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5-Hydroxytryptamine$_{2A}$ Receptors Expressed in Rat Renal Mesangial Cells Inhibit Cyclic AMP Accumulation

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SUMMARY

Second messenger coupling of the 5-hydroxytryptamine (5-HT)$_{2A}$ receptor endogenous to cultured rat glomerular mesangial cells was studied. 5-HT induced an increase in total inositol phosphate levels (EC_{50} = 265 ± 55 nM, maximum stimulation = 150 ± 23%). That effect was sensitive to antagonists of the 5-HT$_{2A}$ receptor and was insensitive to pertussis toxin at doses that eliminated detectable pertussis toxin substrate, as determined by membrane ADP-ribosylation. Surprisingly, 5-HT also induced an inhibition of forskolin-stimulated cAMP accumulation (55 ± 6%, IC_{50} = 5 ± 3 nM). This effect was competitively antagonized by the 5-HT$_{2A}$ receptor antagonists ketanserin, ritanserin, and spiperone and could be produced by the 5-HT$_2$ receptor agonists α-methyl-5-HT (66 ± 13%, IC_{50} = 23 ± 14 nM) and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (65 ± 4%, IC_{50} = 14 ± 7 nM). The inhibition of cAMP accumulation occurred in the presence of a number of agents that either stimulate or inhibit protein kinase C activity, arachidonic acid metabolism, or Ca^{2+} mobilization. In isolated membranes, 5-HT induced a 36 ± 5% inhibition of adenylyl cyclase activity (IC_{50} = 8 ± 4 nM). Inhibition of cAMP accumulation in intact cells and of adenylyl cyclase activity in washed membranes was (>50%) sensitive to pertussis toxin, implicating $G_{i\alpha}$ or $G_{o\alpha}$ subunits in the inhibitory signal. These data suggest that the 5-HT$_{2A}$ receptor can be permissive in its coupling to G proteins and second messengers.

The renal glomerulus is composed of a number of cell types, including endothelial cells, epithelial cells, and mesangial cells. Because glomerular mesangial cells are contractile, they are thought to play a critical role in the ultrafiltration function of the kidney. That function is central to electrolyte, water, and protein homeostasis. Because they are juxtaposed between the vascular compartment and urine compartment, mesangial cells can be influenced by a number of vasoactive or vascular substances. Those include contractile substances such as angiotensin II, arginine-vasopressin, glucose, thromboxane, and serotonin (5-HT) (1–3) and relaxation-inducing substances such as dopamine and PGE_2 (4). Those substances could modulate ultrafiltration either acutely, by inducing contractile changes in the mesangium, or chronically, by leading to mitogenesis or deposition of extracellular matrix, such as might occur in idiopathic mesangiocapillary-type glomerulonephritis or diabetes mellitus. Because the glomerulus could be exposed to 5-HT through local synthesis from the precursor molecule L-5-hydroxytryptophan (5) or by release from platelets or other infiltrating cells, 5-HT from several sources could modulate glomerular function. We have recently identified a rat glomerular 5-HT receptor as the 5-HT$_{2A}$ receptor (6), which is also expressed in brain, aorta, and platelets (see Ref. 7 and references cited therein). Like the 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors, the primary signaling pathway linked to the 5-HT$_{2A}$ receptor is activation of phospholipase C (1, 2). However, in glomerular mesangial cells the signaling pathways linked to the 5-HT$_{2A}$ receptor are quite diverse, including phosphoinositide metabolism (2), liberation of Ca^{2+} derived from intracellular pools (2, 3), activation of PKC (2), stimulation of vasodilator prostaglandin synthesis (1), Cl^- conductance-related membrane depolarization, prolonged cytosolic alkalinization related to activation of electroneutral Na^+/H^+ exchange, enhanced Na^+-independent Cl^-/HCO_3^- countertransport (3), and activation of mitogenesis (2). In particular, the mitogenic effect may be involved either in the pathogenesis of some platelet-associated renal diseases, such as hemolytic-uremic syndrome, or

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HA-1004, *N*-(2'-guanidinoethyl)-5-isoquinolinesulfonamide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; H-7, (±)-1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride; PAPP, 1-[2-(4-aminophenyl)ethyl]-4-(3-trifluoromethylphenyl)piperazine; PBS, phosphate-buffered saline; PDBu, phorbol-12,13-dibutyrate; PGE_2, prostaglandin E_2; PKC, protein kinase(s) C (calcium- and phospholipid-dependent protein kinases); PLA_2, phospholipase A_2; PMA, phorbol-12-acetate-13-myristate; PCR, polymerase chain reaction.

in the normal physiologic response to local platelet activation.

Despite the obvious complexity of signal transduction pathways linked to the 5-HT$_{2A}$ receptor in mesangial cells, there are no reports of the ability of the receptor to modulate adenylyl cyclase in those cells. That issue is particularly important because cAMP induces glomerular relaxation (4) and because of the recent awareness of the complex mechanisms that regulate the activity of at least eight different adenylyl cyclases (8). There are at least four potential ways through which the 5-HT$_{2A}$ receptor could either increase or decrease the activity of adenylyl cyclases. First, low micromolar concentrations of Ca^{2+} can inhibit the activity of adenylyl cyclase types 5 and 6 (9, 10). Second, activation of a Ca^{2+}/calmodulin pathway can activate adenylyl cyclase types 1, 3, and 5 (11–13). Third, PKC can activate type 2 and other adenylyl cyclases (14). Fourth, G protein $\beta\gamma$ dimers can inhibit type 1 adenylyl cyclase (15) or, in concert with $G_{s\alpha}$, can stimulate types 2 and 4 adenylyl cyclases (15, 16).

