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Depolarization-Induced Tyrosine Phosphorylation of p130^{cas}

Shin Kobayashi, Nobuaki Okumura,[1] Masato Okada, and Katsuya Nagai

Division of Protein Metabolism, Institute for Protein Research, Osaka University, 3-2 Yamada-oka, Suita, Osaka 565

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KCl-treatment of PC12 cells induces depolarization of the plasma membrane and Ca^{2+} influx into the cells. We have previously shown that KCl induced tyrosine phosphorylation of cellular proteins of 120, 110, 68, 44, and 42 k, and that the 68 k protein was paxillin. In the present study, we found that the 120 k protein was a Crk-associated Src substrate, p130^{cas}. KCl-induced tyrosine phosphorylation of p130^{cas} was not observed in EGTA-containing medium, suggesting that it was due to Ca^{2+} influx into the cells. Time course experiments showed that tyrosine phosphorylation of p130^{cas} peaked at 5 min after stimulation and returned to the basal level at 60 min, while mobility shift of p130^{cas} was observed within 2 min and lasted over 60 min, indicating that serine or threonine residues, in addition to tyrosine, were phosphorylated on KCl stimulation. *In vitro* kinase assay of immunoprecipitates with anti-p130^{cas} antibody suggested that some protein-tyrosine kinases were associated with p130^{cas}. Using the substrate region of p130^{cas} as the substrate, we found that Fyn and Src were activated on stimulation with KCl. These results indicate that tyrosine phosphorylation of p130^{cas} may be involved in Ca^{2+}-dependent events in neuronal and neuroendocrine cells.

Key words: calcium, PC12, p130^{cas}, Src-family kinases, tyrosine phosphorylation.

Protein tyrosine phosphorylation is involved in a variety of signal transduction systems that modulate cellular functions. In the nervous system, tyrosine kinases have been implicated in neuronal differentiation, axon guidance and synaptic activities. Many growth factor receptors for neuronal cells have tyrosine kinase activity whose activation triggers a signal transduction cascade and results in gene expression of proteins related to neuronal growth and differentiation (*1, 2*).

Intracellular Ca^{2+} is another factor essential for neuronal functions. It is elevated on depolarization of neuronal cells and triggers the release of neurotransmitters from the synaptic membranes. Ca^{2+} influx into neuronal cells is also induced by neurotransmitters, such as glutamate, that stimulate ligand-gated calcium channels (*3-5*), and is suggested to be involved in establishment of the long-term change in synaptic activity and ischemia-induced neuronal cell death.

In adrenal chromaffin cells, depolarization is induced by acetylcholin released from synaptic terminals of the sympathetic nerves, resulting in release of adrenaline into blood (*6*). PC12 cells were originally established from a pheochromocytoma, and depolarization could be induced by treatment with muscarinic agonists or high concentrations of K^+, leading to an increase in the intracellular Ca^{2+} level and secretion of catecholamines, as in the case of normal chromaffin cells (*7*).

PC12 cells have been widely used to study the signal transduction mechanisms of neurotrophic factors, because differentiation to neuron-like cells is induced by nerve growth factor (NGF) and fibroblast growth factor (FGF), while proliferation is induced by epidermal growth factor (EGF). These factors stimulate receptor-tyrosine kinases to trigger intracellular signal transduction pathways. The Ras/MAPK cascade is known to be stimulated by a Ca^{2+}-dependent pathway (*8*). In addition, we have previously reported that depolarization of the cells with KCl induced tyrosine phosphorylation of several proteins including paxillin, a substrate for Src-family kinases localized in the actin-cytoskeleton (*9, 10*). It was also reported that CAKβ/PYK2/RAFTK, a recently identified protein tyrosine kinase (*11-13*), is tyrosine phosphorylated and activated in response to various stimuli that elevate the intracellular Ca^{2+} concentration (*12*). These findings indicate that tyrosine phosphorylation of proteins may be involved in depolarization-dependent events in neuronal cells.

p130^{cas}, which was first identified as a Crk-associated Src substrate (*14*), is a docking protein binding to focal adhesion kinase (FAK), CAKβ/PYK2/RAFTK, and c-Src (*15-19*). In fibroblastic cells, it is recruited to focal contacts and tyrosine phosphorylated depending on integrin-signaling (*20, 21*). In PC12 cells, tyrosine phosphorylation of p130^{cas} is stimulated by growth factor stimulation (*22*) and an increase of cell density (*23*). Here we show that KCl also induces tyrosine-phosphorylation of p130^{cas} in PC12 cells and suggest that, besides its role in cell attachment, p130^{cas} may play an important role in the Ca^{2+}-dependent signal transduction pathway.

