Stimulation of M₃ Muscarinic Receptors Induces Phosphorylation of the Cdc42 Effector Activated Cdc42Hs-associated Kinase-1 via a Fyn Tyrosine Kinase Signaling Pathway*

The tyrosine kinase, activated Cdc42Hs-associated kinase-1 (ACK-1), is a specific effector of the Rho family GTPase Cdc42. GTP-bound Cdc42 has been shown to facilitate neurite outgrowth elicited by activation of muscarinic cholinergic receptors (mACHRs). Because tyrosine kinase activity is a requirement for neuritogenesis in several cell systems, we investigated whether endogenous mACHRs (primarily of the M₃ subtype) expressed in human SH-SY5Y neuroblastoma cells would signal to ACK-1. Incubation of cells with the cholinergic agonist oxotremorine-M (Oxo-M) induced an approximately 6-fold increase in the tyrosine phosphorylation of ACK-1 which was inhibited by atropine. ACK-1 phosphorylation was blocked by Clostridium difficile toxin B, an inhibitor of Rho family GTPases. In contrast, disruption of the actin cytoskeleton with cytochalasin D stimulation of ACK-1 phosphorylation, and moreover, addition of Oxo-M to cells preincubated with this agent elicited a further increase in phosphorylation, indicating that an intact cytoskeleton is not required for mAChR signaling to ACK-1. Although stimulation of M₃ mAChRs induces both an increase in intracellular Ca²⁺ and activation of protein kinase C (PKC), neither of these second messenger pathways was required for receptor-stimulated ACK-1 phosphorylation. Instead, inhibition of PKC resulted in a 2-fold increase in Oxo-M-stimulated ACK-1 phosphorylation, whereas acute activation of PKC with phorbol ester decreased ACK-1 phosphorylation. The agonist-induced tyrosine phosphorylation of ACK-1 was blocked by inhibitors of Src family kinases, and ACK-1 was coprecipitated with Fyn (but not Src) in an agonist-dependent manner. Finally, scrape loaded cells with glutathione S-transferase fusion proteins of either the Fyn-SH2 or Fyn-SH3 domain significantly attenuated mAChR-stimulated ACK-1 tyrosine phosphorylation. The data are the first to show phosphorylation of ACK-1 after stimulation of a receptor coupled to neurite outgrowth and indicate that a Rho family GTPase (i.e. Cdc42) and Fyn are essential upstream elements of this signaling pathway.

Synaptic plasticity plays a critical role in the development of the central nervous system and in mediating the processes of learning and memory. The phenomena of long term potentiation and long term depression are considered in vitro models of the latter (1). Previous studies have demonstrated a requirement for tyrosine kinase activity in mediating both long term potentiation in the hippocampus and long term depression in the cerebellum (2, 3). Furthermore, tyrosine kinase activity has also been shown to play a prominent role in facilitating neurite outgrowth in several in vitro models (4, 5). Currently, ~100 protein tyrosine kinases have been identified (6). These enzymes can be categorized into two distinct classes, receptors with intrinsic tyrosine kinase activity and nonreceptor tyrosine kinases. Examples of receptor tyrosine kinases include classical growth factor receptors such as the receptor for nerve growth factor (7). The nonreceptor tyrosine kinases consist of approximately 10 families including two related families, the activated Cdc42Hs-associated kinase (ACK) family and the focal adhesion kinase (FAK) family (8). Nonreceptor tyrosine kinases of the ACK and FAK families are highly expressed in the brain (9–12) and have been postulated to regulate synaptic changes in the central nervous system (13, 14).

ACK-1 is one of two known members of the ACK family and was originally cloned from a human hippocampal cDNA library (9). ACK-1 is a specific downstream effector of the Rho family small molecular weight GTPase Cdc42. In contrast to many effectors that also act as GTPase-activating proteins for their partner G proteins, the association of ACK-1 with GTP-bound Cdc42 has been shown to inhibit both the intrinsic and GTPase-activating protein-stimulated GTPase activity of Cdc42, suggesting that this interaction may sustain Cdc42 in a GTP-bound (active) state (9). Active Cdc42 promotes the formation of filopodial extensions and actin microspikes (15, 16). These changes in actin cytoskeletal structure are mediated via activation of other Cdc42 effectors, such as the p21-activated kinases (17, 18) and the Wiskott-Aldrich syndrome proteins (19, 20). In neuronal cells, Rho family GTPases regulate changes in