Further impetus for these studies was provided by two recent reports. The first showed that 5-HT$_{2A}$ receptors could amplify increases in forskolin- or adenosine receptor-stimulated cAMP accumulation in the rat neuronal A1A1 cell line through pathways involving PKC-dependent and calcium/calmodulin-dependent mechanisms (17). The second described the ability of bradykinin receptors, which (like the 5-HT$_{2A}$ receptor) primarily activate phospholipase C, to inhibit cAMP accumulation in rat mesangial cells through a pathway involving PKC (18). In light of the studies of bradykinin receptors in mesangial cells, we predicted that the 5-HT$_{2A}$ receptor would also inhibit cAMP accumulation through PKC activation in mesangial cells. Thus, we wanted to examine the possibility that the 5-HT$_{2A}$ receptor expressed in rat renal mesangial cells (6) might modulate the activity of adenylyl cyclase.

Experimental Procedures

Materials. Drugs and reagents were obtained from the following sources. 5-HT and other serotonergic ligands, 3-isobutyl-1-methylxanthine, pertussis toxin, mepacrine, mellitin, phorbol esters, and indomethacin were from Sigma Chemical Co. (St. Louis, MO) or Research Biochemicals (Natick, MA). Forskolin was from Calbiochem (San Diego, CA). Staurosporine, HA-1004, and H-7 were from LC Laboratories (Woburn, MA). *myo*-[^{3}H]Inositol (740 GBq/mmol), [α-^{32}P]ATP (1.11 TBq/mmol), [^{32}P]NAD (1.11 TBq/mmol), [^{3}H]cAMP (555 GBq/mmol), and ^{125}I-cAMP were from DuPont-NEN (Boston, MA). ^{125}I-cAMP and anti-cAMP antiserum were gifts from Dr. Tom Gettys (Department of Medicine, Medical University of South Carolina). Cell culture media, serum, and antibiotics were obtained from Gibco-BRL (Gaithersburg, MD) and culture flasks from Costar (Cambridge, MA). All consumables for microphysiometry were obtained from Molecular Devices (Sunnyvale, CA).

Isolation and primary culture of rat mesangial cells. Mesangial cells were obtained from 150–200-g PVG rats using standard seiving techniques, as described previously (see Ref. 6 and references cited therein). Cells were incubated at 37° in a humidified atmosphere of 95% air/5% CO_2, subcultured every 1–2 weeks by trypsinization, and plated at a density of 2–5 × 10^4 cells/ml in RPMI 1640 medium supplemented with HEPES, pH 7.3, 5 μg/dl insulin, and 20% fetal calf serum. Cells from passages 4–30 were used; after about passage 35, the cells lost the ability for 5-HT to modulate second messengers.

Intact cell cAMP radioimmunoassay and membrane adenylyl cyclase measurements. Intracellular cAMP was measured by radioimmunoassay. 3-Isobutyl-1-methylxanthine (100 μM) was included in the incubations to minimize cAMP degradation. For washed membrane assays, mesangial cells were rinsed with PBS and scraped into 5 ml of the same buffer. After centrifugation (200 × *g* for 10 min), the cell pellet was resuspended in 5 ml of 5 mM Tris, pH 7.4, 5 mM EDTA, and lysed with a glass/glass homogenizer after a 10-min incubation on ice. After a low speed centrifugation step (200 × *g* for 10 min), the pellet containing the nuclear fraction was discarded and the supernatant was centrifuged at 40,000 × *g* for 10 min. The pellet was resuspended in 50 mM sodium-HEPES, pH 8, 2 mM EDTA, and washed in the same buffer. Membranes were resuspended at a concentration of 10 mg of protein/ml for storage at −80°. Adenylyl cyclase activity was then measured by a slight modification of the protocol of Salomon *et al.* (19), using sequential Dowex and alumina chromatography. Conversion of ATP to cAMP was calculated with a personal computer, after correction for recovery of [^{3}H]cAMP tracer.

Measurement of total inositol phosphates. Total inositol phosphates were measured by the technique of Martin (20), as modified previously (21). Cells were maintained in 2 ml of Dulbecco's modified Eagle's medium/F-12 medium, supplemented with 3% fetal calf serum and 8 μCi (~0.3 MBq) of *myo*-[^{3}H]inositol, for 48 hr before studies. Cells layers were washed for 30 min in PBS (with no calcium) and then preincubated for an additional 30 min in PBS containing 2 mM LiCl without calcium. After 30 min, agents were added to the buffer for an additional 30-min incubation. Total inositol phosphates were then extracted on Dowex AG 1-X8 resin in the formate phase.

ADP-ribosylation of membranes. Pertussis toxin-facilitated ADP-ribosylation of membranes was performed exactly as described previously (21), using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography with Kodak XAR film.

Microphysiometry studies. For studies of cell activation, we measured receptor-induced proton efflux using an eight-chamber Cytosensor microphysiometer (Molecular Devices) (22). The night before measurements, cells were plated in regular medium, at 300,000 cells/well, onto polycarbonate membranes (3-μm pore size) in 12-mm inserts. The day of the study, cells were washed with serum-free, bicarbonate-free, Ham's F-12 medium, placed into the microphysiometer chambers, and perfused with the same medium. Proton efflux was measured with a light-addressable silicon sensor. The pump cycle was set to perfuse cells with medium (with or without test agents) for 60 sec, followed by a 23-sec "pump-off" phase during which proton efflux was measured. Cells were typically exposed to the various agents for three or four pump cycles (270 or 360 sec). Valve switches were performed at the beginning of each pump cycle, and data points were accumulated continuously every 90 sec. The peak effect during stimulation was expressed as the percentage of basal efflux rates (averaged from four or five previous measurements) to account for variability between the chambers. Typical basal efflux rates ranged from 100 to 200 μV/sec. Cells were usually responsive for 6–8 hr under the conditions described.

