAGE, transferred to nitrocellulose, and probed either with anti-phosphotyrosine or anti-PYK2 antibodies (upper and lower panels, respectively). *e*, Quiescent PC12 cells were incubated with either 1 μM bradykinin (1 min at 37 °C) or with PMA (1.6 μM, 15 min at 37 °C) in the presence or absence of $CaCl_2$ or EGTA (3 mM) as indicated. In some cases the cells were pretreated with 100 nM PMA for 12 h (indicated by the line above the lanes). PYK2 was immunoprecipitated from stimulated or unstimulated cells (−) and analysed by immunoblot analysis with either anti-phosphotyrosine or anti-PYK2 antibodies.

METHODS. Confluent PC12 cells in 150-mm plates were grown for 18 h in DMEM containing 0.5% horse serum and 0.25% fetal bovine serum. The cells were stimulated at 37 °C with different agonists as indicated, washed with cold PBS and lysed in 800 μl lysis buffer[47]. The cell lysates were subjected to immunoprecipitation with anti-PYK2 antibodies. Following SDS–PAGE and transfer to nitrocellulose, the samples were immunoblotted with either anti-phosphotyrosine (RC20, Transduction Laboratories) or anti-PYK2 antibodies. Antibodies against PYK2 were raised in rabbits immunized with GST fusion protein containing amino acids 362–647 of PYK2 or against a synthetic peptide corresponding to 15 N-terminal residues. For exogenous substrate phosphorylation, PYK2 was immunoprecipitated with anti-peptide antibodies from serum-starved PC12 cells following stimulation for 3 min with carbachol (1 mM), KCl (75 mM) in the absence or presence of EGTA (3 mM) or left unstimulated, as indicated. The immunoprecipitates were washed twice with lysis buffer, once with Tris buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MnCl_2$) and incubated with 30 μl Tris buffer containing 20 μg poly(Glu-Tyr) (4:1) and 5 μCi [γ-^{32}P]ATP (3,000 Ci $mmol^{-1}$; NEN) for 12 min at 23 °C. The reactions were stopped by SDS sample buffer and resolved on 8.5% SDS–PAGE. Chronic treatment with PMA was performed by incubation of the cells with 100 nM PMA for 12 h at 37 °C.

by PKC activation. To test this hypothesis, PMA-sensitive isozymes were down-regulated by prolonged treatment with PMA[20], and the cells were then treated with bradykinin or PMA. This prolonged treatment with PMA completely abolished the subsequent effect of PMA treatment (Fig. 2*e*), but had only a minor effect on bradykinin-stimulated tyrosine phosphorylation of PYK2. This suggests that tyrosine phosphorylation of PYK2 can be induced both by PKC-independent and PKC-dependent mechanisms, and that the effect of bradykinin is likely to be due to calcium release from intracellular stores. Chronic treatment with phorbol ester also had a minor effect on carbachol-induced activation of PYK2 (data not shown), indicating that the effect of carbachol on PYK2 activation is also mediated by calcium influx, and not by PKC isozymes sensitive to phorbol ester.

We have demonstrated that PYK2 is rapidly activated in response to extracellular stimuli that increase intracellular calcium as a result of ion channel activation and membrane depolarization. PYK2 is also activated in response to stimulation of a G-protein-coupled receptor, as well as following stimulation by phorbol ester. Although the molecular mechanisms by which

