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Cell-specific Signaling of the 5-HT1A Receptor

MODULATION BY PROTEIN KINASES C AND A*

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Heterologous expression of the rat 5-HT1A receptor in stably transfected GH4C1 rat pituitary cells (clone GH4ZD10) and mouse Ltk⁻ fibroblast cells (clone LZD-7) (Albert, P. R., Zhou, Q.-Y., VanTol, H. H. M., Bunzow, J. R., and Civelli, O. (1990) *J. Biol. Chem.* 265, 5825–5832) was used to characterize the cellular specificity of signal transduction by the 5-HT1A receptor. We demonstrate that the 5-HT1A receptor, acting via pertussis toxin-sensitive G proteins, can change its inhibitory signaling phenotype and become a stimulatory receptor, depending on the cell type, differentiation state, or intracellular milieu of the cell in which it is expressed. When expressed in pituitary GH4ZD10 cells, activation of 5-HT1A receptors decreased both basal and vasoactive intestinal peptide-enhanced cAMP accumulation and blocked (±)-Bay K8644-induced influx of calcium, inhibitory responses which are typical of neurons which endogenously express this receptor. Similarly, 5-hydroxytryptamine (5-HT) also inhibited adenylyl cyclase in fibroblast LZD-7 cells, reducing the forskolin-induced enhancement of cAMP levels by 50%, but did not alter basal cAMP levels. In contrast to GH4ZD10 cells, where 5-HT had no effect on basal or thyrotropin-releasing hormone-induced phosphatidylinositol turnover, 5-HT enhanced the accumulation of inositol phosphates and induced a biphasic increase in $[Ca^{2+}]_i$ in LZD-7 cells. These dominant stimulatory actions of 5-HT, as well as the inhibitory effects, were absent in untransfected cells and displayed the potency and pharmacological specificity of the 5-HT1A receptor, indicating that the 5-HT1A subtype coupled to both inhibitory and stimulatory pathways in the fibroblast cell. The actions of 5-HT in GH and L cells were blocked by 24-h pretreatment with pertussis toxin, suggesting that inhibitory G proteins (G_i/G_o) mediate both inhibitory and stimulatory signal transduction of the 5-HT1A receptor. However, the 5-HT-induced stimulatory pathway in fibroblasts was blocked selectively by acute (2-min) pretreatment with TPA, an activator of protein kinase C. This action of protein kinase C was potentiated by activation of protein kinase A, indicating that the expression of the stimulatory pathway of the 5-HT1A receptor in LZD-7 cells is modulated by second messengers.

Many hormone and neurotransmitter receptors transduce and amplify their signal via coupling to heterotrimeric G proteins to change the activity of effector enzymes (*e.g.* adenylylcyclase or phospholipase C), which generate intracellular second messengers (Birnbaumer *et al.*, 1990; Gilman, 1987; Ross, 1989). Adenylylcyclase catalyzes the formation of cAMP, a second messenger which activates protein kinase A, inducing the phosphorylation of multiple substrate proteins. Phospholipase C-catalyzed hydrolysis of PIP_2[1] leads to formation of the second messengers IP_3 and DAG (Berridge and Irvine, 1989; Berridge, 1987; Colino and Halliwell, 1987). IP_3 elevates $[Ca^{2+}]_i$ by mobilizing nonmitochondrial calcium stores (Prentki *et al.*, 1984; Streb *et al.*, 1983). DAG induces activation of protein kinase C, and translocation of the enzyme from the cytosol to the membrane compartment, resulting in phosphorylation of a number of protein substrates (Nishizuka, 1986, 1988). PTx-sensitive G proteins are coupled to inhibition of these processes, although in certain cell types, PTx-sensitive enhancement of phospholipase C activity has been reported (Ross, 1989). Thus, there may exist a cellular specificity of the transduction pathways mediated by this class of heterotrimeric G proteins.