Results

Effect of 5-HT on total phosphoinositide metabolism. Treatment of mesangial cells with 5-HT resulted in an increase in total cellular inositol phosphate levels (Fig. 1). The increase in inositol phosphate levels produced by 5-HT was concentration dependent, with an EC_{50} of 265 ± 55 nM and a maximum stimulation of 150 ± 22%. The stimulatory effect of 10 μM 5-HT could be mostly blocked by the antagonists ketanserin and ritanserin, consistent with the involvement of the endogenous 5-HT$_{2A}$ receptor. 5-HT (10 μM) in the presence of 10 μM ketanserin caused only a 24 ± 5% increase in inositol phosphate levels (five experiments) and in the presence of 10 μM ritanserin caused stimulation of only 22 ± 11% (four experiments). In matched control experiments using

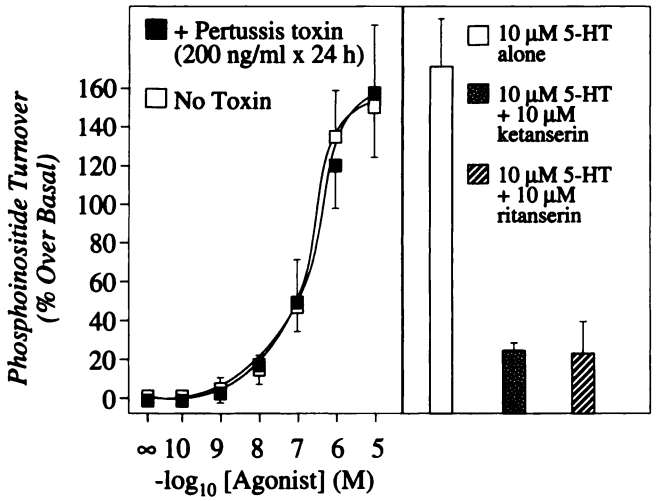

Fig. 1. Increased phosphoinositide turnover induced by 5-HT. *Left*, lack of effect of pertussis toxin on total cellular inositol phosphate levels. 5-HT induced a maximal 150 ± 23% increase in vehicle-treated cells (□) and 155 ± 28% stimulation in pertussis toxin-treated cells (■) (five experiments). *Points*, average of data from the five experiments; *error bars*, interexperiment standard error. Maximal percentage stimulation was calculated for each experiment by least-squares, nonlinear, regression analysis (InPlot program; GraphPAD, San Diego, CA). *Right*, attenuation of 5-HT-stimulated phosphoinositide turnover (□) by ketanserin (▩) and ritanserin (▨). Data were derived from five experiments.

cells with no antagonist, 10 μM 5-HT induced a 165 ± 23% increase in inositol phosphate levels. In intact cells pretreated with pertussis toxin (200 ng, for 24 hr), the subsequent effect of 5-HT to increase inositol phosphate levels was unchanged (EC_{50} = 292 ± 43 nM, 155 ± 28% stimulation). Membranes derived from those cells pretreated with pertussis toxin showed an absence of detectable pertussis toxin-catalyzed ADP-ribosylated substrate, confirming a lack of involvement of a $G_{i\alpha}$ or $G_{o\alpha}$ protein in hydrolysis of phosphoinositides initiated by 5-HT (see Fig. 5).

Effect of 5-HT on cAMP accumulation. The application of 5-HT (0.1 nM to 0.1 mM) to intact mesangial cell monolayers was without effect on basal cAMP levels. However, 5-HT over the same concentration range decreased forskolin-stimulated cAMP accumulation to a large degree. Forskolin at 10 μM elevated cAMP accumulation approximately 10-fold within 10 min. Addition of 5-HT decreased forskolin-stimulated cAMP accumulation by 55 ± 6%, with an IC_{50} value of 5 ± 3 nM (26 experiments). Although we were previously able to amplify cDNA for only the 5-HT_{2A} receptor from mesangial cell mRNA by reverse transcription-PCR, we felt that it was essential to consider the possibility that another receptor type (5-HT_1) might be mediating the inhibition of cAMP accumulation. We examined that possibility in two ways. First, the nonspecific 5-HT_2 receptor agonist α-methyl-5-HT and the specific 5-HT_2 receptor agonist DOI were able to inhibit forskolin-stimulated cAMP accumulation by 66 ± 13% and 65 ± 4%, respectively, with IC_{50} values of 23 ± 14 and 14 ± 7 nM, respectively (three experiments) (Fig. 2A). Second, the 5-HT_{2A} receptor antagonists ketanserin, ritanserin, and spiperone were able to competitively inhibit the effect of 5-HT on cAMP accumulation (Fig. 2B), with K_B values of 0.6, 0.3, and 0.6 nM, respectively, as calculated by the method of Arunlakshana and Schild (23) (two, two, and four experiments, respectively). DOI is a highly potent (≈10