[1] To whom correspondence should be addressed. Tel: +81-6-879-8632, Fax: +81-6-879-8633

Abbreviations: SH, Src homology domain; FAK, focal adhesion kinase; BAP, bacterial alkaline phosphatase.

MATERIALS AND METHODS

Materials—PC12 cells were provided by JCRRB Cell Bank (Japan). Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG and goat anti-rabbit IgG were from Zymed. [γ-^{32}P]ATP and Protein G-Sepharose were from Amersham. Anti-phosphotyrosine (clone 4G10) antibody was from UBI, anti-v-Src antibody (clone 327) was from Oncogene Science, anti-Fyn was from Wako, and anti-phosphotyrosine (clone PY20), anti-Csk, anti-Crk, and anti-FAK (clone 2A7) antibodies were from Transduction Laboratories. Anti-Cas antibody was prepared by immunizing a rabbit with GST-Cas2 fusion protein (*14*).

Cell Culture and Immunoprecipitation—PC12 cells were maintained as described previously (*24*). Cells were stimulated by NGF (50 ng/ml), EGF (10 ng/ml), and KCl (75 mM) for the indicated times, then lysed in 0.5 ml of TNE buffer [10 mM Tris-HCl (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 μM sodium molybdate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. For immunoprecipitation, the lysates were incubated at 4°C with primary antibody for 1 h, then with Protein G-Sepharose for 1 h. The immunecomplexes were washed three times with TNE buffer, resuspended in 20 μl of TNE buffer and 20 μl of 2× SDS-PAGE sample buffer, then boiled for 3 min. Proteins were determined by the method of Bradford (*25*) with bovine serum albumin as a standard.

SDS-PAGE and Immunoblotting—Proteins were separated by SDS-PAGE on an 8% polyacrylamide gel by the method of Laemmli, then electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in Tris-buffered saline containing 0.1% Tween 20 (T-TBS), then incubated with primary antibody for 1 h. After washing with T-TBS, the membrane was incubated with HRP-conjugated rabbit anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG for 1 h. Blots were developed with the Renaissance chemiluminescence Western blotting detection kit (NEN).

In Vitro Kinase Assay—Cells were lysed in RIPA buffer [TNE buffer containing 0.5% sodium deoxycholate and 0.1% SDS] and immunoprecipitated with appropriate antibodies. The immunecomplexes were washed three times with TNE buffer and twice with kinase buffer [10 mM PIPES (pH 7.0), 10 mM $MnCl_2$]. *In vitro* kinase reactions were carried out in 40 μl of kinase buffer containing 10 μM [γ-^{32}P]ATP (10 μCi) at 30°C for 30 min. If necessary, 1 μg of GST-Cas substrate region (201-555 a.a.) was added to the assay mixture. The reaction was stopped by the addition of 40 μl of 2× sample buffer. Then the samples were boiled and separated on an 8% gel. The gel was dried and exposed to an X-ray film for 1 h.

In Vitro Dephosphorylation of p130^{cas}—For phosphatase treatment of proteins, immunoprecipitates were washed twice with TNE buffer and twice with alkaline phosphatase (AP) buffer [10 mM Hepes (pH 8.0), 1 mM $MgCl_2$, and 1 mM $ZnCl_2$]. Proteins were then treated in 40 μl of AP buffer with 4 U bacterial alkaline phosphatase (TAKARA) for 1 h at 37°C. After the reaction, 40 μl of 2× SDS-sample buffer was added to each sample, which was then boiled and analyzed by Western blotting.

RESULTS

In PC12 cells, KCl stimulation induced tyrosine phosphorylation of intracellular proteins of 120, 68, 44, and 42 k (Fig. 1A). We have previously shown that the 68 k phosphoprotein was paxillin, a possible Src-family substrate associated with the actin cytoskeleton (*10*). Since p130^{cas} is, like paxillin, a cytoskeletal component and is tyrosine phosphorylated by Src-family kinases, we investigated whether the 120 k tyrosine-phosphorylated protein was p130^{cas}. As shown in Fig. 1, A and B, the tyrosine phosphorylation level of p130^{cas} was increased by stimulation with KCl, NGF, or EGF for 5 min. The effect of KCl was as strong as that of NGF. In contrast, the effects of KCl on tyrosine phosphorylation of 42 and 44 k proteins, which were probably MAP kinases, were weaker than those of NGF and EGF. NGF and EGF phosphorylated 52 k protein, which was probably SHC, but KCl did not. The strong bands of about 55 k detected in immunoprecipitated materials are signals of the IgG used for immunoprecipitation that reacted with the secondary antibody for Western blotting.

