Published in final edited form as:
Psychopharmacology (Berl). 2011 February ; 213(2-3): 403–412. doi:10.1007/s00213-010-1984-7.

Phospholipase C, Ca^{2+}, and calmodulin signaling are required for 5-HT_{2A} receptor-mediated transamidation of Rac1 by transglutaminase

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Abstract

Rationale—Serotonin and especially serotonin 2A (5-HT_{2A}) receptor signaling are important in the etiology and treatment of schizophrenia and affective disorders. We previously reported a novel 5-HT_{2A} receptor effector, increased transglutaminase (TGase)-catalyzed transamidation, and activation of the small G protein Rac1 in A1A1v cells, a rat embryonic cortical cell line.

Objectives—In this study, we explore the signaling pathway involved in 5-HT_{2A} receptor-mediated Rac1 transamidation.

Methods—A1A1v cells were pretreated with pharmacological inhibitors of phospholipase C (PLC) or calmodulin (CaM), and then stimulated by the 5-HT_{2A} receptor agonist, 2,5-dimethoxy-4-iodoamphetamine (DOI). Intracellular Ca^{2+} concentration and TGase-modified Rac1 transamidation were monitored. The effect of manipulation of intracellular Ca^{2+} by a Ca^{2+} ionophore or a chelating agent on Rac1 transamidation was also evaluated.

Results—In cells pretreated with a PLC inhibitor U73122, DOI-stimulated increases in the intracellular Ca^{2+} concentration and TGase-modified Rac1 were significantly attenuated as compared to those pretreated with U73343, an inactive analog. The membrane-permeant Ca^{2+} chelator, BAPTA-AM strongly reduced TGase-catalyzed Rac1 transamidation upon DOI stimulation. Conversely, the Ca^{2+} ionophore ionomycin, at a concentration that induced an elevation of cytosolic Ca^{2+} to a level comparable to cells treated with DOI, produced an increase in TGase-modified Rac1 without 5-HT_{2A} receptor activation. Moreover, the CaM inhibitor W-7, significantly decreased Rac1 transamidation in a dose-dependent manner in DOI-treated cells.

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Conclusions—These results indicate that 5-HT_{2A} receptorcoupled PLC activation and subsequent Ca^{2+} and CaM signaling are necessary for TGase-catalyzed Rac1 transamidation, and an increase in intracellular Ca^{2+} is sufficient to induce Rac1 transamidation.

Keywords

5-HT_{2A} receptor; Rac1; Transglutaminase; Transamidation; Phospholipase C; Calcium; Calmodulin; Serotonin; Small G proteins; A1A1v cells; Serotonylation

Introduction

Serotonin receptor signaling, especially 5-HT_{2A} receptor signaling, has been implicated in schizophrenia and affective disorders including anxiety, depression, and posttraumatic stress disorder (Roth 1994; Weisstaub et al. 2006). However, the nature of the mechanisms underlying these disorders and their treatments has not been elucidated, hindering the development of better treatment approaches. We previously reported a novel signaling pathway for the 5-HT_{2A} receptor system that has the potential to be involved in these disorders. Stimulation of 5-HT_{2A} receptors causes transamidation of Ras-related C3 botulinum toxin substrate 1 (Rac1) in A1A1v cells, a rat cortical cell line (Dai et al. 2008). However, the underlying molecular mechanisms by which the 5-HT_{2A} receptor signaling regulates transglutaminase (TGase)-catalyzed transamidation and activation of Rac1 are still unclear. TGases are a family of enzymes that catalyze the transamidation, esterification, and deamidation of peptide-bound glutamine residues. The transamidation reaction can result in the addition of a free low-molecular weight amine or the cross-linking of a protein-bound glutamine to a protein-bound lysine residue within or between proteins.

5-HT_{2A} receptor activation can increase inositol 1,4,5-trisphosphate (IP_3) via $G_{q/11}$-mediated activation of phospholipase C (PLC) (Conn and Sanders-Bush 1984). PLC plays an important role in intracellular signal transduction by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP2), a membrane phospholipid, and thereby generating 2 second messengers: IP_3 which diffuses through the cytosol and releases Ca^{2+} from intracellular endoplasmic reticulum (ER) stores and diacylglycerol (DAG). It has been reported that TGase activity can be significantly enhanced in response to increased intracellular Ca^{2+} through IP_3 generation (Zhang et al. 1998). Moreover, serotonin-mediated activation of small G protein Cdc42 was dependent on PLC signaling pathways (Udo et al. 2005).

Rac1, one of the most extensively characterized members in the Rho family of small G proteins, was initially discovered as a substrate for ADP-ribosylation induced by the C3 component of botulinum toxin (Didsbury et al. 1989). Subsequently, Rac1 and its downstream effectors were identified as key signaling molecules in various cell functions, such as cytoskeleton reorganization, cell transformation, axonal guidance, and cell migration (Etienne-Manneville and Hall 2002). Since Rac1 is also an essential mediator in many pathological conditions such as Salmonella invasion, tumor cell migration, and retinal degeneration (Bourguignon et al. 2000; Brown et al. 2007), it is important to define cellular signaling pathways that lead to post-translational modifications and activation of Rac1.

Like all members of the Rho superfamily, Rac1 functions as a molecular switch, cycling between an inactive GDPbound state and an active GTP-bound state. Rho small G proteins can be activated by different signal transduction pathways initiated by extracellular factors such as plateletderived growth factor, insulin, or epidermal growth factor (Bishop and Hall 2000). Serotonin has also been shown to induce activation via TGase-catalyzed transamidation of small G proteins in neurons, platelets, aortic smooth muscle cells, and pancreatic beta cells (Dai et al. 2008; Guilluy et al. 2007; Paulmann et al. 2009; Walther et

al. 2003). TGase catalyzes the transamidation between protein-bound glutamines and primary amines such as serotonin in a Ca^{2+}-dependent manner (Folk and Chung 1985).