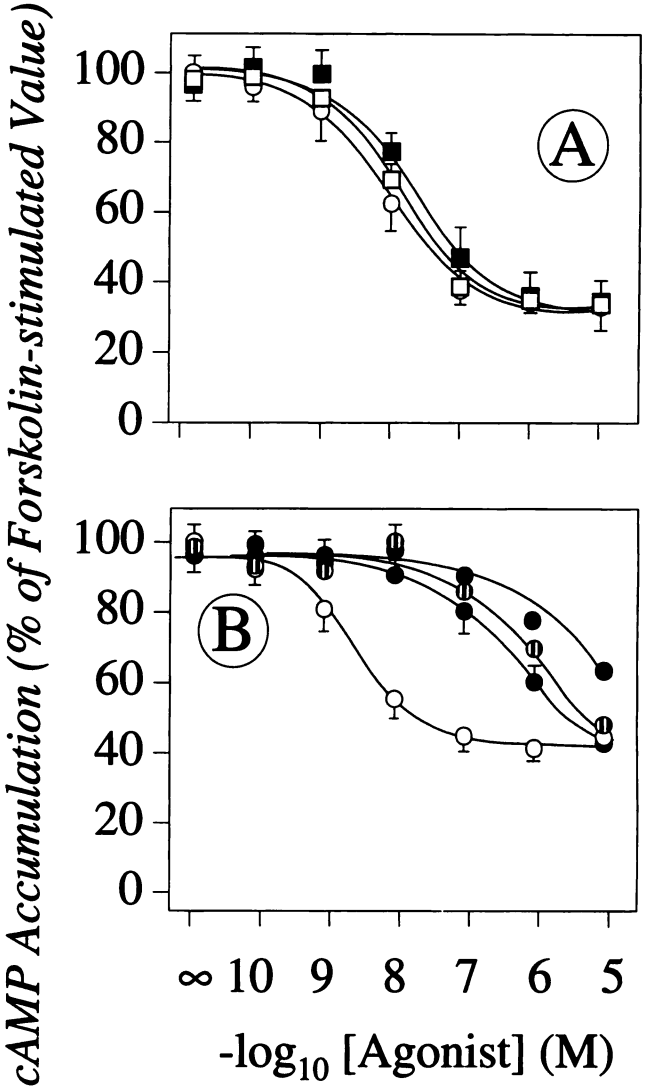

Fig. 2. Involvement of the 5-HT_2 receptor in inhibition of cAMP accumulation in intact mesangial cells. A, 5-HT (○) and the selective 5-HT_2 receptor agonists α-methyl-5-HT (■) and DOI (□) inhibited forskolin-stimulated cAMP accumulation. B, Concentration-response curves for 5-HT alone (○) or in the presence of 100 nM levels of the competitive antagonists ketanserin (●), ritanserin (◍), or spiperone (●) are shown. The plots are representative of three to five separate experiments performed in triplicate. *Standard error bars*, intra-assay variability.

nM) agonist at 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors (24, 25), with much lower potency (>1 μM) at 5-$HT_{1D\beta}$, 5-HT_{1E}, 5-HT_{1F}, and 5-HT_7 receptors (26–29). The agonist α-methyl-5-HT is somewhat less selective, having high affinity for all three subtypes of 5-HT_2 receptors and moderate (100–200 nM) affinities for 5-HT_{1E} and 5-HT_{1F} receptors (27, 28). Ketanserin is a potent antagonist of 5-HT_{2A} (≈1 nM) and 5-HT_{2C} (≈100 nM) receptors (24), with much lower affinity (>1 μM) for 5-$HT_{1D\beta}$, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{5A}, 5-HT_{5B}, and 5-HT_7 receptors (26–30). The antagonist spiperone has high affinity (≈1 nM) for 5-HT_{2A} receptors (24), moderate affinity (≈100 nM) for 5-HT_{1A} and 5-HT_7 receptors (31), and much lower affinity for 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$, 5-HT_{1E}, 5-HT_{1F}, 5-HT_4, and 5-HT_{5B} receptors (26–28, 31–34). Therefore, the data on inhibition of cAMP accumulation are most consistent with an action of an endogenous 5-HT_{2A} receptor.

We next considered the possibility that the effect of 5-HT to

lower forskolin-stimulated cAMP levels was due to activation of cAMP phoshodiesterase activity, resulting in increased degradation of cAMP. 5-HT decreased cAMP accumulation induced by forskolin equally well in the absence and presence of 100 μM isobutylmethylxanthine, which inhibits the activity of a number of cAMP phosphodiesterases (three experiments) (data not shown). Moreover, the inhibition by 5-HT of cAMP accumulation was not due to increased efflux of cAMP from the cells, because the proportions of intracellular (≈20%) and extracellular (≈80%) cAMP were not changed in the presence of 5-HT (three experiments) (data not shown). Thus, it appeared most likely that the inhibition of cAMP accumulation by the 5-HT$_{2A}$ receptor in mesangial cells was mediated by an inhibitory effect on the activity of adenylyl cyclase(s). Additional studies were necessary to establish whether that effect was mediated through an indirect (non-plasma membrane-delimited) pathway or a direct (membrane-delimited) pathway.

Lack of evidence for an indirect pathway leading to inhibition by the 5-HT$_{2A}$ receptor of cAMP accumulation in mesangial cells. Several indirect pathways for the inhibition of cAMP accumulation were considered. First, we tested the possibility that a Ca^{2+}-dependent mechanism was involved. It was necessary to test this possibility because low micromolar concentrations of Ca^{2+} can inhibit the activity of types 5 and 6 adenylyl cyclases (9, 10). We found no evidence to support the involvement of Ca^{2+} in the inhibition by 5-HT of cAMP accumulation in mesangial cells. First, 5-HT was able to inhibit forskolin-stimulated cAMP accumulation equally well in the presence of 5 mM extracellular Ca^{2+} and in its nominal absence. In fact, all of the studies shown in this report were performed in PBS without Ca^{2+} or Mg^{2+} supplementation, unless otherwise noted. Second, 5-HT was able to inhibit cAMP accumulation after treatment with 5 mM extracellular EGTA for up to 1 hr, a treatment that chelates extracellular Ca^{2+} and depletes intracellular Ca^{2+} stores (Fig. 3A). Third, 5-HT was able to inhibit cAMP accumulation in the presence of the Ca^{2+}-ATPase inhibitor thapsigargin, which leads to prolonged elevation of intracellular Ca^{2+} levels in these cells (Fig. 3A).

Because 5-HT activates PLA_2 and increases prostaglandin synthesis in mesangial cells (1) and because prostaglandins can modulate cAMP levels through cell surface receptors, we considered the possibility that the effect of 5-HT was mediated either through a pathway involving PLA_2 activation or through an autocrine loop involving release of prostaglandins. Although PGE_2 has been reported to stimulate cAMP accumulation in rat mesangial cells, prostaglandins have also been reported to inhibit adenylyl cyclase activity in kidney proximal tubules (18). The involvement of PGE_2 in the 5-HT-inhibited cAMP accumulation is unlikely, because PGE_2 stimulated cAMP accumulation by about 40% (three experiments), in keeping with the findings of Bascands *et al.* (18). It is also unlikely that other cyclooxygenase products of arachidonic acid metabolism mediate the effect of 5-HT. Fig. 3B shows that the cyclooxygenase inhibitor indomethacin (10 μM) did not alter the ability of 5-HT to inhibit cAMP accumulation. It is also unlikely that PLA_2 activation plays a role in 5-HT-inhibited cAMP accumulation, because 5-HT inhibited cAMP accumulation in the presence of the nonspecific PLA_2 activator mellitin and the nonspecific PLA_2 inhibitor mepacrine (Fig. 3B).