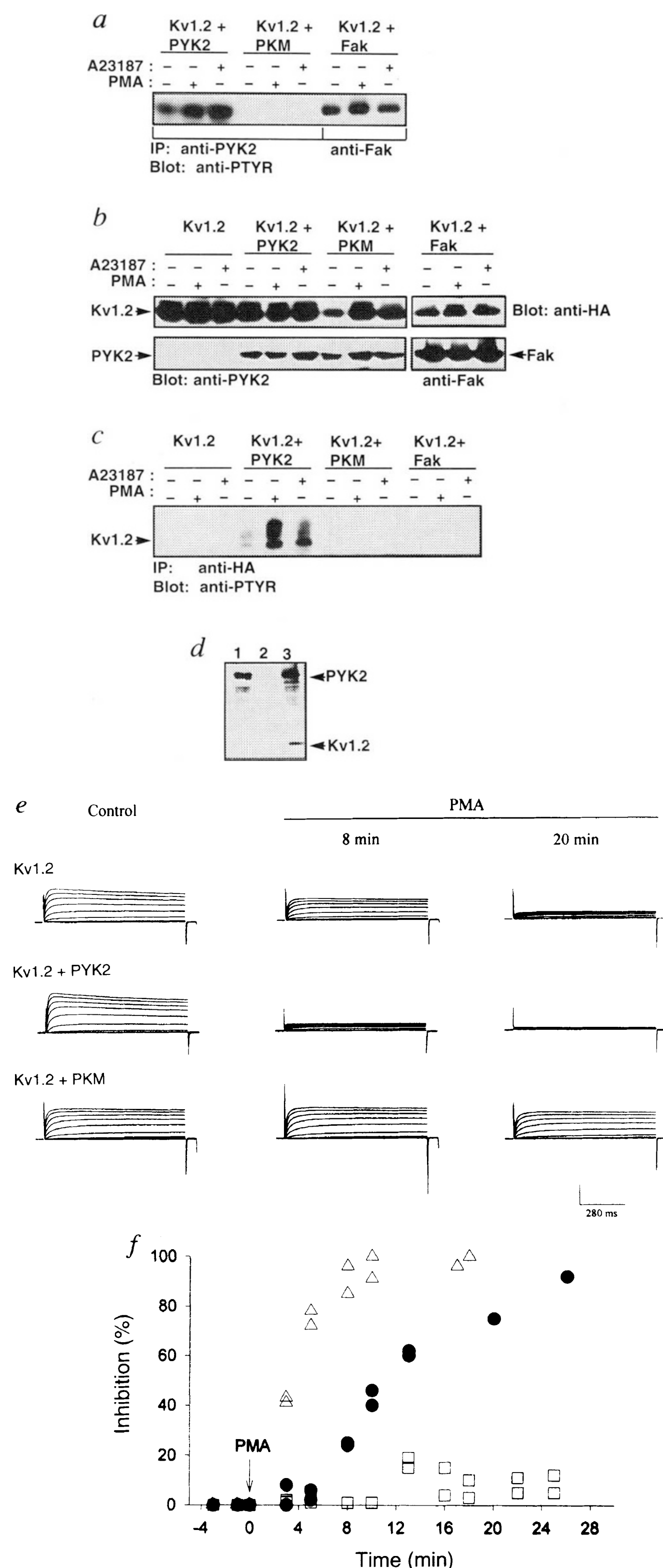

FIG. 3 Stimulation of Kv1.2 potassium channel tyrosine phosphorylation in response to PYK2 activation and suppression of Kv1.2 currents in *Xenopus* oocytes by PMA treatment and PYK2 expression. Embryonic human kidney 293 cells were transiently transfected with different combinations of mammalian expression vectors which direct the synthesis of Kv1.2-HA, PYK2, a kinase-negative PYK2 (PKM) or the protein tyrosine kinase Fak. The cells were grown for 24 h in the presence of 0.2% serum and then either stimulated with PMA (1.6 μM, 10 min at 37 °C), with the calcium ionophore A23187 (6 μM, 10 min at 37 °C) or left unstimulated (−). *a*, Tyrosine phosphorylation of each protein was analysed following immunoprecipitation and immunoblotting with anti-phosphotyrosine antibodies as indicated. *b*, The expression of each protein was determined by immunoblot analysis of total cell lysates from each transfection with anti-PYK2, anti-HA or anti-Fak antibodies. *c*, Tyrosine phosphorylation of Kv1.2 was analysed by immunoprecipitation of Kv1.2-HA protein from each cell line with anti-HA antibodies, followed by immunoblot analysis with anti-phosphotyrosine antibodies. The tyrosine-phosphorylated Kv1.2 protein is indicated by an arrow. *d*, Direct phosphorylation of Kv1.2 by PYK2. Lysates from carbachol-stimulated PC12 cells were subjected to immunoprecipitation with either anti-PYK2 antibodies (lanes 1 and 3) or with preimmune anti-serum (lane 2). The immunoprecipitates were mixed with Kv1.2 protein (lanes 2 and 3), immobilized on Sepharose beads by anti-HA antibodies and subjected to *in vitro* kinase assay in the presence of [γ-^{32}P]ATP. The immunoprecipitates were analysed by SDS–PAGE and autoradiography. The phosphorylated Kv1.2 and PYK2 proteins are indicated by arrows. *e*, Kv1.2 currents from oocytes microinjected with either Kv1.2 mRNA (top), Kv1.2 and PYK2 mRNAs (middle), or Kv1.2 and a kinase-negative mutant of PYK2 mRNAs (PKM) (bottom). Traces of Kv1.2 channels before and after bath application of 100 nM PMA at the annotated time (8 and 20 min) in the same cell are shown. The currents were elicited in response to depolarizing steps from −100 to +30 mV in 10 mV increments every 15 s from a holding potential of −110 mV. Vertical calibration of 1.5 μA (top two rows) or 1 μA (bottom row). *f*, Wild-type PYK2 accelerates and the kinase-negative PYK2 mutant (PKM) suppresses the inhibition of Kv1.2 currents by PMA. Time course of inhibition of Kv1.2 currents following the addition of 100 nM PMA at time 0 in two different oocytes injected with Kv1.2 mRNA alone (circles), oocytes expressing Kv1.2 and PYK2 (triangles) or oocytes expressing Kv1.2 and a kinase-negative mutant of PYK2 (squares). The percentage of current blocked at +30 mV is shown for the times indicated. Recordings were taken under the same conditions as above.

METHODS. 293 cells were transfected by the calcium phosphate technique as described[48]. The influenza virus haemagglutinin peptide (YPYDVPDYAS) tag was added to the C-terminal end of the Kv1.2 cDNA using the following oligonucleotide primers in the PCR; 5′-GCCAGCAG-GCCATGTCACTGG-3′ and 5′-CGGAATTCTTACGATGCGTAGTCAGGGACA-TCGTATGGGTAGACATCAGTTAACATTTTG-3′. The PCR product was digested with *Bal*I and *Eco*RI and used to substitute the corresponding fragment at the C-terminal end of the Kv1.2 cDNA. The Kv1.2-HA cDNA was subcloned into pCMP1 (ref. 49) (a derivative of pCMV-1) downstream of the CMV promoter. A kinase-negative mutant of PYK2 (PKM) was constructed by replacing Lys 475 with an Ala residue by using a site-directed mutagenesis kit (Clontech). The oligonucleotide sequence was designed to create a new *Nru*I restriction site. The nucleotide sequence of the mutant was confirmed by DNA sequencing. The oligonucleotide sequence that was used for mutagenesis is: 5′-CAATGTAGCT-G**TCGCGA**CCTGCAAGAAAGAC-3′ (*Nru*I site, bold; Lys(AAC) substituted by Ala(GCG), underlined). The full-length cDNAs of PYK2, PKM and Fak were subcloned into the mammalian expression vector pCMP1 downstream of the CMV promoter. Human 293 cells were transiently transfected with an expression vector that directs the synthesis of Kv1.2-HA protein and were subjected to immunoprecipitation with anti-HA antibodies. In parallel, PC12 cell lysates were subjected to immunoprecipitation with either anti-PYK2 or preimmune antibodies. The immunoprecipitates were washed, mixed and incubated for 25 min at room temperature with 50 μl kinase buffer[47] containing 5 μCi [γ-^{32}P]ATP (3000 Ci mmol^{-1}; NEN). The samples were washed and analysed by SDS–PAGE and autoradiography. *In vitro* capped RNA transcripts of Kv1.2, PYK2 and PKM were synthesized from linearized plasmids DNA templates using the mMESSAGE mMACHINE kit (Ambion), following the supplier's protocols. The products of the transcription reaction (cRNAs) were diluted in RNAse-free water and stored at −70 °C. Expression of the RNAs was done by injection of 50 nl of RNA into defolliculated stage-V oocytes from *Xenopus laevis*[27]. The injected oocytes were incubated for 2–3 days at 20 °C in L15 solution (1:2 dilution of Gibco's Leibovitz L15 medium in water, with 50 U ml^{-1} nystatin, 0.1 mg ml^{-1} gentamycin, 30 mM HEPES buffer, pH 7.3–7.4, filtered through a 0.45-mm membrane). For electrophysiological recording and anlaysis, ionic currents were recorded with a two-microelectrode voltage-clamp as described[27]. The currents were low-pass filtered at 3 kHz using an eight-pole Bessel filter and stored in a 80286 microcomputer using the pClamp acquisition system (Axon Instruments). The data were analysed with the clamp fit programs of the pClamp system (Axon Instruments). All recordings were performed at room temperature (20–23 °C). The recording chamber was continually perfused with recording solution. To avoid contamination of the oocyte by Ca^{2+}-activated Cl^- currents, low Cl^- recording solution was used (96 mM Na^+ glutamate, 2 mM K^+ glutamate, 0.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM HEPES buffer).

