Cdk5 and Trio modulate endocrine cell exocytosis

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Summary

Hormone secretion by pituitary cells is decreased by roscovitine, an inhibitor of cyclin-dependent kinase 5 (Cdk5). Roscovitine treatment reorganizes cortical actin and ultrastructural analysis demonstrates that roscovitine limits the ability of secretory granules to approach the plasma membrane or one another. Trio, a multifunctional RhoGEF expressed in pituitary cells, interacts with peptidylglycine α-amidating monooxygenase, a secretory granule membrane protein known to affect the actin cytoskeleton. Roscovitine inhibits the ability of Trio to activate Rac, and peptides corresponding to the Cdk5 consensus sites in Trio are phosphorylated by Cdk5. Together, these data suggest that control of the cortical actin cytoskeleton, long known to modulate hormone exocytosis and subsequent endocytosis, involves Cdk5-mediated activation of Trio.

Key words: GDP/GTP exchange factor (GEF), Cytoskeleton, Actin, Peptidylglycine α-amidating monooxygenase (PAM), RhoGTPase, P21-activated kinase (PAK)

Introduction

In neurons and endocrine cells, regulated exocytosis of peptide transmitters and hormones is crucial to function. The final steps of exocytosis are triggered by a rise in intracellular calcium, eventually leading to fusion of docked secretory vesicles with plasma membrane as well as recruitment of vesicles from the reserve pool to the plasma membrane (TerBush and Holz, 1992; Lang et al., 2000). Filamentous actin (F-actin) is associated with both the plasma membrane and secretory granule membranes (Gasman et al., 1999; Gasman et al., 2003; Hong-Geller and Cerione, 2000) and studies in different systems consistently point to the actin cytoskeleton as a player in regulated exocytosis (Carbajal and Vitale, 1997; Schmidt and Hall, 1998; Valentijn et al., 2000; Burke et al., 1997; Ng et al., 2002). Despite its clear importance, the factors coordinating actin dynamics during regulated secretion are not well characterized.

Cdk5 and its activator, p35, are enriched in nerve terminals and localized to vesicular fractions, suggesting a role for Cdk5 in synaptic vesicle recycling (Tan et al., 2003; Svenningsson et al., 2004; Bibb, 2003). Another Cdk5 activator, p39, which displays a spatial and temporal pattern of expression in brain complementary to that of p35, co-localizes with F-actin in neurons and co-fractionates with detergent-insoluble cytoskeleton (Humbert et al., 2000). Phosphorylation of dynaminutes I by Cdk5 is essential for rapid endocytosis of synaptic vesicle proteins (Tan et al., 2003). Phosphorylation of Munc-18 by Cdk5 promotes its dissociation from syntaxin 1, which is then available for SNARE complex formation, potentially facilitating secretion (Shuang et al., 1998). Cdk5 inhibitors decrease insulin secretion by pancreatic β cells and catecholamine secretion from chromaffin cells, while enhancing glutamate release at hippocampal synapses (Lilja et al., 2001; Li et al., 2001; Fletcher et al., 1999). Cdk5 substrates, such as Pak, a Ser/Thr kinase whose activity is regulated by the binding of activated Rac or Cdc42, might play a role in controlling actin dynamics during regulated exocytosis (Parrini et al., 2002; Nikolic et al., 1998). Overexpression of Pak or Rac1 promotes regulated secretion from chromaffin cells (Li et al., 2003).

Small GTPases of the Rho family, key regulators of the actin cytoskeleton, are also known to play important roles in regulated secretion. They are activated by GDP/GTP exchange factors (GEFs) of the Dbl family and inactivated by GTPase activating proteins (GAPs). Chromaffin cells depolarized by exposure to high K^+ exhibit a transient increase in Rac activation, along with alterations in the organization of cortical actin (Li et al., 2003). Dbl family RhoGEFs, such as Cool/Pix, are known to interact with Pak (Bagrodia and Cerione, 1999). Based on genetic evidence from *Drosophila*, Trio, a multifunctional protein with two RhoGEF domains, and Pak are known to participate in a common pathway regulating photoreceptor axon guidance (Newsome et al., 2000). The ability of Kalirin, a *Drosophila* Trio homolog, to stimulate formation of dendritic spines is blocked by inhibitors of Pak (Penzes et al., 2003).

The organization of F-actin in endocrine cells is distinctly different from its organization in fibroblasts. Stress fibers and focal adhesions are rare, with F-actin accumulating under the plasma membrane, on the membranes of the Golgi complex and on secretory granules (Gasman et al., 1999; Gasman et al., 2003; Fucini et al., 2002). In corticotrope tumor cells (AtT-20), inducible overexpression of PAM, a secretory granule membrane protein involved in peptide hormone processing, causes changes in the organization of actin and intermediate filaments, accumulation of secretory product in the trans-Golgi network (TGN) and decreased responsiveness of the cells to secretagogs (Ciccotosto et al., 1999). The cytosolic domain of PAM interacts with Kalirin, a RhoGEF whose overexpression has dramatic effects on cytoskeletal organization (May et al.,

2002; Mains et al., 1999; Alam et al., 2001). While it is tempting to speculate that the interaction of PAM with a RhoGEF could mediate many of these changes in AtT-20 cells, Kalirin levels in pituitary are not high (Alam et al., 1996; Alam et al., 1997; Mains et al., 1999).

Kalirin shares its complex domain structure with Trio, a highly homologous RhoGEF known to be a key player in coordinating cell-matrix interactions and cytoskeletal rearrangements involved in cell movement (Blangy et al., 2000; Seipel et al., 1999). Trio and Kalirin affect actin cytoskeletal dynamics via their RhoGEF domains, which are specific for a subset of the Rho family members (Bellanger et al., 2000; Steven et al., 1998). We show that Cdk5 and p39 are expressed in anterior pituitary and affect both pituitary hormone exocytosis and cortical actin organization. Our data suggest that Cdk5 facilitates regulated exocytosis by phosphorylating Trio, increasing its GEF activity and leading to localized activation of Rac.

Materials and Methods

Cell culture and secretion assays

Anterior pituitaries from adult Sprague Dawley rats (Charles River, MA) were cultured as described (El Meskini et al., 2001). Cells were pre-treated with or without 10 μM roscovitine for 4 hours; treatment was continued during basal collections and the 30 minutes stimulation period with 2 mM $BaCl_2$ (El Meskini et al., 2001). Cells were extracted by sonication in ice-cold TMT buffer, i.e. 20 mM Na[N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] (Na-TES), 10 mM mannitol, pH 7.4, 1% Triton X-100), containing protease inhibitors (0.3 mg/ml PMSF, 50 μg/ml lima bean trypsin inhibitor, 2 μg/ml leupeptin, 16 μg/ml benzamidine and 2 μg/ml pepstatin). Media were analyzed by western blot with antisera to prolactin (IC-5, National Hormone and Peptide Program, NIDDK, A. F. Parlow) and growth hormone (JH89) (Dickerson and Mains, 1990) and by radioimmunoassay for ACTH; secreted PHM activity was assayed as described (El Meskini et al., 2001).