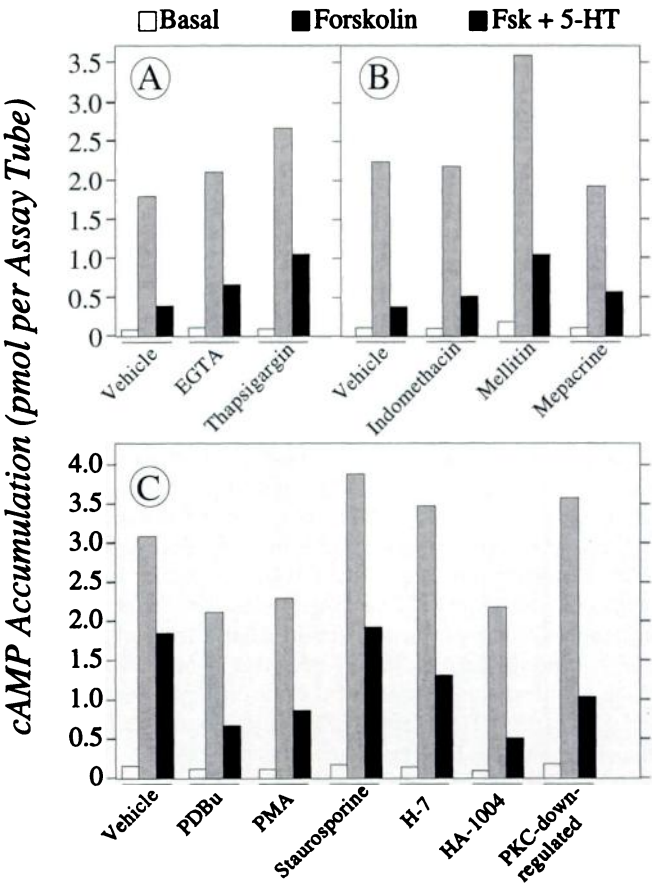

Fig. 3. Lack of evidence for an indirect pathway leading to inhibition by the 5-HT$_{2A}$ receptor of cAMP accumulation in mesangial cells. Cells were pretreated with vehicle or test agents for 30 min before measurement of cAMP accumulation, except when otherwise indicated. A, Effects of chelation of Ca^{2+} with 5 mM EGTA and mobilization of Ca^{2+} with 10 μM thapsigargin on the ability of 5-HT to inhibit forskolin (*Fsk*)-stimulated cAMP accumulation. B, Effects of regulators of arachidonic acid metabolism (10 μM each) on the ability of 5-HT to inhibit forskolin-stimulated cAMP accumulation. C, Effects of regulators of PKC on the ability of 5-HT to inhibit forskolin-stimulated cAMP accumulation. All PKC agents were used at 10 μM, except the phorbol esters PMA and PDBu, which were used at 5 μM for short term (30-min) treatment and at 16 μM for prolonged treatment (PKC down-regulation). Each assay is representative of at least three assays performed in duplicate or triplicate. Data from all experiments are presented in Table 1.

It was important to examine the potential role of PKC in the 5-HT-inhibited cAMP accumulation, because of the recent report that bradykinin inhibits cAMP accumulation in rat renal mesangial cells through activation of a phorbol ester-sensitive PKC (18). Three kinase inhibitors, with a rank order of potency of staurosporine > H-7 > HA-1004, were used to test the involvement of PKC in the ability of 5-HT to inhibit forskolin-stimulated cAMP accumulation. None of those compounds blocked the maximal inhibitory effect of 5-HT. Because staurosporine and HA-1004 can also block the activity of cGMP-dependent protein kinase, its involvement is similarly unlikely. The need for PKC was further tested by prolonged treatment with 16 μM PMA, which should lead to down-regulation of phorbol-sensitive PKC isotypes through increased degradation. These studies suggest that PKC is not necessary for 5-HT to inhibit cAMP accumulation. Moreover, although acute administration of the active

phorbol ester PDBu did slightly lower basal cAMP levels and attenuate forskolin-stimulated cAMP accumulation, as reported previously (18), 5-HT was still able to further suppress cAMP accumulation in its presence (Fig. 3C). Because 5-HT inhibits cAMP accumulation in the presence of PDBu, this finding suggests that the two compounds use distinct pathways to inhibit cAMP accumulation. The ability of 5-HT to inhibit cAMP accumulation was similarly unaffected by treatment with the tyrosine kinase inhibitor genistein (Table 1). In aggregate, the studies discussed in this section led us to examine the possibility that the 5-HT_{2A} receptor inhibits cAMP accumulation through a direct (plasma membrane-delimited) pathway.

Role of a direct (plasma membrane-delimited) pathway through which 5-HT inhibits cAMP accumulation. To assess such a possibility, we used washed membranes to measure forskolin-stimulated adenylyl cyclase activity. Somewhat unexpectedly, 5-HT consistently produced a small (36 ± 5%, six experiments) inhibition of forskolin-stimulated cAMP accumulation (Fig. 4). The IC_{50} (8 ± 4 nM) was similar to that obtained for 5-HT in intact cells. The same degree of inhibition (30 ± 4%) was produced in three experiments using the nonselective 5-HT_{2A} receptor agonist α-methyl-5-HT, supporting the identity of the inhibitory receptor as the 5-HT_{2A} receptor. This degree of inhibtion was similar to that induced by the A1 adenosine receptor agonist (*R*)-N^6-(1-methyl-2-phenylethyl)adenosine (32 ± 3%) (Fig. 4), which has already been shown to inhibit cAMP accumulation and adenylyl cyclase activity in rat mesangial cells (18). Thus, this set of studies supports the hypothesis that the 5-HT_{2A} receptor either interacts directly with an inhibitory G protein α subunit or interacts through release of βγ subunits, which can inhibit sensitive adenylyl cyclases such as types 1, 2, and 4 (15, 16).

