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5-Hydroxytryptamine Type 2A Receptors Regulate Cyclic AMP Accumulation in a Neuronal Cell Line by Protein Kinase C-Dependent and Calcium/Calmodulin-Dependent Mechanisms

KELLY A. BERG, WILLIAM P. CLARKE, YIBANG CHEN, BARBARA J. EBERSOLE, RONALD D. G. McKAY, and SAUL MAAYANI

Departments of Anesthesiology (K.A.B., B.J.E., S.M.) and Pharmacology (W.P.C., Y.C., S.M.), Mount Sinai School of Medicine, City University of New York, New York, New York 10029, and Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892 (R.D.G.M.)

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SUMMARY

The effects of 5-hydroxytryptamine $(5\text{-HT})_{2A}$ receptor activation on cAMP formation were studied in a cell line derived from embryonic rat cortex (A1A1). 5-HT (EC_{50} = 0.87 μM) amplified the amount of cAMP formed in response to 5′-*N*-ethylcarboxamidoadenosine (an adenosine A_2 receptor agonist), cholera toxin, and forskolin after 15 min of coincubation in the presence of the phosphodiesterase inhibitor rolipram. This effect of 5-HT was blocked by 10 nM ketanserin as well as by 10 nM spiperone, indicating a response mediated by the 5-HT_{2A} receptor subtype. Similarly, cAMP accumulation was enhanced by coincubation with the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore A23187. After exposure to PMA for 24 hr (PKC-depleted cells), 5-HT and A23187 still enhanced cAMP formed in response to forskolin and 5′-*N*-ethylcarboxamidoadenosine, whereas the amplifying effects of PMA were abolished. Analysis by Western blots and PKC activity measurements revealed that, of three PKC isoforms detected in A1A1 cells (α, δ, and ε), only the calcium-independent isoform PKC-ε remained in membrane fractions after long term PMA treatment. In PKC-depleted cells, 5-HT-mediated amplification was greatly reduced after treatment with the calcium chelator 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (acetoxymethyl)-ester or the calmodulin antagonists calmidazolium and *N*-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide hydrochloride. In addition, 5-HT-mediated amplification of cAMP accumulation was reduced by the PKC inhibitor staurosporine in normal cells but was unaffected in PKC-depleted cells. In conclusion, these data suggest that 5-HT_{2A} receptor activation can amplify cAMP formation in A1A1 cells by two distinct pathways coupled to the hydrolysis of inositol phosphates, i.e., PKC and calcium/calmodulin.

In vivo, activation of a single receptor system rarely, if ever, occurs in isolation. Instead, it typically occurs amid a barrage of neurotransmitter, neuromodulator, and hormonal stimulation. Because interactions between the signal transduction systems of different receptors ("cross-talk") are common (1), cellular responses to activation of one receptor system may differ depending upon the simultaneous, previous, or subsequent activition of a second receptor system.

Recent evidence suggests that there is considerable cross-talk between the PLC and AC signal transduction systems. Activation of PLC leads to production of the second messengers diacylglycerol and IP_3. Diacylglycerol activates PKC, whereas IP_3 increases $[Ca^{2+}]_i$ (2, 3). Signal transduction through the AC system can be enhanced or depressed, depending on cell type, by activation of PKC (see Ref. 4 and references cited therein). The target (substrate) of PKC action, which also may differ depending on cell type or the PKC isoforms present (5), may be the receptor (6, 7), G_s (8), G_i (9, 10), the AC enzyme itself (11), or the cAMP phosphodiesterase that metabolizes cAMP. Moreover, several studies have reported that activation of PKC alters the agonist affinity (6) or density of receptors (12). In addition, activation of PKC by PMA results in phosphorylation

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ABBREVIATIONS: PLC, phospholipase C; AC, adenylyl cyclase; 5-HT, 5-hydroxytyptamine (serotonin); BAPTA/AM, 1,2-bis(o-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (acetoxymethyl)-ester; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FSK, forskolin; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IP, inositol phosphates; NECA, 5′-*N*-ethylcarboxamidoadenosine; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline containing 0.01% Tween-20; PMA, phorbol 12-myristate 13-acetate; W-7, *N*-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide hydrochloride; $[Ca^{2+}]_i$, intracellular calcium concentration; AM, acetoxymethyl ester; PKC, protein kinase C; BSS, balanced salt solution; IP_3, inositol trisphosphate; PI, phosphatidylinositol.

of the 5-HT$_{1A}$ receptor and a reduction in 5-HT$_{1A}$ agonist inhibition of AC activity in transfected Chinese hamster ovary cells (13). Thus, PKC appears to have multiple sites of action within the AC signaling system, and the effects of PKC can vary with cell type.

Increases in $[Ca^{2+}]_i$ can also result in cell type-specific stimulatory (14) or inhibitory (15) effects on the AC signaling system. The Ca^{2+}-binding protein calmodulin may mediate some of the Ca^{2+}-dependent effects, which include 1) direct stimulation of AC, 2) stimulation of cAMP phosphodiesterase, and 3) activation of calmodulin-dependent kinase or phosphatase (14).

The 5-HT$_2$ receptor family is composed of 5-HT$_{2A}$ (formerly 5-HT$_2$), 5-HT$_{2B}$ (formerly 5-HT$_{2F}$), and 5-HT$_{2C}$ (formerly 5-HT$_{1C}$) receptor subtypes. These receptors are members of the superfamily of G protein-coupled receptors and are known to couple to PLC in many tissues and cell lines (see Ref. 16). We have investigated the effects of activation of the 5-HT$_{2A}$-PLC signal transduction system on stimulation of cAMP accumulation in a novel neuronal cell line (A1A1) derived from embryonic rat cortex. We have found that activation of 5-HT$_{2A}$ receptors increases hydrolysis of PI and amplifies cAMP accumulation in response to stimulation by FSK, cholera toxin, and NECA, an adenosine receptor agonist. The amplification is dependent upon activation of both PKC- and calcium/calmodulin-dependent pathways.

Materials and Methods

Cell line derivation, characterization, and culture. The A1A1 neuronal cell line was derived from retroviral infection of embryonic day 16 rat cortical cultures. The A1A1 clone was selected from G418-resistant colonies after infection with wild-type simian virus 40, as described previously (17). A1A1 cells express nestin, a protein that is expressed transiently during development and is a marker for neuronal precursor cells (18). Cells were maintained in 5% CO_2 at 33°, in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and 100 units/ml penicillin-streptomycin. Unless otherwise indicated, cells were seeded for all experiments at a density of 4×10^4 cells/cm^2. Before each experiment, cells were maintained in serum-free medium (Dulbecco's modified Eagle medium/F12 medium, 1:1, with 5 μg/ml insulin, 5 μg/ml transferrin, 30 nM selenium, 5.35 μg/ml linoleic acid, 0.1% BSA (i.e., ITS$^+$), 20 nM progesterone, and 100 μM putrescine) for 2–3 days.

cAMP accumulation. cAMP accumulation was measured as described previously (19). Briefly, cells grown in 24-well dishes were washed twice with incubation medium (HBSS containing 2 mM Ca^{2+} and 1.0 mM Mg^{2+} and supplemented with 0.1% BSA, 10 mM HEPES, and 2 units/ml adenosine deaminase). In experiments performed in Ca^{2+}-free medium, Ca^{2+}- and Mg^{2+}-free HBSS supplemented with 1 mM Mg^{2+}, 0.1% BSA, 10 mM HEPES, and 2 units/ml adenosine deaminase (Ca^{2+}-free HBSS) was used. Cells were washed once with Ca^{2+}-free HBSS containing 4 mM EGTA, followed by two washes with Ca^{2+}-free HBSS. After the wash, incubation medium containing the phosphodiesterase inhibitor rolipram (0.1 mM) and various concentrations of agonists were added (three wells/concentration) and the cells were incubated at 37° for 15 min. When antagonists were used, they were added 15 min before agonist and/or rolipram administration. Experiments were terminated by replacement of the incubation medium with absolute ethanol. The ethanol extracts (containing intracellular cAMP) were transferred to 12- × 75-mm culture tubes, dried under a stream of air, and reconstituted with the incubation medium that contained extracellular cAMP for that well. Total cAMP (intracellular plus extracellular) accumulation was determined in duplicate by radioimmunoassay. The residue remaining in each well was solubilized with 1 N NaOH and protein content was measured by the method of Lowry *et al.* (20), using BSA as the standard.