these signals induce the activation of PYK2 are not yet known, our results clearly show that elevation of intracellular calcium concentrations is crucial for PYK2 activation. We could not detect any significant effect of Ca^{2+} on the activity of isolated PYK2, indicating that Ca^{2+}-induced PYK2 activator is indirect. The effect of PMA on PYK2 activation indicates that PYK2 can also be activated by a PKC-dependent pathway.

Tyrosine phosphorylation of ion channels

That PYK2 can be activated by an ion channel, such as the nicotinic acetylcholine receptor, as well as by intracellular calcium, raised the possibility that PYK2 may in turn regulate ion channel function by tyrosine phosphorylation. Indeed, tyrosine phosphorylation can regulate the function of several ion channels in the central nervous system[21-23]. The delayed rectifier-type K^+ channel, termed Kv1.2 (also called RAK, RBK2, RCK5, HukIV and NGK1)[24], which is highly expressed in the brain and cardiac muscle[25,26], can be regulated by tyrosine phosphorylation. It has been shown that tyrosine phosphorylation of Kv1.2 is associated with suppression of Kv1.2 currents, inhibition of which has been induced by a variety of stimuli, including carbachol, bradykinin, PMA and calcium ionophore[12]: note that the same stimuli are able to induce tyrosine phosphorylation of PYK2 (Fig. 2). We therefore examined whether PYK2 can phosphorylate the Kv1.2 channel on tyrosine and thus regulate its function. To test this possibility, we expressed in 293 cells the Kv1.2 protein, Kv1.2 together with PYK2, and, as a control, Kv1.2 with a kinase-negative PYK2 mutant (PKM) or the protein tyrosine kinase Fak. The cells were grown for 24 hours in medium containing 0.2% serum and were then stimulated with PMA (1.6 μM), calcium ionophore (6 μM), or were left unstimulated. The expression level of these proteins in each cell line was determined by immunoblotting of total cell lysates with specific antibodies, as shown in Fig. 3*b*. Immunoblot analysis with anti-phosphotyrosine antibodies after immunoprecipitation of transiently expressed PYK2, PKM and Fak by specific antibodies is shown in Fig. 3*a*. PYK2 and Fak but not PKM were phosphorylated on tyrosine in unstimulated cells. Like many protein tyrosine kinases, PYK2 was found to be tyrosine phosphorylated on overexpression in 293 cells. Under these conditions both PMA and A23187 induced weak tyrosine phosphorylation of PYK2 (Fig. 3*a*). It is also possible that under these conditions the activity of PYK2 does not correlate well with phosphotyrosine content.

We next analysed the tyrosine phosphorylation of Kv1.2 channel in each cell line. We added a haemagglutinin (HA) tag to the cDNA expression construct of Kv1.2 and determined the level of Kv1.2 expression by immunoblot analysis with anti-HA antibodies. A similar amount of Kv1.2 protein was expressed in each of the transfected cell lines (Fig. 3*b*, top). Kv1.2 was immunoprecipitated from unstimulated cells, as well as from cells stimulated with PMA or calcium ionophore. The immunoprecipitates were resolved by SDS–PAGE and immunoblotted with anti-phosphotyrosine antibodies. Phosphorylation of Kv1.2 on tyrosine residues was observed only in cells coexpressing PYK2 (Fig. 3*c*). Tyrosine phosphorylation of Kv1.2 was enhanced by PMA or calcium ionophore treatments, indicating that activation of PYK2 is required for PYK2-induced tyrosine phosphorylation of the potassium channel. Moreover, immunoprecipitated PYK2 was able to tyrosine phosphorylate isolated