Calmodulin (CaM) has been shown to increase TGase enzymatic activity. In the presence of CaM, a membraneassociated erythrocyte TGase is activated at physiological and lower than physiological Ca^{2+} concentrations (Billett and Puszkin 1991). Moreover, in the presence of Ca^{2+}, CaM enhanced TGase activity threefold in human platelets and the chicken gizzard (Puszkin and Raghuraman 1985). A CaM inhibitor prevented TGase-catalyzed cross-linking of huntingtin in cells co-transfected with mutant huntingtin and TGase (Zainelli et al. 2004). Taken together, we hypothesize that 5-HT_{2A} receptor stimulated TGase-catalyzed Rac1 transamidation is dependent on PLC-mediated increases in intracellular Ca^{2+} and is regulated by CaM. To test this hypothesis, we examined the effects of PLC inhibition, CaM inhibition, and manipulation of intracellular Ca^{2+} by means of a Ca^{2+} ionophore or a chelating agent on TGase-modified Rac1 in response to 5-HT_{2A} receptor activation in A1A1v cells. Knowing the molecular pathway involved in 5-HT_{2A} receptor-mediated activation of TGase and subsequently Rac1 including the involvement of PLC, $Ca^{2+,}$ and CaM will allow for rationally choosing targets to selectively regulate activation of Rac1 independently of other second messengers activated by 5-HT_{2A} receptors and probe the relevance of each pathway in the treatment and etiology of affective disorders and schizophrenia.

Methods

Cell culture

A1A1v cells, a rat cortical cell line, were grown on 100-mm^2 plates coated with poly-L-ornithine (Sigma, St Louis, MO) and maintained in 5% CO_2 at 33°C, in Dulbecco's modified Eagle medium (DMEM; Fisher Scientific, Pittsburgh, PA) containing 10% fetal bovine serum (FBS; Fisher Scientific, Pittsburgh, PA). Before each experiment, cells were maintained in DMEM with 10% charcoal-treated FBS for 48 h. Charcoal treatment of FBS reduces the concentration of serotonin in the media to approximately 3 nM (Unsworth and Molinoff 1992). Cells from passages 8–15 were used for all experiments.

Chemicals

The following chemicals were used in this study: (–)-1-(2,5-dimethoxy-4-iodoamphetamine HCl (DOI; Sigma, St Louis, MO), U73122 (Tocris Bioscience Ellisville, MO), U73343 (Sigma, St Louis, MO), BAPTA-AM (Invitrogen, Carlsbad, CA), ionomycin (Invitrogen, Carlsbad, CA), and W-7 hydrochloride (Tocris Bioscience Ellisville, MO).

Measurement of intracellular Ca^{2+} concentration

Cells were grown to 90% confluence in black-sided 96-well plates (Fisher Scientific, Pittsburgh, PA). Cells were washed twice with modified Kreb's medium (135 mM NaCl, 5.9 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11.5 mM D-glucose, 11.6 mM Hepes, pH 7.3) and then incubated in the same medium with 5 μM Fura-2 AM (Molecular Probes, Carlsbad, CA), 0.1% bovine serum albumin, and 0.02% Pluronic F127 detergent for 60 min at 33°C in the dark. After loading, the cells were washed twice and incubated in the dark in modified Kreb's medium or pretreated with drugs for 30 min. Following 1 min of equilibration, the cells were stimulated with a single injection of DOI (resulting in a final concentration of 3 μM DOI), and the response was recorded for 3 min in 5-s intervals. Fura-2 fluorescence using 340- and 380-nm excitation and 510-nm emission was measured with a BioTek fluorescence plate reader. After background fluorescence was subtracted, the ratio of fluorescence at 340-nm excitation to that at 380-nm excitation was calculated and used as an

index of the intracellular Ca^{2+} concentration (the 340/380 nm fluorescence ratio is positively correlated with the absolute values of intracellular Ca^{2+} concentration).

Immunoprecipitation of TGase-modified protein

A1A1v cells were harvested and lysed using lysis buffer A (25 mM Tris–HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 1:1,000 protease inhibitor cocktail (Sigma, St Louis, MO) containing 104 μM AEBSF, 0.08 μM aprotinin, 2 μM leupeptin, 4 μM bestatin, 1.5 μM pepstatin A and 1.4 μM E-64). Protein concentration was determined using the BCA Protein Assay kit (Pierce, Rockford, IL). Immunopurification of proteins containing TGase-catalyzed bonds was performed using 81D4 mAb (mouse IgM) prebound to Sepharose beads (Covalab, Lyon, France) using a protocol developed by Covalab and as described previously (Dai et al. 2008). Briefly 20 μl of sepharose-81D4 beads were washed three times in TBS/0.1% Tween 20 with gentle shaking for 15 min, followed by adding cell lysate containing 200 μg of protein (1 μg/μl) to the washed beads and incubating for 2 h at 37°C. After incubation, the pellets were washed four times in TBS/0.1% Tween 20 for 15 min. Then 20 μl of loading buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 5% β-mercaptoethanol) was added to the washed pellets followed by 5-min incubation at 90°C. The samples were then centrifuged at 9,000×*g* for 2 min and the supernatant was transferred and stored at −80°C until immunoblot analysis. It was previously reported that the 81D4 antibody is specific for the Nε-(γ-glutamyl) lysine isopeptide, i.e., the transamidation of a peptide bound lysine residue to a peptide-bound glutamate residue (el Alaoui et al. 1991). Several other substrates similar to the Nε-(γ-glutamyl) lysine isopeptide were found not to cross-react with the 81D4 antibody, however transamidation of a peptide bound lysine to a free low-molecular weight amine was not examined (el Alaoui et al. 1991). Our previous data suggest that the antibody also recognizes transamidation of a peptide bound lysine to serotonin (Dai et al. 2008).

Immunoblot

Immunoaffinity purified proteins and cell lysates were separated on 12% SDS-polyacrylamide gels and then electrophoretically transferred to nitrocellulose membranes. Membranes were then incubated in blocking buffer (5% non-fat dry milk, 0.1% Tween 20, ×1 TBS) for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibodies on a shaker. Primary antibodies (Upstate Biotechnology, NY: anti-Rac1, mouse IgG, 1:700; anti-Na^+/K^+ ATPase, mouse IgG, 1: 10000; Millipore, Billerica, MA: anti-serotonin transporter (SERT) antibodies, rabbit, 1:1,000; Abcam, Cambridge, MA: anti-lactate dehydrogenase, goat antibody conjugated to HRP, 1:4,000) were diluted in antibody buffer (1% non-fat dry milk, 0.1% Tween 20, 1× TBS). The next day, membranes were washed with TBS/0.1% Tween 20 and then incubated with goat-antimouse or goat-anti-rabbit secondary antibody conjugated to HRP (Jackson ImmunoResearch, West Grove, PA) diluted in antibody buffer. Membranes were washed and signal was detected using enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). Using Scion Image for Windows (Scion, Frederick, MD), immunoblots were quantified by calculating the integrated optical density (IOD) of each protein band on the film.