Measurement of IP accumulation. IP accumulation was measured as described previously (21). Cells grown in 12-well plates (initial seed density of 10^5 cells/well) were incubated for 48 hr in serum-free medium containing 2 μCi/ml *myo*-[^{3}H]inositol. Cells were washed and preincubated for 15 min at 37° in HBSS containing 20 mM HEPES and 20 mM LiCl, pH 7.4. Drugs were added to a final volume of 1 ml, and cells were further incubated for 10 min. When antagonists were used, they were added during the preincubation period. The incubation was terminated by the addition of 400 μl of 10% perchloric acid containing 4.8 mM EDTA. After incubation for at least 30 min at 4°, the contents of the wells were neutralized with a solution of 500 mM KOH and 9 mM sodium tetraborate, and the precipitate was allowed to settle. The amount of total IP (inositol monophosphate, inositol bisphosphate, and IP_3) in the supernatant was determined by ion exchange chromatography (22). The tritium content of eluted column fractions was measured by liquid scintillation counting in a Beckman 5000 scintillation counter equipped with automatic quench correction.

PKC activity. PKC activity was measured using the method of Thomas *et al.* (23). After treatment with drugs for the times indicated, cells grown in 15-cm dishes were washed twice with cold PBS, followed by two washes in 20 mM Tris·HCl buffer, pH 7.5, containing 2 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, and 25 μg/ml leupeptin (buffer A), with 333 mM sucrose. Cells were removed from the dish by scraping into buffer A with sucrose. After homogenization at 4°, cytosol and membrane fractions were prepared by centrifugation at 40,000 × *g* for 2 min in an Eppendorf microfuge. For some experiments, before homogenization cells were lysed in buffer A and the nuclei were isolated by centrifugation at 500 × *g* for 10 min. After several washes, the nuclear membrane and remaining membrane fractions were homogenized and solubilized. Membrane fractions (or whole-cell lysates) were solubilized with Triton X-100 (0.5% final concentration). After solubilization, all samples were subjected to DEAE-cellulose chromatography and eluted with buffer A containing 100 mM NaCl. PKC activity was determined after 5 min of incubation at 30°, by measurement of the transfer of [^{32}P]phosphate from [γ-^{32}P]ATP to histone III-S or the oligopeptide substrate pep-ε, which is based on the pseudosubstrate site of PKC-ε (24). The assay mixture (125 μl) contained 20 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 240 μg/ml histone type III-S or 6.25 μg/ml pep-ε, 50 μg/ml leupeptin, 0.48 mM $CaCl_2$, 1.6 μM 1,2-dioctanoylglycerol, 20 μg of phosphatidylserine, 200 μM [γ-^{32}P] ATP, and 50 μl of enzyme (sample) preparation. In experiments designed to measure calcium-dependent activity, $CaCl_2$ was omitted from the assay mixture and 5 mM EGTA was included. The reaction was terminated by spotting a 50-μl aliquot onto Whatman P81 cation exchange paper. This was washed several times with 75 mM H_3PO_4 and dried, and bound radioactivity was counted by liquid scintillation counting (25). Basal PKC activity for each sample was determined in the absence of calcium and lipids. Data are expressed as pmol of ^{32}P incorporated/min/μg of protein. Protein content was measured by the method of Bradford (26), using BSA as the standard.

Measurement of $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ were measured as described previously (21). After a 24-hr incubation with DMSO or PMA (1 μM), cells were gently scraped from 15-cm culture dishes after brief trypsinization and were washed once with BSS (140 mM NaCl, 5.0 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 0.41 mM $MgSO_4$, 1.2 mM $CaCl_2$, 5.5 mM glucose, 10 mM HEPES, pH 7.4 at 37°). The cells were resuspended in BSS containing 0.1% BSA and 5 μM fura-2/AM and were incubated for 60 min at 37° in the dark. The cells were washed once and resuspended in BSS/BSA. For measurement of $[Ca^{2+}]_i$, 2×10^6 cells were placed in a stirred, temperature-controlled (37°) cuvette in a Perkin-Elmer LS50 fluorescence spectrometer equipped with automatic data collection software. After a 5-min preincubation period, data were collected using dual-wavelength excitation at 340 and 380 nm and an emission wavelength of 510 nm. Data points and fluorescence ratios (F_{340}/F_{380}) were calculated every 1.9 sec.

Drugs were added to the cuvette after collection of base-line values for 60 sec. The $[Ca^{2+}]_i$ was calculated from the fluorescence ratios after calibration with 30 μM digitonin (to obtain F_{max}), followed by 10 mM EGTA, pH > 9 (to obtain F_{min}) (27). Basal $[Ca^{2+}]_i$ values were calculated as the average value obtained during 30 sec before drug addition.

Western blots. Purified rat brain PKC was a gift from Dr. Maria Pereira (Department of Pharmacology, Mount Sinai School of Medicine, New York) and was prepared as described (28). Subcellular fractions of A1A1 cells were prepared as described above for PKC activity measurements. After separation on 10% sodium dodecyl sulfate-polyacrylamide gels, samples were electroblotted onto nitrocellulose membranes using a Transblot SD electrophoretic transfer cell (Bio-Rad). The membranes were incubated overnight at 4° in PBS-T with 5% fetal bovine serum, 5% dry milk, and 1% ovalbumin to block nonspecific sites. After several washes with PBS-T supplemented with 0.01% dry milk and 0.01% ovalbumin (wash buffer), membranes were 1) incubated overnight at 4° with isoenyme-specific monoclonal antibodies to α, β, or γ diluted 1/100 in wash buffer or 2) incubated for 1 hr at room temperature with rabbit antisera diluted in wash buffer (anti-δ, 1/1000; anti-ϵ, 1/5000; or anti-ζ, 1/1000). After incubation with the primary antibody, membranes were washed three times for 15 min with wash buffer, followed by three 15-min washes with PBS-T. Horseradish peroxidase-conjugated goat anti-rabbit or donkey anti-mouse IgG second antibody (1/3000 dilution in PBS-T; Amersham) was added and the membranes were incubated for 1 hr at room temperature. After several washes with PBS-T, blots were made visible with the enhanced chemiluminescence detection system (Amersham), according to the manufacturer's instructions.

Data analysis. Concentration-response data were fit by nonlinear regression to the model:

$$E = E_{max}/(1 + (EC_{50}/(A)^n) \quad (1)$$

where E is the measured response at a given concentration of agonist (A), E_{max} is the maximal response, EC_{50} is the concentration of agonist producing half-maximal response, and n is the slope factor.

For calculation of IP accumulation, data were expressed relative to basal values according to the equation:

$$\% \text{ stimulation} = [(\text{experimental dpm} - \text{basal dpm})/\text{basal dpm}] \times 100 \quad (2)$$

The accumulation of cAMP induced by NECA and FSK varied between experiments. For calculation of amplification of cAMP levels, data were normalized by defining the pmol of cAMP/mg of protein accumulated in the absence of amplifying agonist as the control value and the pmol of cAMP/mg of protein accumulated in the presence of amplifying agonist as the experimental value. The amplification (%) was calculated according to the equation:

$$\% \text{ amplification} = [\text{experimental} - \text{control})/\text{control}] \times 100 \quad (3)$$

Values for accumulation of cAMP (in pmol of cAMP/mg of protein) are provided in the figure legends.

The Student t test was used for statistical comparisons.

Materials. All cell culture reagents were purchased from GIBCO (Grand Island, NY). ITS$^+$ was purchased from Collaborative Biomedical Products (Bedford, MA). Rolipram was a generous gift of Berlex Laboratories (Cedar Knolls, NJ). FSK, calmidazolium, W-7, anti-PKC-δ, -ϵ, and -ζ antisera, and BAPTA/AM were purchased from Calbiochem (La Jolla, CA). Anti-PKC-α, -β, and -γ monoclonal antibodies were purchased from Seikagaku America, Inc. (Rockville, MD). Pep-ϵ was purchased from Bachem (Torrance, CA). Fura-2/AM was purchased from Molecular Probes (Eugene, OR). *myo*-[^{3}H]Inositol, ^{125}I-cAMP tracer, and [γ-^{32}P]ATP were from New England Nuclear (Boston, MA). Anti-cAMP antibody was purchased from ICN Immunobiologicals (Lisle, IL). NECA was from Research Biochemicals, Inc. (Natick, MA). All other drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

5-HT$_{2A}$ receptor-mediated amplification of cAMP accumulation. Incubation of A1A1 cells with the adenosine receptor agonist NECA increased cAMP accumulation in a concentration-dependent and saturable manner (Fig. 1A). In the presence of 10 μM 5-HT, NECA-stimulated cAMP accumulation was enhanced. In the experiments shown in Fig. 1A, 5-HT increased the maximal response (E_{max}) of NECA by approximately 50%, without changing the EC_{50} or slope factor of the NECA concentration-response curve. 5-HT did not alter basal cAMP accumulation (5.3 ± 2.8 versus 6.65 ± 4.06 pmol/mg of protein with 10 μM 5-HT, eight experiments). 5-HT (10 μM) also increased cAMP accumulation in response to stimulation by FSK (1 μM, five experiments) and cholera toxin (2.5 μg/ml, three experiments), by 132 ± 38% and 100 ± 23%, respectively. The concentration-response curves for 5-HT to enhance cAMP accumulation in response to stimulation by NECA (five experiments) and FSK (two experiments) are shown in Fig. 1B. The E_{max}, pEC_{50}, and slope index values (mean ± standard error) for 5-HT were 179 ± 20%, 6.15 ± 0.19 (0.87 μM), and 0.87 ± 0.25 and 154 ± 14%, 6.1 ± 0.22 (0.85 μM), and 0.81 ± 0.17 for NECA and FSK, respectively. The effect of 5-HT on NECA- and FSK-stimulated cAMP accumulation was insensitive to prior treatment with 100 ng/ml pertussis toxin for 24 hr (data not shown). Amplification by 5-HT of NECA- and FSK-stimulated cAMP accumulation was antagonized by spiperone (10 nM) and ketanserin (10 nM) (Fig. 1B), which suggests that the 5-HT effect is mediated by the 5-HT$_{2A}$ receptor subtype.