Tissue extraction and western blot analysis

Anterior and neuro-intermediate lobes of pituitary, superior cervical ganglia, and cerebral cortex were extracted in ice-cold TMT buffer containing protease inhibitors. Proteins from each tissue (20 μg) were fractionated by SDS-PAGE (5% gels for Trio; 4-15% for the lower molecular weight proteins), transferred to PVDF and blots probed for Trio (CT35 antiserum 1:1000, to the C-terminus of Trio) (McPherson et al., 2002), Cdk5 (C-8 antibody 1:1000, Santa Cruz) and p35 (C-19 antibody 1:1000, Santa Cruz).

Yeast two-hybrid screen

A fragment of Trio was identified as an interactor with a bait that consisted of a mixture of wild-type PAM truncated cytosolic domain (PAM-CDt) and PAM-CDt in which both casein kinase II sites had been mutated to Asp (PAM-1;Thr^{946}Asp and Ser^{949}Asp; TS/DD) (Alam et al., 1997; Alam et al., 2001). The rat pituitary library was prepared with random primed RNA from whole pituitary using pDBLeu and 10 million clones were screened, yielding a single $lacZ^+$ clone (Alam et al., 1997). The fragment (clone 24) was identical in amino acid sequence to human $Trio^{695\text{-}1136}$ (O75962); owing to three nucleotide differences, this sequence differs at three positions from that predicted for rat $Trio^{729\text{-}1170}$ (XP_226888.2). This region of Trio is homologous to rat $Kalirin^{719\text{-}1160}$, which encompasses part of spectrin-repeat 5 through the end of spectrin-repeat 9.

GST pull-down assays

A stable AtT-20 cell line expressing tetracycline-inducible PAM was induced by adding 4 μg/ml of Doxycycline for 48 hours (Ciccotosto et al., 1999). Cells were extracted in binding buffer (20 mM PIPES, pH 6.5, 2 mM Na_2 EDTA, 10 mM Na_4 pyrophosphate, 50 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, and protease inhibitor cocktail). A GST-Trio-spectrin fusion protein (amino acid residues 695-1136) was constructed. PAM-1 cell extracts were incubated with glutathione-Sepharose adsorbed GST-Trio-spectrin and the material bound to the beads was eluted with Laemmli sample buffer. Proportional amounts of eluted samples were analyzed by western blot with PAM antibody (JH629 1:1000) (El Meskini et al., 2000).

RT-PCR analysis

Total RNA was extracted and RT-PCR was performed for p39 with specific primers (forward: 5′ AGCCCCGCAGGCGGCACCGC 3′; reverse: 5′ AGCGCTGCCAGAAGCGCTCCT 3′) and β-actin as control.

Constructs for transfection

A pMT2 vector encoding HA-tagged human Trio was kindly provided by Michel Streuli (Debant et al., 1996). The NH_2-terminal HA-tag was replaced with a His_6-Myc epitope tag and full-length human Trio was subcloned into the pEAK10 expression vector. The pEAK10-Trio-GEF1 expression vector was created as described for pEAK10-Kalirin-GEF1 (May et al., 2002). A mammalian expression vector encoding full-length mouse Cdk5 was generated using a 5′ primer with an added *Eco*RI site (underlined) and a consensus translational initiation sequence (bold) (5′ CCGAATTC**GCCGCCACC**ATGCA-GAAATACGAGAAACTG 3′) and a 3′ primer with an added *Not*I site (5′ AAGCGGCCGCTACGGGGGACAGAAGTCAGA 3′). An expression vector encoding full-length mouse p35 was created in a similar manner (5′ primer: 5′ CCGAATTC**GCCGCCACC**ATG-GGCACGGTGCTG 3′; 3′ primer: 5′ AAGCGGCCGCTCACC-GATCCAGCCCCAG 3′). PCR products were subcloned into the TA-cloning vector (Invitrogen) and inserts were subcloned into the pCMS-EGFP expression vector (Clontech). Plasmid DNAs were prepared and their sequence confirmed.

Immunoprecipitation and Cdk5 kinase assay

Vectors encoding Cdk5 and p35 were separately transfected into hEK-293 cells. Cell extracts were mixed and incubated with p35 antibody on ice. Following centrifugation, the antibody/p35/Cdk5 complex was isolated by incubation with protein A agarose. The resin was rinsed and incubated with peptide substrate (500 μM) plus 1 μCi ^{32}P-γATP (3000 Ci/mmol; NEN, Boston, MA) in kinase assay buffer (20 mM MOPS, 30 mM $MgCl_2$, 1 mM EDTA, 0.1 mM DTT, 2 mM Na_3VO_4, pH 7.4) and bovine serum albuminutes (Qi et al., 1995) and tumbled for 30 minutes at 30°C. Supernatants were spotted on 2 cm^2 P18 ion exchange paper (Whatman, Maidstone, UK). After three rinses in 1% phosphoric acid solution, samples were analyzed in a liquid scintillation counter using Cerenkov counting. Peptides were synthesized, purified by HPLC and verified by mass spectroscopic analysis (Henry Keutmann, Massachusetts General Hospital, Boston, MA).

Transient transfection and Rac activation assays

Stably transfected hEK-293-PAM-1 cells in 35 mm dishes were transfected with pEAK10 expression vector encoding human Trio or Trio-GEF1 or with empty pEAK10 vector. Transfected cells were treated or not with 10 μM roscovitine for 4 hours before harvest. Cells were lysed in Mg^{2+} Lysis Buffer (MLB) (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 2%

glycerol; Upstate, Lake Placid, NY) containing protease inhibitors, 2 mM Na_3VO_4 and Pak-CRIB-GST fusion protein. After centrifugation, supernatants were incubated with glutathione-Sepharose beads with constant agitation. Beads were washed twice with MLB buffer and eluted with Laemmli sample buffer. Eluted samples were fractionated by SDS-PAGE and transferred to PVDF membranes. For positive and negative controls, pooled cell extracts were incubated with GTPγS or GDP, respectively, before incubation with Pak-CRIB-GST fusion protein.

Sub-cellular fractionation

Primary anterior pituitary cultures were either treated with 10 μM roscovitine for 4 hours or maintained without drug. Control and treated cells were then processed with a ball-bearing homogenizer (H&Y Enterprises, Redwood City, CA) in ice-cold homogenization buffer (150 mM sucrose, 60 mM KCl, 2.5 mM $MgCl_2$, 20 mM HEPES-KOH, pH 7.5) containing protease inhibitors. Differential centrifugation yielded a P1 (crude nuclear, pellet 1) fraction (900 ***g*** for 5 minutes), a fraction enriched in secretory granules and (P2, 14,200 ***g*** for 15 minutes), a fraction enriched in lighter membrane compartments (P3, 35,5000 ***g*** for 15 minutes), and the final supernatant (cytosol). Equal proportions of the fractions were subjected to western blot analysis.