Statistical analyses

Data are presented as mean±the standard error of the mean (SEM) and analyzed by one- or two-way ANOVA. Post hoc tests were conducted using Bonferroni's multiple comparison tests. GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA) was used for all statistical analyses. A probability level of $p<0.05$ was considered to be statistically significant for all statistical tests.

Results

PLC inhibition

We previously reported that 5-HT_{2A} receptor stimulation induced TGase-catalyzed Rac1 transamidation and activation in A1A1v cells (Dai et al. 2008). To test the dependence of Rac1 transamidation on PLC activation, cells were pretreated with 5 μM of the PLC inhibitor, U73122, or an inactive analog, U73343, for 30 min prior to the stimulation of 5-HT_{2A} receptors by 3 μM DOI. U73122 inhibits both PLC-mediated hydrolysis of PIP2 to IP_3 and the coupling of G protein-PLC activation. Changes in intracellular Ca^{2+} were monitored by the 340/380 nm fluorescence ratio of the Fura-2-loaded cells. DOI stimulation induced a robust increase (by at least 200%) in the intracellular Ca^{2+} concentration within cells pretreated with U73343, whereas PLC inhibition by U73122 reduced the intracellular Ca^{2+} response to DOI (Fig. 1a). In parallel experiments, TGase-modified Rac1 was evaluated by immunoprecipitation using the 81D4 antibody directed against the TGase-catalyzed covalent bond and immunoblots. There were significant increases in TGase-modified Rac1 within DOI-stimulated cells, but pretreatment with U73122 significantly attenuated DOI-induced Rac1 modification by TGase (Fig. 1b, c). These results suggest a specific role of PLC upstream of Rac1 transamidation in A1A1v cells. Two-way ANOVA indicates a significant main effect of DOI [$F(1,8)=108.3$; $p<0.001$] and U73122 [$F(1,8)=29.99$; $p<0.001$] on TGase-modified Rac1. There was also a significant interaction between DOI and U73122 [$F(1,8)=17.40$; $p<0.01$]. The ubiquitously expressed Na^+/K^+ ATPase is a well established plasma membrane marker, and its function underlies essentially all of mammalian cell physiology. To verify that the PLC inhibitor-mediated decrease in Rac1-transamidation by TGase was not due to U73122-induced cellular toxicity, cell lysates from the same experiment were examined on immunoblot with antibodies for Na^+/K^+ATPase and lactate dehydrogenase. There are no significant differences in Na^+/K^+AT Pase levels or lactate dehydrogenase among cells with different treatments (Fig. 1b). Similar measurements were used in all of the following experiments.

Ca^{2+} chelation

In order to determine if TGase-modified Rac1 specifically depends on an increase in intracellular Ca^{2+} upon 5-HT_{2A} receptor activation, A1A1v cells were pretreated with increasing concentrations of the membrane-permeant Ca^{2+} chelator, BAPTA-AM (0, 5, 10, 20 μM) for 30 min, then the cells were challenged by DOI. Dose-dependent reductions in the DOI-mediated Ca^{2+} response were observed by measuring Fura-2 fluorescence (Fig. 2a). Since 20 μM BAPTA caused the most significant inhibition in Ca^{2+} increases, it was used in the parallel experiments to evaluate TGase-modified Rac1. DOI stimulation of cells without BAPTA-pretreatment significantly increases the amount of TGase-modified Rac1 about twofold compared with vehicle-stimulated cells, whereas pretreatment with BAPTA has a notable inhibitory effect on DOI-induced Rac1 modification by TGase (Fig. 2b, c), suggesting that an increase in intracellular Ca^{2+} is required for DOI-mediated Rac1 transamidation. Two-way ANOVA indicates a significant main effect of DOI [$F(1,8)=37.09$; $p<0.001$] and BAPTA [$F(1,8)=9.85$; $p<0.05$] on TGase-modified Rac1. There was also a significant interaction between DOI and BAPTA [$F(1,8)=9.50$; $p<0.05$].

Ca^{2+} ionophore

To determine if an increase in intracellular Ca^{2+} is sufficient to enhance Rac1 modification by TGase, a Ca^{2+} ionophore, ionomycin was used to produce an elevation of cytosolic Ca^{2+} concentration without 5-HT_{2A} receptor activation. Ionomycin is an effective and specific Ca^{2+} carrier, so it has been used in studies of Ca^{2+} flux across plasma and ER membranes. To determine the concentration of ionomycin that raises the levels of cytosolic Ca^{2+} to levels comparable to 3 μM DOI (the concentration of DOI used to induce TGase-modified

Rac1), serial dilutions of ionomycin (from 1,000 nM 12.5 nM) were tested for their ability to induce increases in intracellular Ca^{2+}. As shown in Fig. 3a, 25 nM ionomycin was able to produce a comparable increase in cytosolic Ca^{2+} in comparison to 3 μM DOI, so this concentration was used in the parallel experiments to measure the effects on TGase-modified Rac1. After cells were treated with either 3 μM DOI or 25 nM ionomycin for 15 min, ionomycin mediated an increase in TGase-modified Rac1 to levels similar to those induced by DOI. One-way ANOVA indicated there is no significant difference in TGase-modified Rac1 between DOI- and ionomycin-treated cells. Taken together, these data suggest an increase in intracellular Ca^{2+} via an ionophore can mimic 5-HT_{2A} receptor-induced cytosolic Ca^{2+} increases and is sufficient to induce TGase-catalyzed Rac1 transamidation.

CaM inhibition

TGase is not only a Ca^{2+}-dependent enzyme, but its activity is also positively regulated by CaM. Moreover, Ca^{2+} signaling in cells is mainly mediated by CaM, a potent Ca^{2+}-binding protein. The CaM inhibitor, W-7, binds with high affinity to CaM in the presence of Ca^{2+} and thereby inhibits the activation of CaM-dependent enzymes. To test whether CaM modulates TGase-catalyzed transamidation of Rac1, cells were pre-incubated for 1 h with 0, 0.5, or 5 μM W-7 hydrochloride, and then stimulated with 3 μM DOI or vehicle for 15 min. We found that in DOI-stimulated cells, the levels of TGase-modified Rac1 were significantly higher than in vehicle-stimulated cells, whereas pretreatment with W-7 decreased DOI-stimulated Rac1 modification by TGase in a dose-dependent manner (Fig. 4). Two-way ANOVA indicates a significant main effect of DOI [F (1,12)=144.6; p<0.001] and W-7 [F(2,12)=8.152; p<0.01] on TGase-modified Rac1. There was also a significant interaction between DOI and W-7 [F(2,12)=5.01; p<0.05].

