Subcellular Localization of Cyclic Nucleotide Phosphodiesterase Type 10A Variants, and Alteration of the Localization by cAMP-dependent Protein Kinase-dependent Phosphorylation*

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Our previous studies have suggested that two phosphodiesterase type 10A (PDE10A) variants, PDE10A1 and PDE10A2, transcripts, are mainly expressed in humans and that PDE10A2 and PDE10A3 transcripts are major variants in rats. In the present study, immunoblot analysis demonstrated that PDE10A proteins, especially PDE10A2, are more abundant in membrane fractions than in cytosolic fractions of rat striatum. Recombinant PDE10A1 and PDE10A3 were produced only in cytosolic fractions of transfected PC12h cells. By contrast, recombinant PDE10A2 was present mainly in subcellular fractions. This finding agreed well with the result of subcellular fractionation of PDE10A in rat striatum. Immunocytochemical analysis showed that PDE10A2 was localized in the Golgi apparatus of transfected PC12h cells. PDE10A2 was phosphorylated by cAMP-dependent protein kinase (PKA) at Thr\textsuperscript{16}. Interestingly, recombinant protein of wild-type PDE10A2, but not PDE10A2 mutant with an Ala replacement at Thr\textsuperscript{16}, was distributed to cytosolic fractions by co-transfection with a plasmid encoding the catalytic subunit of PKA. A PDE10A2 mutant with Glu substitution at Thr\textsuperscript{16}, which can be a mimic of phosphorylation, was localized in the cytosolic fractions of transfected PC12h cells. These observations implied that phosphorylation of PDE10A2 at Thr\textsuperscript{16} by PKA caused alteration of subcellular localization of PDE10A2 from the Golgi apparatus to cytosol. It is hypothesized that cAMP signaling in the Golgi area and the cytosol in neurons is controlled through alteration of subcellular localization of PDE10A2 by activation of PKA in response to intracellular elevations of cAMP.

Cyclic nucleotides, cAMP and cGMP, control physiological functions in neuronal networks. Dopamine, adenosine, and vasoactive intestinal peptide are neuronal transmitters and cause elevation of intracellular cAMP levels in striatal neurons (1–3). Cyclic AMP-dependent protein kinase (PKA),

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The abbreviations used are: PKA, cAMP-dependent protein kinase; PDE, cyclic nucleotide phosphodiesterase; AKAP, A-kinase-anchoring protein; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; FITC, fluorescence-activated cell sorting; CHAPS, 3-[3-cholamidopropyl(dimethylammonio)]-1-propanesulfonic acid; WT, wild type.
 Localization of PDE10A

EXPERIMENTAL PROCEDURES

Materials—[3H]cGMP, [3H]cAMP, and [γ-32P]ATP were from Amersham Biosciences. Cyclic GMP, cAMP, Crotalus atrox snake venom, erythro-9(2-hydroxy-3-nonyl)-adenine, rolipram, and calmodulin were purchased from Sigma. Dowex (1×8 200–400) was purchased from Dow Chemical Co.

Generation of Antiserum—Rabbit polyclonal antisera were raised against the poly-L-lysine-based multiple antigen peptide (for PDE10A2 and PDE10A3) complexes containing the sequences MRIEERKSQH-LTGL (residues 1–14 of human PDE10A1), MEDGPSNASCFFRRLTE (residues 1–18 of human and rat PDE10A2), and MSNSDGPEGAVGSCNA (residues 1–15 of rat PDE10A3). The peptides were synthesized by a PSSM-8 peptide synthesizer (Shimadzu, Japan) or purchased from Sawady Technology. The complexes were mixed with Freund’s complete adjuvant (Sigma) for the first immunization and with Freund’s incomplete adjuvant when boosted. After immunizing five times, antisera were collected and stored at −80°C until use. Purified PDE10A antibody, which recognizes the common region in PDE10A variants, was prepared from the antiserum reported previously (12), using an antigen affinity chromatography from a HiTrap N-hydroxysuccinimide-activated column (Amersham Biosciences), according to the manufacturer’s instructions. The antibody was eluted with 0.1 M glycine-HCl (pH 2.7) and then neutralized immediately.