In cell types where receptor-induced enhancement of PI turnover was blocked by pretreatment with PTx, a parallel PTx-sensitive enhancement of cell proliferation was sometimes reported (Kavanaugh *et al.*, 1988; Seuwen *et al.*, 1988; Van Corven *et al.*, 1989), a response not observed in cells without this pathway (*e.g.* nondividing neuronal cells). Although it is sometimes unclear which intracellular messenger (*e.g.* cAMP, DAG, $[Ca^{2+}]_i$, or others) generates the proliferative signal (Van Corven *et al.*, 1989), these second messengers are known to play important roles in the proliferation and differentiation of cells (Berridge, 1987; Mendoza *et al.*, 1986; Weinstein, 1988). The capacity of a family of receptors to assume different signaling pathways depending on the cell type may play a role in determining the proliferative state of a cell or may participate in the differentiation process of dividing stem cells. At the very least, signal transduction switching would alter the response of a target cell to a given stimulus, possibly even reversing the response. Such altered responsiveness could influence an entire network of cells,

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[1] The abbreviations used are: PIP_2, phosphatidylinositol (4,5)-bisphosphate; 5-HT, 5-hydroxytryptamine, serotonin; $[Ca^{2+}]_i$, cytosolic free calcium concentration; PI, phosphatidylinositol; IP_3, inositol (1,4,5)-trisphosphate; DAG, diacylglycerol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; TRH, thyrotropin-releasing hormone; VIP, vasoactive intestinal peptide; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; G protein, guanine nucleotide-binding regulatory protein; G_i, inhibitory G protein; G_o, G protein identified as a PTx substrate in brain; PTx, pertussis toxin; 8-Br-cAMP, 8-bromo-cyclic adenosine adenosine 3′:5 monophosphate; PLC, phospholipase C; EGTA, [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid.

leading to subtle or perhaps great alterations in organism as a whole.

These studies were undertaken to determine whether a specific receptor, the 5-HT1A receptor, is coupled via PTx-sensitive G proteins to the same signal transduction pathway independent of the cell type in which it is expressed or whether there exists a cell specificity to the signal transduction pathway of the receptor. When expressed in GH4C1 pituitary cells, which have several properties of neuronal cells including pituitary hormone receptors, voltage-gated ion channels, and regulated secretion of hormones, the 5-HT1A receptor displayed a signaling phenotype characteristic of neurons in which the receptor is endogenously expressed. That is, 5-HT inhibited adenylylcyclase (Albert *et al.*, 1990; Clark *et al.*, 1987; Dumuis *et al.*, 1988; Fargin *et al.*, 1989; Zgombick *et al.*, 1989) and decreased calcium influx via voltage-gated calcium channels (Colino and Halliwell, 1987; Innis and Aghajanian, 1987; Pennington and Kelly, 1990; Ropert, 1988; Sprouse and Aghajanian, 1988; Zgombick *et al.*, 1989). When expressed in Ltk$^-$ fibroblast cells, a model of undifferentiated cells, the 5-HT1A receptor exhibited an additional stimulatory pathway, switching its signaling pathway from inhibition to stimulation of $[Ca^{2+}]_i$. Thus the 5-HT1A receptor displayed either the inhibitory or the dominant stimulatory signal transduction phenotype, depending on the cell in which it was expressed. The choice of signaling phenotype was modulated by activation of protein kinases C and A, converting the fibroblast signaling phenotype to neuron-like signaling.

EXPERIMENTAL PROCEDURES

Materials

TPA, 8-Br-cAMP, forskolin, and 5-HT, IBMX, and VIP were purchased from Sigma; TRH and ionomycin were from Calbiochem; *myo*-[2-^{3}H]inositol was obtained from Amersham Corp. Spiperone and (±)-Bay K8644 were from Research Biochemical Inc., Natick, MA. Fura 2-AM was from Molecular Probes, Eugene, OR. GH4C1 cells were from Dr. A. H. Tashjian, Harvard School of Public Health, Boston, MA. Ltk$^-$ cells were from Dr. O. Civelli, Oregon Health Sciences University, Portland, OR.