Immunofluorescence

Primary anterior pituitary cells grown on glass slides were fixed in pre-warmed 4% formaldehyde in PBS, permeabilized and incubated with primary antibodies (Cdk5 monoclonal antibody 1:100, J-3, Santa Cruz; prolactin polyclonal antibody 1:100,000, IC-5, National Hormone and Peptide Program, NIDDK, A. F. Parlow). Antisera to ACTH (Kathy and JH44) and growth hormone (JH89) were characterized previously (Dickerson and Mains, 1990). The Rac1 antibody was from BD Biosciences. TRITC-phalloidin (Sigma P1957) was used to detect filamentous actin. Stacks of images were acquired from cover-slipped slides under oil using a 60× lens. The images were obtained using OpenLab and deconvolved using Volocity software (Improvision). The images shown are at comparable distances above the culture dish.

Electron microscopy

Anterior pituitary cultures were fixed, dehydrated and embedded in a thin layer of plastic. Somatotropes were identified based on morphological criteria (Stachura et al., 1986); identification was confirmed using immunoelectron microscopy with an antiserum to GH (JH89) (Dickerson and Mains, 1990). Somatotropes were systematically photographed and two parameters were measured: plasma membane/granule and granule/granule distance. All morphologically identified somatotropes were photographed, with no regard to granule density in the individual cells. For both types of granule measurements, sampling was performed starting from the left hand corner of the section, using an overlay grid; the first five peripheral granules hit by the grid were chosen. Both types of measurements thus only analyze granules in the peripheral zone of the cell and give no information about overall granule density in the cell. Measurements from two experiments were pooled and analyzed blindly. Cell membrane-granule distance: the distance between the cell membrane and all peripheral granules within 400 nm of the cell membrane was measured (4 nm resolution) in 18 control cells (81 granules) and 21 roscovitine treated cells (104 granules). Granule-granule distance: a transparent overlay was placed over each print; the first five granules hit by a cross were chosen and the distance from each granule to all of the surrounding granules was measured at a resolution of 4 nm, up to a maximum of 400 nm. Eight prints of control cells (40 granules) and seven prints of roscovitine treated cells (35 granules) were analyzed, with 2-7 measurements/granule. Data were analyzed using the Kolmogorov-Smirnov distribution (Conover, 1999).

Results

Cdk5 has a role in endocrine cell exocytosis

Although Cdk5 is often described as a nervous-system-specific kinase (Nikolic et al., 1998; Dhavan and Tsai, 2001; Tomizawa et al., 2002), we tested the hypothesis that Cdk5 plays a role in the regulated exocytosis of pituitary hormones. Roscovitine, a relatively selective inhibitor of Cdk5 (Meijer and Raymond, 2003; Tomizawa et al., 2002), was used to assess the role of Cdk5 in regulated secretion. $BaCl_2$ was used as a secretagog because of its ability to mimic Ca^{2+} in the late steps of regulated secretion (TerBush and Holz, 1992; El Meskini et al., 2001). Peptidylglycine α-amidating monooxygenase (PAM), an enzyme involved in the maturation of peptide hormones, is a membrane protein present in the secretory pathway. PAM is present at varying levels in all hormone-secreting pituitary cells, so quantification of PHM activity in the medium allows a general assessment of secretory activity in resting and stimulated pituitary cells (El Meskini et al., 2000). During secretory granule maturation, endoproteolytic cleavage of PAM produces two functional domains, soluble secreted peptidylglycine α-hydroxylating monooxygenase (PHM) and membrane bound peptidyl-α-hydroxyglycine α-amidating lyase (PAL) (Eipper et al., 1993). Consistent with previous studies, we found that basal release of PHM activity was increased 10-fold by barium stimulation (Fig. 1A, white bars). In roscovitine-treated cells, the ability of barium to stimulate secretion of PHM activity was reduced more than 50% compared with control cells (Fig. 1A, black bars), with no change in cell content of the enzyme (data not shown).

To determine whether Cdk5 was expressed in the pituitary, western blot analysis was performed. Similar levels of Cdk5 were detected in the anterior pituitary, neurointermediate lobe (NIL) and sympathetic neurons (SCG), with only slightly higher levels of Cdk5 in the cerebral cortex (Fig. 1B, top). One of two alternative essential activators of Cdk5, p35, was detected in the cortex and NIL, but not in the anterior pituitary (Fig. 1B, bottom). The p35 detected in the neurointermediate lobe of the pituitary presumably originates from the neurosecretory terminals of hypothalamic neurons. As we lacked antibodies specific for p39, the other known Cdk5 activator, tissues were probed for expression of p39 by RT-PCR using p39-specific primers. The levels of p39 mRNA were similar in anterior pituitary, superior cervical ganglion and cerebral cortex, but clearly lower in neurointermediate lobe (Fig. 1C, top).

We next examined the role of Cdk5 in exocytosis of specific pituitary hormones (Fig. 2). Upon incubation with $BaCl_2$, prolactin release increased between 6- and 8-fold with respect to basal secretion (Fig. 2A). Incubation with roscovitine reduced the $BaCl_2$-stimulated release of prolactin to 42±18% of control. Basal release of prolactin, which is high in dissociated pituitary cell cultures due to the absence of tonic dopaminergic inhibition, was also reduced by roscovitine treatment (Fig. 2A). Roscovitine exerted an inhibitory effect of similar magnitude on the barium-stimulated exocytosis of

growth hormone (Fig. 2B) and adrenocorticotropic hormone (ACTH) (Fig. 2C).

Next, we probed for Cdk5 in specific pituitary endocrine cell types. Using immunocytochemistry, we identified Cdk5 in lactotropes (Fig. 2D, upper), somatotropes (Fig. 2D, lower) and corticotropes (not shown). Cdk5 staining was non-uniform, suggesting that the majority of the enzyme is associated with some structure, but its subcellular distribution was distinct from that of prolactin or GH (Fig. 2D). These data suggest that Cdk5 plays a direct role in regulated secretion and that the inhibitory effect of roscovitine occurs in hormone-secreting cells, not indirectly via an effect on non-endocrine cells (e.g. folliculostellate cells) present in our cultures. Together, these results point to a role for Cdk5 in regulated secretion from anterior pituitary endocrine cells.

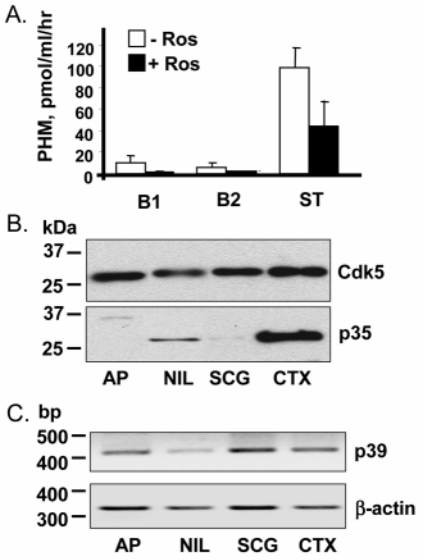


Fig. 1. Cdk5 plays a role in exocytosis in pituitary endocrine cells. Cultured rat anterior pituitary cells were pre-incubated with (+Ros) or without (-Ros) roscovitine (10 μM) for 4 hours and then incubated with the same medium for two collection periods of 30 minutes each (B1 and B2) followed by incubation in medium containing secretagog (2 mM $BaCl_2$, ST) with or without drug for 30 minutes. The media were collected and assayed for peptidylglycine α-hydroxylating monooxygenase activity (PHM) (A). Extracts of rat anterior pituitary (AP), neurointermediate lobe (NIL), superior cervical ganglion (SCG) and cerebral cortex (CTX) were probed for Cdk5 (B, top) and p35 (B, bottom) by western blot. The same tissues were tested for p39 expression by RT-PCR (C, top). β-actin amplification was carried out in parallel to provide a loading control (C, bottom). One representative experiment (of at least three) is shown.