Preparation of PDE Isozymes from Rat Striatum—Male Sprague-Dawley rats obtained from Japan SLC at 10 weeks of age were anesthetized with diethyl ether before striatum was excised and stored at −80°C until use. A 0.36-g sample of rat striatum was disrupted in 7.2 ml of ice-cold homogenization buffer HB-A (20 mM Tris-HCl, pH 7.5, 2 mM magnesium acetate, 0.3 mM CaCl₂, 1 mM dithiothreitol, 1.3 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride) by a sonicator (TOMY Seiko, Japan). The homogenates were centrifuged at 100,000 g for 60 min at 4°C, and the resultant supernatants were designated as cytosolic fractions. The pellets were resuspended with a volume of ice-cold HBT-A buffer equal to that of the cytosolic fractions, designating detergent-insoluble fractions. Lactate dehydrogenase (LDH) activity in each fraction was assayed using the LDH-cytotoxic test (Wako Pure Chemical Industries). Approximately 20% of the total LDH activity was present in membrane fractions in these experiments. The membrane and cytosolic fractions were applied to a HiTrap Q column (Amersham Biosciences) equilibrated in elution buffer A (20 mM Tris-HCl, pH 7.5, 1 mM dithiotheriol, 1 mM CaCl₂, and 5 mM benzamidine), with and without 0.5% Triton X-100, respectively. The column was washed with 20 ml of the above buffer used for equilibration, and proteins were then eluted from the column by running a linear NaCl gradient (0–0.5 M, 100 ml) in the buffer. Fractions (2 ml each) collected on ice were assayed for cGMP and cAMP hydrolytic activities.

PDE Assay—The PDE assay was performed by the radiolabeled nucleotide method. The assay buffer contained 50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 4 mM 2-mercaptoethanol, 0.33 mg/ml bovine serum albumin (Sigma), enzyme solution, unlabeled cGMP or cAMP, and 12.5 nM [3H]cGMP or 4.88 nM [3H]cAMP. The reaction was started by mixing the substrate into 500 µl of the assay buffer, and tubes were incubated at 37°C for 30 min. After boiling for 1.5 min, the mixtures were added to 100 µl of 1 mg/ml Crotalus atrox snake venom and incubated at 37°C for 30 min. The reaction was stopped by the addition of 500 µl of methanol. The resultant solutions were applied to a Dowex (1×8 200–400) column (5 × 12 mm), and flow-through fractions containing [3H]adenosine or [3H]guanosine were collected. After washing the column with 1.0 ml of methanol, the solutions passing through the column were mixed with the flow-through fractions. Aqueous scintillation mixtures were added to the mixtures, and the radioactivities were then measured.

Chemical compounds were dissolved in Me₂SO. For studies of the inhibition of PDE activities, inhibitors in Me₂SO (final concentration 1% (v/v)) were added to the assay buffer containing enzyme and preincubated for 5 min before the reactions were initiated by the addition of substrate.

Construction of Expression Plasmids—A DNA fragment for the amino-terminal region of human PDE10A1 was amplified by PCR using a primer set: 5’-AAAAACGTTATGAGATGAAGAGAGG-3’ plus 5’-GCGTTTTAGTTCATATACAATCC-3’ and pBlue-PDE10A encoding human PDE10A1 cDNA (10). The HindIII-KpnI fragment of an amplified DNA and the 2.4-kb KpnI fragment of pBlue-PDE10A encoding human PDE10A1 cDNA (10). The HindIII-KpnI fragment of an amplified DNA and the 2.4-kb KpnI fragment of pBlue-PDE10A encoding human PDE10A1 cDNA (10). The HindIII-KpnI fragment of an amplified DNA and the 2.4-kb KpnI fragment of pBlue-PDE10A encoding human PDE10A1 cDNA (10).
Localization of PDE10A

Expression of PDE10A Variants in PC12h Cells—PC12h cells, a subclone of rat pheochromocytoma PC12 cells, were kindly provided by Drs. Hiroshi Hatanaka and Masashi Yamada of Osaka University (34). PC12h cells (6.3 × 10⁶ cells/well) were cultured in poly-l-lysine-coated 6-well plates containing Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and 5% horse serum at 37°C in an atmosphere of 5% CO₂. The cells were washed with ice-cold PBS and scraped in ice-cold homogenization buffer HB-C (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 10 mM leupeptin, 1 mM Aprotinin, 100 µM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40). The cells were disrupted by a sonicator, and homogenates were centrifuged at 100,000 × g for 60 min at 4°C. High speed supernatants, which provided equal amounts of wild-type and mutant-type FLAG-tagged human PDE10A2 proteins (FLAG-PDE10A2 and FLAG-PDE10A2-T16A, respectively) were mixed with protein G-Sepharose (PDI, Huntington, NY) and a Quantity One Program (PDI). After washing with PBS, and then the cells were incubated with FITC-conjugated anti-FLAG antibody (1:500 dilution; Sigma) for 3 h or anti-FLAG rabbit polyclonal antibody (1:100 dilution; Sigma) for 1 h and/or anti-TCN38 (trans-Golgi network 38) monoclonal antibody (1:150 dilution; Transduction Laboratories) for 1 h at room temperature. Primary antibodies were washed with PBS, and then the cells were incubated with FITC-conjugated anti-IgG antibody and/or rhodamine-conjugated anti-IgG antibody (Jackson Laboratories). The cells were washed with PBS and viewed by a confocal microscope (TCS-NT, Leica). Geometric images shown in the figures are representative of those obtained from at least three independent immunostaining experiments. At least two images per experiment were taken, and more than 10 fields in each experiment were observed.