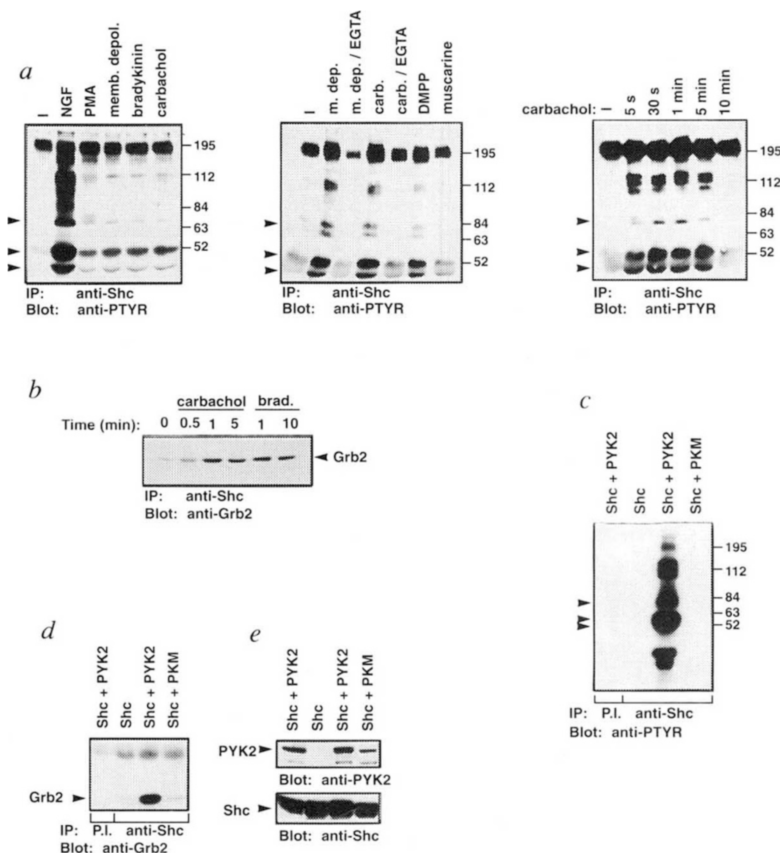

FIG. 4 Tyrosine phosphorylation of Shc and its association with Grb2 in response to activation of PC12 cells and by overexpression of PYK2. *a*, Tyrosine phosphorylation of Shc in response to bradykinin, carbachol, PMA and other stimuli. Quiescent PC12 cells were stimulated for 5 min at 37 °C with bradykinin (1 μM), carbachol (1 mM), KCl (75 mM), PMA (1.6 μM), NGF (100 ng ml^{-1}), or left unstimulated (–) (left). The cells were also stimulated with carbachol (1 mM) or potassium chloride (75 mM) in the presence of 3 mM EGTA as indicated. Stimulations with DMPP (100 μM) or muscarine (1 mM) were performed under the same conditions (middle). Time course of carbachol-induced tyrosine phosphorylation of Shc was performed by incubation of the cells with 1 mM carbachol for the indicated periods of time (right). The Shc proteins were immunoprecipitated with anti-Shc antibodies, the immunoprecipitates were resolved by SDS–PAGE (8%), transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine antibodies. *b*, Association between Grb2 and Shc in carbachol or bradykinin-stimulated PC12 cells. PC12 cells were treated with carbachol or bradykinin and then subjected to immunoprecipitation with anti-Shc followed by immunoblotting with Grb2 antibodies. *c*, Tyrosine phosphorylation of Shc in 293 cells that express Shc alone or coexpress Shc together with either PYK2, or PKM. The cells were lysed and subjected to immunoprecipitation with anti-Shc antibodies or preimmune serum (PI) as indicated. The immunocomplexes were washed, run on an SDS gel and immunoblotted with anti-phosphotyrosine antibodies. Shc proteins (of M_r 46K, 52K and 66K) are marked by arrows. *d*, PYK2 induces association of Shc with Grb2. Shc proteins were immunoprecipitated from each cell line using anti-Shc antibodies. As a control, the lysates of cells that coexpress PYK2 and Shc were subject to immunoprecipitation with preimmune serum (P.I.). The presence of Grb2 in the immunocomplexes was determined by immunoblotting with anti-Grb2 antibodies. *e*, The expression level of PYK2, PKM (top) and Shc (bottom) in each cell line was determined by immunoblot analysis of total cell lysates with specific antibodies as indicated.
METHODS. PC12 cells were grown in DMEM containing 0.25% fetal bovine serum and 0.5% horse serum for 18 h before stimulation. After stimulation, the cells were washed with cold PBS and lysed in 0.8 ml lysis buffer[47]. PYK2 was immunoprecipitated by anti-PYK2 antibodies, the immunoprecipitates were resolved by 7.5% SDS–PAGE and immunoblotted either with anti-phosphotyrosine antibodies (RC20, Transduction Laboratories, Lexington, Kentucky) or with anti-PYK2 antibodies.

Kv1.2 channel protein (Fig. 3*d*), suggesting that tyrosine phosphorylation of the channel by PYK2 is direct.

Suppression of channel currents

Because tyrosine phosphorylation of Kv1.2 is known to be associated with the suppression of Kv1.2 currents[12], and PYK2 can induce tyrosine phosphorylation of Kv1.2 (Fig. 3*c*), we investigated whether stimulation of PYK2 can suppress Kv1.2 currents. We explored the effect of PYK2 expression on currents generated by Kv1.2 expression in *Xenopus* oocytes. Stage V oocytes were microinjected either with Kv1.2 mRNA alone or with Kv1.2 together with PYK2 or PKM mRNAs. After 2–3 days of incubation at 20 °C, macroscopic currents exhibited by the oocytes were recorded with a two-microelectrode voltage clamp as previously described[27]. Large outward rectifier currents were recorded upon membrane depolarization above −40 mV, indicating that functional Kv1.2 channels are expressed in the oocytes. The expression of Kv1.2, PYK2 and PKM in the frog oocytes was confirmed by immunoblot analysis with anti-HA or anti-PYK2 antibodies (data not shown).

It has previously been shown that Kv1.2 currents in *Xenopus* oocytes can be inhibited in response to PMA treatment or elevation of intracellular calcium concentration[12]. This suppression is mediated by an unknown endogenous protein tyrosine kinase(s) that can phosphorylate the Kv1.2 channels[12]. Because PYK2 can be activated by PMA, we examined the effect of PYK2 expression on Kv1.2 currents in oocytes in the absence or presence of PMA. We also examined the effect of the kinase-negative mutant PKM on PMA-induced suppression of Kv1.2 currents mediated by the endogenous protein tyrosine kinase. PMA treatment of oocytes that were injected with Kv1.2 mRNA were found to cause inhibition of Kv1.2 currents (Fig. 3*e*). As previously shown, the inhibition of the currents developed gradually after application of PMA, reaching 80–90% inhibition after approximately 20 min incubation[12]. Moreover, the rate of channel blockade was found to be dependent on the concentration of PMA applied (data not shown). Coexpression of PYK2 resulted in an acceleration of Kv1.2 current inhibition (Fig. 3*e,f*), which was observed at every concentration of PMA tested. For example, 8 min after the addition of 100 nM PMA, 25% inhibition of outward current was observed in oocytes expressing Kv1.2 alone, as compared with 85% inhibition observed in oocytes coexpressing Kv1.2 and PYK2 (Fig. 3*e,f*). Current inhibition by PMA treatment in the absence or presence of PYK2 expression did not result in changes in both the kinetics or voltage dependence of the remaining currents (Fig. 3*e*). Interestingly, coexpression of Kv1.2 and PKM led to nearly complete inhibition of PMA-induced potassium channel blockage (Fig. 3*e,f*). It is possible that the endogenous protein tyrosine kinase activated by PMA responsible for suppression of Kv1.2 currents in oocytes represents the *Xenopus* homologue of PYK2 or a closely related protein tyrosine kinase that can be affected by a dominant interfering mutant of PYK2.