Methods

Cell Culture—All cells were grown as monolayer in α-minimum Eagle's medium supplemented with 5% fetal bovine serum (L cells) or Ham's F-10 medium with 8% fetal bovine serum (GH cells), at 37 °C in a humidified atmosphere, with 5.0% carbon dioxide. Media were changed 12–24 h prior to experimentation. The concentration of 5-HT present in the medium was at most 10 ± 4 nM (based on serum 5-HT concentrations provided by GIBCO). Chronic (24-h) treatment with 1 μM 5-HT did not alter the acute responses measured in 5-HT-free HBBS buffer (see below).

Measurement of $[Ca^{2+}]_i$—Cells were harvested by incubation in calcium-free HBBS containing 5 mM EDTA and 0.05% trypsin and centrifuged at 200 × *g* for 3 min at room temperature. The cells were washed once with HBBS (118 mM NaCl, 4.6 mM KCl, 1.0 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, 20 mM Hepes, pH 7.2) and then resuspended in 0.5 ml of HBBS and incubated for 30 min at 37 °C in the presence of 2 μM Fura-2AM (dissolved in dimethyl sulfoxide to 5 mM). They were then diluted to 10 ml, centrifuged, washed twice with HBBS buffer, and finally resuspended in 2 ml of HBBS and placed in a fluorescence cuvette. Change in fluorescence ratio (λ_{ex} = 340/380 nm, λ_{em} = 500 nm) was recorded on a Perkin-Elmer (Buckinghamshire, Great Britain) LS-50 spectrofluorometer and analyzed by computer, based on a K_D of 227 nM for the Fura 2·Ca^{2+} complex (Grynkiewicz *et al.*, 1985). Calibration of R_{max} was performed by addition of 0.1% Triton X-100 and 20 mM Tris base and of R_{min} by addition of 10 mM EGTA (Albert and Tashjian, 1984; Grynkiewicz *et al.*, 1985). All experimental compounds were added directly to the cuvette from 200-fold concentrated test solutions at times indicated in the figures.

cAMP Assay—Measurement of cAMP was performed as described previously (Albert *et al.*, 1990). In brief, cells were plated in six-well 35-mm dishes; medium was changed 12–24 h prior to experimentation. After removal of the medium, cells were preincubated in 2 ml/well HBBS for 5–10 min at 37 °C, the buffer was replaced by 1 ml of HBBS containing 100 μM IBMX, and the incubation was continued for another 5 min. Then, the various test compounds were added to the wells, and the cells were incubated at room temperature for 30 min. The buffer was collected and stored at −20 °C until assay for cAMP. cAMP was assayed by a specific radioimmunoassay (ICN) as described (Albert *et al.*, 1990) with antibody used at 1:500 dilution. After 16 h of incubation at 4 °C, 20 μl of 10% bovine serum albumin and 1 ml of 95% ethanol were added consecutively to precipitate the antibody-antigen complex. Standard curves showed IC_{50} of 0.5 ± 0.2 pmol by using cAMP as standard. Data for cAMP assay are described as mean ± S.E. for triplicate wells.

Assessment of PI Hydrolysis—An adaptation of the protocols of Raymond *et al.* (1989) was used. In brief, cells grown in six-well dishes were equilibrated for 48 h in the regular medium supplemented with 3 μCi/ml of [^{3}H]*myo*-inositol. After washing three times with HBBS, cells were incubated for 2 h in HBBS containing 10 mM LiCl. The medium was then replaced by 1 ml of fresh medium supplemented by various concentrations of test compounds, and the cells were incubated for 25 min. The reaction was terminated by aspiration followed by the addition of 0.3 ml of ice-cold 10% trichloroacetic acid. The lysates were collected and centrifuged 10,000 × *g* for 15 min. The supernatants were extracted three times with 2.5 ml of diethyl ether, while the pellets were discarded. The samples were neutralized by addition of 10 μl of 1 N NaOH, and their radioactivity was measured. In our assay, basal IP levels were 480 ± 169 cpm/dish in GH cells and 29,000 ± 3000 cpm/dish (mean ± S.D. of five independent experiments).