In Vitro Phosphorylation of PDE10A by PKA—Relative amounts of FLAG-PDE10A2 proteins produced in COS-7 cells were estimated by immunoblotting using anti-FLAG monoclonal antibody as described previously (14). The amounts of the recombinant proteins were also confirmed by measuring their PDE activities. 48 h after transfection, the cells were washed with ice-cold PBS and scraped in ice-cold homogenization buffer HB-B (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 10 mM leupeptin, 100 µM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40). The cells were disrupted by a sonicator, and cytosolic and membrane fractions were prepared by ultracentrifugation using the HB-B buffer and then added to each tube. After centrifugation, the supernatants were centrifuged at 5,000 × g for 1 min, and the pellets were washed three times with ice-cold HB-B buffer. The resultant pellets were resuspended with the HB-B buffer and then added to each tube. After centrifugation, the immunoprecipitated proteins were subjected to immunoblotting and PDE activity assays. FLAG-tagged PDE10A2 proteins bearing equal cellular densities by immunoblotting exhibited similar enzymatic activities (data not shown). The immunoprecipitated proteins showing equal amounts were phosphorylated at 30°C for various times in reaction buffer (20 mM Tris-HCl, pH 7.5, 20 mM magnesium acetate, 200 µM ATP, 6.7 nM [γ-32P]ATP, 5 mM β-glycerophosphoric acid disodium salt, and 1 mM sodium orthovanadate) containing various concentrations of the catalytic subunit of bovine heart PKA (Promega). The phosphorylated mixtures were centrifuged at 5,000 × g for 1 min. The pellets were resuspended in the Laemmli buffer for SDS-PAGE (35) and boiled. The samples were centrifuged at 5,000 × g for 1 min, and the supernatants were subjected to SDS-PAGE and autoradiography.

Expression of Phosphorylated PDE10A2 in Vivo—First, the specificity of isolation of phosphorylated PDE10A2 with a PhosphoProtein™ affinity column (Qiagen) was verified. FLAG-tagged PDE10A2 proteins produced in transfected COS-7 cells were immunoprecipitated with or without FLAG-tagged PKA catalytic subunit showing equal amounts of proteins by an anti-FLAG monoclonal antibody and protein G-Sepharose (Amersham Biosciences) and anti-FLAG monoclonal antibody and incubated at 4°C overnight by rotation. The samples were centrifuged at 5,000 × g for 1 min. The pellets were resuspended in the Laemmli buffer for SDS-PAGE (35) and boiled. The samples were centrifuged at 5,000 × g for 1 min, and the supernatants were subjected to SDS-PAGE and autoradiography.

In Vitro Phosphorylation of PDE10A2 by PKA—Relative amounts of FLAG-PDE10A2 proteins produced in COS-7 cells were estimated by immunoblotting using anti-FLAG monoclonal antibody as described previously (14). The amounts of the recombinant proteins were also confirmed by measuring their PDE activities. 48 h after transfection, the cells were washed with ice-cold PBS and scraped in ice-cold homogenization buffer HB-B (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 10 mM leupeptin, 100 µM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40). The cells were disrupted by a sonicator, and cytosolic and membrane fractions were prepared by ultracentrifugation using the HB-B buffer and then added to each tube. After centrifugation, the supernatants were centrifuged at 5,000 × g for 1 min, and the pellets were washed three times with ice-cold HB-B buffer. The resultant pellets were resuspended with the HB-B buffer and then added to each tube. After centrifugation, the immunoprecipitated proteins were subjected to immunoblotting and PDE activity assays. FLAG-tagged PDE10A2 proteins bearing equal cellular densities by immunoblotting exhibited similar enzymatic activities (data not shown). The immunoprecipitated proteins showing equal amounts were phosphorylated at 30°C for various times in reaction buffer (20 mM Tris-HCl, pH 7.5, 20 mM magnesium acetate, 200 µM ATP, 6.7 nM [γ-32P]ATP, 5 mM β-glycerophosphoric acid disodium salt, and 1 mM sodium orthovanadate) containing various concentrations of the catalytic subunit of bovine heart PKA (Promega). The phosphorylated mixtures were centrifuged at 5,000 × g for 1 min. The pellets were resuspended in the Laemmli buffer for SDS-PAGE (35) and boiled. The samples were centrifuged at 5,000 × g for 1 min, and the supernatants were subjected to SDS-PAGE and autoradiography.
Characterization of PDE10A Variants and PDE Isozymes in Rat Striatum—HiTrap Q chromatography was carried out to identify PDE isozymes in both cytosolic and membrane fractions of rat striatum under conditions similar to those reported previously (12). Fig. 2. A and B, is shown the elution profiles of cytosolic and membrane fractions, respectively. Proteins immunoreactive with purified anti-PDE10A antibody were observed only in fractions 16–20 (cytosolic fractions) and fractions 14–20 (membrane fractions), indicating that cAMP hydrolytic activities in these fractions contained PDE10A (Fig. 2, B and C). Moreover, immunoreactive signals in membrane fractions are shown. Elution profiles of PDE activities after HiTrap Q Sepharose chromatography of cytosolic fractions and membrane fractions are shown (B and C). Cysolic fractions (high speed supernatants) (B) and membrane fractions (detergent-soluble fractions) (C) of rat striatal homogenates were prepared as described under "Experimental Procedures." Proteins were eluted from the column with a linear gradient of NaCl from 0 to 0.5 M. Cyclic AMP and cGMP hydrolytic activities in these fractions contained PDE10A (Fig. 2). A and B, respectively. The percentages of PDE10A and LDH activities in each fraction were calculated by comparing optical densities of immunoblotting and LDH activities in each fraction to the sum of optical densities and LDH activities from three fractions (C + M + I as 100%). Typical results of at least two independent experiments are shown.