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¶ The abbreviations used are: ACK, activated Cdc42Hs-associated kinase; FAK, focal adhesion kinase; GST, glutathione S-transferase; mACHR, muscarinic cholinergic receptor; MARCKS, myristoylated alanine-rich C-kinase substrate; Oxo-M, 2-butyln-1-ammonium, N,N,N-trimethyl-4-(2-oxo-1-pyrrolidinyl)iodide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PKC, protein kinase C; pMA, phospholipid; PMA, phorbol 12-myristate 13-acetate; PP1, PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyI)pyrazolo[3,4-d]pyrimidine; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyI)pyrazolo[3,4-d]pyrimidine; PVDF, polyvinylidene fluoride; PTK-2, proline-rich kinase-2; Tyr(P), phosphorysorine.

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growth cone morphology (21, 22). In particular, Rac and Cdc42 facilitate neurite outgrowth, whereas Rho induces neurite retraction and growth cone collapse (23–25). Although ACK family proteins have been postulated to play a role in growth cone remodeling (14), the activation of this class of nonreceptor tyrosine kinase has not yet been investigated in a neuronal cell model.

Cholinergic transmission is a critical element involved in the maintenance of cognitive functions in the central nervous system such as learning and memory (26). For example, deficits in muscarinic cholinergic receptor (mACHR) signaling contribute to the pathophysiology of Alzheimer’s disease and other types of age-related dementia (27–29). The SH-SY5Y cell line is a human neuroblastoma derived as a subclone from the parental SK-N-SH line (30, 31). SH-SY5Y cells express a high density of mACHRs, principally of the M₄ subtype (32, 33), which are coupled to the phospholipase C-dependent production of inositol 1,4,5-trisphosphate and diacylglycerol (34, 35). These second messenger molecules lead to an increase in intracellular Ca²⁺ and activation of protein kinase C (PKC), respectively. Stimulation of mACHRs on SH-SY5Y cells has been shown to elicit morphological changes characteristic of neurite outgrowth (36, 37) and induces a marked increase in the tyrosine phosphorylation of the nonreceptor tyrosine kinase FAK (38). Given that Cdc42 has been shown to regulate mACHR-mediated growth cone remodeling (23), we investigated whether mACHR stimulation in SH-SY5Y cells couples to activation of the Cdc42 effector ACK-1.

The results demonstrate that agonist occupancy of mACHRs on SH-SY5Y neuroblastoma cells elicits an enhanced tyrosine phosphorylation of ACK-1 which is dependent on Cdc42 function, Fyn tyrosine kinase activity, and an interaction between ACK-1 and Fyn. The last may require both the Fyn-SH2 and SH3 domains. The results are the first to show a receptor-mediated activation of ACK-1 in a neuronal cell system and support a role for this nonreceptor tyrosine kinase in signaling during growth cone remodeling.

EXPERIMENTAL PROCEDURES

Materials—Phorbol 12-myristate 13-acetate (PMA), bisindolylmaleimide I (GF 109203X), PP1, PP2, PP3, thapsigargin, and cycloheximide D were obtained from Calbiochem. Atropine and mecamylamine were from Sigma. OXtremorine-M (Oxo-M) was purchased from Research Biochemicals International (Natick, MA). Clostridium difficile toxin B was generously provided by Dr. Klaus Aktories and Dr. Fred Hofmann (Albert-Ludwigs-Universität, Freiburg, Germany). All other chemicals were of reagent grade. [³²P] ATP (10 mCi/ml), reagents for enhanced chemiluminescence, and peroxidase-conjugated sheep anti-mouse IgG were purchased from Amer sham Pharmacia Biotech. Monoclonal antibodies to phosphotyrosine Tyr(P) and myristoylated alanine-rich protein kinase C substrate (MARCKS) were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Polyclonal antibodies to FAK and ACK1, blocking peptide for ACK-1, monoclonal antibodies to Src, Fyn, and glutathione S-transferase (GST), agaro-conjugated protein A/G, and purified GST, GST-Fyn-SH2, and GST-Fyn-SH3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Tissue culture supplies were purchased from Corning Glass Works (Corning, NY) and Sarstedt (Newton, NC). Dulbecco’s modified Eagle’s medium was from Life Technologies, Inc. Fetal calf serum was from Summit Biotechnology (Ft. Collins, CO). Human SH-SY5Y neuroblastoma cells were obtained from Dr. June Biedler (Sloan-Kettering Institute, New York).