5-HT$_{2A}$ receptor-mediated increase in IP accumulation and activation of PKC. Incubation of A1A1 cells with 5-HT increased the accumulation of (total) IP in a concentration-dependent and saturable manner (Fig. 2). The E_{max}, pEC_{50}, and slope index values (mean ± standard error, four experiments) for 5-HT to increase IP accumulation were 66 ± 11% over basal, 6.22 ± 0.27 (0.71 μM), and 1.03 ± 0.08, respectively. The effect of 5-HT on IP accumulation was insensitive to prior treatment with 100 ng/ml pertussis toxin for 24 hr (data not shown). 5-HT-stimulated IP accumulation was antagonized by spiperone (10 nM) and by ketanserin (10 nM) (Fig. 2), which suggests that, like the amplification of NECA-stimulated cAMP accumulation, the 5-HT-induced increase in hydrolysis of PI is mediated by the 5-HT$_{2A}$ receptor subtype.

In addition, we measured PKC activity (as defined by phosphorylation of histone III-S) in cytosol and membrane fractions of A1A1 cells in response to incubation with 10 μM 5-HT. As shown in Table 1, 5-HT transiently increased PKC activity in both the membrane and cytosol fractions but did not cause translocation of enzyme activity. After 15 min of incubation with 5-HT, PKC activity in both fractions approached control levels. This transient activation without measurable translocation is consistent with other reported hormone and neurotransmitter effects on PKC activity in other cell lines (29, 30).

Activation of PKC-amplified cAMP accumulation in response to stimulation by NECA, FSK, and cholera toxin. Coincubation with the phorbol ester PMA (1 μM) enhanced NECA stimulation of cAMP accumulation (Fig. 3A) in a manner similar to that of 5-HT, but greater in magnitude. In the experiments shown in Fig. 3A, PMA increased the E_{max} of NECA by approximately 300%, without altering its EC_{50} or slope index. PMA did not alter basal cAMP accumulation (5.3

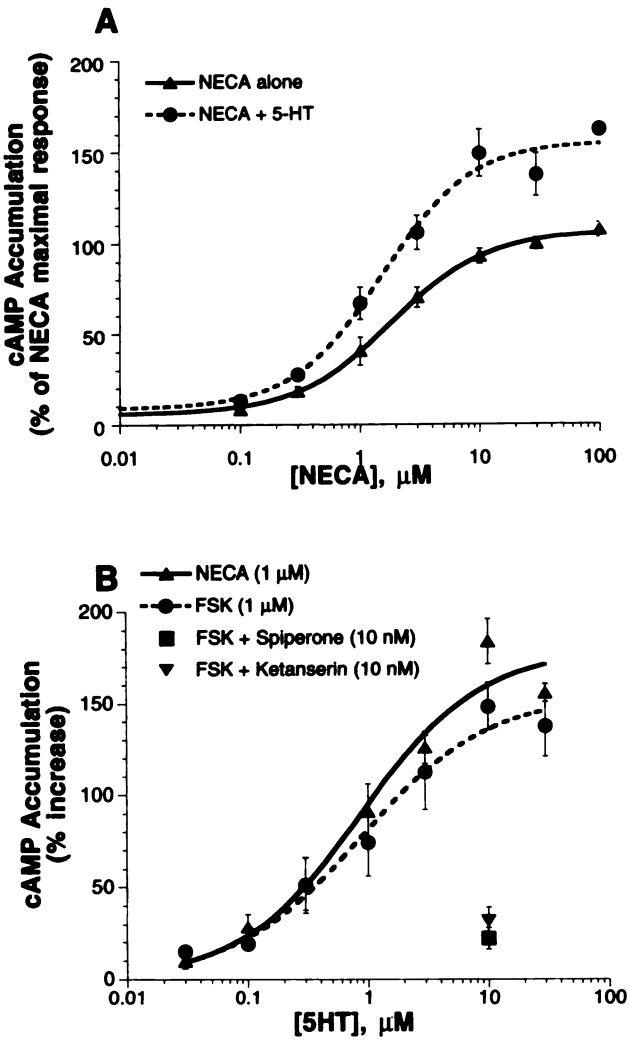

Fig. 1. Effect of 5-HT on NECA- and FSK-stimulated cAMP accumulation. A, Concentration-response curves for NECA in the presence and absence of 5-HT (10 μM). cAMP accumulation was measured in response to incubation (15 min) with NECA alone or with NECA and 5-HT together in paired experiments. Concentration-response data were fit by nonlinear regression to eq. 1, as described in Materials and Methods. Data shown are mean ± standard error from three experiments and are expressed as a percentage of the maximal response to NECA alone. The E_{max}, pEC_{50}, and slope index values (mean ± standard error, three experiments) for NECA alone and NECA in the presence of 5-HT were 174 ± 58 pmol/mg of protein, 5.75 ± 0.88 (1.8 μM), and 1.16 ± 0.07 and 247 ± 63 pmol/mg of protein, 5.82 ± 0.11 (1.5 μM), and 1.13 ± 0.08, respectively. In paired experiments, the increase in NECA-stimulated cAMP accumulation by 5-HT averaged 149.4 ± 10.9%. B, Concentration-response curves for 5-HT amplification of NECA (1 μM)-stimulated and FSK (1 μM)-stimulated cAMP accumulation. Each data point represents the mean ± standard error of five (NECA) or two (FSK) experiments. Data are expressed as a percentage of the cAMP accumulation in response to NECA or FSK in the absence of 5-HT. The E_{max}, pEC_{50}, and slope index values (mean ± standard error) with 5-HT were 179 ± 20%, 6.15 ± 0.19 (0.87 μM), and 0.87 ± 0.25 and 154 ± 14%, 6.1 ± 0.22 (0.85 μM), and 0.81 ± 0.17 for NECA and FSK, respectively. In the absence of 5-HT, NECA- and FSK-stimulated cAMP accumulation was 40.5 ± 31 pmol of cAMP/mg of protein and 318 ± 18 pmol of cAMP/mg of protein, respectively. Also shown is the antagonism by ketanserin (10 nM, four experiments) and spiperone (10 nM, four experiments) of the 5-HT (10 μM)-induced amplification of FSK (1 μM)-stimulated cAMP accumulation.

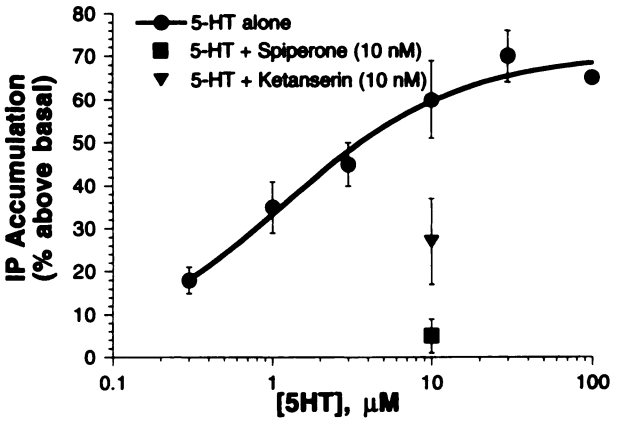

Fig. 2. Effect of 5-HT on IP accumulation. Cells were labeled with 2 μCi/ml *myo*-[^{3}H]inositol for 48 hr. After washing, cells were preincubated with LiCl (20 mM) for 15 min and then stimulated with various concentrations of 5-HT. Accumulation of [^{3}H]IP was measured after 10 min. Data are expressed as a percentage of IP accumulation in the absence of 5-HT (basal, 1611 ± 80 dpm) and represent the mean ± standard error of four experiments. Data were fit by nonlinear regression to eq. 1, as described in Materials and Methods. The E_{max}, pEC_{50}, and slope index values (mean ± standard error) for 5-HT were 66 ± 11%, 6.22 ± 0.27 (0.71 μM), and 1.03 ± 0.08, respectively. Also shown is the antagonism of 5-HT (10 μM) by ketanserin (10 nM, four experiments) and spiperone (10 nM, four experiments).