RESULTS

Distribution of PDE10A in Rat Striatum—The presence of PDE10A in high speed supernatants (cytosol), detergent-soluble fractions (membrane), and detergent-insoluble fractions of rat striatum were investigated by immunoblot analysis. Using affinity chromatography, we first isolated anti-PDE10A antibody recognizing several PDE10A variants from the anti-PDE10A antiserum produced previously (12). The proteins in cytosolic, membrane, and insoluble fractions were subjected to immunoblot analysis with purified antibody, according to their original protein contents in each fraction. The anti-PDE10A antibody showed strong staining of an ∼87-kDa band in the proteins from membrane fractions (Fig. 2A). A moderate signal of the same protein size was observed in both cytosolic and insoluble fractions. Thus, PDE10A protein was particularly abundant in membrane fractions. No signal was observed in the fractions by purified anti-PDE10A antibody preadsorbed with antigen peptide. LDH activity was predominantly detected in cytosolic fractions as shown in Fig. 2A.

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Insoluble fractions (lane I) of rat striatum were separated by ultracentrifugation as described under Experimental Procedures.
fractions were much stronger than those in cytosolic fractions. These results indicated that PDE10A protein was highly abundant in membrane fractions of rat striatum compared with that in cytosolic fractions. With regard to other PDE isozymes in cytosolic fractions, cAMP hydrolytic activities (fractions 28–42) eluting at −0.3 M NaCl were inhibited by a PDE4 inhibitor, rolipram (10 μM), but not by 1 μM cGMP, demonstrating that these fractions contained PDE4. The cGMP hydrolytic activities in fractions 32–46 (cytosolic fractions) were increased ~3–20-fold by the addition of Ca²⁺ and calmodulin, indicating that these fractions contained Ca²⁺/calmodulin-dependent PDE (PDE1). With regard to membrane fractions of rat striatum, cAMP hydrolytic activities in fractions 29–30 were activated slightly by 1 μM cGMP and inhibited by a PDE2 inhibitor, erythro-9-(2-hydroxy-3-nonyl)-adenine (10 μM), indicating the presence of PDE2 in these fractions. The cGMP hydrolytic activities in fractions 29–35 of membrane fractions were stimulated slightly by Ca²⁺ and calmodulin, suggesting that these fractions contained PDE1.

Detection of PDE10A Variants in Rat Striatum—Polyclonal antisera toward the synthetic peptides corresponding to the unique amino-terminal sequences of human PDE10A1, human and rat PDE10A2, and rat PDE10A3 were produced. The antigenic specificity was tested using PDE10A variants produced in COS-7 cells. The cAMP-PDE activities of the cells expressing each PDE10A variant were ~70-fold higher than those of mock-transfected cells. Production of the PDE10A proteins seemed to be almost the same among the transfected cells carrying each of the three PDE10A constructs. Extracts of transfected COS-7 cells showing equal levels of PDE10A activities were electrophoretically separated, blotted onto membranes, and then incubated with a purified anti-PDE10A antibody and each PDE10A variant-specific antiserum. Results using purified anti-PDE10A antibody showed that all PDE10A variants gave equal optical densities (Fig. 3A). Each antiserum specifically detected a PDE10A variant carrying an antigen peptide but did not detect other PDE10A variants. The equal optical densities were obtained after 15-s exposure with anti-PDE10A antibody and anti-PDE10A1 antiserum and 5-s exposure with anti-PDE10A2 and anti-PDE10A3 antisera in immunoblot analysis, indicating that titers of antisera against PDE10A2 and PDE10A3 were about 3-fold higher than those of the anti-PDE10A antibody and the anti-PDE10A1 antiserum.