Roscovotine induces cortical actin rearrangement

Studies in different cellular systems have consistently revealed an essential role for F-actin in regulated exocytosis (Carbajal and Vitale, 1997; Lilja et al., 2001; Muallem et al., 1995; Valentijn et al., 2000). Nevertheless, the mechanisms through which actin dynamics affect exocytosis are poorly understood. To investigate the role of the actin cytoskeleton in the inhibition of regulated secretion by roscovitine, control and roscovitine-treated cells were stained simultaneously with fluorescently tagged phalloidin and antiserum to prolactin. Control cells showed the characteristic prolactin perinuclear staining, with granules appearing as scattered puncta (Fig. 3A, center left). As expected if the secretory granules accumulated due to the reduced basal level of exocytosis, roscovitine treatment seemed to increase the number of puncta, (Fig. 3A, center right). Interestingly, roscovitine treatment consistently caused a marked increase in phalloidin staining immediately adjacent to the plasma membrane (Fig. 3A, compare top right with left). Increased phallodin staining adjacent to the plasma membrane was also observed in somatotropes after roscovitine treatment (Fig. 3B, top), suggesting an accumulation of cortical filamentous actin.

To understand the effects of roscovitine treatment, primary anterior pituitary cells were examined by electron microscopy (Fig. 4A). Somatotropes are the most prevalent cell type in these cultures and were identified morphologically; their

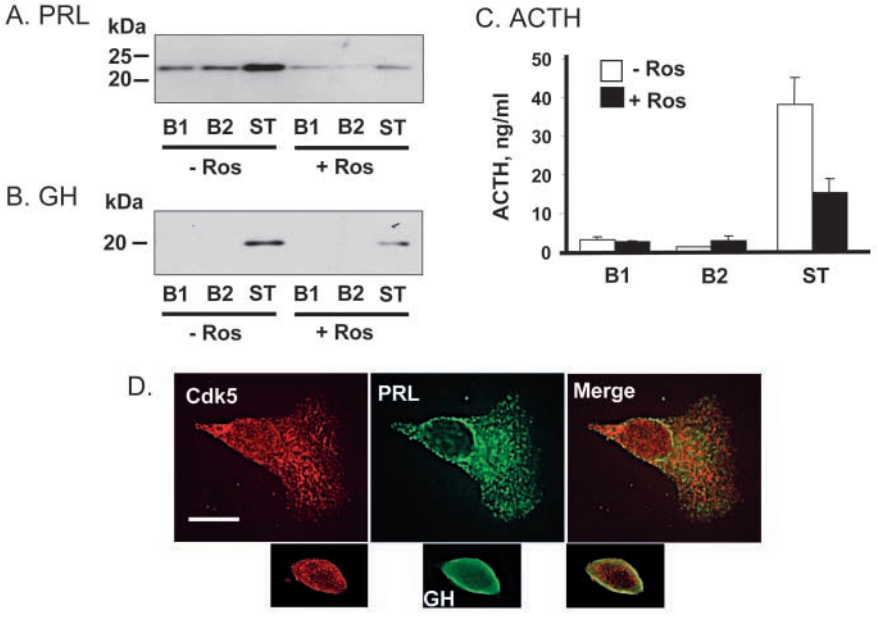


Fig. 2. Cdk5 plays a role in exocytosis of multiple pituitary hormones. Media from cultured rat anterior pituitary cells treated with or without roscovitine (as in Fig.1A) were analyzed for prolactin (PRL) (A) or growth hormone (GH) (B) by western blot and for ACTH (C) by radioimmunoassay after a basal-basal-stimulation regimen as in Fig. 1A. One representative experiment (of at least three) is shown.
(D) Immunocytochemical analysis was performed by co-staining fixed cultures for prolactin and Cdk5 (upper) or GH and Cdk5 (lower); bar, 10 μm.

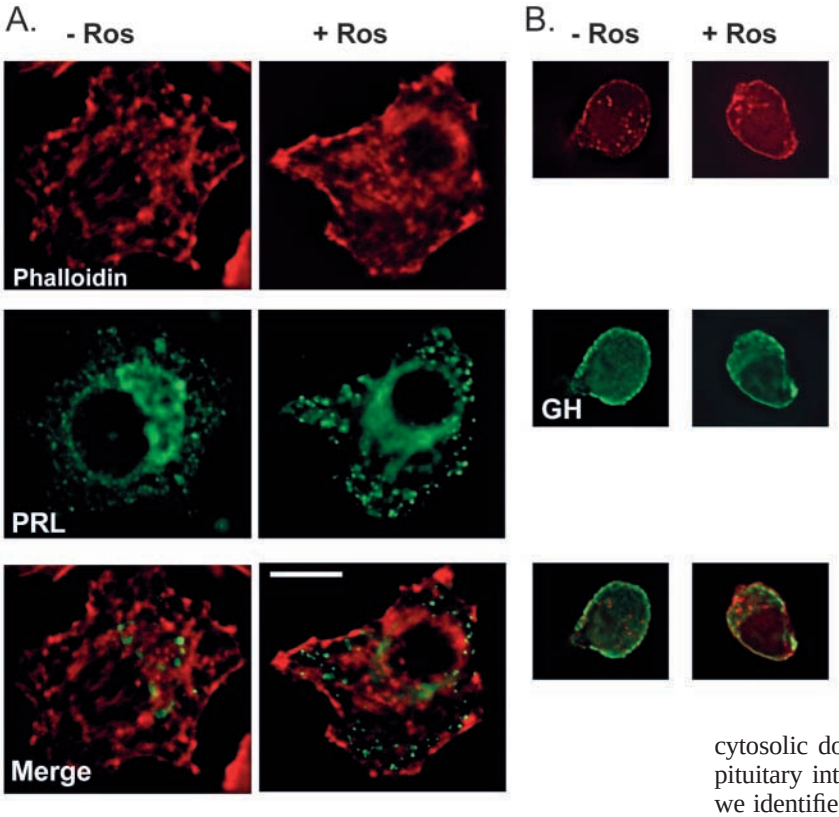


Fig. 3. Roscovitine causes cortical actin rearrangement. Anterior pituitary cells maintained in control medium (–Ros) or treated with 10 μM roscovitine (+Ros) were fixed and visualized simultaneously for F-actin using TRITC-phalloidin (top) and prolactin (center) (A) or F-actin (top) and growth hormone (center) (B). Bottom, merged images; bar, 10 μm.

identity was confirmed by immunoelectron microscopy using a GH antibody (Fig. 4C). F-actin is not well preserved by conventional fixation, and neither modified fixation methods (Maupin-Szamier and Pollard, 1978), negative staining of cytoskeletal preparations nor scanning electron microscopy of gold-platinum-shadowed cytoskeletal preparations proved suitable for examining the cortical actin network in rounded cells. Therefore the effects of roscovitine treatment on the cortical region were assessed by measuring two parameters: the distance between granules and the plasma membrane (Fig. 4B, top left) and the distance between neighboring granules (Fig. 4B, top right). In both cases, the histograms revealed a roscovitine-mediated increase in the mean distance (asterisks). Statistical analysis using the Kolmogorov-Smirnov distribution (Conover, 1999) confirmed the conclusion that secretory granules in roscovitine treated cells are not as closely apposed to the plasma membrane or each other as observed in control cells (Fig. 4B, bottom). Roscovitine treatment did not affect the morphology of granules in the trans-Golgi network (TGN) area (not shown). Although indirect, these data are consistent with an accumulation of cortical filamentous actin following inhibition of Cdk5 with roscovitine.