TABLE 1

Time course for the effect of 5-HT on PKC activity in cytosol and membrane fractions from A1A1 cells

Cytosol and membrane fractions were prepared from A1A1 cells after treatment with 5-HT (10 μM) for various times. Activity of PKC was determined by measuring incorporation of [^{32}P]phosphate into histone III-S. Data represent pmol of [^{32}P] phosphate incorporated into histone III-S/min/μg of protein and are means ± standard errors of three experiments.

Time	PKC activity	
	Cytosol	Membrane
min	*pmol/min/μg*	
0	5.64 ± 0.39	2.21 ± 0.40
5	6.87 ± 0.34*	3.46 ± 0.65*
15	6.21 ± 0.49	3.25 ± 1.03

* Significantly different from corresponding time 0 point ($p < 0.05$).

± 2.8 versus 6.3 ± 4.5 pmol/mg of protein with 1 μM PMA, eight experiments). Fig. 3B shows that PMA induced almost complete translocation of PKC activity from the cytosol to the membrane fraction after 15 min of incubation. Coincubation with PMA (1 μM) for 15 min also increased FSK (1 μM)-stimulated and cholera toxin (2.5 μg/ml)-stimulated cAMP accumulation, by 145 ± 30% (three experiments) and 200 ± 47% (three experiments), respectively.

Other compounds were tested to confirm the role of PKC in the PMA-induced amplification of NECA- and FSK-stimulated cAMP accumulation. The phorbol ester phorbol 12,13-dibutyrate (1 μM) enhanced cAMP accumulation in response to 3 μM NECA by 228 ± 32% (two experiments) and increased the response to 1 μM FSK by 92 ± 29% (two experiments), whereas the inactive phorbol ester 4α-phorbol didecanoate (1 μM) had no effect on either NECA- or FSK-stimulated cAMP levels (three experiments) (data not shown). In addition, staurosporine (1 μM), an inhibitor of PKC, reduced the potentiation of NECA-stimulated cAMP levels by both PMA and 5-HT, by 83% and 57%, respectively.

Evidence that depletion of PKC reduced but did not

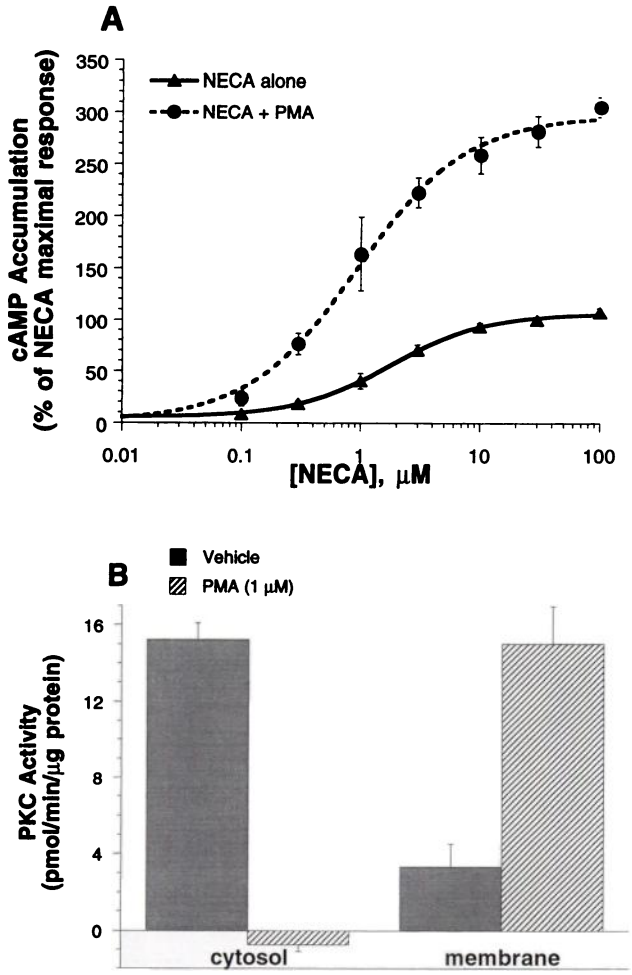

Fig. 3. Effect of PMA on NECA-stimulated cAMP accumulation (A) and PKC activity (B) in A1A1 cells. A, cAMP accumulation was measured in response to incubation (15 min) with NECA alone or with NECA and PMA (1 μM) together in paired experiments (three experiments). Data shown for the NECA-only concentration-response curve are repeated from Fig. 1, because the experiments with NECA plus PMA were done along with those for NECA plus 5-HT. Data were fit by nonlinear regression to eq. 1, as described in Materials and Methods, and are expressed as a percentage of the maximal response to NECA alone. The E_{max}, pEC_{50}, and slope index values (mean ± standard error, three experiments) for NECA in the presence of PMA were 518 ± 191 pmol/mg of protein, 6.02 ± 0.12 (0.95 μM), and 1.03 ± 0.10, respectively. In paired experiments, the increase in NECA-stimulated cAMP accumulation by PMA averaged 293 ± 11.0%. B, PKC activity was measured in cytosol and membrane fractions using histone III-S as a substrate, after incubation with 1 μM PMA or vehicle (0.01% DMSO) for 15 min. Data shown are mean ± standard error (three experiments).

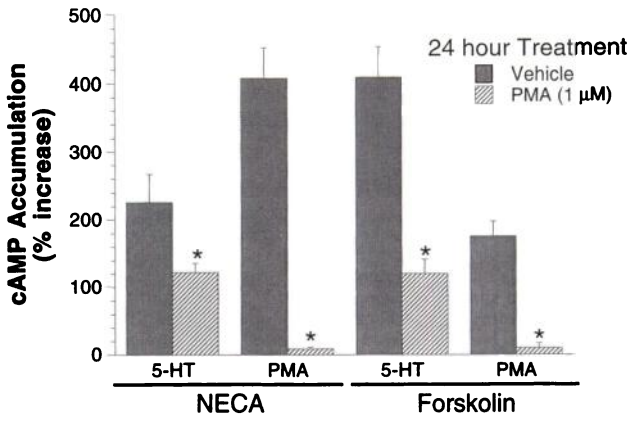

Fig. 4. Effect of long term (24-hr) PMA incubation on the amplification by 5-HT of NECA- and FSK-stimulated cAMP accumulation. A1A1 cells, incubated in the presence of 1 μM PMA or vehicle (0.01% DMSO) for 24 hr, were coincubated for 15 min with NECA (1 μM) or FSK (1 μM) and with either 5-HT (10 μM) or PMA (1 μM). All non-PMA-containing conditions included vehicle (0.01% DMSO). *Bars*, mean ± standard error (four experiments) of percentage increase above NECA- or FSK-stimulated cAMP accumulation (as calculated with eq. 3 in Materials and Methods), which averaged 92.8 ± 6.2 and 53.0 ± 28.5 pmol/mg of protein, respectively, after 24-hr DMSO treatment and 156.8 ± 35.3 and 68.0 ± 25.4 pmol/mg of protein, respectively, after 24-hr PMA treatment. *, Significantly different from corresponding 24-hr vehicle treatment ($p \leq 0.05$; Student's unpaired *t* test).

abolish the amplification by 5-HT of NECA-stimulated cAMP accumulation. To further establish that 5-HT amplification of cAMP levels in A1A1 cells was mediated by PKC, we attempted to abolish the 5-HT response with long term PMA treatment, which is known to down-regulate PKC in a variety of cells (31–33). When A1A1 cells were incubated with PMA (1 μM) for 24 hr to deplete PKC activity, the amplification of NECA- and FSK-stimulated cAMP accumulation by a second application (15 min) of PMA was abolished (Fig. 4). In contrast, amplification by 5-HT was reduced, but still present, after 24-hr PMA treatment. The 5-HT-mediated accumulation of IP was enhanced after treatment for 24 hr with 1 μM PMA (64 ± 16% after 24-hr DMSO treatment versus 128 ± 31% after 24-hr PMA treatment). Therefore, the reduced amplification of NECA- and FSK-stimulated cAMP accumulation observed after PKC depletion was not due to impaired hydrolysis of IP by 5-HT receptor activation.