The time-course of tyrosine phosphorylation of cellular proteins induced by KCl is shown in Fig. 2A. Tyrosine phosphorylation of the 120 k protein was enhanced within 2 min after stimulation, peaked at 5 min, and returned to the basal level in 30 min. The proteins of about 68 k, which seemed to be different phosphorylated forms of paxillin, were phosphorylated with a similar time course to p130^{cas}, but were dephosphorylated more slowly. Phosphorylation of MAP kinases was much slower than that of these two

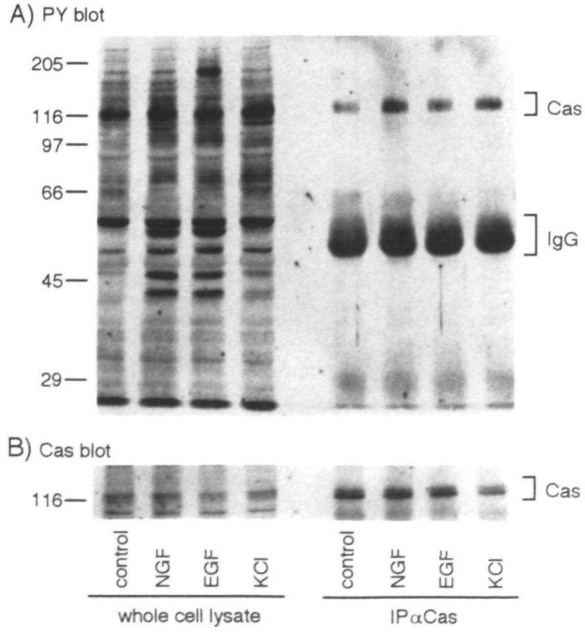

Fig. 1. **p130^{cas} was tyrosine phosphorylated in response to KCl and growth factors.** PC12 cells were treated for 5 min with NGF (50 ng/ml), EGF (10 ng/ml), or KCl (75 mM) as indicated. Then cell lysates were prepared, incubated with anti-p130^{cas} antibody, and subsequently precipitated with Protein A-Sepharose. The whole cell lysates and the immunoprecipitates were analyzed by Western blotting with anti-phosphotyrosine (Panel A) and anti-p130^{cas} (Panel B) antibodies. The positions of p130^{cas} and IgG are marked.

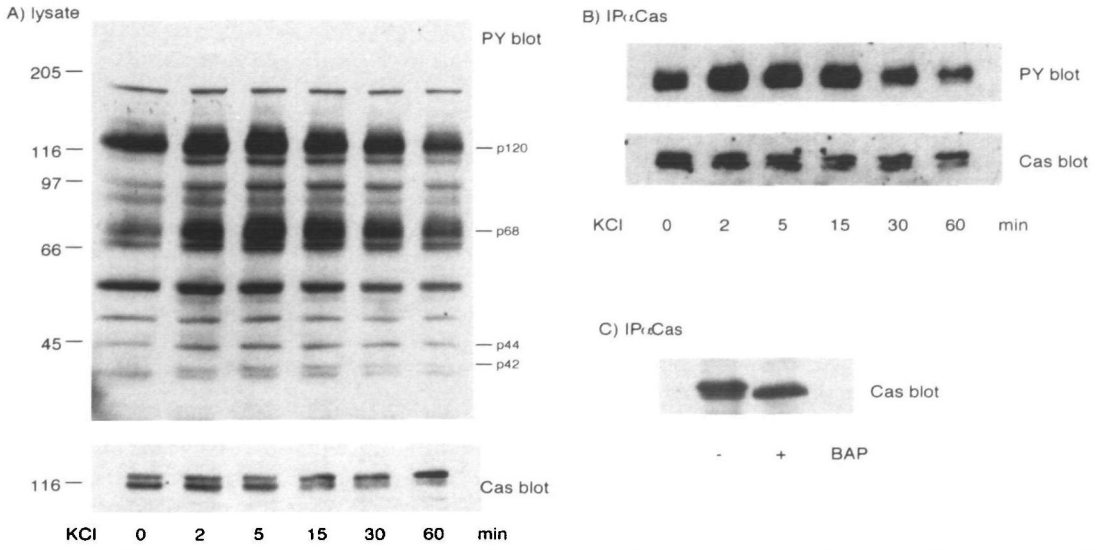

Fig. 2. **Time-course of tyrosine phosphorylation of p130^{cas} by KCl stimulation.** PC12 cells were stimulated with KCl for the indicated periods, then immunoprecipitated with anti-p130^{cas} antibody. The whole cell lysates (A) and the immunoprecipitates (B) were analyzed by Western blotting with anti-phosphotyrosine (upper panels) and anti-p130^{cas} (lower panels) antibodies. (C) PC12 cells were stimulated with KCl for 60 min, lyzed, and immunoprecipitated with anti-p130^{cas} antibody. The immunoprecipitates were treated with or without bacterial alkaline phosphatase (BAP) and analyzed by Western blotting with anti-p130^{cas} antibody.