Trio, a pituitary PAM interactor, is a Cdk5 substrate

Our data indicate that Cdk5 activity plays a role in regulating cortical actin organization. In neurons, Cdk5 is known to inactivate Pak, a Rac- and Cdc42-activated kinase that modulates actin polymerization (Nikolic et al., 1998). In earlier studies, we found that inducible overexpression of PAM, like exposure to roscovitine, caused both an accumulation of cortical actin and a decrease in the ability of $BaCl_2$ to stimulate hormone secretion (Ciccotosto et al., 1999). While the lumenal domains of PAM catalyze the C-terminal amidation of glycine-extended peptides (Eipper et al., 1993), its cytosolic domain is essential for its routing and identified cytosolic interactors include Kalirin (Fig. 5A) and P-CIP2 (Alam et al., 1996). Kalirin, a GEF for Rac and RhoG, plays a central role in neurons, regulating axon initiation and outgrowth as well as dendritic branching and spine formation, but is poorly expressed in the pituitary (Penzes et al., 2001; May et al., 2002).

A RhoGEF able to interact with a secretory pathway membrane protein might play a role in regulated exocytosis. Therefore, we used the cytosolic domain of PAM as bait to search for a homologous pituitary interactor. By screening a rat pituitary cDNA library, we identified Trio, a Rho GEF that is a paralog of Kalirin. The region of Trio identified as a PAM interactor is composed entirely of spectrin-like repeats and corresponds to the region of Kalirin that interacts with PAM (Alam et al., 1996; Alam et al., 1997) (Fig. 5A).

We expressed the PAM-interacting fragment of rat Trio as a GST fusion protein to examine the physiological significance of the interaction. In a previous study, we showed that PAM aggregates at slightly acidic pH, the pH in maturing secretory granules and in the endocytic pathway (Bell-Parikh et al., 2001). The binding of membrane PAM to GST-Trio spectrin was therefore evaluated in pull-down assays after solubilization of membranes from PAM-1 expressing cells at pH 7.5 or pH 6.6. At pH 7.5 (not shown), PAM did not bind to GST-Trio-spectrin immobilized on glutathione-Sepharose. In contrast, mildly acidic conditions enabled binding (Fig. 5B). Western blot analysis of the input sample reveals the expected mixture of intact PAM-1, membrane PAL (PALm) and soluble PHM (Ciccotosto et al., 1999). Approximately 1% of the intact PAM-1 binds specifically to resin containing GST-Trio-spectrin. Soluble PHM lacks a cytosolic domain and its lack of binding serves as a control for specificity. Despite the fact that PALm contains the cytosolic domain, it does not bind to GST/Trio-spectrin (Fig. 5B), suggesting important differences between full-length PAM and fragments derived from it.

Western blot analysis indicated that full-length Trio is expressed in adult rat anterior pituitary, with detectable amounts in the neurointermediate lobe and superior cervical ganglion, but undetectable amounts in adult cerebral cortex (Fig. 5C). Its distribution suggests a physiological role for Trio as a pituitary RhoGEF. Endogenous pituitary Trio could not be localized reliably by immunostaining with antibodies currently available. However, when exogenously expressed in AtT-20

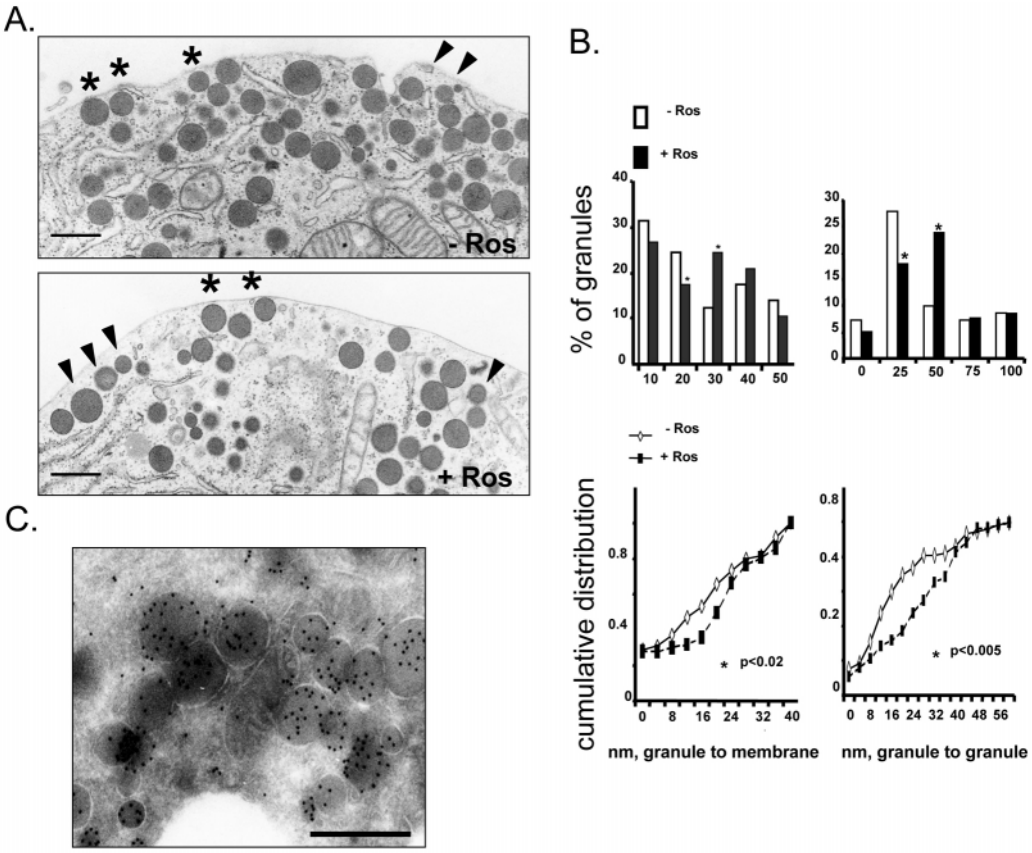


Fig. 4. Treatment with roscovitine alters granule localization beneath the plasma membrane. (A) Representative somatotropes from cultures treated with roscovitine as in Fig. 3 (– or +Ros) and then prepared for electron microscopy. Asterisks mark granules closely apposed to the plasma membrane; arrowheads mark granules separated from the plasma membrane; bar, 500 nm.(B) Granules were pooled based on their distance from the plasma membrane (top left) or from other granules (top right) in somatotropes treated with (filled bars) or without roscovitine (empty bars). Kolmogorov-Smirnov cumulative distributions for granule-to-membrane (bottom left) and granule-to-granule (bottom right) distances are shown. (C) Morphological identification of somatotropes in electron micrographs was confirmed by immuno-gold labeling of growth hormone; bar, 500 nm.

cells (a model for pituitary), Myc-tagged recombinant Trio is most concentrated at the plasma membrane (Fig. 5D).