It has been shown that some PKC isoforms are differentially resistant to down-regulation by long term phorbol ester treatment (e.g., PKC-δ, PKC-ε, and PKC-ζ) (34) and some may be insensitive to stimulation by phorbol esters (e.g., PKC-ζ) (35). Because the effect of 5-HT was not completely abolished, it is possible that any PKC activity that may remain after long term PMA treatment could be responsible for the effects of 5-HT. Therefore, we used PKC activity measurements and Western blots to evaluate the effectiveness of long term PMA treatment in A1A1 cells. We measured PKC activity in three subcellular fractions of A1A1 cells, using as substrates either histone III-S or pep-ε (substrates for various isoforms of PKC, with overlapping selectivity). As shown in Table 2, with histone III-S as a substrate PKC activity was greatly reduced in the cytosol and membrane fractions after long term PMA treatment; however, substantial activity remained in the nuclear fraction. With pep-ε as a substrate PKC activity in the cytosol was reduced but activity was still present in the membrane and nuclear fractions.

To determine which isoforms of PKC remained after long term PMA treatment, we next prepared Western blots against six PKC isoforms (α, β, γ, δ, ε, and ζ, for which antibodies were commercially available) in three subcellular fractions of A1A1 cells. As shown in Fig. 5, immunopositive results were obtained only with anti-α, -δ, and -ε antisera. After 24-hr treatment of cells with PMA, the α and δ isoforms were no longer detectable in the cytosol or membrane fractions; however, a small amount of δ immunoreactivity was found in the nuclear fraction. In addition, immunoreactivity with PKC-ε was still present and actually increased in the nuclear membrane and cytosol fractions (Fig. 5).

TABLE 2

Comparison of PKC activity in subcellular fractions of A1A1 cells treated for 24 hr with either DMSO or PMA

Cytosol, nuclear membrane, and remaining membrane fractions were prepared from A1A1 cells incubated in the presence of 1 μM PMA or DMSO vehicle for 24 hr, as described in Materials and Methods. Activity of PKC was determined by measuring incorporation of [^{32}P]phosphate into histone III-S or the synthetic substrate pep-ε. Data shown are pmol incorporated/min/μg of protein and are means ± standard deviations of two experiments performed in duplicate.

	PKC activity			
	Histone III-S		Pep-ε	
	DMSO	PMA	DMSO	PMA
	pmol/min/μg			
Cytosol	6.65 ± 0.25	1.12 ± 0.29	6.55 ± 0.65	2.00 ± 0.50
Membrane	3.79 ± 0.53	1.31 ± 0.78	9.36 ± 0.51	5.26 ± 0.66
Nuclear membrane	4.12 ± 0.28	2.90 ± 0.56	10.2 ± 2.8	7.79 ± 0.09

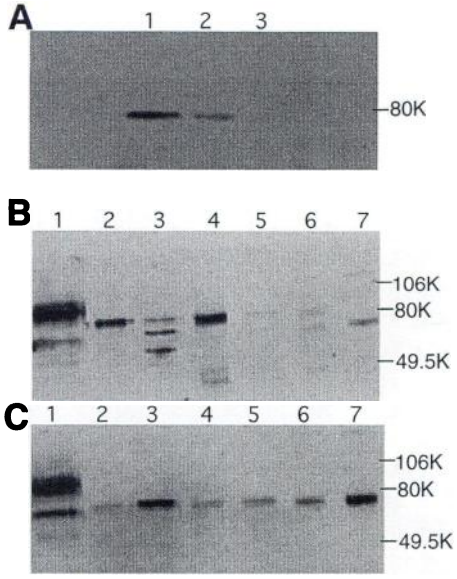

Fig. 5. Western blot analysis of PKC-α, -δ, and -ε isoforms in A1A1 cells after 24-hr incubation with vehicle (0.01% DMSO) or PMA (1 μM). Proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membranes, and probed for PKC isoenzymes as described in Materials and Methods. Purified PKC from rat brain was used as a control. A, PKC-α immunoreactivity in whole-cell homogenates (10 μg of protein/lane). *Lane 1*, purified rat PKC; *lane 2*, A1A1 cells after 24-hr vehicle treatment; *lane 3*, A1A1 cells after 24-hr PMA treatment. Nitrocellulose membranes were incubated overnight at 4° with anti-PKC-α monoclonal antibody (1/100). PKC-α immunoreactivity was undetectable in whole-cell lysates after 24-hr PMA treatment. B and C, PKC-δ (B) and PKC-ε (C) immunoreactivity in subcellular fractions (1.5 μg protein/lane) of A1A1 cells (see Materials and Methods). Nitrocellulose membranes were incubated for 1 hr at room temperature with rabbit anti-rat PKC-δ antiserum (1/1000 dilution) or rabbit anti-rat PKC-ε antiserum (1/5000 dilution). B and C, *Lane 1*, purified rat PKC; *lane 2*, nuclear membranes after 24-hr vehicle treatment; *lane 3*, nuclear membranes after 24-hr PMA treatment; *lane 4*, membranes after 24-hr vehicle treatment; *lane 5*, membranes after 24-hr PMA treatment; *lane 6*, cytosol after 24-hr vehicle treatment; *lane 7*, cytosol after 24-hr PMA treatment. In 24-hr vehicle-treated cells, immunoreactivity with the anti-PKC-δ antibody was found in both membrane fractions as a single band. In PMA-treated cells, three bands appeared in nuclear membrane fractions, whereas no immunoreactivity was detected in the remaining membrane fraction. Immunoreactivity with the anti-PKC-ε antibody was found in all fractions after 24-hr PMA treatment and appeared to be increased in the nuclear membrane and cytosol fractions.

The Western blots and PKC activity measurements indicated that some PKC activity remained after long term PMA treatment. The PKC isoforms that have been shown to be differentially resistant to down-regulation by phorbol esters fall into a class of novel forms of PKC that do not require calcium for activation (36). Both the δ and ε isoforms of PKC, which remained after long term PMA treatment, are known to be calcium independent (see Ref. 37 and references cited therein). Therefore, we evaluated the calcium dependence of the PKC activity that remained after 24-hr PMA treatment. PKC activity in whole-cell lysates (as defined by incorporation of [^{32}P] phosphate into pep-ε) was 17.6 ± 2.2 pmol of ^{32}P incorporated/min under normal assay conditions (with calcium) and 17.5 ± 4.5 pmol of ^{32}P incorporated/min under assay conditions in the absence of added calcium and in the presence of 5 mM EGTA. These data were consistent with the Western blot data and indicated that the PKC activity remaining after 24-hr PMA treatment was Ca^{2+} independent.

Calcium dependence of the amplification by 5-HT of cAMP accumulation in cells after long term PMA treatment. Because activation of 5-HT$_{2A}$ receptors is known to increase $[Ca^{2+}]_i$ levels in many cell types (see Ref. 16), we investigated the role of Ca^{2+} in the enhancement by 5-HT of cAMP accumulation in cells after long term PMA treatment. Fig. 6A shows that application of 5-HT to A1A1 cells caused a rapid (<10 sec) increase in $[Ca^{2+}]_i$, as measured directly with fura-2. This increase averaged 88 ± 8 nM (mean ± standard error, six experiments) over resting levels in cells after treatment with vehicle (0.01% DMSO) for 24 hr. Interestingly, in PKC-depleted cells the rise in $[Ca^{2+}]_i$ in response to 5-HT was >2-fold greater in magnitude (203 ± 50 nM over resting levels, six experiments), which is in agreement with long term PMA effects on 5-HT-mediated accumulation of IP (see above). Long term PMA treatment did not affect resting levels of $[Ca^{2+}]_i$ (239 ± 41 nM in PKC-depleted cells versus 202 ± 33 nM in controls, six experiments). 5-HT also increased $[Ca^{2+}]_i$ in cells studied in Ca^{2+}-free medium containing EGTA (data not shown).