To determine which type of PDE10A variant is present in rat striatum, we performed immunoblotting using the above antibody and antiserum (Fig. 3B). The antibody prepared against a common region of all PDE10A variants showed immunoreactivity mainly in membrane fractions. Interestingly, immunoblotting using anti-PDE10A2 serum revealed that PDE10A2 was predominantly expressed in membrane fractions but not in cytosolic fractions. No signal was observed in the fractions using either a purified anti-PDE10A antibody or anti-PDE10A2 antiserum preadsorbed with the antigen peptides. By contrast, PDE10A1 and PDE10A3 proteins were not detected in rat striatum using anti-PDE10A1 and anti-PDE10A3 antisera under these conditions.

Distribution of Recombinant PDE10A Variants in Mammalian Cells—PC12 and PC12h cells are preferred to investigate signal transduction in nigra and striatal neurons, because these cell lines respond to several neurotransmitters including dopamine and adenosine (36, 37). We examined the possible presence of the three variants of PDE10A transcripts in untransfected PC12h using PCR analysis. PDE10A2 and PDE10A3 transcripts, but not PDE10A1 transcripts, were observed in this cell line, whereas the activity of PDE10A separated by chromatography was too low to be detected (data not shown). In order to examine subcellular distribution of PDE10A variants, expression plasmids for recombinant FLAG-tagged PDE10A variants (human PDE10A1, human PDE10A2, human PDE10AΔ13, rat PDE10A2, and rat PDE10A3; see Fig. 1) were transfected into PC12h cells, and the cytosolic and membrane fractions of the cells were separated by ultracentrifugation using buffers lacking or containing detergent (0.5% Triton X-100), respectively. As shown in Fig. 4A, recombinant human PDE10A1 and rat PDE10A3 expressed in transfected PC12h cells were present only in cytosolic fractions. Human and rat PDE10A2 proteins were primarily localized in membrane fractions. Human and rat PDE10A2 proteins contain the same amino-terminal sequence, which is distinct from those of PDE10A1 and PDE10A3. To study the role of the amino-terminal sequences of PDE10A variants in the subcellular distribution of these proteins, we produced a mutant of human PDE10A, termed PDE10A1Δ13 (Fig. 1), lacking the unique amino-terminal sequence. It is intriguing that PDE10A1Δ13 expressed in transfected PC12h cells was detected in cytosolic fractions, suggesting that the unique amino-terminal portion of PDE10A2 serves as an anchor necessary for association with the membrane.

The membrane fractions from PDE10A2-expressing PC12h
Infections. Cytosolic and membrane fractions are represented by experiments were performed at least four times with different trans-
ti-FLAG antibody and peroxidase-conjugated anti-IgG antibody. The transferred onto membranes. Immunoblotting was performed with an-
lane of membrane fractions) were subjected to 7.5% SDS-PAGE and suspended in equal volumes of the solution. 

All fractions prepared from the same samples were sus-

Interactions. 

Membrane fractions prepared from 1.5 × 10⁶ PC12h cells transiently expressing human PDE10A2 and from 10 mg of rat striatum. Membrane fractions were double-stained with anti-FLAG rabbit polyclonal and anti-TGN38 mouse antibodies and then visualized with anti-FITC-conjugated rabbit IgG (E) and anti-rhodamine-conjugated mouse IgG (F) antibodies, respectively. G, a merged image of E and F. No specific immunofluorescence was observed in untransfected PC12h cells as shown in H. All images were taken with a confocal microscope.

Alteration of Subcellular Localization of PDE10A Variants by Active and Inactive PKA—Our previous report demonstrated that PDE10A2 has a motif for PKA phosphorylation (Arg-Arg-Leu-Thr¹⁶) in its amino terminus and that PDE10A2, but not another PDE10A variant having a different amino terminus, is phosphorylated by PKA (14). We thus examined the effects of PKA-mediated phosphorylation on subcellular localization of recombinant PDE10A variants. First, to confirm the PKA-phosphorylation site of PDE10A2, an in vitro kinase assay was performed using the wild-type human PDE10A2 (PDE10A2-WT) and its mutant with an Ala substitution at Thr¹⁶ (PDE10A2-T¹⁶A). PDE10A2-WT was phosphorylated by the catalytic subunit of PKA in a concentration-dependent manner (Fig. 6A). PKA-mediated ³²P incorporation of PDE10A2-T¹⁶A was significantly reduced compared with that of PDE10A2-WT using various concentrations of PKA. Moreover, phosphorylation of PDE10A2-WT was progressively increased during a 30-min incubation, and the phosphorylation level of PDE10A2-T¹⁶A was still low even at 30 min of incubation (Fig. 6B). These observations indicated that Thr¹⁶ of PDE10A2 was a significant target for phosphorylation by PKA in vitro.