Cell Culture Conditions—Human SH-SY5Y neuroblastoma cells (passages 68–78) were routinely grown in 75-cm² tissue culture flasks containing 20 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were grown for 7–14 days at 37 °C in a humidified atmosphere consisting of 90% air and 10% CO₂. Cells were washed once with 2 ml of prewarmed (37 °C) treatment buffer consisting of 138 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM NaH₂PO₄, 5 mM glucose, and 20 mM HEPES (pH 7.4) and allowed to equilibrate at 37 °C in 1 ml of treatment buffer for 20 min. During the equilibration period cells were preincubated with inhibitors as described previously (37). Cells were then incubated for the specified duration at 37 °C with either buffer or 1 mM Oxo-M. After incubation, the buffer was aspirated, and the cells were rinsed once with 2 ml of ice-cold phosphate-buffered saline (PBS; pH 7.4). Cells were then incubated on ice and scraped into lysis buffer (200 μl/well) containing 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 50 mM NaCl, 1 mM EGTA, 1 mM β-glycerophosphate, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cell debris was removed by centrifugation at 6,000 × g for 3 min, and the protein concentration of the supernatant was determined using a commercially available protein assay kit (Pierce Chemical Co.). Aliquots (~50 μg) of supernatant protein were diluted to a final volume of 500 μl with lysis buffer and transferred to tubes containing either 2 μg of polyclonal anti-ACK1 or 2 μg of polyclonal anti-FAK. In some experiments, varying amounts of the ACK1 blocking peptide were included during immunoprecipitation. Samples were then incubated with mixing for 16–20 h at 4 °C. 25 μl of agaro-conjugated protein A/G was added for an additional 4 h with mixing. Immune complexes were pelleted by centrifugation and washed three times with ice-cold lysis buffer. The final agarose pellet was resuspended in an equal volume of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, boiled for 5 min, and electrophoresed through 7.5% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA) and processed for immunoblot analysis.

Coimmunoprecipitation of Src Family Kinases and ACK1—Cells were incubated for 5 min with either buffer or Oxo-M and lysed as described above. Lysates were then immunoprecipitated with 2 μg of either polyclonal anti-ACK1 or monoclonal anti-Src or anti-Fyn. Immune complexes were resolved by SDS-PAGE, transferred to PVDF, and processed for immunoblot analysis.

Immunoblot Analysis—Non-specific binding sites were blocked in PBS (pH 7.4) containing 0.1% Tween 20 (PBS-T) and 1% bovine serum albumin for 1 h at room temperature. Primary monoclonal antibodies were diluted in blocking solution (final concentration of 0.5–1.0 μg/ml) and incubated with the membranes for 1 h. Excess primary antibody was removed by washing the membranes three times in PBS-T. The blots were then incubated in peroxidase-conjugated, anti-mouse second- antibody (1:10,000) diluted in PBS-T for 1 h. The blots were subsequently washed three times in PBS-T. Immunoreactive proteins were detected by enhanced chemiluminescence. In some experiments, membranes were reprobed after stripping in 0.1 M Tris-HCl (pH 8.0), 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 52 °C. The blots were rinsed twice in PBS-T and processed as above with a different primary antibody. Autoradiograms shown are representative of two to four independent replicate experiments. In all cases the first antibody to the epitope of interest was detected by computer-assisted imaging densitometry (MCID; Imaging Research, St. Catharines, Ontario, Canada).

³²P Labeling of ACK1 and MARCKS—Cells were incubated in isotopic equilibrium at 37 °C in 1 ml of phosphate-free treatment buffer containing 1 mM [³²P] ATP, for 4 h. The labeling solution was aspirated, and unincorporated [³²P] ATP was removed by washing three times with 2 ml of the same buffer. The cells were then incubated with either vehicle, Oxo-M or PMA, as described in the legend to Fig. 4, and either ACK1- or MARCKS was immunoprecipitated from cell lysates. Immune complexes were resolved by SDS-PAGE and transferred to PVDF membranes. [³²P] Labeled proteins were visualized by PhosphoImager analysis (Cyclone Storage Phosphor System; Packard Instrument Company, Meriden, CT). Membranes containing ACK1- or MARCKS immunoprecipitates were subsequently immunoblotted for Tyr(P) as described above.