Statistical Method—Data were analyzed for statistical significance using the Student's *t* test.

RESULTS

Inhibitory Signaling by 5-HT1A Receptors in Pituitary Cells—GH4ZD10 rat pituitary cells expressing the transfected rat 5-HT1A receptor (Albert *et al.*, 1990) were used to characterize the signal transduction pathways of the 5-HT1A receptor in an electrically excitable cell model. As shown previously (Albert *et al.*, 1990), treatment of these cells with 100 nM 5-HT significantly decreased both basal and VIP-stimulated cAMP accumulation; these actions of 5-HT were abolished by 24-h pretreatment with 25 ng/ml PTx (Fig. 1*A*). Concurrent addition of 100 nM TPA attenuated 5-HT-induced inhibition of basal cAMP accumulation but was without effect on inhibition of VIP-stimulated cAMP levels. Neither PTx nor TPA significantly decrease basal cAMP levels. Since accumulation of cAMP in our experiment was measured in the presence of 100 μM IBMX to inhibit cAMP degradation by phosphodiesterases (Albert *et al.*, 1990), the observed change in cAMP accumulation presumably represents a change in its synthesis. Thus, the 5-HT1A receptor induced a G_i/G_o-mediated inhibition of adenylate cyclase as observed in other cell types (Clark *et al.*, 1987; Dumuis *et al.*, 1988; Fargin *et al.*, 1989; Frazer *et al.*, 1990; Zgombick *et al.*, 1989).

In order to examine the actions of 5-HT1A receptor activation on voltage-dependent calcium influx, the opening of L-type calcium channels was enhanced using dihydropyridine channel agonist (±)-Bay K8644 (Enyeart *et al.*, 1987, 1990; Triggle and Janis, 1987), and changes in $[Ca^{2+}]_i$ were measured using intracellularly trapped fluorescent calcium indicator Fura-2 (Grynkiewicz *et al.*, 1985) (see "Methods"). (±)-Bay K8644 (200 nM) increased $[Ca^{2+}]_i$ by 1.4-fold in GH4ZD10 cells (Fig. 1*B*). Acute pretreatment with 1 μM 5-HT did not alter basal $[Ca^{2+}]_i$ but completely blocked the action of 200 nM (±)-Bay K8644 (Fig. 1*B*). This inhibitory action of 5-HT was abolished by 10 μM spiperone (not shown) or by 24-h pretreatment with 25 ng/ml PTx (Fig. 1*C*). In untransfected GH4C1 cells no effect of 5-HT (1 μM) on (±)-Bay K8644 actions was observed (not shown). Thus, as observed in neuronal systems, 5-HT1A receptor activation in GH4ZD10 cells