The expression plasmid coding for PKA catalytic subunit was co-transfected with the PDE10A plasmids, and subcellular localization of PDE10A in cytosolic and membrane fractions was analyzed by immunoblotting. Co-expression of a FLAG-tagged
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catalytic subunit of PKA with recombinant PDE10A variants in PC12h cells changed subcellular localization of human and rat PDE10A2 from membranes to cytosol, whereas PDE10A2 was present in membrane fractions in the absence of PKA (Fig. 6C). By contrast, subcellular localization of PDE10A1 and PDE10A3 lacking a PKA phosphorylation motif was not affected by co-expression of PKA. PDE10A2-T16A was also localized to membranes of transfected PC12h cells. Interestingly, subcellular localization of PDE10A2-T16A was not changed by co-expression of PKA in cells, suggesting that PKA-mediated phosphorylation of Thr16 in the unique amino terminus of PDE10A2 alters subcellular localization of the protein.

The effect of inactive PKA catalytic subunit having Ala substitution at Lys168 in the ATP-binding site on subcellular localization of recombinant PDE10A2 protein in PC12h cells was examined. In contrast to the case of the active PKA catalytic subunit, the inactive PKA catalytic subunit did not alter the membrane localization of PDE10A2 in cells (Fig. 6D).

Phosphorylation of PDE10A2 by PKA Co-expression in Transfected PC12h Cells—To determine whether PDE10A2 is phosphorylated by co-expression of the PKA catalytic subunit in transfected PC12h cells, a PhosphoProtein™ purification column was used to separate phosphorylated proteins. First, the specificity of PhosphoProtein™ purification procedures for phosphorylated PDE10A2 was verified using FLAG-tagged PDE10A2 phosphorylated by PKA catalytic subunit in vitro. Whereas nonphosphorylated PDE10A2 was found in flow-through fractions, phosphorylated PDE10A2 that was labeled with 32P was eluted in phosphorylated protein fractions (Fig. 7A), indicating that the affinity column is applicable for separation of phosphorylated PDE10A2.

In vitro phosphorylation of PDE10A2 was confirmed as follows. After transfection into PC12h cells, cytosolic, membrane, and insoluble fractions were prepared with 0.25% CHAPS buffer as described under “Experimental Procedures” and subjected to immunoblotting. Fig. 7B shows that PDE10A2 was predominantly localized in cytosol and membrane fractions in the presence and absence of PKA catalytic subunit, respectively, under this condition. A 0.25% CHAPS buffer provided the same results as a buffer containing 0.5% Triton X-100, as described in the legends to Figs. 2A and 4A. After cytosolic fractions were mixed with membrane fractions, the mixture was loaded onto the PhosphoProtein™ purification column. PDE10A2 proteins in flow-through fractions and eluates were analyzed by immunoblotting. As shown in Fig. 7C, PDE10A2 proteins produced without PKA co-expression appeared mainly in flow-through fractions, which contained nonphosphorylated proteins, whereas PDE10A2 co-expressed with PKA catalytic subunit was eluted from the column with elution buffer. These results indicated that PDE10A2 was phosphorylated by PKA catalytic subunit in vivo.