Scrape Loading with GST Fusion Proteins—Either GST alone or GST fusion proteins (Fyn-SH2 or Fyn-SH3) were scrape loaded into cells at a concentration of 20 μg/ml in scrape-loading buffer containing 10 mM Tris-HCl (pH 7.0), 114 mM KCl, 15 mM NaCl, and 5.3 mM MgCl₂ (39). Scrape-loaded cells were incubated for 30 min in the presence of 10 μM Oxo-M. The cells were subsequently washed in serum-free medium, and seeded into 35-mm culture dishes. After further incubation at 37 °C in 10% CO₂ for 3 h, the cells had reattached and were incubated with either buffer or Oxo-M for 5 min. ACK1 immune complexes were isolated and probed for Tyr(P). The post-ACK1 immunoprecipitate lysates were also resolved by SDS-PAGE and were probed for GST to estimate the efficiency of scrape loading.
A cells enhances the tyrosine phosphorylation of ACK-1. Buffer alone or 1 mM Oxo-M. Triton X-100-soluble extracts were immunoprecipitated (IP) with 2 μg of polyclonal anti-ACK-1 in either the absence or presence of varying concentrations of ACK-1-blocking peptide. Immune complexes were resolved by SDS-PAGE, transferred to PVDF, and immunoblotted (IB) for Tyr(P) (PTyr), as described under “Experimental Procedures.” B, SH-SY5Y cells were preincubated for 10 min with either 10 μM atropine (ATR) or 10 μM mecamylamine (MEC) prior to incubation with 1 mM Oxo-M for a further 5 min. ACK-1 immune complexes were probed for Tyr(P). C, SH-SY5Y cells were incubated with 1 mM Oxo-M for varying times, and ACK-1 immune complexes were probed for Tyr(P). D, SH-SY5Y cells were incubated with varying concentrations of Oxo-M for 5 min, and ACK-1 immune complexes were blotted for Tyr(P) (upper panel) or ACK-1 (lower panel).

**RESULTS**

**Agonist Occupancy of mAChRs Enhances the Tyrosine Phosphorylation of ACK-1—** Incubation of SH-SY5Y neuroblastoma cells with the cholinergic agonist Oxo-M elicited a marked increase in the tyrosine phosphorylation of ACK-1 (6.2 ± 0.7-fold increase over basal, n = 6, p < 0.001, Fig. 1A, first lane versus second lane). Immunoprecipitation of ACK-1 was competitively antagonized by coin cubation with a blocking peptide that mimicked the antigenic site on ACK-1 (Fig. 1A). Agonist-stimulated ACK-1 phosphorylation was blocked by preincubation with the muscarinic selective antagonist, atropine, but was unaffected by the nicotinic selective antagonist, mecamylamine (Fig. 1B). The increased tyrosine phosphorylation of ACK-1 elicited by Oxo-M was time- and dose-dependent. The agonist-stimulated phosphorylation of ACK-1 was rapid and persistent, increasing within 1 min of agonist exposure, peaking at 5–10 min, and remaining elevated above the basal level for at least 1 h (Fig. 1C). Furthermore, Oxo-M induced the tyrosine phosphorylation of ACK-1 with an EC50 of ~100 μM (Fig. 1D, upper panel). The addition of agonist had no effect on the immunoprecipitation efficiency of ACK-1 (Fig. 1D, lower panel).

mAChR-stimulated ACK-1 Tyrosine Phosphorylation Is Dependent on Rho Family GTPase Activity but Not on an Intact Actin Cytoskeleton—ACK-1 has been shown to interact specifically with the Rho family GTPase Cdc42 (9). To determine whether the mAChR-induced increase in ACK-1 tyrosine phosphorylation was dependent on Cdc42 activity, cells were preincubated with C. difficile toxin B. Toxin B is a monoglycosyltransferase that selectively inhibits the function of Rho family GTPases (40). After 24 h of incubation with toxin B, the subsequent C3 exoenzyme-catalyzed ADP-ribosylation of Rho proteins in SH-SY5Y cell lysates is significantly decreased, a result indicating that toxin B efficiently enters intact SH-SY5Y cells and inhibits Rho family GTPases in these cells (37). Moreover, SH-SY5Y cells become rounded, and a marked disruption of the actin cytoskeleton is observed after incubation with toxin B (37). The latter morphology is characteristic of cells in which Rho family GTPase function has been compromised (40). Preincubation with toxin B essentially abolished the agonist-induced tyrosine phosphorylation of ACK-1 (Fig. 2A), a result consistent with a requirement for Cdc42 function in signaling from the M5 mAChR to ACK-1.