To test whether CaM modulates 5-HT_{2A} receptor-mediated Ca^{2+} release, cells were pre-incubated for 1.5 h with 0, 0.5, or 5 μMW-7 hydrochloride, and then stimulated with 3 μM DOI or vehicle for 3 min. We found pretreatment with W-7, did not alter DOI-stimulated Ca^{2+} release (Fig. 4a).

SERT expression in A1A1v cells

Western blots were prepared with the membrane fraction of rat dorsal raphe and frontal cortex and whole cell lysates from A1A1v cells. A single major band is detected with an antibody directed against the amino-terminus of SERT in A1A1v cells comparable to the band in rat dorsal raphe and frontal cortex which are used as positive controls (Fig. 5a).

Discussion

Several lines of evidence indicate $G_{q/11}$-protein-coupled receptors, such as bradykinin or endothelin-1 receptor, are able to stimulate the activation of Rac1 (Clerk et al. 2001; van Leeuwen et al. 1999), but the mechanisms by which Rac1 becomes activated through these receptors remains poorly characterized. Nevertheless, it is worth noting that the activation of Rac1 is highly regulated by physiological stimuli, and aberrant activation occurs under many pathological conditions such as Salmonella invasion, tumor cell migration, and retinal degeneration (Bourguignon et al. 2000; Brown et al. 2007). Therefore, further studies that delineate signaling pathways involved in Rac1 activation may uncover new potential therapeutic targets for these diseases. We have previously demonstrated that the 5-HT_{2A} receptor, another $G_{q/11}$-protein-coupled receptor, is responsible for TGase-catalyzed transamidation and activation of Rac1 in A1A1v cells based on the fact that selective 5-$HT_{2A/2C}$ receptor agonist, but not 5-HT_{1A} receptor agonist, induces transamidation of Rac1 by TGase. Moreover, expression of 5-HT_{2C} receptors is not detectable in this cell line and a

selective 5-HT_{2A} receptor antagonist blocks Rac1 transamidation (Dai et al. 2008). In the present study, we investigated the mechanisms involved in Rac1 transamidation in response to 5-HT_{2A} receptor stimulation. Our experimental results identified that PLC-mediated increases in intracellular Ca^{2+} and subsequent CaM activation are required for 5-HT_{2A} receptor-mediated Rac1 transamidation. 5-HT_{2A} receptor stimulation activates PLC which catalyzes the hydrolysis of PIP2 into IP_3. IP_3 mobilizes Ca^{2+} from ER stores and thereby increases the intracellular Ca^{2+} and leads to the activation of CaM. Our data suggest that both Ca^{2+} and CaM activate TGase, which catalyzes transamidation of Rac1 to bioamines, such as serotonin (Fig. 5).

Except for the 5-HT_3 receptor, which is a ligand-gated ion channel, the other 5-HT receptors belong to the G protein-coupled receptor (GPCR) superfamily. G proteins such as $G\alpha_{12}$ and $G\alpha_{13}$, have been shown to transduce signals from GPCR to activate Rho small G proteins (Suzuki et al. 2003). One of the guanine nucleotide exchange factors (GEFs) for Rho, p115RhoGEF, has served as a direct link between $G\alpha_{12}/G\alpha_{13}$ and Rho (Kozasa et al. 1998). However, the signaling pathways between 5-HT_{2A} receptors and Rac1 transamidation and activation are not well understood. 5-HT_{2A} receptor is primarily coupled to $G_{q/11}$ and activates various isoforms of PLC (Conn and Sanders-Bush 1984). It also induces the activation of phospholipase A2 and the release of arachidonic acid (Felder et al. 1990). Moveover, through interaction with ADP-ribosylation factor 1 via its C-terminal domain, 5-HT_{2A} receptor is able to signal through the phospholipase D pathway (Robertson et al. 2003). Our results suggest that 5-HT_{2A} receptor activation triggers TGase-catalyzed Rac1 transamidation predominantly through a pathway involving PLC (Fig. 1).

Although it has been reported that PLC activates Rac1 in a manner dependent on Iba1, a Ca^{2+}-binding protein specifically expressed in macrophage/microglia (Kanazawa et al. 2002), Rac1 transamidation and activation in A1A1v cells, a neuronal cell line, could be mediated by other signaling pathways. Cleavage of PIP2 by PLC yields two important second messengers, IP_3 and DAG, to initiate a variety of cellular functions. Since Ca^{2+} chelation reduces DOI-stimulated Rac1 transamidation to basal levels (Fig. 2), IP_3-induced Ca^{2+} release rather than DAG signaling plays a pivotal role in TGase-catalyzed Rac1 transamidation. These results are in agreement with an earlier study suggesting that in thrombin- and collagen-stimulated human blood platelets, PLC activity and an increase in intracellular Ca^{2+} concentration were required for Rac1 activation, whereas PKC inhibition had no effect (Soulet et al. 2001). Increases in intracellular Ca^{2+} concentration upon 5-HT_{2A} receptor stimulation are achieved by releasing intracellular Ca^{2+} stores and/or by activating Ca^{2+} channels, depending on the cell of interest. 5-HT_{2A} receptor-induced contraction of rat aorta may require the concerted action of voltage-gated Ca^{2+} channels and phosphoinositide turnover (Nakaki et al. 1985). More interestingly, stimulation of 5-HT_{2A} receptors on astrocytes caused Ca^{2+} influx through voltage independent Ca^{2+} channels which were dependent of the IP_3 production and subsequent Ca^{2+} release from internal stores (Hagberg et al. 1998). Therefore, both Ca^{2+} mobilization from internal stores and influx of extracellular Ca^{2+} could contribute to the PLC/IP_3-mediated intracellular Ca^{2+} increase in A1A1v cells.

CaM and TGase are Ca^{2+}-dependent proteins. CaM activation is coupled to intracellular Ca^{2+} elevation and also correlates with the spatial pattern of increased Ca^{2+} (Hahn et al. 1992). Upon Ca^{2+} binding to the EF hand motifs of CaM, both N- and C-terminal domains adopt an open confirmation, exposing hydrophobic surfaces, which then interact with a variety of target proteins including TGase (Puszkin and Raghuraman 1985; Zainelli et al. 2004). TGases contain a cysteine in their active site that is unmasked only in the presence of Ca^{2+} (Hand et al. 1993) and Ca^{2+} activation of TGase is further regulated by other signal modulators such as GTP and CaM (Puszkin and Raghuraman 1985; Zainelli et al. 2004). In

order for TGase to become receptive to the glutamine substrate, Ca^{2+} first must bind to TGase, inducing its conformational change which permits the glutamine substrate binding to the catalytic center (Folk 1983). A rise of Ca^{2+} concentration increases TGase activity in a dose-dependent manner in vitro and in cells (Shin et al. 2004; Siefring et al. 1978). Consistent with these results, the elevation of intracellular Ca^{2+} induced via a Ca^{2+} ionophore lead to increased TGase-catalyzed Rac1 transamidation (Fig. 3).