Recruitment of Shc/Grb2/Sos complexes

The results presented so far suggest that PYK2 activation provides an element that translates increases in intracellular calcium induced by neuronal activity into functional changes. It has been shown that calcium influx by voltage-sensitive calcium channels leads to MAP kinase activation in PC12 cells[28]. We therefore analysed the role of PYK2 in MAP kinase activation.

The stimuli that induce the activation of PYK2 also induce tyrosine phosphorylation of the adaptor protein Shc. Treatment of PC12 cells with bradykinin, carbachol, 75 mM potassium

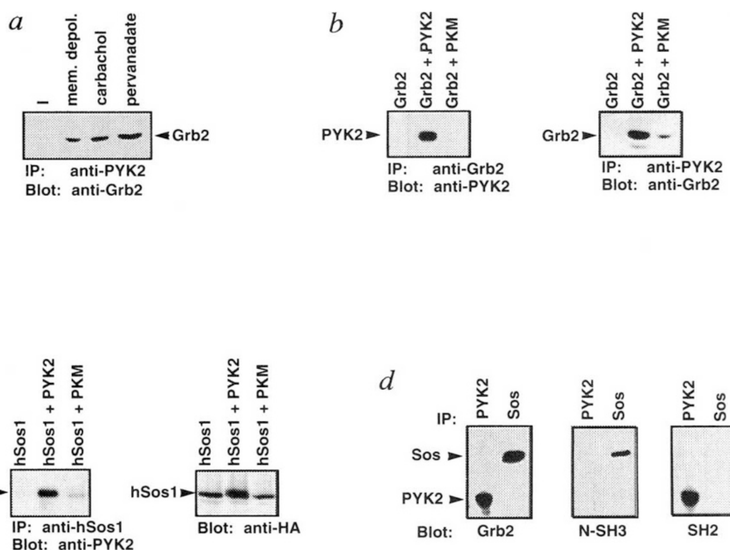

FIG. 5 Association of PYK2 with Grb 2 and Sos1 in intact cells and *in vitro*. *a*, Association between Grb2 and PYK2 in stimulated PC12 cells. PC12 cells were stimulated with KCl, carbachol and pervanadate and subjected to immunoprecipitation with anti-PYK2 antibodies followed by immunoblotting with anti-Grb2 antibodies. *b*, Embryonic human kidney 293 cells were transiently transfected with different combinations of mammalian expression vectors that direct the synthesis of Grb2, PYK2 and a kinase-negative PYK2 mutant (PKM). The cells were made soluble and immunoprecipitated with anti-Grb2 (left) or anti-PYK2 antibodies (right). The immunocomplexes were washed, resolved by SDS–PAGE, transferred to nitrocellulose, and immunoblotted with either anti-PYK2 (left) or anti-Grb2 antibodies (right). *c*, Embryonic human kidney 293 cells were transiently transfected with mammalian expression vectors encoding hSos1-HA, hSos1-HA together with PYK2, or hSos1-HA together with PKM. hSos1 was immunoprecipitated with anti-hSos1 antibodies from each cell line, and the presence of PYK2 in the immunocomplexes was determined by immunoblotting with anti-PYK2 antibodies (left). Expression levels of hSos1 (right), was determined by immunoblot analysis of total cell lysates with anti-HA antibodies. *d*, Direct binding of Grb2 SH2 domain to PYK2. Human embryonic kidney 293 cells were transfected with expression vectors that direct the synthesis of PYK2 or hSos1. These proteins were immunoprecipitated as indicated, resolved by SDS–PAGE and transferred to nitrocellulose filters. The filters were incubated with GST-fusion proteins of Grb2, Grb–SH2 domain as well as Grb2–N–SH3 and C–SH3 domains. The binding of the fusion proteins to the filters was detected by anti-GST antibodies followed by protein A conjugated to horseradish peroxidase. The binding of Grb2, Grb–SH2 domain and Grb2–N–SH3 domain to PYK2 and Sos1 are shown. C–SH3 domain does not bind to PYK2 or to Sos1.

METHODS. Quiescent PC12 cells were stimulated with carbachol (1 μM), KCl (75 mM) or pervanadate (100 μm) for 4 min at 37 °C or left unstimulated. PYK2 was immunoprecipitated with a mixture of anti-PYK2 and anti-peptide antibodies (see Fig. 2) and the presence of Grb2 in the immunocomplex was indicated by immunoblotting with anti-Grb2 antibodies (Transduction Laboratories). 293 cells were transiently transfected with the full-length cDNAs of PYK2, PKM Grb2 and hSos1-HA cloned into the mammalian expression vector pCMP1 downstream of the CMV promoter, using the calcium phosphate precipitation method[48]. Full details about the antibodies and protocols used for the immunoprecipitation of Grb2 and Sos have been described previously[50]. A mammalian expression vector that encodes the hSos1-HA was constructed as described[50]. The recombinant fusion proteins GST–Grb2, GST–SH2, GST–N–SH3 and GST–C–SH3 (5 μg ml^{-1}) were incubated with PYK2 or Sos1 immobilized on nitrocellulose filters for 1 h at room temperature. Following extensive washing, filters were incubated with rabbit polycloned antibodies against GST and probed with protein A conjugated to horseradish peroxidase.

chloride (to induce membrane depolarization), PMA and, as a control, nerve growth factor (NGF) resulted in tyrosine phosphorylation of Shc as well as several associated proteins (Fig. 4*a*). Tyrosine phosphorylation of Shc in response to membrane depolarization and carbachol treatment was dependent on the presence of extracellular calcium (Fig. 4*a*, middle), indicating that calcium influx is involved in the regulation of Shc phosphorylation by these stimuli. Tyrosine phosphorylation of Shc in response to carbachol treatment is induced by means of stimulation of the nicotinic acetylcholine receptor, as determined by pharmacological analysis (Fig. 4*a*, middle panel). The effect of carbachol on tyrosine phosphorylation of Shc was transient with maximum tyrosine phosphorylation detected after one minute, followed by a rapid decline (Fig. 4*a*, right). However, NGF induced persistent stimulation of Shc phosphorylation for at least 5 hours after factor addition[29]. The duration of Shc phosphorylation may have an important impact on the Ras signalling pathway and gene expression induced by these stimuli[30,31].