Role of inhibitory G proteins. The involvement of pertussis toxin-sensitive G proteins in 5-HT_{2A} receptor-mediated inhibition of cAMP accumulation was investigated first in washed membranes. Basal cyclase activity was no different in cells that had been pretreated with pertussis toxin (200 ng, for 24 hr) than in cells that had been treated with vehicle. The ability of 5-HT to inhibit adenylyl cyclase activity was attenuated by more than half (34 ± 6% versus 14 ± 5% inhibition, $p < 0.05$, three experiments) under conditions that eliminated almost all detectable substrate from subsequent pertussis toxin-catalyzed ADP-ribosylation experiments (Fig. 5B). The same approach was used in intact cells, where basal cAMP accumulation was not significantly altered by pertussis toxin pretreatment. However, pertussis toxin attentuated 5-HT-mediated cAMP inhibition to an even greater degree than that shown in the membrane assay (Figs. 4 and 5A). In control cells 5-HT induced a 53 ± 8% decrease in forskolin-stimulated cAMP levels (IC_{50} = 4 ± 2 nM, seven experiments), whereas in pertussis toxin-treated cells it induced a 6 ± 5% decrease in forskolin-stimulated cAMP levels (seven experiments). These results suggest at least a partial role for pertussis toxin-sensitive G proteins in

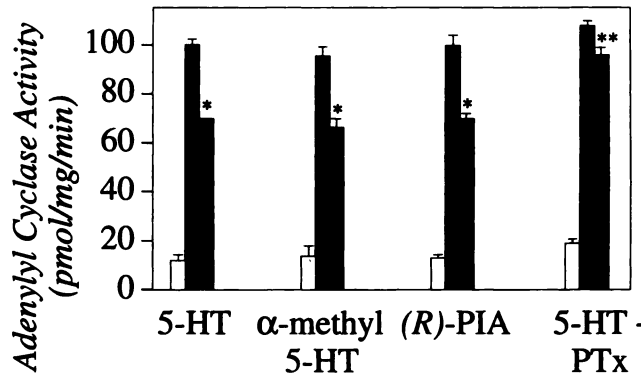

Fig. 4. Role of a direct (plasma membrane-delimited) pathway through which 5-HT inhibits cAMP accumulation. Membrane adenylyl cyclase activity was measured for 30 min at 37°, using approximately 20 μg of protein/tube. Data (mean ± standard error) are derived from three experiments performed in triplicate, in which basal levels of cAMP (□), 100 μM forskolin-stimulated levels (▩), and levels in the presence of forskolin plus test agent (10 μM) (■) were measured. *(R)-PIA*, (*R*)-N^6-(1-methyl-2-phenylethyl)adenosine. *5-HT + PTx*, cells were pretreated with 200 ng/ml pertussis toxin for 24 hr before the assay. Inhibition of forskolin-stimulated adenylyl cyclase activity was significant in each case (*, $p < 0.01$; **, $p < 0.05$; reverse Bonferroni correction used). Experiments using full concentration-response curves are discussed in the text.

TABLE 1

5-HT-induced inhibition of mesangial cell cAMP accumulation under various conditions

Data are presented as mean ± standard error of *n* experiments. Experimental details are provided in Experimental Procedures and in the legends to Fig. 3 and 5.

Condition	Inhibition	*p* versus control	IC_{50} for 5-HT	*p* versus control	*n*
	%		nM		
All vehicle	55 ± 6		5 ± 3		26
Pertussis toxin	6 ± 5	0.00005	NA[a]	0.003	7
+10 μM Indomethacin	60 ± 9	NS[b]	3 ± 1	NS	3
+10 μM Mepacrine	43 ± 12	NS	2 ± 1	NS	3
+10 μM Mellitin	67 ± 13	NS	10 ± 7	NS	3
+5 mM EGTA	56 ± 11	NS	12 ± 7	NS	3
+10 μM Thapsigargin	47 ± 12	NS	102 ± 101[c]	NS	5
+10 μM Staurosporine	44 ± 6	NS	41 ± 30[c]	NS	4
+10 μM H-7	40 ± 8	NS	13 ± 11	NS	7
+10 μM HA-1004	42 ± 8	NS	82 ± 65[c]	NS	6
+5 μM PMA or PDBu	46 ± 9	NS	101 ± 103[c]	NS	4
PKC-down-regulation	58 ± 19	NS	136 ± 135[c]	NS	6
+10 μM Genistein	53 ± 11	NS	51 ± 49[c]	NS	4

[a] NA, not applicable.

[b] NS, not significant.

[c] There is a great deal of variability in the data presented in this table, none of which reflects a statistically significant variation from control values, due to large standard errors. The variability is due in each case to a single aberrant IC_{50} value (~500 nM to 5 μM). When the "aberrant" assays were excluded from data analyses, all of these mean IC_{50} values fell in the range of 2–10 nM.

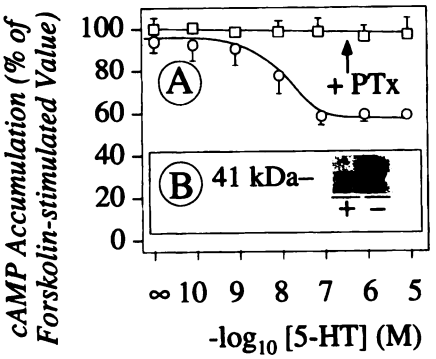

Fig. 5. Role of a pertussis toxin-sensitive pathway for 5-HT-inhibited cAMP accumulation in intact cells. A, Data (mean ± standard error) are derived from a single experiment (representative of seven experiments performed in triplicate) in which increasing concentrations of 5-HT were used in assays with 100 μM forskolin after treatment with vehicle (○) or pertussis toxin (*PTx*) (200 ng/ml, for 24 hr) (□). Full details of the experimental data are provided in the text. B, Pertussis toxin pretreatment eliminated nearly all detectable pertussis toxin substrate, as determined by ADP-ribosylation assay of membranes (three experiments). This representative gel used 20 μg of membranes for each condition and was exposed to film for 24 hr at room temperature. +, pretreatment with PTx; −, vehicle treatment.