The ACK family of nonreceptor tyrosine kinases (9, 12) shows some homology to the FAK family, which includes two principal members, FAK and PYK-2 (11, 41). Stimulation of an enhanced tyrosine phosphorylation status of either FAK or PYK-2 requires an intact actin cytoskeleton (39, 42). Therefore, we analyzed the effects of the actin cytoskeleton-destabilizing agent cytochalasin D on mAChR-stimulated ACK-1 tyrosine phosphorylation. After a 30-min preincubation with cytochalasin D, SH-SY5Y cells displayed a significant disruption of actin stress fibers (results not shown). As documented previously (38), cytochalasin D pretreatment suppressed the basal tyrosine phosphorylation of FAK and completely inhibited the mAChR-induced increase in FAK phosphorylation (Fig. 2B, lower panel). In contrast, the basal tyrosine phosphorylation of ACK-1 was increased dramatically after incubation with cytochalasin D, and the agonist-induced increase in ACK-1 phosphorylation was sustained after disruption of the cytoskeleton.
identified a PKC-dependent Negative Feedback Loop That Limits mAChR Signaling to ACK-1—Agonist occupancy of M₃ mAChRs leads to the phospholipase C-dependent generation of inositol 1,4,5-trisphosphate and sn-1,2-diacylglycerol which, in turn, increase intracellular Ca²⁺ and activate PKC, respectively. Given that increases in intracellular Ca²⁺ and/or PKC activity can elicit an increased tyrosine phosphorylation of either PYK-2 or FAK (42, 43), the potential involvement of these second messengers in mAChR signaling to ACK-1 was investigated. First, the effects of depletion of intracellular Ca²⁺ stores on agonist-induced ACK-1 phosphorylation were assessed. As described previously (44), a 30-min preincubation of SH-SY5Y cells with 1 µM thapsigargin completely abolished the inositol 1,4,5-trisphosphate-mediated increase in intracellular Ca²⁺ observed after mAChR stimulation. However, thapsigargin pretreatment did not have a statistically significant effect on agonist-induced ACK-1 phosphorylation (690 pg ± 296 pg in control versus 426 pg ± 194 pg in thapsigargin-treated, n = 4, p = 0.48, data not shown). This result suggests that agonist-induced Ca²⁺ release does not play a significant role in mAChR signaling to ACK-1.

Next, we evaluated the effects of agents that modulate PKC activity on mAChR-stimulated ACK-1 tyrosine phosphorylation. Preincubation with the PKC inhibitor bisindolylmaleimide I, resulted in a statistically significant 2-fold increase in the mAChR-induced tyrosine phosphorylation of ACK-1 (Fig. 3, A and B). In contrast, activation of PKC, via an acute preincubation with phorbol ester, significantly inhibited agonist-stimulated ACK-1 tyrosine phosphorylation by ~50% (Fig. 3, A and B). This latter inhibitory effect of PMA on ACK-1 tyrosine phosphorylation was prevented by preincubation with the PKC inhibitor bisindolylmaleimide I (Fig. 3, A and B). These data indicate that, in general, PKC activity is inhibitory for ACK-1 tyrosine phosphorylation, and more specifically, receptor-mediated PKC activity limits the ability of the mAChR to stimulate ACK-1 tyrosine phosphorylation. To determine whether the inhibitory effects of PKC on ACK-1 tyrosine phosphorylation were mediated via direct serine or threonine phosphorylation of ACK-1 on some inhibitory site(s), the potential of PMA to increase ³²P incorporation into ACK-1 was assessed in ³²P-labeled SH-SY5Y cells. Incubation of ³²P-prelabeled cells with Oxo-M induced an increase in the tyrosine phosphorylation of ACK-1 (Fig. 4A) and a corresponding increase in ³²P incorporation into ACK-1 (Fig. 4B). In contrast, the addition of PMA resulted in a decrease in the basal tyrosine phosphorylation of ACK-1 (Fig. 4A, see also Fig. 3A, first lane versus fifth lane) with no detectable increase in ³²P incorporation into ACK-1 (Fig. 4B). Under the latter conditions, incubation with PMA induced phosphorylation of the PKC substrate MARCKS in SH-SY5Y cells as indicated by the increase in ³²P incorporation into this protein (Fig. 4C). Thus, activation of PKC blunts tyrosine phosphorylation of ACK-1 without inducing a corresponding increase in either serine or threonine phosphorylation of ACK-1.