The activated EGF receptor can recruit Grb2 directly, and also indirectly by tyrosine phosphorylation of Shc[32–34]. We have therefore investigated whether PYK2 can induce tyrosine phosphorylation of Shc, and explored its association with Grb2 in PC12 cells stimulated with carbachol or bradykinin. These treatments induced complex formation between Grb2 and Shc only in stimulated PC12 cells (Fig. 4*b*). Similar results were obtained in 293 cells expressing PYK2 (Fig. 4). In this experiment, Shc proteins were immunoprecipitated with anti-Shc antibodies from Shc, from Shc and PYK2, or from Shc and PKM-expressing cells. The samples were resolved by SDS–PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine (Fig. 4*c*) or anti-Grb2 antibodies (Fig. 4*d*). This experiment reveals dramatic tyrosine phosphorylation of Shc in cells that overexpress PYK2. Moreover, several phosphotyrosine-containing proteins were found in Shc immunoprecipitates from cells overexpressing PYK2. Similar results were also obtained in cells expressing endogenous Shc proteins that were transfected with PYK2 cDNA and subjected to immunoprecipitation analysis with anti-Shc antibodies (data not shown). Immunoblot analysis with Grb2 antibodies of Shc immunoprecipitates (Fig. 4*d*) indicated that Grb2 associates with tyrosine-phosphorylated Shc in cells overexpressing PYK2. However, direct association between PYK2 and Shc was not detected.

Shc was shown to be involved in the coupling of both receptor and non-receptor tyrosine kinases to the Ras/MAPK signalling pathway[33,35]. Tyrosine-phosphorylated Shc can activate the Ras signalling pathway by binding to the SH2 domain of the adaptor protein Grb2 that is complexed to the guanine-nucleotide-releasing factor Sos by means of its SH3 domains[32,33]. The MAP kinase signalling pathway in PC12 cells can be activated by NGF[36], by peptide hormones that activate G-protein-coupled receptors[37], by phorbol ester[38], and by calcium influx following membrane depolarization[28]. However,the mechanism underlying activation of the Ras/MAP kinase signalling pathway by G-protein-coupled receptors and by calcium influx are not known. To explore the possibility that calcium-induced PYK2 activation is responsible for activation of the Ras/MAPK signalling pathway, we examined the interaction between the adaptor protein Grb2 with PYK2 in PC12 cells stimulated by carbachol or KCl. Carbachol or KCl treatments led to complex formation between Grb2 and PYK2 in stimulated PC12 cells (Fig. 5*a*). Similar results were obtained in 293 cells transfected with different combinations of expression vectors that direct the synthesis of PYK2, PKM and the adaptor protein Grb2. Grb2 is directly associated with wild-type PYK2 but not with PKM (Fig. 5*b*). Experiments with glutathione *S*-transferase (GST) fusion proteins indicate that the association is mediated by the SH2 domain of Grb2 (Fig. 5*d*). Inspection of the PYK2 primary structure shows that Tyr 881 is followed by an LNV sequence that was shown to be a canonical binding site for the SH2 domain of Grb2 (ref. 33).

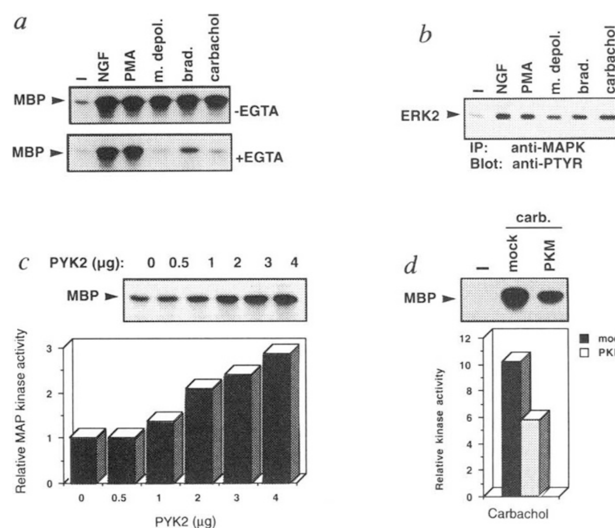

FIG. 6 Activation of MAP kinase in PC12 cells by different agonists. Quiescent PC12 cells were stimulated for 5 min at 37 °C with bradykinin (1 μM), carbachol (1 mM), KCl (75 mM), PMA (1.6 μM), NGF (100 ng ml^{-1}) or left unstimulated (−) in the absence or presence of EGTA (3 mM). The cells were lysed and ERK1,2 were immunoprecipitated with specific antibodies. The immunocomplexes were washed and subjected to a standard MBP phosphorylation assay (*a*) or resolved by SDS–PAGE, transferred to nitrocellulose filters and immunoblotted with anti-phosphotyrosine antibodies (*b*). *c*, Activation of MAP kinase by overexpression of PYK2. Human embryonic kidney 293 cells were transfected with increasing concentrations of a mammalian expression vector that directs the synthesis of PYK2. ERK2 protein was immunoprecipitated from each cell line, and the immunocomplexes were washed and subjected to MBP phosphorylation assay (top). Quantification of MAP kinase activity for each cell line was determined by phosphorimager and Image Quant software (Molecular Dynamics). MAP kinase activity in transfected cells is compared to activity detected in control mock-transfected cells (bottom). *d*, Suppression of carbachol-induced MAP kinase activation by overexpression of kinase-negative mutant PKM in PC12 cells. Stimulation of PC12 with carbachol, immunoprecipitation and MBP phosphorylation are described in *a*. MBP phosphorylation (top); quantification of MAP kinase activity (bottom).
METHODS. PC12 cells were starved for 18 h as described. The cells were stimulated for 5 min at 37 °C with the indicated stimuli, lysed and subjected to immunoprecipitation with anti-ERK 1 or 2 antibodies as indicated (Santa Cruz Biotechnology, SC-93 or SC 154). The immunoprecipitates were washed twice with lysis buffer[47], and once with Tris buffer containing 10 mM Tris-HCl, pH 7.2, 100 mM NaCl, 1 mM Na-vanadate and 5-mM benzamidine. The immunocomplexes were resuspended in 40 μl MAP kinase buffer containing 30 mM Tris-HCl, pH 8, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 15 μg MBP, 10 μM ATP and 5 μCi [γ-^{32}P]ATP (Amersham). The samples were incubated for 3 min at 30 °C and the reactions were stopped by the addition of SDS-sample buffer. The samples were resolved on 15% SDS–PAGE and analysed by autoradiography. Human embryonic kidney 293 cells were transiently transfected with increasing concentrations of pCMP1-PYK2 DNA (0.5–4 μg). Then, 12 h after transfection, the cells were grown in medium containing 0.2% serum for 24 h. The cells were lysed, immunoprecipitated with ERK2 antibodies and subjected to MBP phosphorylation assay as described above. PC12 cells in 65-mm plates were transiently transfected with 10 μg DNA of pCMP1 or pCMP1-pKM using lipofectamine reagents (GIBCO-BRL). The cells were starved for 24 h, stimulated with 1 mM carbachol for 5 min and subjected to MAP kinase activity assay as described.