Fig. 6B shows that incubation of A1A1 cells with the Ca^{2+} ionophore A23187 enhanced NECA-stimulated cAMP accumulation in both normal and long term PMA-treated cells. This enhancement was abolished when the ionophore was added to cells in the absence of extracellular Ca^{2+}. As expected, amplification of NECA-stimulated cAMP accumulation by PMA was abolished after 24-hr treatment with PMA and the amplification in normal cells was unaffected by removal of extracellular Ca^{2+}. As shown earlier, the enhancement by 5-HT was reduced, but not abolished, by depletion of PKC in cells in normal medium. Removal of extracellular Ca^{2+} did not affect the enhancement produced by 5-HT in nondepleted or depleted cells. In contrast, the amplification by 5-HT in cells treated with PMA for 24 hr was greatly reduced by preincuba-

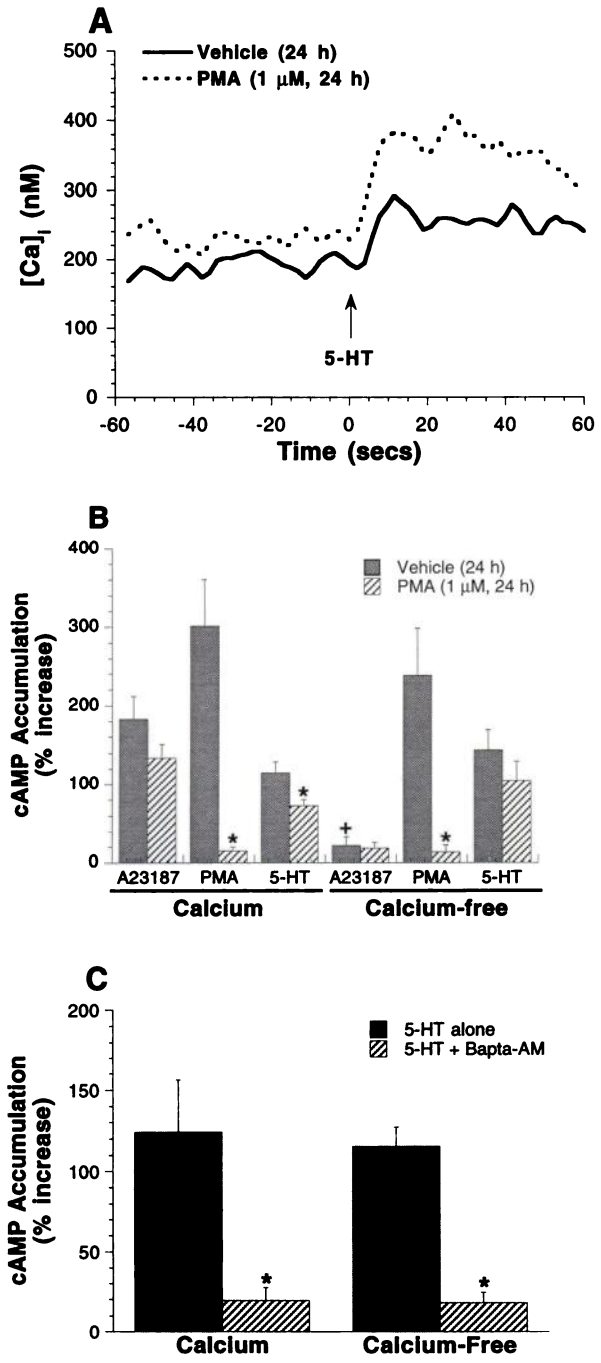

Fig. 6. A, Effect of long term PMA treatment of A1A1 cells on increases in $[Ca^{2+}]_i$ by 5-HT. Changes in $[Ca^{2+}]i$ were measured in cells that had been loaded with fura-2, as described in Materials and Methods. ——,24-hr vehicle (0.01% DMSO) treatment; – – –, 24-hr PMA (1 μM) treatment. Data shown are representative of six replicate determinations done on two separate occasions. Resting $[Ca^{2+}]_i$ values were 202 ± 33 nM in vehicle-treated cells and 239 ± 41 nM in PMA-treated cells (mean ± standard error, six experiments). B, Effect of Ca^{2+} on enhancement of NECA-stimulated cAMP accumulation by the Ca^{2+} ionophore A23187, 5-HT, and PMA. After 24-hr treatment with either PMA (1 μM) or vehicle (0.01% DMSO), cAMP accumulation was measured in cells that had been coincubated for 15 min with NECA (1 μM) and either A23187 (1 μM), 5-HT (10 μM), or PMA (1 μM), in either Ca^{2+}-containing (normal) or Ca^{2+}-free medium. The percentage increase in cAMP accumulation was calculated as described in eq. 3 in Materials and Methods. In Ca^{2+}-containing medium, NECA-stimulated cAMP accumulation was 75.6 ± 19 pmol of cAMP/mg of protein after 24-hr treatment with vehicle and 77.0 ± 25 pmol of cAMP/mg of protein after 24-hr treatment with PMA. In Ca^{2+}-free medium, NECA-stimulated cAMP accumulation was 132.0 ± 29 pmol of cAMP/mg of protein after 24-hr treatment with vehicle and 119.6 ± 22 pmol of cAMP/mg of protein after 24-hr treatment with PMA. *Bars*, mean ± standard error of five experiments. *, Significantly different from the corresponding 24-hr vehicle treatment group ($p \leq 0.05$; Student's unpaired *t* test); +, significantly different from corresponding group in normal (Ca^{2+}-containing) medium ($p \leq 0.05$; Student's unpaired *t* test). C, Effect of the membrane-permeable Ca^{2+} chelator BAPTA/AM on the amplification by 5-HT of NECA-stimulated cAMP accumulation in A1A1 cells treated for 24 hr with PMA. After 24-hr treatment with PMA (1 μM), cells were preincubated for 30 min at 37° with BAPTA/AM (10 μM) or vehicle (medium), in either Ca^{2+}-containing or Ca^{2+}-free medium. cAMP accumulation was measured in cells that had been incubated for 15 min with NECA (1 μM) alone or NECA and 5-HT (10 μM). The percentage increase in cAMP accumulation produced by 5-HT was calculated as described by eq. 3 in Materials and Methods. In Ca^{2+}-containing medium, NECA-stimulated cAMP accumulation was 51.0 ± 9 pmol of cAMP/mg of protein after 30-min treatment with vehicle and 45.7 ± 8 pmol of cAMP/mg of protein after 30-min treatment with BAPTA/AM. In Ca^{2+}-free medium, NECA-stimulated cAMP accumulation was 46.2 ± 17 pmol of cAMP/mg of protein after 30-min treatment with vehicle and 33.7 ± 15 pmol of cAMP/mg of protein after 30-min treatment with BAPTA/AM. *Bars*, mean ± standard error of six or seven experiments. *, Significantly different from the corresponding vehicle group.

tion with the membrane-permeable calcium chelator BAPTA/AM (10 μM) in either Ca^{2+}-containing or Ca^{2+}-free medium (Fig. 6C). 5-HT-mediated accumulation of IP was not reduced by incubation with BAPTA/AM (data not shown), indicating that reduced amplification by the calcium chelator was not due to impaired hydrolysis of IP. Similarly, amplification produced by incubation with A23187 (1 μM) in PKC-depleted cells was reduced from 178.7 ± 1.9 pmol/mg of protein (163%) to 133.0 ± 9.8 pmol/mg of protein (118%) by preincubation with BAPTA/AM ($p < 0.005$, three experiments). NECA-stimulated cAMP accumulation was unaltered by BAPTA/AM (68.3 ± 3.5 versus 61.3 ± 8.8 pmol/mg of protein, respectively, three experiments). Chelation of Ca^{2+} with BAPTA/AM to evaluate the calcium dependence of amplification by 5-HT in normal cells (i.e., not PMA treated) was not done because the Ca^{2+}-sensitive PKC-α present in normal cells (Fig. 5) would be affected, therefore confounding interpretation of the results.

Insensitivity to staurosporine of the amplification by 5-HT of cAMP accumulation after long term PMA treatment. As shown in Fig. 7A, staurosporine (50–1000 nM) had no effect on 5-HT-mediated amplification of NECA-stimulated cAMP levels in PKC-depleted cells. In contrast, when included in the PKC assay mixture, 1000 nM staurosporine completely inhibited all PKC activity (presumably PKC-ε) in cells treated with PMA for 24 hr. Moreover, the IC_{50} of staurosporine for inhibition of PKC-ε activity in A1A1 cells (100 nM) (data not shown) correlated well with previous reports of staurosporine inhibition of PKC-ε activity (190 nM) (38).

Calmodulin antagonist blockade of the amplification by 5-HT and A23187 of NECA-stimulated cAMP levels in cells after 24-hr PMA treatment. The calmodulin inhibitors calmidazolium and W-7 were used to determine the role of calmodulin in the Ca^{2+}-sensitive, 5-HT-mediated potentiation of cAMP accumulation in long term PMA-treated cells. Both calmidazolium and W-7 increased NECA-stimulated cAMP accumulation, by an average of 45%. NECA-stimulated cAMP accumulation averaged 50.2 ± 6.5 pmol/mg of protein (six experiments) in the absence of antagonists versus 72 ± 13.9 pmol/mg of protein (four experiments) in the presence of calmidazolium and 74 ± 6.7 pmol/mg of protein (four experi-

ments) in the presence of W-7. Calmidazolium and W-7 reduced the amplification by 5-HT by 67% and 92%, respectively (Fig. 7B). Similarly, calmidazolium and W-7 reduced the enhancement of NECA-stimulated cAMP levels by the calcium ionophore A23187, by 64% and 89%, respectively (Fig. 7B).