These data suggest that Trio, a known regulator of the actin cytoskeleton, could function in a pathway through which secretory pathway membrane proteins coordinate cytoskeletal organization and the processes of exocytosis and endocytosis. Both genetic and biochemical studies place Trio and Pak in the same regulatory pathway (Newsome et al., 2000; Penzes et al., 2003; Lin and Greenberg, 2000). Pak kinase is activated by the binding of activated Rac or Cdc42 and inactivated when phosphorylated by Cdk5 (Nikolic et al., 1998). The ability of both PAM overexpression and exposure to roscovitine to inhibit $BaCl_2$-elicited hormone exocytosis and cause the accumulation of cortical actin suggests a shared pathway for PAM and Cdk5 (Ciccotosto et al., 1999; Mains et al., 1999). We therefore explored the possibility that Trio might be a Cdk5 substrate. Human, mouse, rat and *Xenopus* Trio contain three conserved consensus Cdk5 phosphorylation sites, (Ser/Thr)-Pro-X-(Arg/Lys/His): S^{1720}PVR, S^{2358}PRK and T^{2454}PRH (Fig. 5A).

We tested the ability of synthetic peptides containing these sites to serve as Cdk5 substrates (Fig. 5E). As a control, the essential proline residue in the first peptide was replaced with an alanine (designated SAVR). Only the first potential Cdk5 site in Trio (SPVR) has an equivalent site in Kalirin. In vitro kinase assays demonstrated that all three peptides were phosphorylated when incubated with Cdk5/p35 complex. In contrast, although retaining the target serine residue, the SAVR peptide was not phosphorylated by Cdk5/p35. As expected, phosphorylation of the three substrate peptides was inhibited by the addition of roscovitine (Fig. 5E).

Roscovitine treatment decreases Trio GEF activity

The N-terminal GEF domain (GEF1) of Trio catalyzes the activation of Rac and RhoG (Blangy et al., 2000). Its structure has been determined by NMR, revealing a critical role for the pleckstrin homology domain (Liu et al., 1998). To date, the GEF activity of full-length Trio has not been reported. Because Trio interacts with PAM, hEK-293 cells stably expressing PAM-1 were used to ask whether full-length Trio activates Rac and, if so, whether roscovitine treatment alters this ability. Treatment with roscovitine did not alter the level of expression of full-length Trio or its isolated first GEF domain (T-GEF1) (Fig. 6A, top) or the level of endogenous Rac (Fig. 6A, center). As assessed by binding to the CRIB domain of Pak, expression of full-length Trio or T-GEF1 resulted in the activation of Rac (Fig. 6A, bottom). Roscovitine treatment decreased the GEF activity of full-length Trio, but did not affect the ability of T-GEF1 to activate Rac (Fig. 6A, bottom). Data from several similar experiments were quantified (Fig. 6B). The baseline activation of Rac was 5.4±0.5% of the level of Rac activation observed following expression of Trio and decreased to 3.3±0.6% after pre-treatment with roscovitine. Roscovitine pre-treatment reduced Rac activation by Trio to 45±2% of control levels (Fig. 6B).

Roscovitine treatment modifies Rac localization

We used subcellular fractionation to ask whether roscovitine treatment affected the localization of Rac in primary cultures of pituitary cells (Fig. 7A, top). Under control conditions, most