This site is equivalent to Tyr 925 of Fak, a known Grb2 binding site[39].

We next examined the interaction of PYK2 with the guanine nucleotide releasing factor Sos1. Human embryonic kidney 293 cells were transfected with expression vectors encoding Sos1 together with either PYK2 or PKM, and subjected to immunoprecipitation/immunoblotting analysis with anti-Sos1 or anti-

PYK2 antibodies, respectively. Wild-type PYK2, but not the kinase-negative mutant PKM, was co-immunoprecipitated with the Sos1 protein. Hence Grb2 is bound to Sos1 by means of its SH3 domains, and to PYK2 via its SH2 domain (Fig. 5*d*), leading to the recruitment of Sos by tyrosine-phosphorylated PYK2. Growth-factor-induced activation of receptor tyrosine kinases leads to a shift in the electrophoretic mobility of Sos protein. The mobility shift is due to phosphorylation by serine and threonine kinases that are dependent on Ras activation, including the MAP kinase[33,40]. Sos1 protein from PYK2 transfected cells exhibits reduced electrophoretic mobility compared to Sos1 protein from PKM-expressing cells (Fig. 5*c*, right). This shows that PYK2 overexpression leads to the activation of Ser/Thr kinases that are responsible for the phosphorylation of Sos1. We therefore concluded that tyrosine-phosphorylated PYK2 can directly and indirectly recruit Grb2 by means of tyrosine phosphorylation of Shc, revealing at least two alternative routes for PYK2-induced activation of the Ras signalling pathway.

We next examined the ability of these stimuli to induce the activation of MAP kinase in PC12 cells. Quiescent PC12 cells were stimulated with different agonists in the absence or presence of EGTA (Fig. 6*a*). Lysates from stimulated cells were immunoprecipitated with anti-MAP kinase (ERK1,2) antibodies, and MAP kinase activity was analysed by using myelin basic protein (MBP) as an exogenous substrate (Fig. 6*a*). In parallel, ERK2 immunoprecipitates were transferred to nitrocellulose filters and probed with phosphotyrosine antibodies (Fig. 6*b*). This demonstrated that the addition of various agonists to PC12 cells induced a similar profile of tyrosine phosphorylation and MAP kinase activation. It is clear that MAP kinase activation is induced by both calcium-influx-dependent and independent mechanisms.

Because activation of MAP kinase was observed in response to stimuli that induce PYK2 phosphorylation, we examined the possibility that PYK2 overexpression could induce MAP kinase activation. Human embryonic kidney 293 cells were transiently transfected with increasing concentrations of mammalian expression vector that directs the synthesis of PYK2. The cells were grown for 24 hours in the presence of 0.2% serum, and ERK2 proteins were immunoprecipitated, washed and subjected to MBP phosphorylation assay. PYK2 overexpression induced MBP phosphorylation in a concentration-dependent manner (Fig. 6*c*). However, overexpression of the kinase negative mutant PKM suppressed carbachol-induced activation of MAP kinase (Fig. 6*d*), probably by dominant negative suppression of agonist-induced activation of endogenous PYK2 in PC12 cells.

Discussion

We have discovered a protein tyrosine kinase termed PYK2 that is highly expressed in the adult rat brain. PYK2 is a second member of the Fak family of non-receptor protein tyrosine kinases. We present data demonstrating that PYK2 is activated in response to bradykinin, a neuropeptide hormone that mediates its biological effects by activation of a G-protein-coupled receptor and by stimulation of phosphatidylinositol hydrolysis. PYK2 is also tyrosine-phosphorylated in response to activation of the nicotinic acetylcholine receptor, as well as by membrane depolarization and calcium-ionophore treatment. All of these stimuli result in enhanced levels of cytosolic calcium influx. PYK2 is thus activated by extracellular signals that lead to calcium influx or calcium release from internal stores. Moreover, PYK2 appears to be regulated by both PKC-dependent and independent mechanisms. Our experiments show that PYK2 may also link G-protein-coupled receptors and calcium influx to the MAP kinase signalling pathway, which relays signals from the cell surface to regulate transcriptional events in the nucleus. Moreover, the effects of PYK2 on tyrosine phosphorylation and action of the Kv1.2 potassium channel provide a mechanism for heterologous regulation of ion channel function by activation of an intermediate protein tyrosine kinase. PYK2 can, therefore, link neuropeptide hormones that act by means of G-protein-coupled receptors, stimulating phosphatidylinositol hydrolysis with the action of target channel molecules. Similarly, PYK2 may modulate the action of ion channels, such as voltage-gated calcium channels, that mediate their responses by, and are sensitive to, intracellular calcium concentration. PYK2 may therefore provide an autoregulatory role for the same channel that is responsible for PYK2 activation. One potential target of PYK2 is the nicotinic acetylcholine receptor.