Requirement for Fyn Tyrosine Kinase in the mAChR-induced Phosphorylation of ACK-1—Previous studies have demonstrated that FAK and PYK-2 each physically interacts with Src family kinases, and both are prominent substrates for this class of tyrosine kinase (45–48). Similarly, preincubation of SH-SY5Y cells with the Src family kinase inhibitor PP1 significantly attenuated the mAChR-stimulated tyrosine phosphorylation of ACK-1 (Fig. 5A). The inhibitory effects of PP1 were maximal at 10 µM, a concentration that essentially abolished Oxo-M-induced ACK-1 phosphorylation. In addition, a second Src family kinase inhibitor, PP2, also blocked agonist-induced ACK-1 tyrosine phosphorylation, whereas a negative control compound, PP3, had no effect (Fig. 5B). Moreover, Fyn tyrosine kinase, but not Src, was detected in ACK-1 immunoprecipitates obtained from lysates of agonist-stimulated SH-SY5Y cells (Fig. 6, first versus second lane of each panel). In contrast, tyrosine-phosphorylated ACK-1 was not detectable in Fyn immunoprecipitated ACK-1 tyrosine phosphorylation by ~50% (Fig. 3, A and B). This latter inhibitory effect of PMA on ACK-1 tyrosine phosphorylation was prevented by preincubation with the PKC inhibitor bisindolylmaleimide I (Fig. 3, A and B). These data indicate that, in general, PKC activity is inhibitory for ACK-1 tyrosine phosphorylation, and more specifically, receptor-mediated PKC activity limits the ability of the mAChR to stimulate ACK-1 tyrosine phosphorylation. To determine whether the inhibitory effects of PKC on ACK-1 tyrosine phosphorylation were mediated via direct serine or threonine phosphorylation of ACK-1 on some inhibitory site(s), the potential of PMA to increase ³²P incorporation into ACK-1 was assessed in ³²P-labeled SH-SY5Y cells. Incubation of ³²P-prelabeled cells with Oxo-M induced an increase in the tyrosine phosphorylation of ACK-1 (Fig. 4A) and a corresponding increase in ³²P incorporation into ACK-1 (Fig. 4B). In contrast, the addition of PMA resulted in a decrease in the basal tyrosine phosphorylation of ACK-1 (Fig. 4A, see also Fig. 3A, first lane versus fifth lane) with no detectable increase in ³²P incorporation into ACK-1 (Fig. 4B). Under the latter conditions, incubation with PMA induced phosphorylation of the PKC substrate MARCKS in SH-SY5Y cells as indicated by the increase in ³²P incorporation into this protein (Fig. 4C). Thus, activation of PKC blunts tyrosine phosphorylation of ACK-1 without inducing a corresponding increase in either serine or threonine phosphorylation of ACK-1.

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A stimulated tyrosine phosphorylation of ACK-1. Confluent cultures of SH-SY5Y cells were incubated for 5 min alone or 1 mM Oxo-M. ACK-1 immune complexes were resolved by SDS-PAGE, transferred to PVDF, and immunoblotted for Tyr(P). B, SH-SY5Y cells were preincubated for 15 min with either vehicle (VEH, 0.2% dimethyl sulfoxide) or 10 μM PP1, PP2, or PP3 prior to the addition of Oxo-M for a further 5 min. ACK-1 immune complexes were probed for Tyr(P). IP, immunoprecipitation; IB, immunoblotted.

FIG. 6. Coprecipitation of ACK-1 and Fyn after mACHR stimulation. Confluent cultures of SH-SY5Y cells were incubated for 5 min with either buffer alone or buffer containing 1 mM Oxo-M. Cell lysates were immunoprecipitated (IP) with either polyclonal anti-ACK-1 or monoclonal anti-Src or anti-Fyn as shown. Immune complexes were resolved by SDS-PAGE, transferred to PVDF, and sequentially immunoblotted (IB) for Tyr(P) (PTyr), Src, and Fyn. The blots shown are representative of results obtained in three independent experiments.

The monoclonal antibody to Fyn used in the previous experiments recognizes an epitope within the SH2 domain and/or SH3 domains. The latter result suggested that ACK-1 may interact with Fyn at one or both of these sites, thus preventing the Fyn antibody from recognizing Fyn that is associated with ACK-1 in this manner. To test this hypothesis, SH-SY5Y cells were scrape loaded with GST fusion proteins of either the Fyn-SH2 or Fyn-SH3 domain, and mACHR-stimulated ACK-1 tyrosine phosphorylation in these cells was compared with control cells scrape loaded with GST alone. Agonist-induced ACK-1 phosphorylation was inhibited significantly in cells scrape loaded with either the Fyn-SH2 domain (approximately a 60% decrease compared with the GST control) or the Fyn-SH3 domain (approximately a 90% decrease compared with the GST control) (Fig. 7, A and C). Immunoblotting of cell lysates for GST showed that scrape loading of the Fyn-SH3 domain (which is smaller than the Fyn-SH2 domain) may have been slightly more efficient than that of the Fyn-SH2 domain (Fig. 7B). Collectively, these data indicate that Fyn tyrosine kinase is required for mACHR signaling to ACK-1, and moreover, Fyn may associate with ACK-1 via both SH2 domain- and SH3 domain-mediated interactions.