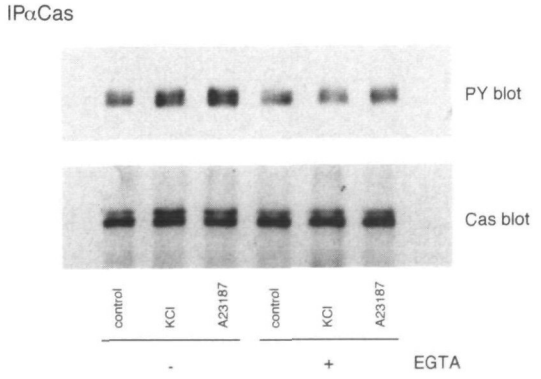

Fig. 3. **KCl-induced tyrosine phosphorylation is Ca^{2+}-dependent.** PC12 cells were pretreated with or without 3 mM EGTA for 5 min, then stimulated with KCl (75 mM) or A23187 (10 μM) for 5 min as indicated. Then the cells were lyzed and immunoprecipitated with anti-p130^{cas} antibody. The immunoprecipitates were analyzed by Western blotting with anti-phosphotyrosine (upper panel) or anti-p130^{cas} (lower panel) antibodies.

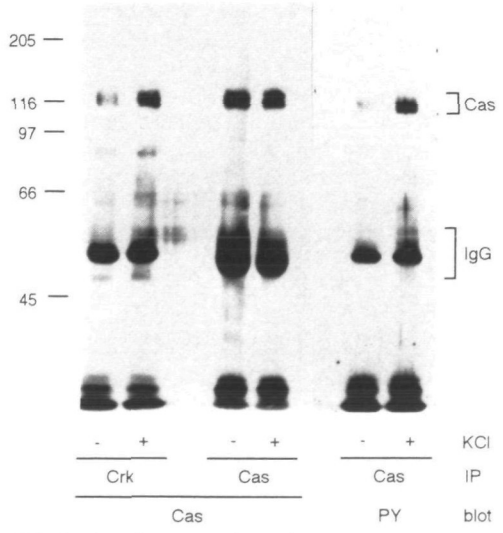

Fig. 4. **KCl-induced association of p130^{cas} with Crk.** PC12 cells were stimulated with KCl (75 mM) for 5 min, lyzed, and immunoprecipitated with anti-p130^{cas} or anti-Crk antibodies. The immunoprecipitates were then analyzed by Western blotting with anti-phosphotyrosine (PY) or anti-p130^{cas} (Cas) antibodies. Positions of p130^{cas} and IgG are marked.

proteins, peaking at 15 min.

The time-course of tyrosine phosphorylation of p130^{cas} was further examined by immunoprecipitation with anti-p130^{cas} antibody followed by Western blotting with anti-phosphotyrosine antibody (Fig. 2B). Consistent with the result shown in Fig. 2A, tyrosine phosphorylation of p130^{cas} was increased 2 min after stimulation and peaked at 5 min. In addition, KCl stimulation resulted in a mobility shift of p130^{cas}. This occurred 2 min after stimulation, but, in contrast to its tyrosine phosphorylation, lasted over 60 min, indicating that the mobility shift would be mainly due to serine or threonine phosphorylation. It was further confirmed that BAP treatment of p130^{cas} restored the mobility of p130^{cas} to the basal level (Fig. 2C). These results indicate that KCl induced phosphorylation of not only tyrosine but also serine and/or threonine residues of p130^{cas}.

KCl is known to induce depolarization of PC12 cells, resulting in calcium influx into the cells. The effect of KCl on tyrosine phosphorylation was mimicked by A23187, a calcium ionophore, which also results in calcium influx (Fig. 3). Also, addition of EGTA to the medium prior to stimulation eliminated the effect of KCl or A23187 on tyrosine phosphorylation of p130^{cas}. These results indicate that the effect of KCl was mediated by Ca^{2+} influx.