Because calmidazolium and W-7 have been reported to inhibit PKC activity (39), we tested the capacity of these calmodulin antagonists to inhibit PKC-ε activity remaining in PKC-depleted cells. When the antagonists were included in the PKC assay mixture, phosphorylation of pep-ε was not changed by calmidazolium (10 μM) but was reduced 31% by W-7 (100 μM).

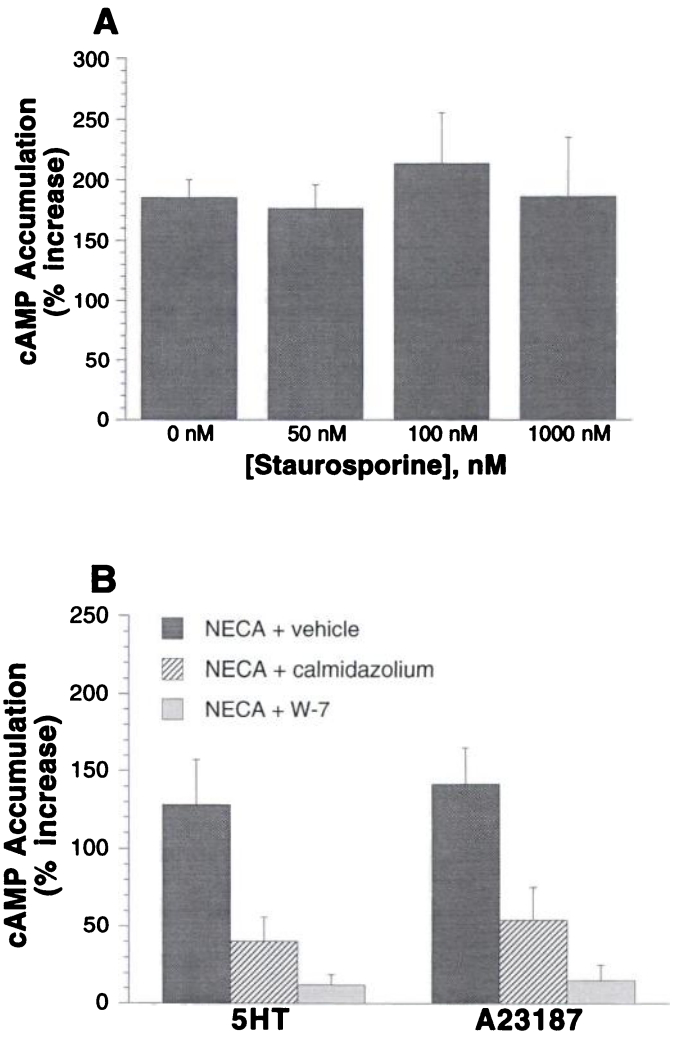

Fig. 7. A, Effect of staurosporine on the amplification by 5-HT of NECA-stimulated cAMP accumulation in A1A1 cells treated with PMA for 24 hr. cAMP accumulation was measured in response to stimulation with NECA (1 μM) alone (30.7 ± 14 pmol of cAMP/mg of protein) or with NECA and 5-HT (10 μM), in the absence or presence of various concentrations of staurosporine. Staurosporine had no effect on NECA-stimulated cAMP accumulation. The percentage increase in cAMP accumulation produced by 5-HT was calculated as described by eq. 3 in Materials and Methods. *Bars*, mean ± standard error of five experiments. B, Effect of calmodulin antagonists calmidazolium and W-7 on amplification by 5-HT and A23187 of NECA-stimulated cAMP accumulation in A1A1 cells treated with PMA for 24 hr. cAMP accumulation was measured in response to stimulation with NECA (1 μM) alone or with NECA and either 5-HT (10 μM) or A23187 (1 μM), in the absence or presence of calmidazolium (10 μM) or W-7 (100 μM). The percentage increase in cAMP accumulation produced by 5-HT was calculated as described by eq. 3 in Materials and Methods. NECA-stimulated cAMP accumulation was 50.2 ± 7 pmol/mg of protein in the absence of antagonists versus 72 ± 14 pmol/mg of protein in the presence of calmidazolium and 74 ± 7 pmol/mg of protein in the presence of W-7. *Bars*, mean ± standard error of four experiments.

Discussion

Of the many receptor subtypes for 5-HT, only the 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ subtypes have been shown to couple to PLC in cells and tissues where the receptors are naturally expressed. PLC-induced hydrolysis of inositol lipids liberates diacylglycerol and IP$_3$, which activate PKC and increase $[Ca^{2+}]_i$, respectively. Clonal cell lines have proven to be valuable systems for investigations of receptor-mediated signal transduction pathways. Currently, naturally expressed 5-HT$_{2A}$ receptors are found in few cell lines, which, with one exception (C6 glioma), are derived from peripheral tissues such as smooth muscle (e.g., A7r5), mammary gland (WRK1), kidney (NRK), sciatic nerve (RT4-B), and pituitary (P11). A1A1 cells are derived from neurons of embryonic rat cortex, an area known to contain high densities of 5-HT$_{2A}$ receptors in adults (40). In A1A1 cells, 5-HT increases the hydrolysis of PI (Fig. 2) and increases PKC activity (Table 1) and $[Ca^{2+}]_i$ (Fig. 6A). The 5-HT-induced PI hydrolysis is blocked by nanomolar concentrations of spiperone and ketanserin (Fig. 2), which suggests that this response is mediated by 5-HT$_{2A}$ receptors and not 5-HT$_{2B}$ or 5-HT$_{2C}$ receptors (16, 41).

Unlike treatment with the phorbol ester PMA (Fig. 3B), 5-HT did not cause translocation of PKC activity but transiently increased total PKC activity (both in the cytosol and in the membrane) (Table 1). Although 5-HT$_{2A}$ receptor activation has been found to produce translocation of PKC activity from the cytosol to the membrane in slices of rat cortex (42) and in platelets (43), there are several reports of agonist-induced increases in PKC activity without measurable translocation (29, 30, 44, 45). Agonist-induced PKC activation without translocation may represent the transient (within minutes) nature of agonist-induced diacylglycerol production and $[Ca^{2+}]_i$ increase from hydrolysis of inositol phospholipids, even in the continued presence of agonist (46). It may also reflect differences in characteristics of the PKC isoforms expressed in given tissues or cells (46). The reasons for the difference in 5-HT$_{2A}$ receptor effects on PKC activity in A1A1 cells versus those in cortical slices or platelets are unknown.

Application of 5-HT to A1A1 cells also amplified cAMP accumulation (increased E_{max} with no change in EC$_{50}$ or slope index and no change in basal cAMP accumulation) in response to stimulation by adenosine receptors with NECA (Fig. 1A). The EC$_{50}$ of 5-HT to enhance NECA-stimulated cAMP accumulation (0.87 μM) (Fig. 1B) was similar to that for the hydrolysis of IP (0.71 μM) (Fig. 2). The 5-HT-mediated amplification of cAMP accumulation is also likely mediated by 5-HT$_{2A}$ receptors, because it was blocked by nanomolar concentrations of spiperone and ketanserin (Fig. 1B). Furthermore, as discussed below, this effect of 5-HT was mediated by both activation of PKC and increases in $[Ca^{2+}]_i$. It is noteworthy that this secondary response (i.e., enhancement of cAMP accumulation) to activation of 5-HT$_{2A}$ receptors is greater in magnitude (E_{max} ≈ 150%) than the primary response of hydrolysis of PI (E_{max} ≈ 70%), which suggests an amplification step between the pathways. 5-HT has also been shown to augment cAMP accumulation stimulated by histamine and vasoactive intestinal polypeptide in brain slices from guinea pig cerebral cortex (47) and by isoproterenol in slices from rat cerebral cortex (48).

However, Magistretti and Schorderet (49) were unable to demonstrate an effect of 5-HT on vasoactive intestinal polypeptide-stimulated cAMP accumulation in mouse cerebral cortical slices. Whether methodological or species differences account for this discrepancy is unknown.

The main finding of this work is that in A1A1 cells 5-HT_{2A} receptor activation enhanced stimulated cAMP accumulation by two distinct mechanisms, i.e., a PKC-dependent pathway and a calcium/calmodulin-dependent pathway. Several findings support a role for PKC in the amplification of cAMP accumulation by 5-HT. First, 5-HT increased PKC activity (Table 1). Second, activation of PKC with the phorbol esters PMA and phorbol 12,13-dibutyrate mimicked the effects of 5-HT on NECA-, cholera toxin-, and FSK-stimulated cAMP accumulation (Fig. 3A), whereas the inactive phorbol ester 4α-phorbol didecanoate was without effect. Third, long term treatment of A1A1 cells with phorbol ester, a treatment known to down-regulate PKC (31, 33), abolished amplification of NECA-stimulated cAMP accumulation by PMA and reduced that by 5-HT (Fig. 4).