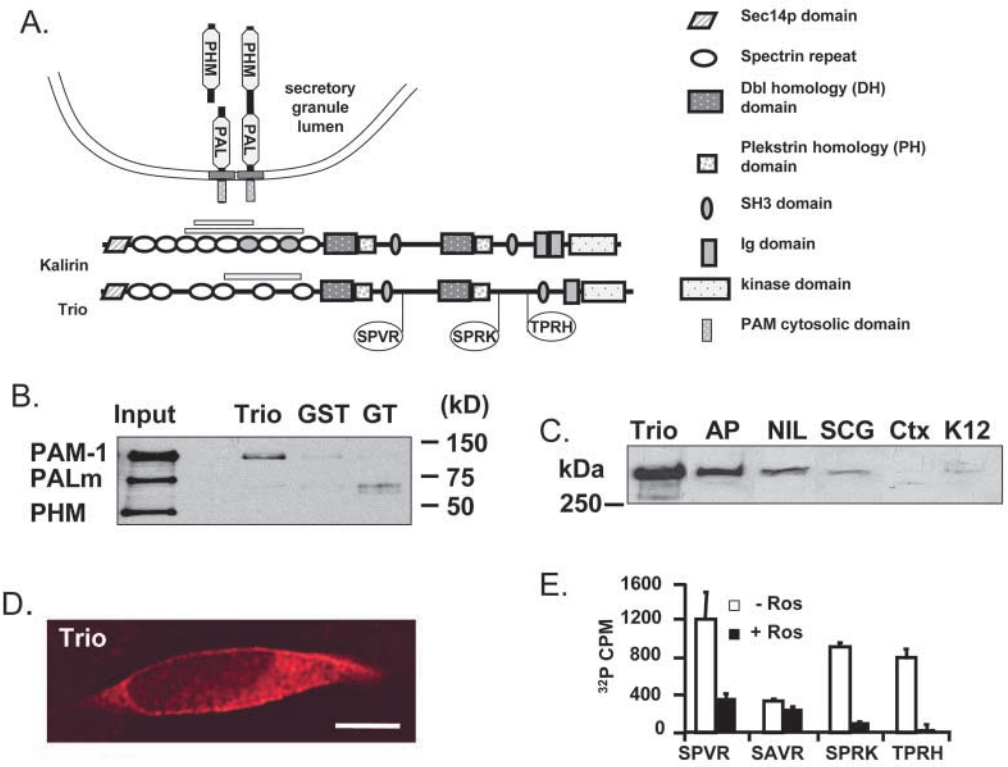


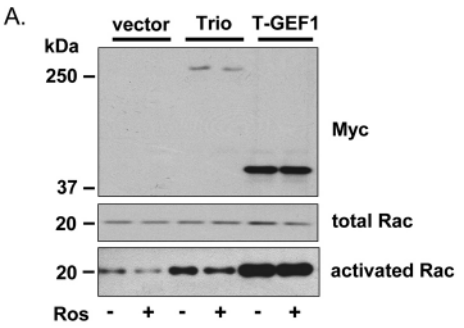
Fig. 5. Trio, a RhoGEF that interacts with a secretory pathway membrane protein, is highly expressed in anterior pituitary and is a Cdk5 substrate. (A) Data obtained from a yeast two-hybrid screen of a pituitary cDNA library, using the cytosolic domain of PAM as bait, are summarized. The products of PAM processing and their membrane topology are shown. Kalirin, a paralog of Trio, was previously identified as a PAM interactor. The region of rat Trio identified is identical in amino acid sequence to human Trio, residues 695-1136. The predicted structures of Kalirin and Trio, and the region of each that interacts with PAM, are shown. Consensus sites for Cdk5 phosphorylation are indicated for Trio. (B) Extracts of an AtT-20 cell line stably expressing PAM-1 were incubated with glutathione-Sepharose beads that had been incubated with GST-Trio-spectrin fusion protein (Trio), GST or buffer (GT). Adsorbed proteins were eluted and analyzed by immunoblotting using an antibody specific for PAM; the amount of input was 10% of that of bound fractions. (C) Extracts (20 μg protein) of rat anterior pituitary (AP), neurointermediate lobe (NIL), superior cervical ganglion (SCG) and cerebral cortex (Ctx) were probed with a Trio antibody; extracts of cells transiently expressing Trio or Kalirin-12 (K12) were analyzed at the same time. (D) AtT-20 cells transiently expressing myc-tagged Trio were visualized with antibody to the myc epitope; bar, 10 μm. (E) Three peptides encompassing the potential Cdk5 phosphorylation sites in Trio (shown in A) were synthesized; the essential Pro residue in the first site was mutated to Ala, yielding a control peptide, SAVR. The peptides were incubated for 30 minutes with immunoprecipitated p35/Cdk5 and ^{32}P-γ-ATP in kinase assays in the absence (white bars) or presence (black bars) of 10 μM roscovitine. Three independent experiments were performed.

of the Rac was recovered in the cytosol fraction (SN). The secretory granule enriched P2 fraction contained only 13±4% of the total Rac. Upon incubation with roscovitine, the amount of Rac recovered in the secretory granule fraction was reduced to 4±2% of the total (Fig. 7A, top). Despite the fact that phalloidin staining revealed a roscovitine-mediated alteration in filamentous actin, no roscovitine-mediated changes in the distribution of actin were apparent upon subcellular fractionation (Fig. 7A, bottom), probably because F-actin is not sufficiently stabilized under our homogenization conditions. We next used immunocytochemistry to evaluate the localization of Rac in pituitary endocrine cells. Under control conditions, the Rac in corticotropes concentrates at the plasma membrane (Fig. 7B left) and is not associated with the ACTH-containing secretory granules. Roscovitine treatment produced small discontinuities in the previously continuous ring of Rac staining in the sub-plasmalemmal region (Fig. 7B, right). Similar subtle shifts in the localization of Rac were observed in somatotropes and mammotropes (data not shown). Although both subcellular fractionation and immunofluorescence localization demonstrate an effect of roscovitine treatment on Rac, it is difficult to compare the behavior of Rho proteins, which interact reversibly with Rho GDI and with membranes via their prenyl tails, using these approaches (Gasman et al., 2004).

Discussion

Secretory granule release is a carefully orchestrated process. Despite several studies demonstrating a role for F-actin in exocytosis, the mechanisms that regulate the complex changes in the cortical actin cytoskeleton that occur during exocytosis and the subsequent endocytosis that must follow are poorly understood. Our study demonstrates that inhibition of Cdk5 activity diminishes exocytosis from several types of pituitary endocrine cell. We identify Trio, a RhoGEF that is prevalent in the pituitary and localizes to the sub-plasmalemmal region, as a Cdk5 substrate whose ability to activate Rac is decreased by treatment with the Cdk5 inhibitor roscovitine (Figs 5 and 6). The ability of Trio to interact with PAM, a secretory pathway membrane protein, suggests a mechanism through which cytoskeletal changes might be coordinated with specific exocytotic or endocytotic events (Fig. 8).

Many of our conclusions are based on the assumption that roscovitine acts by inhibiting Cdk5. Cdk5 is as prevalent in the pituitary as it is in the CNS and is present in most pituitary endocrine cells. At the concentration used, roscovitine would also inhibit Cdk1 and Cdk2 (Meijer and Raymond, 2003), but the low levels of Cdk2 in the anterior pituitary and the lack of expression of Cdk1 in corticotropes (data not shown) make Cdk5 the most likely target of roscovitine. Our data also show that p39 is highly expressed in the anterior pituitary. In



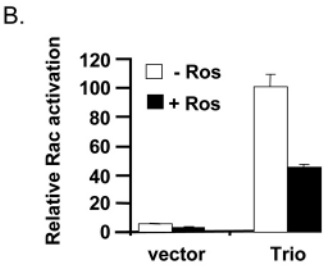


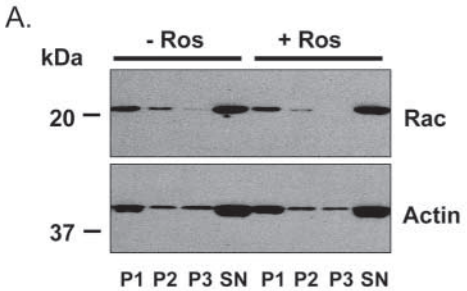
Fig. 6. Roscovitine inhibits Trio-GEF activity. (A) hEK293 cells stably expressing PAM-1 were transiently transfected with vector, myc-tagged Trio (Trio) or Myc-tagged Trio-GEF1 (T-GEF1). Control cells (–) and cells pre-treated with roscovitine (+) were extracted and aliquots were probed for Myc-tagged products (top) and total Rac (center); activated Rac was assessed following adsorption to GST-Pak-CRIB bound to glutathione agarose (bottom). A representative experiment is shown. (B) Rac activation was quantified from three independent assays; error bars show standard deviation.

neurons, p39 co-fractionates with the cytoskeleton (Humbert et al., 2000); its subcellular localization in pituitary endocrine cells has not yet been evaluated. Cdk5 substrates known to play a role in regulated secretion include amphiphysin 1 (Floyd et al., 2003), Munc18 (Shuang et al., 1998) and Pak, a Rac/Cdc42 activated serine/threonine kinase that affects the actin cytoskeleton through its ability to activate LIMK (Gohla and Bokoch, 2002). In neurons, hyperphosphorylation of Pak by Cdk5 inhibits its kinase activity (Nikolic et al., 1998).

We found that roscovitine inhibits the ability of pituitary cells to release hormone in response to secretagog (Figs 1 and 2) and suggest that this is due to the appearance of a continuous cortical ring of filamentous actin (Fig. 3). Although it is widely accepted that the cortical actin cytoskeleton acts as a physical barrier, keeping secretory granules from reaching the plasma membrane (Burke et al., 1997; Ng et al., 2002; Valentijn et al., 2000) direct ultrastructural visualization of cortical actin in pituitary endocrine cells proved difficult. Despite use of the approaches that allowed direct analysis of cortical actin in flattened cells (Small and Herzog, 1994; Svitkina and Borisy, 1998), we had to rely on measurements of granule spacing to quantify the effects of roscovitine treatment on the spacing of cortical granules (Fig. 4). Secretory granules were depleted from a 50 nm region subjacent to the plasma membrane following roscovitine treatment (Fig. 4C). In chromaffin cells, this region contains the granules thought to undergo exocytosis (Lang et al., 2000; Muallem et al., 1995; Vitale et al., 1995). Coupled with the changes in phalloidin staining, our data support a role for Cdk5 in regulating cortical F-actin (Fig. 8).