In addition to Ca^{2+}, CaM also interacts with TGase and regulates its cross-linking activity. CaM has been shown to coimmunoprecipitate with TGase2 in transfected cells (Zainelli et al. 2004). CaM increased TGase activity in human erythrocyte (Billett and Puszkin 1991), platelets, and chicken gizzard (Puszkin and Raghuraman 1985). Interestingly, exogenous CaM with a binding site on the α-subunits of the hexadecameric phosphorylase kinase molecule $(\alpha\beta\gamma\delta)_4$, stimulates TGase-catalyzed incorporation of putrescine into the β- and γ-subunits, but inhibits incorporation of amines into the α-subunit (Nadeau and Carlson 1994). Some of the CaM antagonists, such as dansylcadaverine, inhibit TGase (Lorand et al. 1979) and its protein cross-linking ability. We have previously reported that a CaM antagonist or inhibitory CaM fragments, attenuated TGase-modified mutant huntingtin in cell and animal models of Huntington's disease (Dai et al. 2009; Dudek et al. 2009; Zainelli et al. 2004). CaM antagonists and Ca^{2+} chelators also have been shown to depress the motile progression of sperm cells possibly mediated by TGase-catalyzed cross-linking of the contractile proteins (Cariello and Nelson 1985). Our present results revealed that the CaM inhibitor, W-7, reduced TGase-modified-Rac1 in dose-dependent fashion (Fig. 4), indicating that TGase-catalyzed Rac1 transamidation, at least in part, was positively regulated by CaM. Moreover, in the presence of a 5-HT_{2a} receptor agonist, CaM has been shown to interact with 5-HT_{2a} receptors at the sites that are important for G protein coupling, suggesting a putative role for CaM in regulating 5-HT_{2a} receptor function (Turner and Raymond 2005). However, since the CaM inhibitor had no effect on DOI-stimulated calcium release, the effect of CaM inhibition is not likely mediated by alterations in signaling at the 5-HT_{2A} receptor but by modulation of TGase activity.

The significance of TGase in Rac1 transamidation was confirmed by TGase siRNA and a chemical inhibitor in our previous studies (Dai et al. 2008). In addition to small G proteins such as Rac1, RhoA, Rab3a, and Rab27a (Dai et al. 2008; Paulmann et al. 2009; Walther et al. 2003) that could undergo TGase-catalyzed transamidation to serotonin (serotonylation), more recently, cytoskeletal proteins including actin, myosin heavy chain, and filamin A have been identified as serotonylated proteins (Watts et al. 2009). However, serotonin may not be the only amine that could be incorporated in these proteins. Neuronal differentiation of SH-SY5Y cells induced by retinoic acid increases the expression/activation of TGases, resulting in transamidation of putrescine to RhoA and activation of RhoA (Singh et al. 2003). Moreover, spermidine and putrescine are suitable substrates for transglutaminase-catalyzed transamidation of RhoA in vitro (Schmidt et al. 2001). In addition to amine incorporation, another important TGase-catalyzed modification of small G proteins is deamidation of glutamine residues. However, this reaction has only been demonstrated by bacterial toxins, not in mammalian systems and transglutaminases. The site-specific deamidation of Gln53 and Gln61 of RhoA and Ras, respectively, by bacterial toxins with TGase activity inhibits the GTPase activity and renders small G proteins constitutively active (Lorand and Graham 2003).

In conclusion, our study provides important new insights into the signaling mechanisms underlying 5-HT_{2A} receptor-induced Rac1 transamidation and activation by TGase in A1A1v cells, and highlights the necessity of PLC-mediated Ca^{2+} mobilization and CaM in TGase-catalyzed Rac1 transamidation. As an activating signal for both TGase and CaM, an elevation of intracellular Ca^{2+} is sufficient to induce Rac1 transamidation. Accumulating

evidence suggests that Rho and Rab family of small G proteins are subject to TGase-mediated modification and activation. The essential role of Rac1 in neuronal development, especially in the regulation of dendritic spines (Corbetta et al. 2009; Tsai et al. 2009), axon formation and guidance (Ng et al. 2002), neurite outgrowth and differentiation (Li et al. 2002) is well characterized, and perturbations in Rac1 signaling have been associated with cognitive disorders such as mental retardation (Govek et al. 2005). Given the importance of Rac1 in various pathophysiological processes, the biological functions of Rac1 activation by TGase-catalyzed transamidation in neurons are worth exploring in more depth.

Abbreviations

DOI	2,5-Dimethoxy-4-iodoamphetamine
CaM	Calmodulin
DAG	Diacylglycerol
ER	Endoplasmic reticulum
GDIs	GDP-dissociation inhibitors
GAPs	GTPase-activating proteins
GEFs	Guanine nucleotide exchange factors
IP_3	Inositol 1,4,5-trisphosphate
PIP2	Phophatidyl inositol-4,5-bisphosphate
SERT	Serotonin transporter
TGase	Transglutaminase

Acknowledgments

This work was supported by US Public Health Service [Grant MH068612].

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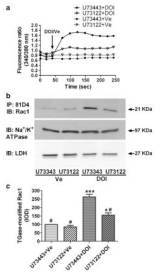

Fig. 1.