p130^{cas} has multiple SH2-binding motifs, each of which

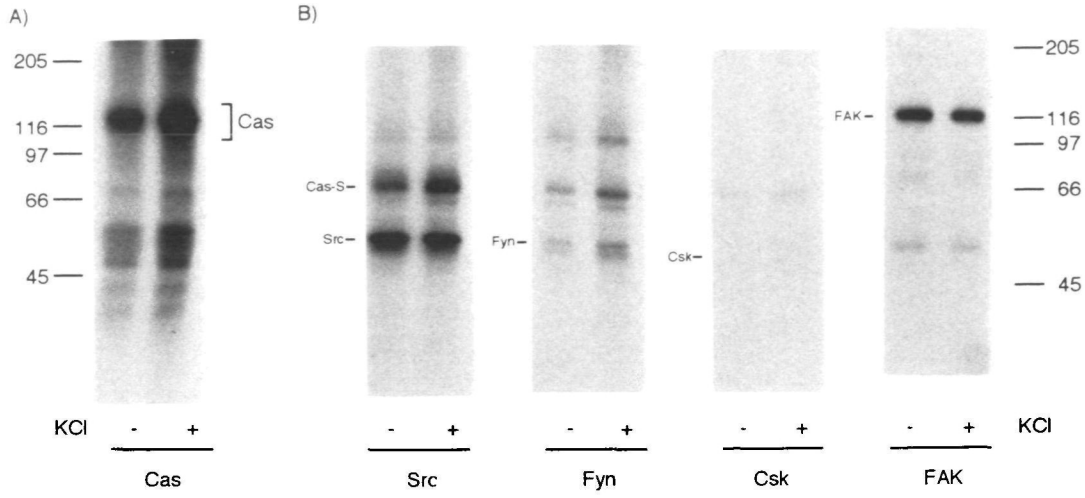

Fig. 5. **KCl-dependent activation of Src-family kinases.** PC12 cells were stimulated with KCl for 5 min and cell lysates were prepared. (A) The lysates were immunoprecipitated with anti-p130^{cas} antibody and incubated in a buffer containing [γ-^{32}P]ATP (10 μCi) at 30°C for 30 min. (B) Lysates were immunoprecipitated with anti-Src, anti-Fyn, anti-Csk, and anti-FAK antibodies as indicated at the bottom of the panels and *in vitro* kinase assay was carried out with GST-Cas-substrate region as the substrate. The samples were separated on an 8% SDS-polyacrylamide gel and exposed to an X-ray film. The positions of p130^{cas} (Cas), GST-Cas-substrate region (Cas-S), Src, Fyn, CSK, and FAK are marked.

contains a tyrosine phosphorylation site. It is known that the SH2 domain of c-Crk binds to the phosphorylated form of some of these tyrosine residues of p130^{cas} (*14, 22*). Thus we next examined whether p130^{cas} was associated with c-Crk after KCl stimulation. As shown in Fig. 4, the immunoprecipitated materials from KCl-treated cells with anti-Crk antibody contained p130^{cas} , while those from untreated cells did not. The change in tyrosine phosphorylation levels of p130^{cas} by KCl treatment was confirmed by immunoprecipitation with anti-cas antibody followed by Western blotting with anti-Cas or anti-phosphotyrosine antibody. The strong bands of about 55 k are signals of IgG used for immunoprecipitation. These results indicate that KCl-stimulation results in association of p130^{cas} with c-Crk.

p130^{cas} also has a binding site for Src-family kinases, which may phosphorylate p130^{cas}. Indeed, immunoprecipitated materials with anti-p130^{cas} antibody contained kinase activity for p130^{cas} (Fig. 5A), and this activity was higher in immunecomplexes from KCl-treated cells. To examine what kind of kinases were activated by KCl stimulation and phosphorylated p130^{cas}, Src, Fyn, CSK, and FAK were immunoprecipitated and their activities were measured with GST-fusion protein of the substrate region of p130^{cas} as the substrate. As shown in Fig. 5B, Src and Fyn could phosphorylate p130^{cas}, while CSK and FAK would not. In addition, the activities of Src and Fyn seemed to be stimulated by KCl.

To confirm that Src-family kinases were activated in response to KCl, we immunoprecipitated with anti-Src and Fyn antibodies and measured their activities with the substrate region of p130^{cas} as the substrate. As shown in Fig. 6, the activity of Src was weakly enhanced by KCl stimulation. On the other hand, activity of Fyn was clearly enhanced within 2 min after stimulation, peaked at 5 min, then slowly decreased. At 5 min, Fyn activity was about twofold higher than the control level.