When over-expressed, PAM, a secretory pathway membrane protein essential to the maturation of many bioactive peptides, causes changes in the organization of the cortical actin cytoskeleton and in the stimulated release of hormone (Ciccotosto et al., 1999). We show here that the cytosolic domain of PAM interacts with Trio, which is abundant in the anterior pituitary (Fig. 5). The interaction of PAM with Trio may localize Trio or alter its catalytic activity (Fig. 8). When assessed in a GST pull-down assay, only a small fraction of the total PAM-1 bound to Trio; despite having the same cytosolic domain, a cleaved form of PAM-1 (PALm), did not bind Trio (Fig. 5). Because the properties of PAM-1 are sensitive to pH (Bell-Parikh et al., 2001) and phosphorylation (Steveson et al., 2001), these same factors may influence the ability of PAM to interact with Trio. Similarly, the endoproteolytic cleavage that generates PALm from PAM-1 occurs in maturing secretory granules, and could serve as a readout for lumenal events.

Both Rac and exogenous Trio localize to the cortical region of pituitary endocrine cells (Figs 5 and 7). Because roscovitine treatment decreased the ability of Trio to produce activated Rac in transfected fibroblasts (Fig. 6), we propose that phosphorylation of Trio by Cdk5 stimulates its GEF activity. A change in the activity of either Trio-GEF domain could affect the dynamics of actin polymerization through the actions of activated RhoGTPases on effector proteins like Pak and ROCK (Schmidt and Hall, 1998) (Fig. 8). Filaminutes A (also known as filaminutes 1 or ABP280), an actin filament cross-linking protein, interacts with the first PH domain of Trio and is essential to the ability of Trio-GEF1 to induce ruffling (Seipel et al., 1999). Filaminutes organizes F-actin into a gel-like structure and links the actin cytoskeleton to the plasma



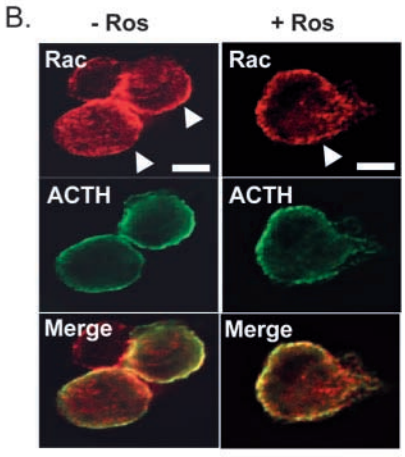


Fig. 7. Roscovitine reduces the fraction of Rac associated with secretory granules and the plasma membrane. (A) Proportional aliquots of the crude nuclear (P1), secretory granule/plasma membrane enriched (P2), microsomal (P3) and cytosol (SN) fractions of cultured pituitary cells treated or not with roscovitine (+ or –Ros) were analyzed by immunoblotting for Rac (top) and actin (bottom); similar results were observed in three experiments. (B) Cultured anterior pituitary cells were either treated with roscovitine (+Ros) or not (–Ros), fixed and stained with a Rac antibody and an ACTH antibody. Arrowheads indicate Rac staining under the plasma membrane; bars, 5 μm.

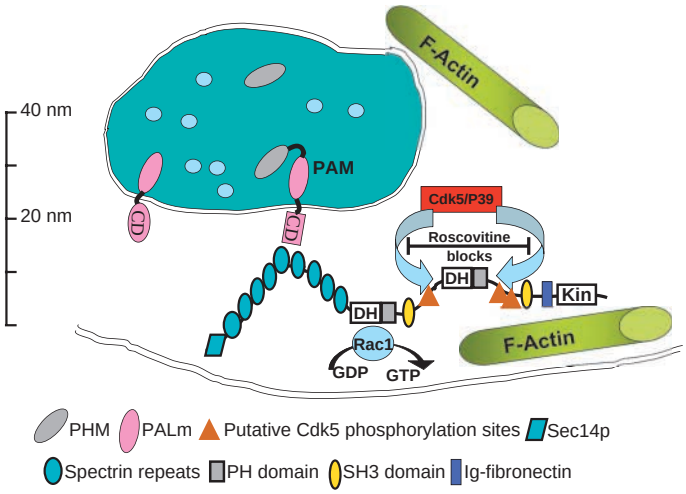


Fig. 8. A Role for Cdk5 and Trio in regulated secretion from pituitary endocrine cells. Cdk5 and p39 are highly expressed in the pituitary. Inhibition of Cdk5 by roscovitine results in a decrease in the ability of $BaCl_2$ to stimulate hormone release, in the accumulation of subplasma membrane filamentous actin and in a decrease in the ability of cortical large dense core vesicles to approach the plasma membrane or each other. Trio, a substrate for Cdk5 and Rho GEF, interacts with the cytosolic domain of PAM, a membrane protein localized to the regulated secretory pathway in pituitary endocrine cells. The ability of Trio to activate Rac is decreased following roscovitine treatment. Phosphorylation of Trio by Cdk5/p39 and local activation of Rac may play a role in coordinating the complex changes in the organization of the subplasma membrane actin cytoskeleton that must accompany regulated secretion and subsequent endocytosis (Bokoch, 2003).

membrane by binding an assortment of membrane proteins (Robertson et al., 2003). The interaction of Trio with filaminutes may orient the former within the highly organized cortical actin mesh.

The activity of many GEFs of the Dbl family, to which Trio and Kalirin belong, is regulated by phosphorylation (Zheng, 2001). Under basal conditions, Trio is Ser- and Tyr-phosphorylated in COS-7 cells (Medley et al., 2003; Debant et al., 1996). Peptides corresponding to the three consensus Cdk5 phosphorylation sites in Trio are each substrates for recombinant Cdk5 in vitro (Fig. 5E). Because roscovitine treatment decreases the GEF activity of full-length Trio but not that of the isolated Trio GEF1 domain (Fig. 6), the simplest explanation is that phosphorylation of Trio by Cdk5 alters its conformation or its ability to interact with effectors, thus increasing its GEF1 activity. Studies with purified proteins will be needed to distinguish these possibilities.

The concept that the state of polymerization of cortical actin is crucial in secretory granule docking, exocytosis and endocytosis is well accepted. Nevertheless, studies on the roles of the RhoGTPases in these processes have produced contradictory results, only in part explained by the different methods used or the different tissues analyzed (Carbajal and Vitale, 1997; Schmidt and Hall, 1998; Gasman et al., 1999; Hong-Geller and Cerione, 2000; Lilja et al., 2001; Muallem et al., 1995; Vitale et al., 1995). We suggest that the inhibitory effect of roscovitine on stimulated secretion of hormone is mediated in part by rearrangement of the cortical actin cytoskeleton, with formation of a sub-plasmalemmal barrier that prevents access of secretory granules to the plasma membrane and inhibits endocytosis. Our results are consistent with a cellular cascade in which Trio phosphorylation by Cdk5 activates its GEF activity, facilitating secretory granule access to the plasma membrane. The identification of effectors upstream of Cdk5 and downstream of activated Rac is required to elucidate the pathway and validate the model proposed.

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