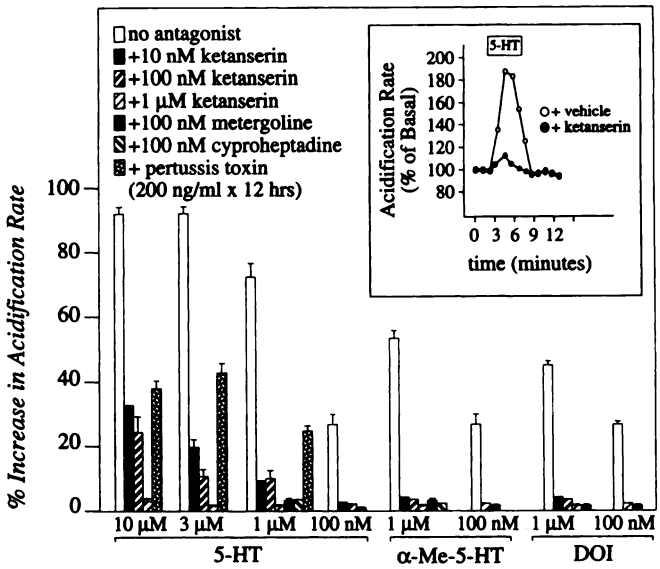

Fig. 6. Microphysiometry studies supporting the presence of 5-HT$_{2A}$ receptors in rat mesangial cells. The 5-HT$_{2A}$ receptor agonists 5-HT, DOI, and α-methyl-5-HT (*α-Me-5-HT*) increased the cellular acidification rate in a concentration-responsive pattern. Increased acidification was blocked by a number of 5-HT$_{2A}$ receptor antagonists. The inhibition by ketanserin was also dependent on concentration. Pertussis toxin treatment also attenuated the 5-HT-generated signal by >50%; similar results for α-methyl-5-HT are not shown. *Inset*, representative tracings from two different microphysiometer chambers. All experiments were performed in at least four chambers in two to six different experimental sessions, and data (mean ± standard error) presented are pooled from all experiments performed for each condition.

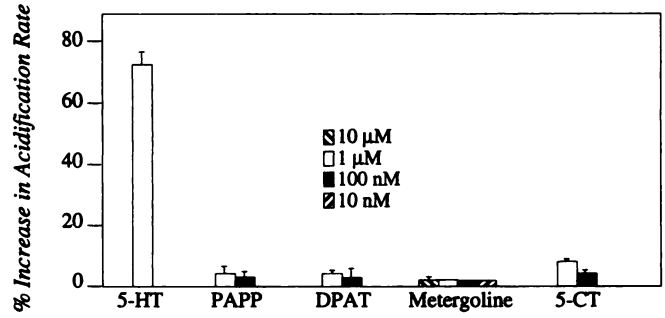

Fig. 7. Microphysiometry studies not supporting the presence of 5-HT$_1$ receptors in rat mesangial cells. In contrast to 5-HT, the 5-HT$_{1A}$ receptor agonists PAPP, 8-hydroxy-2-dipropylaminotetralin (*DPAT*), metergoline, and 5-carboxamidotryptamine (*5-CT*) had minimal effects on the acidification rate of rat renal mesangial cells. All experiments were performed in at least four chambers in two to six different experimental sessions, and data (mean ± standard error) presented are pooled from all experiments performed for each condition.

carrying the inhibitory effect of the 5-HT$_{2A}$ receptor to the adenylyl cyclase(s) through a membrane-delimited pathway.

Lack of evidence for the involvement of other 5-HT receptors in signaling in mesangial cells. We have previously been able to demonstrate mRNA or PCR products for only the 5-HT$_{2A}$ receptor in rat mesangial cells (6). However, due to the surprising finding that the 5-HT$_{2A}$ receptor apparently inhibits cAMP accumulation in mesangial cells with high potency and efficacy, we were compelled to further examine the possibility that an endogenous 5-HT$_1$ receptor is involved, particularly in light of the observed, unexpectedly higher potency of 5-HT to inhibit cAMP accumulation than to stimulate hydrolysis of inositol polyphosphates. We chose to use microphysiometry, an extremely sensitive means of assessing cellular activation by receptors (22). Fig. 6 shows that 5-HT, DOI, and α-methyl-5-HT induced concentration-dependent increases in the acidification rate and that those increases were antagonized by the 5-HT$_2$ receptor antagonists ketanserin, metergoline, and cyproheptadine. Those results are consistent with the predominant or exclusive presence of 5-HT$_2$ receptors in mesangial cells. The signal was also >50% blocked by pretreatment with pertussis toxin, reminiscent of the effect of similar treatment on the ability of 5-HT to inhibit cAMP accumulation (Fig. 6). Fig. 7 shows that a number of prototypical 5-HT$_1$ receptor agonists (8-hydroxy-2-dipropylaminotetralin, 5-carboxamidotryptamine, PAPP, and metergoline) had minimal effects on acidification. Importantly, in the current studies metergoline served as an antagonist without detectable agonist properties (Figs. 6 and 7). Metergoline has been shown to be a 5-HT$_2$ receptor antagonist while being a full or partial agonist for 5-HT$_{1D\alpha}$, 5-HT$_{1D\beta}$, and 5-HT$_{1F}$ receptors and being inactive at 5-HT$_{1E}$ and 5-HT$_4$ receptors (26, 28, 31, 32).