DISCUSSION

In neuronal and neuroendocrine cells, stimulation with neurotransmitters triggers membrane depolarization resulting in exocrine of circulating hormones or neurotransmitters. We have previously shown that KCl-induced depolarization of PC12 cells stimulated tyrosine phosphorylation of several cellular proteins of 42, 44, 68, and 120 k (*9*). The 68 k protein was identified as paxillin, a cytoskeletal protein associated with the actin filament (*10*). In the present study, we showed that p130^{cas}, a cytoskeletal protein of 120 k, was also tyrosine phosphorylated on KCl stimulation.

p130^{cas} is a docking protein containing an SH3 domain, multiple tyrosine phosphorylation sites in SH2 binding motifs and an SH3 binding site (*14, 19*). In fibroblasts, it is associated with focal contacts and tyrosine phosphorylated on integrin-mediated cell adhesion (*20, 21*). p130^{cas} is also highly expressed in PC12 cells and neurons in the central nervous system (*14*). Its function in the nervous system is not fully understood, but, since p130^{cas} is tyrosine phosphorylated upon KCl stimulation, it may be involved in activity-dependent modifications of neuronal networks.

Like paxillin, p130^{cas} was originally identified as a highly tyrosine phosphorylated protein in v-src-transformed cells (*26*). In addition, it is associated with Src-family kinases through their SH2 and SH3 domains (*19*). The present results thus indicate that Src-family kinases and their substrate proteins might act in the Ca^{2+}-dependent pathway in neuroendocrine cells. We detected protein kinase activity that was coimmunoprecipitated with anti-p130^{cas} antibody and was increased on KCl stimulation (Fig. 5), suggesting that some kinases could be associated with p130^{cas} upon stimulation with KCl. We tried to identify these kinases by Western blotting with anti-Src, Fyn, and Yes antibodies, but without success (data not shown). However, this may due to low sensitivity of the antibodies,

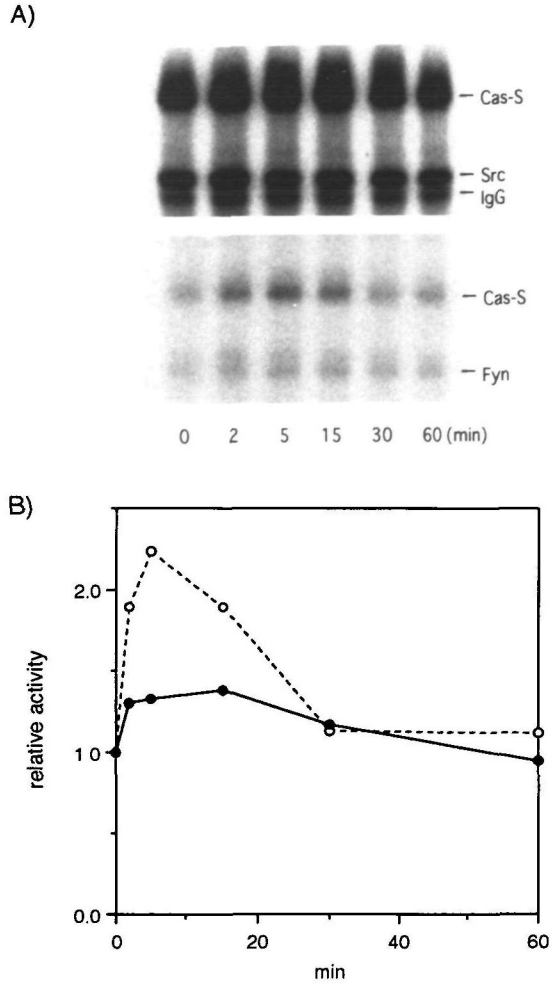

Fig. 6. **Time-course of activation of Src and Fyn by KCl stimulation.** (A) PC12 cells were stimulated with KCl for the indicated periods and immunoprecipitated with anti-Src (upper panel) or anti-Fyn (lower panel) antibodies. The immunoprecipitates were then washed, and *in vitro* kinase assay was carried out in a buffer containing [γ-^{32}P]ATP (10 μCi) and GST-Cas-substrate region. Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 8% gel and the radioactivities were detected by autoradiography. The positions of pp60$^{c\text{-}src}$, pp59^{fyn}, IgG, and GST-Cas-substrate region (Cas-S) are marked. (B) Signals of the autoradiogram were quantified by densitometric analysis as Src (closed circle) and Fyn (open circle).

and it is still possible that Src-family kinases were associated with p130^{cas} on stimulation with KCl.

We showed that KCl stimulation induced tyrosine phosphorylation of the Cas substrate region, where the Crk-SH2 domain can bind (Fig. 4). Indeed, tyrosine phosphorylated form of p130^{cas} was co-immunoprecipitated with anti-Crk antibody. Crk is another docking protein that has one SH2 domain and two SH3 domains (*27*). Its SH3 domains bind to several proteins such as C3G (*28*) and DOCK180 (*29*) and stimulates signaling cascades including the Crk-C3G-JNK pathway (*30*). These might be activated on depolarization and act downstream of p130^{cas}.