**DISCUSSION**

A role for the nonreceptor tyrosine kinases, FAK and PYK-2, in the regulation of synaptic plasticity in the central nervous system has recently been reviewed (13). Similarly, members of the related ACK family of nonreceptor tyrosine kinases have also recently been postulated to regulate neurite outgrowth (14), given their high levels of expression in brain and their potential capacities to perpetuate the actin cytoskeletal remodeling activity of the small molecular weight GTPase Cdc42 (9, 12). In the current study, we demonstrate that stimulation of neurotropic M₃ mACHRs on SH-SY5Y human neuroblastoma cells elicits an enhanced tyrosine phosphorylation of ACK-1 which occurs rapidly (within 1 min) on a time course that precedes that observed previously for mACHR-mediated neurite outgrowth (within minutes) in this same cell system (36) (Fig. 1). Given that a requirement for Cdc42 in the regulation of mACHR-induced neurite outgrowth has been described previously (20), the results of the present investigation are consistent with a role for ACK-1 tyrosine kinase in the process of growth cone remodeling. The mACHR-stimulated increase in the tyrosine phosphorylation of ACK-1 was dependent on the activity of a Rho family GTPase, most likely Cdc42, as demonstrated by its inhibition after preincubation with C. difficile toxin B (Fig. 24). Recently, we have also described a novel requirement for Cdc42 function in the regulation of mACHR signaling to FAK (37). This latter observation, coupled with the recent findings of Eisenmann et al. (49) that Cdc42 and ACK-1 also regulate the tyrosine phosphorylation of the FAK substrate, p130Cas, suggests the potential for cross-talk between these two families of nonreceptor tyrosine kinases.

In the present study, the actin cytoskeletal disruptive effects of toxin B were dissociated from its ability to inhibit ACK-1...
signaling by the observation that preincubation with cytochalasin D actually promoted ACK-1 phosphorylation (Fig. 2B).

Moreover, addition of a mAChR agonist to cells preincubated with cytochalasin D elicited a further increase in ACK-1 tyrosine phosphorylation (Fig. 2B), suggesting that an intact actin cytoskeleton is not required for mAChR signaling to ACK-1. The above findings are in direct contrast to the absolute requirement for an intact actin cytoskeleton in mAChR signaling to FAK (Fig. 2B; Ref. 38). However, they are in agreement with a previous report that demonstrated that disruption of the actin cytoskeleton with cytochalasin leads to disassembly of the extracellular matrix (50). Matrix disassembly has, in turn, been shown to promote a compensatory activation of Cdc42, an enhanced formation of filopodia, and an increased tyrosine phosphorylation of ACK-1 (51). These last findings are further supported by our observation that ACK-1 phosphorylation induced by disruption of the cytoskeleton with cytochalasin D was blocked by inhibition of Rho family GTPase activity with toxin B (Fig. 2C). Furthermore, our results corroborate the observations of Yang et al. (14) who demonstrated that integrin-mediated ACK-2 tyrosine phosphorylation also does not depend on the integrity of the actin cytoskeleton, although it is uncertain why these authors did not observe an increase in ACK-2 phosphorylation after incubation with cytochalasin D alone.

Although stimulation of M3 mAChRs elicits both an increase in intracellular Ca2+ and an activation of PKC, neither of these second messenger pathways was required for mAChR signaling to ACK-1 in SH-SY5Y cells. Instead, receptor-mediated PKC activation was found to act as a negative feedback loop to limit signaling from mAChRs to ACK-1 (Fig. 3). The ability of PKC activity to blunt ACK-1 tyrosine phosphorylation was apparently not caused by direct phosphorylation of ACK-1 on inhibitory serine or threonine residues (Fig. 4). This result suggests that the PKC-mediated negative regulation of ACK-1 tyrosine phosphorylation may occur further upstream in the signaling pathway, perhaps at the level of the Cdc42 GTPase. In this context, it is interesting to note that serine phosphorylation of the small molecular weight GTPase, Rac1, by Akt kinase has recently been shown to inhibit the ability of Rac1 to bind GTP (52). This observation suggests the potential for negative regulation of Rho family GTPase function by serine-threonine kinases. In addition, activation of PKC in neuronal cells has been shown to induce a transient shift in actin cytoskeletal organization from predominantly filopodial projections to lamellipodial protrusions, indicating that PKC may be a negative regulator of Cdc42 function in vivo (53).