Effect of PLC inhibition on DOI-induced Ca^{2+} signals and TGase-modified Rac1 in A1A1v cells. **a** After pretreatment with 5 μM U73122 or U73343 (negative control) for 30 min, and stimulation with either 3 μM DOI or vehicle (*Ve*) for 15 min, changes in the fluorescence ratio (340/380 nm) were recorded from cells loaded with Fura-2. A representative example from three independent experiments conducted in triplicate is shown. *Arrows* indicate the time of drug application. **b** Upper, immunoprecipitation (*IP*) and immunoblot (*IB*) analyses revealed that U73122 pretreatment inhibited the increase in TGase-modified Rac1 upon DOI stimulation. Middle and lower, cells lysates from the same experiment were examined on IB with Na^+/K^+ ATPase and lactate dehydrogenase (*LDH*) antibodies respectively, which indicate that U73122 had no effect on cell viability. **c** Quantitation of effects of U73122 and DOI on TGase-modified Rac1 in three separate experiments. Data shown were the mean IOD±S.E. M., and they were normalized to U73343- and Ve- treated cells. Two-way ANOVA followed by Bonferroni test: *one asterisk and three asterisks* indicated $p<0.05$ and 0.001 as compared to U73343- and Ve-treated cells, respectively; *one number sign* indicated $p<0.001$ as compared to U73343- and DOI- treated cells

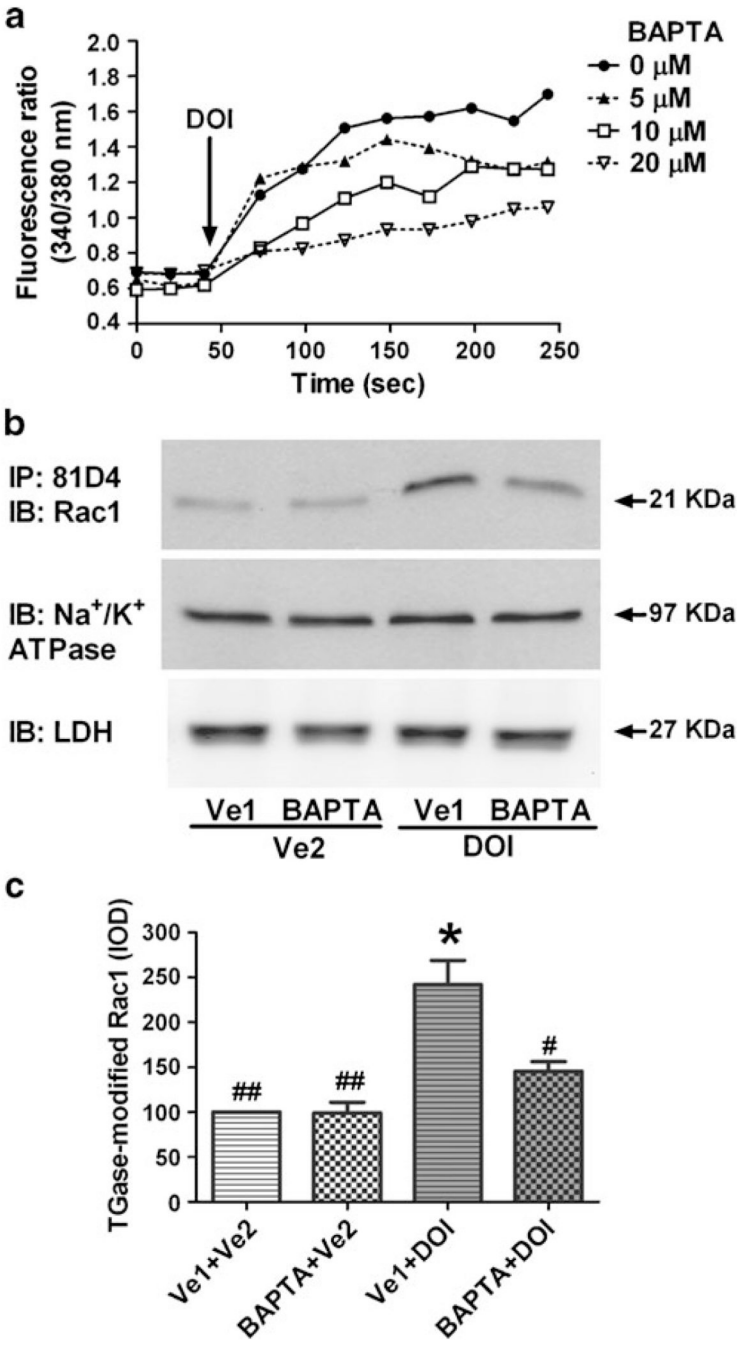

Fig. 2.

Pre-incubation with a Ca^{2+} chelator attenuated the DOI-mediated elevation of cytosolic Ca^{2+} and TGase-modified Rac1. **a** Dynamic changes in the fluorescence ratio (340/380 nm) in Fura-2 loaded A1A1v cells. The Ca^{2+} response to exposure to 3 μM DOI was gradually reduced with the increasing concentrations of BAPTA. A representative example of three independent experiments conducted in triplicate is shown. **b** *Upper*, cells were pretreated with 20 μM BAPTA or the vehicle for BAPTA (*Ve1*) for 30 mins, and then stimulated with 3 μM DOI or the vehicle for DOI (*Ve2*) for 15 mins. TGase-modified Rac1 was detected by IP and IB analysis. BAPTA inhibited the increase in TGase-modified Rac1 in DOI-stimulated cells. *Middle and lower*, cells lysates from the same experiment were examined

on IB with Na^+/K^+ATPase and LDH antibodies respectively. There was no significant difference among various treatments. **c** Quantitation of effects of BAPTA and DOI on TGase-modified Rac1 in three separate experiments. Data shown were the mean IOD±S.E. M., and they were normalized to Ve1- and Ve2- treated cells. Two-way ANOVA followed by Bonferroni test: *one asterisk* indicated $p<0.001$ as compared to Ve1- and Ve2- treated cells; *one number sign* and *two number signs* indicated $p<0.05$ and $p<0.001$ as compared to Ve1- and DOI- treated cells, respectively