Because p130^{cas} and paxillin were tyrosine phosphorylated on KCl treatment, it seems likely that some tyrosine kinases were activated by this stimulation. It is also known that CAKβ/PYK2/RAFTK can be activated by Ca^{2+} (*12*). We showed in this report that Fyn and Src were activated by stimulation with KCl (Fig. 6). Activation of Fyn was more prominent than that of Src and Fyn may act as an important component of Ca^{2+}-dependent signaling pathways. The mechanism underling the activation of Src-family kinases remains to be determined.

Stimulation of PC12 cells with KCl also induced a mobility shift of p130^{cas} on SDS-polyacrylamide electrophoresis (Fig. 2). But the time-course of the mobility shift was different from that of tyrosine phosphorylation. The result indicated that KCl induced not only tyrosine but also serine/threonine phosphorylation of p130^{cas}. It remains to be determined which kinases are responsible for this phosphorylation.

In conclusion, we have shown that p130^{cas}, in addition to paxillin, is tyrosine phosphorylated on depolarization of PC12 cells. This indicates that substrates for Src-family kinases are involved in depolarization-induced events of neuroendocrine cells. Further studies are needed to determine the role of tyrosine phosphorylation of these proteins on cellular functions.

REFERENCES

1. Greenberg, M.E., Hermanowski, A.L., and Ziff, E.B. (1986) Effect of protein synthesis inhibitors on growth factor activation of c-fos, c-myc, and actin gene transcription. *Mol. Cell. Biol.* **6**, 1050-1057
2. Sheng, M., Dougan, S.T., McFadden, G., and Greenberg, M.E. (1988) Calcium and growth factor pathways of c-fos transcriptional activation require distinct upstream regulatory sequences. *Mol. Cell. Biol.* **8**, 2787-2796
3. Mehta, P.P., Battenberg, E., and Wilson, M.C. (1996) SNAP-25 and synaptotagmin involvement in the final Ca^{2+}-dependent triggering of neurotransmitter exocytosis. *Proc. Natl. Acad. Sci. USA* **93**, 10471-10476
4. Lovinger, D.M., Merritt, A., and Reyes, D. (1994) Involvement of N- and non-N-type calcium channels in synaptic transmission at corticostriatal synapses. *Neuroscience* **62**, 31-40
5. Hegstad, E., Haugstad, T.S., Hauglie, H.E., and Langmoen, I.A. (1992) Calcium-dependent release of glutamate from human cerebral cortex. *Brain Res.* **585**, 340-342
6. Kidokoro, Y. and Ritchie, A.K. (1980) Chromaffin cell action potentials and their possible role in adrenaline secretion from rat adrenal medulla. *J. Physiol. (Lond.)* **307**, 199-216
7. Salton, S.R., Margolis, R.U., and Margolis, R.K. (1983) Release of chromaffin granule glycoproteins and proteoglycans from potassium-stimulated PC12 pheochromocytoma cells. *J. Neurochem.* **41**, 1165-1170
8. Rosen, L.B., Ginty, D.D., Weber, M.J., and Greenberg, M.E. (1994) Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. *Neuron* **12**, 1207-1221
9. Okumura, N., Okada, M., and Nakagawa, H. (1994) Depolarization-induced tyrosine phosphorylation in PC12h cells. *J. Biochem.* **116**, 346-350
10. Khan, M.A., Okumura, N., and Okada, M. (1996) Depolarization-induced tyrosine phosphorylation of paxillin in PC12h cells. *Eur. J. Biochem.* **235**, 579-584
11. Sasaki, H., Nagura, K., Ishino, M., Tobioka, H., Kotani, K., and Sasaki, T. (1995) Cloning and characterization of cell adhesion kinase beta, a novel protein-tyrosine kinase of the focal adhesion kinase subfamily. *J. Biol. Chem.* **270**, 21206-21219
12. Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J.M., Plowman, G.D., Rudy, B., and Schlessinger, J. (1995) Protein tyrosine kinase PYK2 involved in Ca^{2+}-induced regulation of ion channel and MAP kinase functions. *Nature* **376**, 737-745
13. Avraham, S., London, R., Fu, Y., Ota, S., Hiregowdara, D., Li, J., Jiang, S., Pasztor, L.M., White, R.A., Groopman, J.E., and