Calcium concentration inside cells is highly localized because of a variety of calcium-binding proteins that provide a strong buffer[41]. Moreover, in excitable cells the level of calcium can be regulated by voltage-dependent calcium channels that induce a large and transient increase in intracellular calcium concentration, leading to calcium oscillations and calcium waves[41]. Because PYK2 activity is regulated by intracellular calcium level, both the temporal and spatial pattern of PYK2 activation may replicate the spatial and temporal profile of intracellular calcium concentration. An intriguing possibility, therefore, is that PYK2 and other calcium-dependent tyrosine kinases[42] may provide a mechanism for rapid and highly localized control of ion channel function, as well as localized activation of the MAP kinase signalling pathway. The effect of PMA on PYK2 activation also suggests a tight association between PKC and PYK2 activation.

Potassium channels are frequent targets for phosphorylation by protein kinases that are activated by neurotransmitters or neuropeptides[43]. Phosphorylation of these and other voltage-gated channels or neurotransmitter receptors provides an important regulatory mechanism for modulation of neuronal activity[5]. *In situ* hybridization and preliminary immunolocalization analyses indicate that PYK2 is expressed in hippocampal pyramidal cell dendrites (unpublished results), suggesting a role for this kinase in synaptic plasticity mediated by calcium influx after postsynaptic depolarizaton. In summary, PYK2 may represent an important signalling imtermediate molecule between neuropeptides that activate G-protein-coupled receptors, or neurotransmitters that increase Ca^{2+} influx, and downstream signalling events that regulate neuronal activity. Moreover, our results suggest a mechanism for the coupling between various signals that elevate intracellular calcium and the MAP kinase signalling pathway: activation of MAP kinase leads to translocation of the enzyme to the nucleus to phosphorylate TCF/Elk-1, resulting in induction of c-*fos* transcription[44] and activation of AP-1 transcriptional factor[45]. PYK2 may also be involved in calcium-mediated regulation of gene expression in neuronal cells induced by NMDA receptor or voltage-sensitive calcium channels[46]. PYK2 therefore may control a broad array of processes in the central nervous system, including short- and long-term neuronal plasticity. □

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Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1

Jonathan Goldberg[*], Hsien-bin Huang[†], Young-guen Kwon[†], Paul Greengard[†], Angus C. Nairn[†‡] & John Kuriyan[*‡]

[*] Howard Hughes Medical Institute, [†] The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA

The crystal structure of mammalian protein phosphatase-1, complexed with the toxin microcystin and determined at 2.1 Å resolution, reveals that it is a metalloenzyme unrelated in architecture to the tyrosine phosphatases. Two metal ions are positioned by a central β–α–β–α–β scaffold at the active site, from which emanate three surface grooves that are potential binding sites for substrates and inhibitors. The carboxy terminus is positioned at the end of one of the grooves such that regulatory sequences following the domain might modulate function. The fold of the catalytic domain is expected to be closely preserved in protein phosphatases 2A and 2B (calcineurin).

PROTEIN phosphatases were first discovered about fifty years ago as a cellular activity that converts glycogen phosphorylase from the active *a* form to the inactive *b* form (for a review, see ref. 1). The critical role that reversible phosphorylation is now known to play in cellular signal transduction places the protein phosphatases in a position of central importance because of their ability to reverse the action of protein kinases. Phosphatases that act on phosphorylated serine and threonine residues are present in all eukaryotic cell types and participate in the control of a wide range of cellular processes, including cell-cycle progression, cell proliferation, protein synthesis, transcriptional regulation and neurotransmission[2,3]. In contrast to the protein kinases, for which several three-dimensional structures have been elucidated, the serine/threonine phosphatases have as yet eluded crystallographic analysis and are less well understood in terms of structure and mechanism.

The serine/threonine phosphatases are unrelated in sequence to the protein tyrosine phosphatases, and have been classified into four groups (1, 2A, 2B, 2C) on the basis of the differences in their biochemical properties[4]. Protein phosphatases (PP) 1, 2A and 2B (also known as calcineurin) have highly homologous catalytic domains (Fig. 1), but differ in their substrate specificities and interactions with regulatory molecules. PP-2C is unrelated to these enzymes, and we shall here use the term Ser/Thr phosphatase to refer specifically to relatives of PP-1. Included with the Ser/Thr phosphatases is an enzyme encoded by bacteriophage λ (λ phosphatase), which is homologous in sequence to the N-terminal half of PP-1 (Fig. 1) and has been a useful model for mechanistic studies[5].

Ser/Thr phosphatases are subject to complex regulation. Proteins known as 'targeting subunits' form heterodimers with the catalytic subunit of PP-1, resulting in its specific localization and modulation of its inhibition by other factors[6]. The heat-stable inhibitors, inhibitor-1 and its homologue DARPP-32 (dopamine- and cyclic AMP-regulated phosphoprotein of apparent M_r 32K), when phosphorylated by cAMP-dependent protein kinase, bind to PP-1 and inhibit it. DARPP-32 is highly enriched in certain types of neurons, where its phosphorylation links the dopamine activation of receptors to the control of Na^+,K^+-ATPase and the membrane potential[7]. PP-1 is also inhibited by inhibitor-2 (ref. 2) and by phosphorylation in a C-terminal region by a cyclin-dependent protein kinase[8,9]. Calcineurin is a heterodimer formed by a catalytic A subunit and a calmodulin-like B subunit[10], and is activated by the binding of Ca^{2+}-calmodulin to a regulatory region that follows the catalytic domain[11].

Certain natural toxins of agricultural and medical importance are potent inhibitors of the Ser/Thr phosphatases[12]. PP-1 and PP-2A are inhibited by the membrane-permeable polyether okadaic acid and by cyclic heptapeptides known as microcystins. Both these toxins are powerful inducers of tumours[13,14], which suggests a role for PP-1 and PP-2A in growth suppression. The immunosuppressants cyclosporin and FK506 inhibit calcineurin when bound to the proteins cyclophilin and FKBP, respectively, and the discovery of this phenomenon has revealed that calcineurin has a key role in T-cell signalling[15].

‡ To whom correspondence should be addressed.