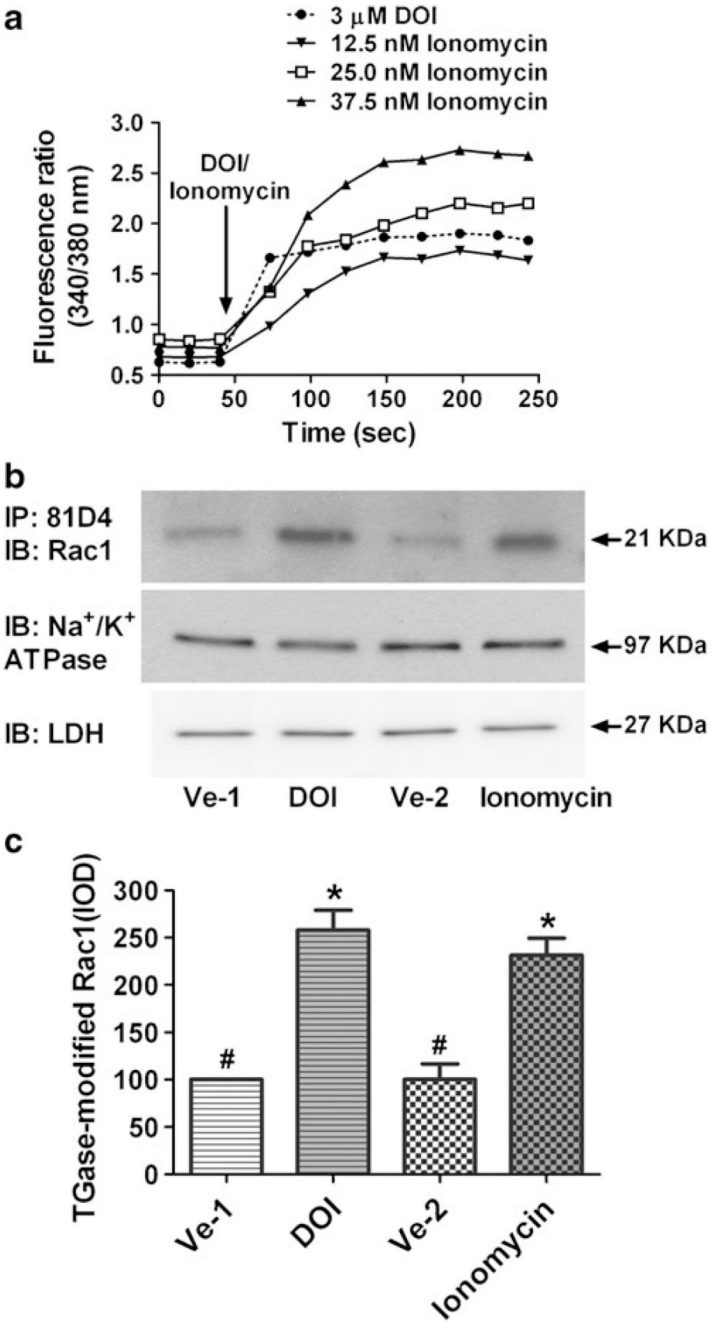

Fig. 3.

The Ca^{2+} ionophore mimicked the DOI-induced increase in cytosolic Ca^{2+} and TGase-catalyzed transamidation of Rac1. **a** Fura-2-loaded cells were stimulated with either 3 μM DOI or different concentrations of ionomycin. The changes in the fluorescence ratio (340/380 nm) were recorded. A representative example from three independent experiments conducted in triplicate is shown. **b** *Upper*, cells were treated for 15 mins with 3 μM DOI, 25 nM ionomycin or vehicle (*Ve*). Ionomycin caused an increase in TGase-modified Rac1 comparable to that induced by DOI. *Middle and lower*, cell viability, as indicated by Na^+/K^+ATPase and LDH levels was not significantly different among the various treatments. **c** Quantitation of effects of ionomycin and DOI on TGase-modified Rac1 in three separate

experiments. Data shown were the mean IOD±S.E.M., and were normalized to Ve-1-treated cells. One-way ANOVA followed by Bonferroni test: *one asterisk* indicates $p<0.01$ as compared to Ve-1-treated cells; *one number sign* indicates $p<0.01$ as compared DOI-treated cells

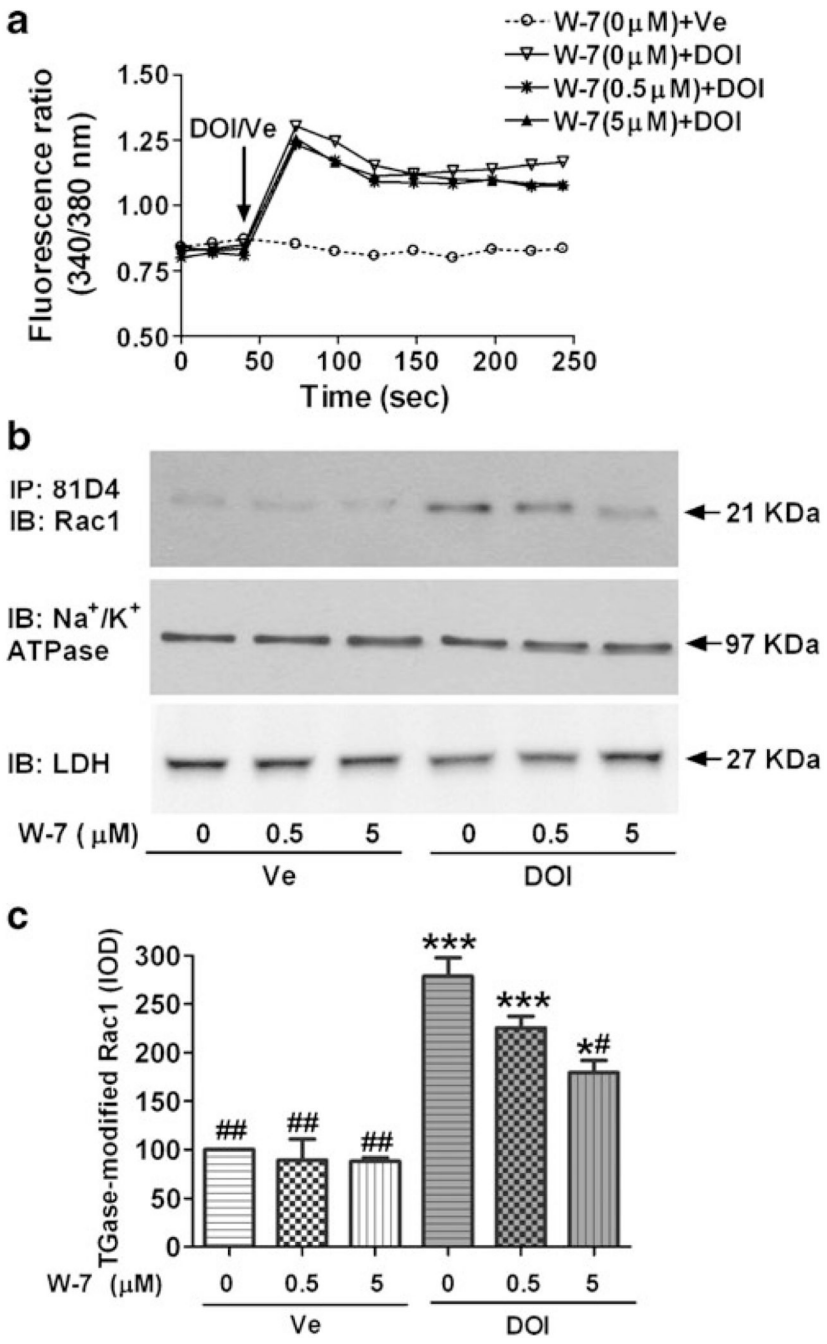

Fig. 4.