Avraham, H. (1995) Identification and characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain. *J. Biol. Chem.* **270**, 27742-27751
14. Sakai, R., Iwamatsu, A., Hirano, N., Ogawa, S., Tanaka, T., Mano, H., Yazaki, Y., and Hirai, H. (1994) A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. *EMBO J.* **13**, 3748-3756
15. Burnham, M.R., Harte, M.T., Richardson, A., Parsons, J.T., and Bouton, A.H. (1996) The identification of p130^{cas}-binding proteins and their role in cellular transformation. *Oncogene* **12**, 2467-2472
16. Harte, M.T., Hildebrand, J.D., Burnham, M.R., Bouton, A.H., and Parsons, J.T. (1996) p130^{cas}, a substrate associated with v-Src and v-Crk, localizes to focal adhesions and binds to focal adhesion kinase. *J. Biol. Chem.* **271**, 13649-13655
17. Polte, T.R. and Hanks, S.K. (1995) Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130^{cas}. *Proc. Natl. Acad. Sci. USA* **92**, 10678-10682
18. Astier, A., Avraham, H., Manie, S.N., Groopman, J., Canty, T., Avraham, S., and Freedman, A.S. (1997) The related adhesion focal tyrosine kinase is tyrosine-phosphorylated after beta1-integrin stimulation in B cells and binds to p130^{cas}. *J. Biol. Chem.* **272**, 228-232
19. Nakamoto, T., Sakai, R., Ozawa, K., Yazaki, Y., and Hirai, H. (1996) Direct binding of C-terminal region of p130^{cas} to SH2 and SH3 domains of Src kinase. *J. Biol. Chem.* **271**, 8959-8965
20. Hamasaki, K., Mimura, T., Morino, N., Furuya, H., Nakamoto, T., Aizawa, S., Morimoto, C., Yazaki, Y., Hirai, H., and Nojima, Y. (1996) Src kinase plays an essential role in integrin-mediated tyrosine phosphorylation of Crk-associated substrate p130^{cas}. *Biochem. Biophys. Res. Commun.* **222**, 338-343
21. Nakamoto, T., Sakai, R., Honda, H., Ogawa, S., Ueno, H., Suzuki, T., Aizawa, S., Yazaki, Y., and Hirai, H. (1997) Requirements for localization of p130^{cas} to focal adhesions. *Mol. Cell. Biol.* **17**, 3884-3897
22. Ribon, V. and Saltiel, A.R. (1996) Nerve growth factor stimulates the tyrosine phosphorylation of endogenous Crk-II and augments its association with p130^{cas} in PC-12 cells. *J. Biol. Chem.* **271**, 7375-7380
23. Kobayashi, S., Okumura, N., Nakamoto, T., Okada, M., Hirai, H., and Nagai, K. (1997) Activation of pp60$^{c\text{-}src}$ depending on cell density in PC12h cells. *J. Biol. Chem.* **272**, 16262-16267
24. Okumura, N., Miyatake, Y., Takao, T., Tamaru, T., Nagai, K., Okada, M., and Nakagawa, H. (1994) Vasoactive intestinal peptide induces differentiation and MAP kinase activation in PC12h cells. *J. Biochem.* **115**, 304-308
25. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
26. Kanner, S.B., Reynolds, A.B., Vines, R.R., and Parsons, J.T. (1990) Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc. Natl. Acad. Sci. USA* **87**, 3328-3332
27. Matsuda, M., Tanaka, S., Nagata, S., Kojima, A., Kurata, T., and Shibuya, M. (1992) Two species of human CRK cDNA encode proteins with distinct biological activities. *Mol. Cell. Biol.* **12**, 3482-3489
28. Tanaka, S., Morishita, T., Hashimoto, Y., Hattori, S., Nakamura, S., Shibuya, M., Matuoka, K., Takenawa, T., Kurata, T., Nagashima, K., and Matsuda, M. (1994) C3G, a guanine nucleotide-releasing protein expressed ubiquitously, binds to the Src homology 3 domains of CRK and GRB2/ASH proteins. *Proc. Natl. Acad. Sci. USA* **91**, 3443-3447
29. Hasegawa, H., Kiyokawa, E., Tanaka, S., Nagashima, K., Gotoh, N., Shibuya, M., Kurata, T., and Matsuda, M. (1996) DOCK180, a major CRK-binding protein, alters cell morphology upon translocation to the cell membrane. *Mol. Cell. Biol.* **16**, 1770-1776
30. Tanaka, S., Ouchi, T., and Hanafusa, H. (1997) Downstream of Crk adapter signaling pathway: activation of Jun kinase by v-Crk through the guanine nucleotide exchange protein C3G. *Proc. Natl. Acad. Sci. USA* **94**, 2356-2361