Discussion

5-HT was previously shown to be a mitogen in rat glomerular mesangial cells (2), probably through a 5-HT$_2$ receptor subtype, although the precise 5-HT receptor subtype was not clearly delineated. Because members of both the 5-HT$_1$ (5-HT$_{1A}$ and 5-HT$_{1B}$) and 5-HT$_2$ (5-HT$_{2A}$ and 5-HT$_{2C}$) receptor subfamilies have been shown to mediate growth and malignant transformation of fibroblast cells (35–38), we previously sought to identify the 5-HT$_2$ receptor subtype expressed on mesangial cells (6). Those studies showed by reverse transcription-PCR and RNA blot assays that mesangial cells express a serotonin receptor of the 5-HT$_{2A}$ (and not 5-HT$_{2C}$ or 5-HT$_{1A}$) subtype, which is therefore the primary candidate receptor to mediate mitogenesis, ultrafiltration, and other effects in those cells.

In the current study, we characterized some of the signal-

ing pathways of the mesangial cell 5-HT_{2A} receptor, as an initial step toward identifying the candidate mitogenic and contractile signaling pathways linked to this receptor. Our results confirm that phospholipase C is activated as proposed (1, 2) and shows a 2-fold increase in total cellular inositol phosphate levels, with an EC_{50} of 265 ± 55 nM for serotonin. We further characterized that effect of serotonin as being independent of $G_{i\alpha}$ proteins, as demonstrated by persistent 5-HT-induced inositol phosphate increases in cells pretreated with pertussis toxin in amounts sufficient to completely eliminate pertussis toxin substrate, as measured by *in vitro* ADP-ribosylation assays with washed cell membranes.

Unexpectedly, the present study also demonstrated that 5-HT efficiently inhibits cAMP accumulation induced by forskolin in mesangial cells (55 ± 6%, with an IC_{50} value of 5 ± 3 nM, 26 experiments). This effect appeared to be mediated by the 5-HT_{2A} receptor, because it was blocked by the 5-HT_{2A} receptor antagonists ketanserin, ritanserin, and spiperone. Additional evidence for the predominant or exclusive involvement of the 5-HT_{2A} receptor was provided by microphysiometry studies. In contrast to the effects on phosphoinositide hydrolysis, the inhibition of cAMP accumulation by 5-HT was sensitive to pertussis toxin, arguing for at least a partial role of $G_{i\alpha}$ or $G_{o\alpha}$ subunits in the regulation of cAMP levels by the 5-HT_{2A} receptor. This finding was also unexpected, because all members of the 5-HT_2 receptor subfamily share structural and functional properties, including a nearly exclusive coupling to pertussis toxin-insensitive G proteins.

Recently, several unconventional pathways leading to the inhibition of cellular cAMP levels have been described. Those pathways appear to involve indirect routes, requiring cross-talk between various signaling pathways formerly thought to be highly compartmentalized. In general, receptors that activate those novel pathways classically couple to phospholipase C activation and, hence, to elevation of intracellular Ca^{2+} levels and/or activation of PKC. Elevation of intracellular Ca^{2+} levels has been shown to inhibit cAMP accumulation in a number of cell types, including NCB-20 cells (39), C6–2B cells (9), and cardiac myocytes (40). Activation of PKC leads to inhibition of cAMP accumulation in other cell types, including several types of renal cells (41). In contrast, in D384 human astrocytoma cells bradykinin inhibits cAMP accumulation via a calcium-dependent inhibition of adenylyl cyclase, which does not involve a G_i protein, calmodulin, or PKC (42). With respect to mesangial cells, bradykinin inhibits cAMP accumulation through a pathway requiring phospholipase C, PKC, and a pool of pertussis toxin-insensitive G proteins (18).

Because, to our knowledge, a "direct" (membrane-delimited) coupling of any 5-HT_2 receptor to $G_{i\alpha}$ or $G_{o\alpha}$ subunits has not been described, we first studied the possible involvement of indirect candidate pathways in the ability of the 5-HT_{2A} receptor to inhibit cAMP accumulation. We were not able to confirm the involvement of a number of cytosolic factors, such as Ca^{2+} and PKC, in the ability of 5-HT to inhibit cAMP accumulation. Similarly, we found no evidence for the involvement of an autacrine loop involving cyclooxygenase metabolites of arachidonic acid. In membranes, 5-HT was shown to inhibit adenylyl cyclase activity through a mechanism that was partially sensitive to pertussis toxin. Those studies implicate pertussis toxin-sensitive G proteins as one factor in the ability of the 5-HT_{2A} receptor to inhibit cAMP accumulation. It is possible that other factors that are contained within or associated directly with washed mesangial cell membranes also mediate a portion of the inhibitory signal. One likely possibility would be the involvement of $\beta\gamma$ G protein pairs in inhibiting a sensitive adenylyl cyclase type. Those $\beta\gamma$ subunits could be contributed by any G protein α subunit that is activated by the 5-HT_{2A} receptor.

Our studies stimulate an intriguing speculation. In mesangial cells, agents that increase intracellular cAMP levels have been shown to be both antiproliferative and relaxation inducing (1–3). In contrast, agents that activate phospholipase C and either Ca^{2+} mobilization or PKC generally promote proliferation and/or cause contraction. Because the 5-HT_{2A} receptor in mesangial cells both inhibits cAMP accumulation and activates phospholipase C, it is likely that those signaling pathways may cooperate either to induce a mitogenic response or to cause contraction. Such a scheme may provide a mechanism for distal signal amplification by cooperation of two proximal signals generated through a single receptor.

In summary, the current studies present data that support a surprising dual coupling of the 5-HT_{2A} receptor in rat mesangial cells 1) to phosphoinositide hydrolysis through a pertussis toxin-insensitive pathway and 2) to the inhibition of adenylyl cyclase activity through a pathway that is mainly sensitive to pertussis toxin. In that sense, the 5-HT_{2A} receptor may be similar to the thrombin receptor in its ability to transmit cellular signals through both pertussis toxin-sensitive and -insensitive G proteins. Further studies will be needed to determine the full range of potential G protein coupling partners for the 5-HT_{2A} receptor.

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