Fig. 6. Effects of co-expression of the catalytic subunit of PKA on subcellular distribution of FLAG-tagged PDE10A variants and mutant. A, phosphorylation of wild-type PDE10A2 (h10A2) and a PDE10A2-T16A mutant (h10A2-T16A) was examined by an in vitro kinase assay with the catalytic subunit of PKA. Whole cell lysates of transfected COS-7 cells showing equal levels of cAMP hydrolytic activities were immunoprecipitated (IP) with an anti-FLAG antibody, and the resultant immunoprecipitants were subjected to an in vitro kinase assay (see “Experimental Procedures”) (lower panel). To monitor the amount of each protein, immunoprecipitants were immunoblotted (IB) with an anti-FLAG antibody (upper panels). B, time course for 32P incorporation of the above PDE10A2 proteins by the catalytic subunit of PKA (50 nM). Immunoprecipitants showing equal cAMP hydrolytic activities were subjected to an in vitro kinase assay for various incubation times. Positions of phosphorylated proteins are indicated by arrows. C, effects of the catalytic subunit of PKA on subcellular distribution of FLAG-tagged PDE10A variants and mutant in transiently transfected PC12h cells. PC12h cells were co-transfected with expression plasmids for FLAG-tagged proteins of human PDE10A1 (h10A1), human PDE10A2 (h10A2), its mutant (h10A2-T16A; Ala-substitution at Thr16), rat PDE10A2 (r10A2), and rat PDE10A3 (r10A3) in combination with (+) or without (−) an expression plasmid for a FLAG-tagged catalytic subunit of PKA (see “Experimental Procedures”). 24 h after transfection, cytosolic and membrane fractions were prepared in equal volumes of the solution, subjected to SDS-PAGE, and analyzed by immunoblotting using an anti-FLAG antibody. 15 μg of each fraction (approximately 2.5 μg of protein/lane of cytosolic fractions and 0.5 μg of protein/lane of membrane fractions) were loaded. Positions of proteins are indicated as arrows. D, effects of a PKA mutant lacking kinase activity on subcellular distribution of FLAG-tagged PDE10A2 in transiently transfected PC12h cells. PC12h cells were co-transfected with an expression plasmid for a FLAG-tagged catalytic subunit of PKA or for a FLAG-tagged PKA mutant having Ala substitution at Lys169 in the ATP-binding site. 24 h after transfection, cytosolic and membrane fractions were prepared, subjected to SDS-PAGE, and analyzed by immunoblotting using an anti-FLAG antibody. The experiments were performed three times with independent transfections, and a representative result is shown. Cytosolic and membrane fractions are represented by C and M, respectively. Positions of proteins are indicated as arrows.
findings strongly implied that cytosolic PDE10A2 produced in the presence of PKA catalytic subunit was phosphorylated, whereas PDE10A2 localized in membranes without PKA co-expression was not phosphorylated.

**Localization of PDE10A2 Mutants at Thr**<sup>16**</sup> **in Transiently Transfected PC12h Cells**—In order to confirm the effect of phosphorylation of PDE10A2 at Thr**<sup>16**</sup> on subcellular localization, a mutant PDE10A2 (T16E) with Glu replacing Thr**<sup>16**</sup>, which mimics phosphorylation of this site, was examined. The mutant protein was identified in cytosolic fractions of transfected PC12h cells (Fig. 8), whereas wild type PDE10A2 was expressed in membrane fractions. Replacement of Thr**<sup>16**</sup> of PDE10A2 with Ser (T16S), Asp (T16D), Lys (T16K), or Gly (T16G) did not affect membrane localization of the protein.

PDE10A2. These findings suggested that PDE10A2 is localized to the cytosolic fractions by PKA-mediated phosphorylation of Thr**<sup>16**</sup>.

**Immunocytochemical Analysis of Intracellular Localization of PDE10A2 Co-expressed with PKA in Transfected PC12h Cells**—Intracellular localization of FLAG-tagged wild-type and mutant-type PDE10A2 proteins in transfected PC12h cells was investigated using immunofluorescent staining analysis. Wild-type PDE10A2 and a PDE10A2 mutant with Ala substitution at Thr**<sup>16**</sup> were localized in the Golgi apparatus of transfected cells (Fig. 9, A–F). The Golgi signal of PDE10A2 overlapped with that of TGN38 (Fig. 9, C and F). Wild-type PDE10A2 co-expressed with histidine-tagged PKA catalytic subunit and a PDE10A2 mutant with a Glu substitution at Thr**<sup>16**</sup> exhibited cytosolic localization of PDE10A2 (Fig. 9, G and H). Immunoblotting showed that wild type PDE10A2 co-expressed with histidine-tagged PKA catalytic subunit was present in cytosolic fractions (data not shown). These results were in accordance with those obtained by immunoblotting.

**DISCUSSION**

We demonstrated here that PDE10A protein is abundant in membrane fractions compared with that in the cytosolic fractions of rat striatum. Moreover, PDE10A1 was revealed to be a major cAMP-hydrolyzing PDE in membrane fractions of rat striatum under these conditions. On the other hand, levels of PDE10A1 activities were almost equal to those of PDE4 in cytosolic fractions. Regarding cGMP-hydrolyzing activities, Ca**<sup>2**+**</sup>- and calmodulin-activated PDE (PDE1) was rich in rat striatum. PDE1A2 and PDE1A3 transcripts are major variants in rat brain (12). The presence of a PDE1A2 protein exclusively in membrane fractions of rat striatum indicated that this variant is a membrane-associated PDE. By contrast, an immunoreactive protein of PDE1A3 was detected in neither cytosolic nor membrane fractions using the anti-PDE1A3 antibody, although PDE1A3 transcripts were detected in rat striatum by PCR (data not shown). This inconsistency concerning the existence of PDE1A3 transcripts but lack of enzyme protein in the striatum may be due to exclusion by translational regulation, protein degradation of PDE1A3, or translation of PDE1A3 from another initiation site downstream of the putative first methionine of PDE1A3.