Perhaps the most significant result to come from the current study is the finding that Fyn, a Src family kinase, is required for receptor signaling to ACK-1. Inhibition of Src family kinase activity blunted mAChR-stimulated ACK-1 tyrosine phosphorylation (Fig. 5), and ACK-1 coprecipitated with Fyn tyrosine kinase, but not Src, in an agonist-dependent manner (Fig. 6). However, we observed a selective interaction of ACK-1 with Fyn rather than Src after mAChR stimulation in SH-SY5Y cells, this result does not preclude an interaction between ACK-1 and Src in other cell or receptor systems. In fact, there is often significant overlap in the interactions of multiple Src family members with a given substrate. For example, FAK has been shown to interact in vitro with both Src and Fyn, although Fyn is the principal regulator of FAK function in vivo as demonstrated by hypophosphorylation of FAK in neural tissue isolated from Fyn knockout mice (54). Thus, ACK-1 is similar to FAK and PYK-2 in that it interacts with Src family kinases and is a likely substrate for phosphorylation by this class of nonreceptor tyrosine kinase (45–48). The agonist dependence of the interaction between ACK-1 and Fyn observed in the present study suggests that receptor stimulation of Cdc42 activity and the subsequent binding of Cdc42 to ACK-1 may localize ACK-1 to the plasma membrane in close proximity to Fyn. This hypothesis is consistent with previous results of Yang and Cerione (12) who demonstrated that a constitutively active mutant of Cdc42 failed to stimulate ACK-2 tyrosine phosphorylation in vivo but that coexpression of this mutant with ACK-2 resulted in a marked enhancement of ACK-2 tyrosine phosphorylation and coassociation with Cdc42 in vivo. Collectively, these results suggest that the activation of ACK proteins by Cdc42 may be indirect, perhaps via localization of ACK to a site at which it can interact with other proteins that modulate its tyrosine phosphorylation and/or activity.

Finally, scrape loading SH-SY5Y cells with GST fusion proteins of either the Fyn-SH2 or Fyn-SH3 domain significantly attenuated mAChR signaling to ACK-1. The inhibitory effect was most pronounced in cells scrape loaded with the Fyn-SH3 domain (Fig. 7). These fusion proteins lack kinase activity and therefore likely inhibit ACK-1 phosphorylation by competing with endogenous Fyn for binding sites on the ACK-1 protein. Fyn may associate with ACK-1 via an interaction between the Fyn-SH3 domain and a C-terminal proline-rich region on ACK-1 which is exposed after its association with GTP-bound Cdc42. Previous work has suggested that Cdc42 binding to the Cdc42/Rac-interacting binding domain of ACK-2 may dissociate an intramolecular interaction between the SH3 domain and a C-terminal proline-rich region of this protein (14). A similar process may occur after the association of ACK-1 with GTP-bound Cdc42, thus allowing for ACK-1 to interact with other target proteins via these structural motifs. Indeed an interaction between ACK-1 and the SH3 domain(s) of the adaptor protein GRB-2 after epidermal growth factor receptor stimulation has been described previously (55). Fyn may also interact with phosphorylated tyrosine residues on ACK-1 via the Fyn-SH2 domain.

In summary, stimulation of mAChRs on SH-SY5Y human neuroblastoma cells elicited an enhanced tyrosine phosphorylation of the Cdc42 effector ACK-1. Agonist-stimulated ACK-1 phosphorylation was dependent on Fyn tyrosine kinase activity and the association of ACK-1 with Fyn via both SH2 domain- and SH3 domain-mediated interactions. The results suggest that the previously documented role of Cdc42 in the regulation of neurite outgrowth after cholinergic stimulation may also involve signaling via the nonreceptor tyrosine kinase ACK-1.

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Stimulation of M₃ Muscarinic Receptors Induces Phosphorylation of the Cdc42 Effector Activated Cdc42Hs-associated Kinase-1 via a Fyn Tyrosine Kinase Signaling Pathway

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