Although in many other systems activation of PKC has been shown to influence cAMP production, this is the first demonstration of such an effect produced by activation of 5-HT_{2A} receptors. The effect of PKC on the AC signaling system appears to be highly dependent upon the type of cell studied and the method used to stimulate AC. For example, in Swiss 3T3 fibroblasts activation of PKC enhances cAMP production in response to adenosine A_2 and prostaglandin E_1 receptor stimulation but not in response to β-adrenergic receptor stimulation or stimulation by FSK or cholera toxin (50, 51). β-Adrenergic receptor-stimulated cAMP production is increased by PKC activation in rat pinealocytes (52) and is reduced in rat reticulocytes (9). These differential effects may be related to the complement of PKC isoforms present within particular cell types and their substrate specificities. Just as the effect of PKC on the AC signaling system can vary, so can the target of PKC action. Activation of PKC has been shown to affect the AC enzyme itself (11) and the receptors (6, 50) and G proteins (8, 10) that couple to AC. In A1A1 cells, we found that 5-HT and PKC enhanced cAMP accumulation in response to stimulation by NECA (which acts through the adenosine receptor), cholera toxin (which activates G_s), and FSK (which directly activates AC). This suggests that, in A1A1 cells, PKC may regulate a component that is common to each of these methods of stimulating cAMP production. A likely candidate therefore is the AC enzyme itself.

Although PKC mediates part of the effect of 5-HT on amplification of cAMP accumulation, it is not responsible for all of the action of 5-HT. Long term treatment of A1A1 cells with phorbol ester completely eliminated the PMA-induced enhancement of NECA- and FSK-stimulated cAMP accumulation (Fig. 4), suggesting that, with respect to amplification of cAMP accumulation, PKC was functionally down-regulated. Under these conditions, 5-HT still enhanced NECA- and FSK-stimulated cAMP accumulation (Fig. 4). Certain isoforms of PKC have been shown to be differentially resistant to down-regulation (34, 53, 54). Therefore, it is conceivable that, if present in A1A1 cells, one of these isoforms may mediate the amplification elicited by 5-HT, although this PKC isoform would also have to be insensitive to activation by phorbol ester, because PMA was without effect after PKC down-regulation. Measurement of PKC activity (Table 2), using two substrates with overlapping specificities, revealed that some PKC activity still remained after down-regulation. Most of this activity was confined to the nuclear membrane, which is unlikely to participate (within a short time frame, i.e., <15 min) in receptor signal transduction events in the cell membrane. However, we did find residual PKC activity in the cytosol and cell membrane compartments.

Analysis of Western blots (Fig. 5) revealed that A1A1 cells expressed the α, δ, and ϵ isoforms of PKC and that, after long term PMA treatment, the α isoform was no longer detectable. However, a small amount of PKC-δ immunoreactivity remained in the nuclear fraction and immunoreactivity to PKC-ϵ was still present in the membrane and cytosol fractions and actually increased in the nuclear membrane fraction. Both PKC-δ and PKC-ϵ are members of a novel class of PKC isoforms that have been shown to be resistant to down-regulation by long term phorbol ester treatment and that do not require Ca^{2+} for activation (37, 38). Because the PKC-δ and PKC-ϵ isoforms are sensitive to activation by phorbol ester (37) but amplification by PMA of cAMP accumulation was completely abolished, these isoforms are unlikely to mediate the amplifying effects of 5-HT after long term PMA treatment. These findings suggest that the PKC-dependent amplification in A1A1 cells is likely mediated by PKC-α.

Because, in addition to activation of PKC by diacylglycerol, hydrolysis of PI liberates IP_3, which increases $[Ca^{2+}]_i$, we evaluated the role of Ca^{2+} in the amplification by 5-HT of cAMP accumulation after long term phorbol ester treatment. Application of 5-HT to A1A1 cells rapidly (<10 sec) increased $[Ca^{2+}]_i$ (Fig. 6A). The 5-HT-mediated accumulation of IP and increase in $[Ca^{2+}]_i$ were enhanced 2-fold after long term treatment with phorbol ester. Long term phorbol ester treatment has also been shown to increase receptor-mediated hydrolysis of PI in Swiss 3T3 cells (55) and in a rat liver epithelial cell line, WB (56). Like the effect of 5-HT, treatment of A1A1 cells with the Ca^{2+} ionophore A23187 also enhanced NECA-stimulated cAMP accumulation and, in contrast to that of PMA, this effect was not reduced by long term phorbol ester treatment (Fig. 6B). In Ca^{2+}-free medium, the effect of the ionophore was abolished, whereas those of PMA and of 5-HT were not affected. However, the amplification of NECA-stimulated cAMP accumulation by 5-HT in long term PMA-treated cells was almost completely abolished after treatment with the membrane-permeable calcium chelator BAPTA/AM (Fig. 6C). In addition, the ionophore-induced amplification of NECA-stimulated cAMP accumulation was reduced 45% by pretreatment with BAPTA/AM. Although we were unable to evaluate the calcium sensitivity of 5-HT_{2A} receptor-mediated amplication of cAMP accumulation in normal cells due to the presence of calcium-dependent PKC-α, these data suggest that increases in $[Ca^{2+}]_i$ can mediate amplification of NECA-stimulated cAMP accumulation in cells in which PKC activity is functionally down-regulated. Furthermore, the effect of 5-HT in long term PMA-treated cells is Ca^{2+} dependent, likely from intracellular Ca^{2+} sources. Finally, the effect of 5-HT is not due to activation of PKC isoforms that remain after long term treatment with phorbol ester, because these isoforms are known to be Ca^{2+} independent and the PKC inhibitor staurosporine, at concentrations that completely block PKC activity *in vitro*

(1000 nM), did not affect the 5-HT-mediated amplification (Fig. 7A).

The Ca^{2+}-dependent effect of 5-HT is likely due to the activation of calmodulin, because two calmodulin antagonists (calmidazolium and W-7) blocked the amplification by both 5-HT and A23187 of NECA-stimulated cAMP accumulation in long term PMA-treated cells (Fig. 7B). Although these calmodulin antagonists have been shown to be capable of inhibiting Ca^{2+}-sensitive PKC activity (39), their effect in blocking the 5-HT and A23187 amplification in long term PMA-treated cells is unlikely to be due to antagonism of remaining Ca^{2+}-independent PKC, because they had little or no effect on PKC activity measurements in long term PMA-treated cells. However, because these calmodulin antagonists are effective inhibitors of Ca^{2+}-sensitive PKC and there are no established methods currently available to "down-regulate" the calmodulin pathway without also affecting the PKC pathway, we cannot evaluate the extent of the contribution of the Ca^{2+}/calmodulin-dependent 5-HT pathway in normal cells.

The effect of calmodulin on the AC system is unlikely to be due to stimulation of calmodulin-dependent AC (type 1), because 5-HT did not increase basal cAMP accumulation and we have not detected calmodulin-sensitive AC activity in membrane preparations from A1A1 cells.[1] Experiments to measure changes in calmodulin levels and calmodulin-dependent protein kinase activity in response to 5-HT are in progress.

In conclusion, the results reported here suggest that activation of 5-HT$_{2A}$ receptors in the A1A1 neuronal cell line results in amplification of the AC/cAMP signaling system by two distinct pathways coupled to PI hydrolysis-PKC and Ca^{2+}/calmodulin. However, these two mechanisms may not be the only means of 5-HT-mediated amplification in these cells. Recent reports indicate that G protein $\beta\gamma$ units can stimulate the type II form of AC in the presence of G_s activation (57, 58). The involvement of $\beta\gamma$ subunits in 5-HT$_{2A}$-mediated enhancement of cAMP levels in A1A1 cells is under investigation.

The A1A1 cell line was generated by retroviral transduction of simian virus 40 T-antigen into embryonic day 16 cultures of rat cerebral cortex. Many cell lines have been derived from the developing mammalian neuroepithelium, using a variety of immortalization methods (59). These cell lines generally represent embryonic stages of neuronal differentiation. Cell lines derived from hippocampus and cerebellum by T-antigen immortalization differentiate into neurons and glia when implanted into the developing brain (60, 61). The differentiation of an immortal cell provides a strategy for the genetic analysis of signal transduction mechanisms *in vivo* in both developing and mature neurons. The use of the A1A1 cell line to study 5-HT$_{2A}$ receptor function raises the issue of whether A1A1 cells may express signaling pathways found in mature cortex. Determination of the biological function of 5-HT$_{2A}$ receptors may be aided through genetic perturbations of A1A1 cells followed by transplantation into the developing brain.

Acknowledgments

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[1] K.A. Berg and W.P. Clarke, unpublished observations.

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Send reprint requests to: Kelly A. Berg, Department of Anesthesiology, Mail Stop 1010, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029–6574.