Three major PDE10A variants, PDE10A1, PDE10A2, and PDE10A3, possess unique amino termini. Many splice variants of PDEs, other than PDE10A, having unique amino and carboxyl termini show distinct subcellular localization (17, 23, 25, 39). We demonstrated here that PDE10A2 was predominantly present in membrane fractions of transiently transfected PC12h cells. This observation was in accordance with results obtained by chromatography of extracts of striatum. By con-
Antibody (mouse IgG and B cell), pFLAG-h10A2 and pHisMax-cAK-C–PC12h cells were transfected with pFLAG-h10A2 (wild type; lytic subunit of PKA in transiently transfected PC12h cells.

and mutant-type PDE10A2 proteins with or without the catalytic subunit of PKA in PC12h cells. PDE10A1 and PDE10A3, which were transfected PC12h cells demonstrated that localization of PDE10A2 is recruited from the Golgi apparatus to the cytosol in response to its phosphorylation by PKA.

The presence of PDE10A2 in the Golgi area of transfected PC12h cells and alteration of subcellular localization of PDE10A2 to cytosol in response to its phosphorylation by PKA might modulate PKA activation in the Golgi area of neuronal cells (41).

The α isoform catalytic subunit of PKA translocates from the Golgi area to the nucleus in neuronal cells via intracellular cAMP elevation induced by 24-h stimulation by ethanol, and phosphorylates the cAMP response element binding factor in the nucleus (42, 43). There are some reports concerning cAMP signaling via PKA activation in the Golgi apparatus of neurons.

Some studies of translocation and alteration of localization of PDEs have been reported. After prolonged thyroid-stimulating hormone stimulation, PDE4D present in the perinuclear region of FRTL-5 thyroid cells diffuses throughout the cytoplasm (25). In rat aortic vascular smooth muscle cells, PDE4D3 is translocated from particulate to cytosolic fractions by co-stimulation with forskolin and phorbol 12-myristate 13-acetate (23). It is plausible that phosphorylation of PDE10A2 by PKA in PC12h cells causes alteration of PDE10A2 localization to the cytosol.

Subcellular localization of PDE10A2-T16A lacking a PKA phosphorylation site was not changed to cytosol in the presence of the catalytic subunit of PKA, and as expected, PDE10A2-T16E, which contains a negatively charged amino acid residue at Thr16, showed cytosolic localization in transfected PC12h cells. However, another PDE10A2 mutant (PDE10A2-T16D) having a negatively charged amino acid, aspartic acid, replacing Thr16 failed to change the subcellular localization of the protein. From these observations, glutamic acid is likely to be structurally more suitable for mimicking phosphorylated threonine of PDE10A2 than is aspartic acid. In addition, cytosolic PDE10A2 co-expressed with the catalytic subunit of PKA in PC12h cells was eluted in phosphoprotein fractions using a PhosphoProtein™ affinity column. Thus, phosphorylation of Thr16 in the amino-terminal region of PDE10A2 is required for the alteration of subcellular localization.

The physiological meaning of compartmentalization of PDEs in the Golgi area of neurons is not well understood. One role of PDE10A2 in the perinuclear region of cells is coordination of cyclic nucleotide signaling in this local space. The RII subunit of PKA binds to Neurobeachin, which anchors PKA to the Golgi/centrosome of PC12 cells and neuronal cells (41).

Intracellular localization of FLAG-tagged wild-type and mutant-type PDE10A2 proteins with or without the catalytic subunit of PKA in transiently transfected PC12h cells.

PC12h cells were transfected with pFLAG-h10A2 (wild type; A–C), pFLAG-h10A2-T16A (D–F), pFLAG-h10A2 and pHisMax-cAK-C (G), and pFLAG-h10A2-T16E (H). Immunocytochemical analyses were performed using an anti-FLAG rabbit polyclonal antibody (A and D) and an anti-TGN38 mouse antibody (B and E) or anti-FLAG mouse monoclonal antibody (G and H) as described under “Experimental Procedures.” The cells were visualized with FITC-labeled anti-rabbit IgG (A and D), anti-rhodamine-conjugated mouse IgG (B and E), or FITC-labeled anti-mouse IgG (G and H) antibodies. A and D were merged with B and E (resulting in C and F), respectively. All images were taken with a confocal microscope.

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Subcellular Localization of Cyclic Nucleotide Phosphodiesterase Type 10A Variants, and Alteration of the Localization by cAMP-dependent Protein Kinase-dependent Phosphorylation

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