The CaM inhibitor W-7 caused a dose-dependent reduction in DOI-stimulated TGase-modified Rac1. **a** Pretreatment with the CaM inhibitor W-7 had no effect on DOI-stimulated Ca^{2+} release. Data shown from one representative experiment performed in triplicate and repeated two additional times. **b** Cells were pretreated with increasing concentrations of W-7 for 1 h and then stimulated with either 3 μM DOI or vehicle (*Ve*). TGase-modified Rac1, Na^+/K^+ATPase and LDH were measured by IP and/or IB. W-7 decreased the levels of TGase-modified Rac1 upon DOI stimulation, but did not significantly affect Na^+/K^+ATPase or LDH levels at the concentrations of W-7 applied. c Quantitation of effects of W-7 on TGase-modified Rac1 in three separate experiments. Data shown were the mean IOD

±S.E.M., and were normalized to Ve-stimulated cells without W-7 pretreatment. Two-way ANOVA followed by Bonferroni test: *one asterisk* and *three asterisks* indicate $p<0.05$ and $p<0.001$ as compared to Ve-stimulated cells without W-7 pretreatment, respectively; *one number sign* and *two number signs* indicate $p<0.01$ and $p<0.001$ as compared to DOI-stimulated cells without W-7 pretreatment, respectively

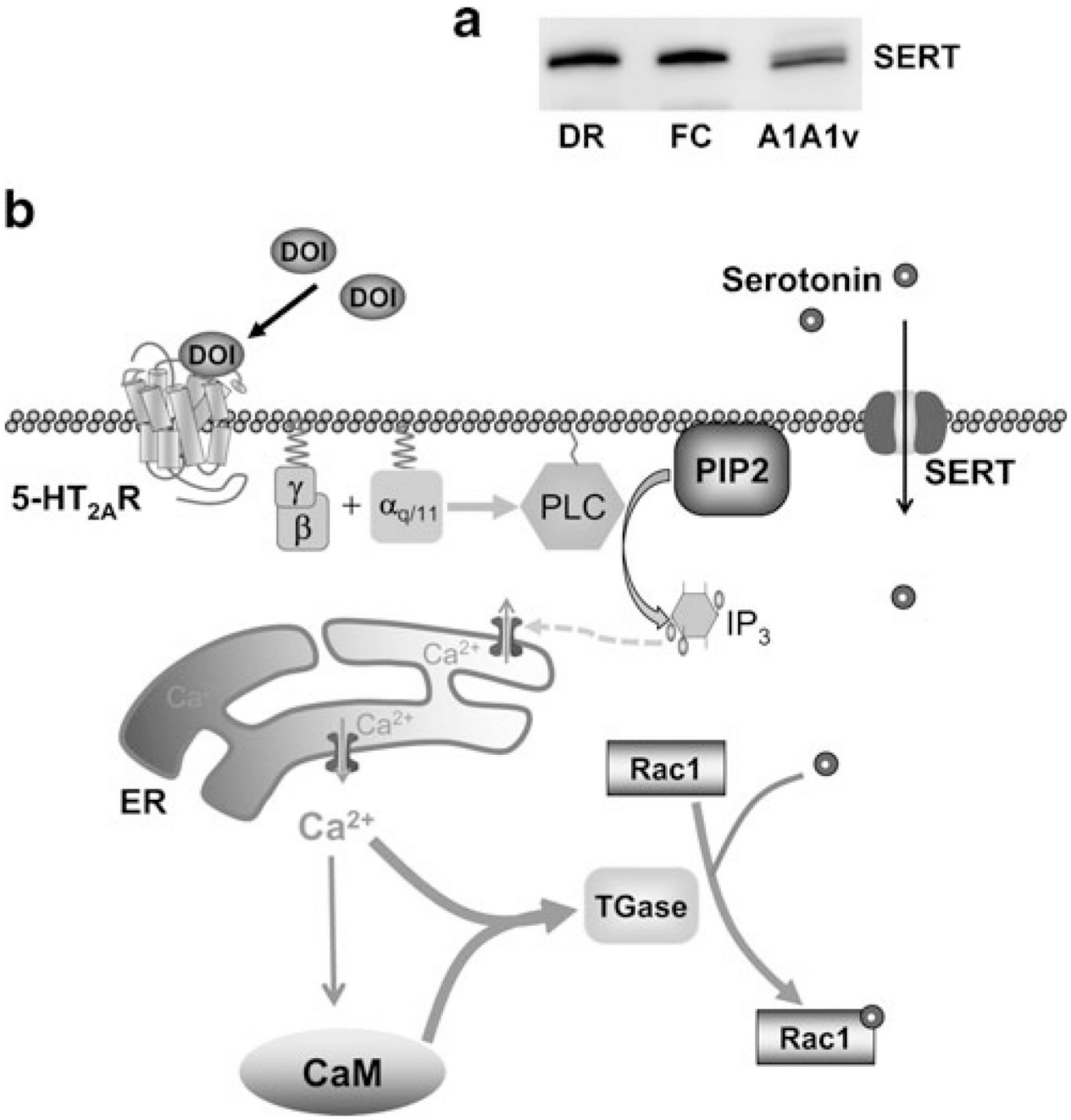

Fig. 5.

a Western blot demonstrating the presence of SERT in A1A1v cells. A Western blot was prepared with 2.5 μg of the membrane fraction of rat dorsal raphe (*DR*) and frontal cortex (*FC*) and compared to 20 μg of lysate from A1A1v cells. **b** Schematic representation showing $_{5\text{-HT}_{2A}}$ receptor signal-mediated transamidation of Rac1 by TGase. DOI stimulates $G_{q/11}$ proteins-coupled $_{5\text{-HT}_{2A}}$ receptors, leading to the activation of PLC, which in turn hydrolyses PIP2 into IP3. IP3 releases Ca^{2+} from ER and results in the activation of Ca^{2+}-dependent proteins, such as CaM and TGase. Positively regulated by both Ca^{2+} and CaM, TGase catalyzes transamidation of Rac1 to bioamines, such as serotonin (Dai et al. 2008) transported into the cytoplasm by SERT. Abbreviations: *5-HT*$_{2A}$*R*$_{5\text{-HT}_{2A}}$ receptor; *CaM*,

calmodulin; *ER*, endoplasmic reticulum; *PLC*, phospholipase C; *PIP2*, phophatidyl inositol-1,4-bisphosphate; *IP3*, inositol-1,4,5-triphosphate; *SERT*, serotonin transporter; *TGase*, transglutaminase; α, β, γ, G protein α,β,γ subunits