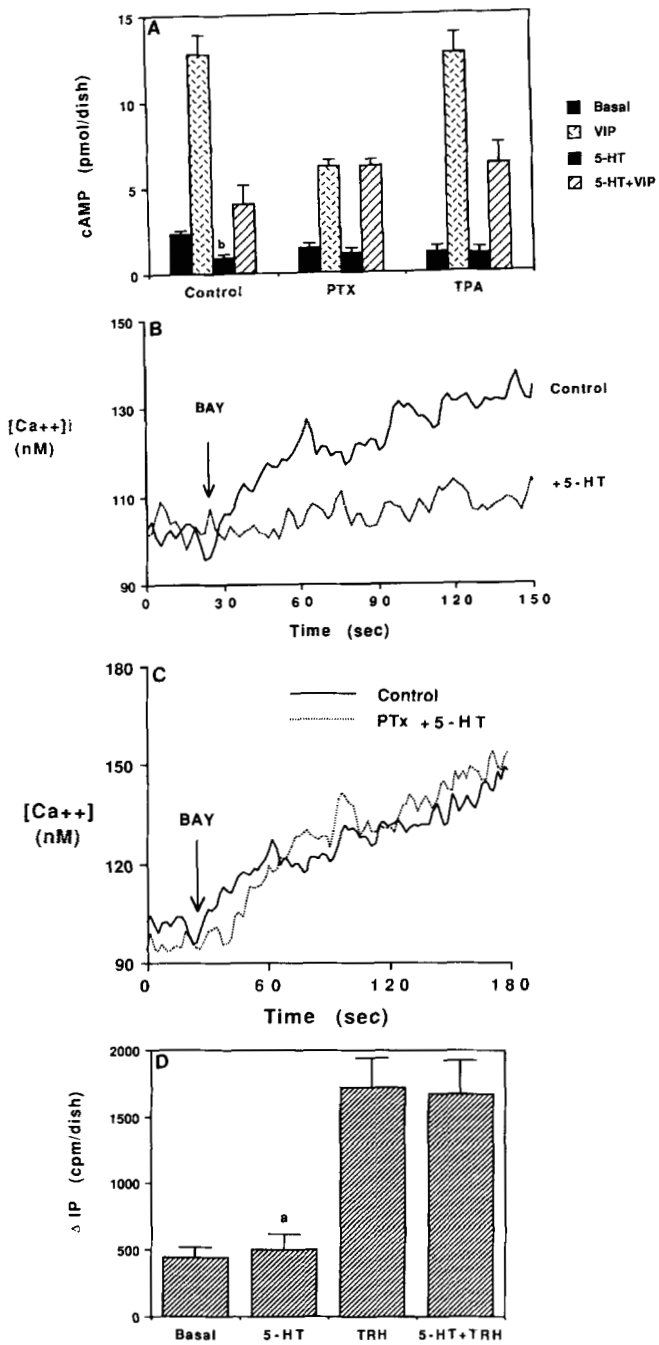

FIG. 1. **5-HT1A receptor-mediated signal transduction in GH4ZD10 cells.** *A*, 5-HT-induced inhibition of cAMP accumulation in GH4ZD10 cells. cAMP accumulation was measured in media harvested from cells 20 min after addition of regulators. The error bars indicate S.D. of triplicate determinations from one experiment which was repeated two additional times with similar results. "*b*" designates $p < 0.05$ by Student's *t* test compared with basal levels; the significance of other data points is readily apparent. Concentrations used were: 5-HT, 100 nM; VIP, 200 nM; TPA, 100 nM. PTx (25 ng/ml) was added 24 h prior to experimentation and was absent during assay. *B*, Inhibition of (±)-Bay K8644-induced changes in $[Ca^{2+}]_i$ by 5-HT in GH4ZD10 cells. The data are representative of three independent experiments. $[Ca^{2+}]_i$ was measured using calcium indicator Fura-2, and (±)-Bay K8644 (200 nM) or 5-HT (100 nM) added as indicated. (±)-Bay K8644 increased $[Ca^{2+}]_i$ by 1.41 ± 0.08-fold basal level (mean ± S.D., $n = 3$). *C*, blockade of 5-HT action on (±)-Bay K8644 by 25 ng/ml PTx. Concentrations of 5-HT and (±)-Bay K8466 were as in *B*, and PTx treatment was as in *A*. The data are representative of three independent experiments. *D*, 5-HT1A receptor activation does not alter PI turnover in GH4ZD10 cells. Intracellular levels of [^{3}H]inositol and inositol phosphates were measured as described under "Methods." Error bars represent S.D. of triplicate samples, and one of three experiments is shown. "*a*" denotes $p > 0.05$ compared with basal level. TRH (100 nM) or 5-HT (1 μM) were used.

suppressed calcium channel opening and inhibited increase in $[Ca^{2+}]_i$.

Finally, activation of the 5-HT1A receptor in GH4ZD10 cells induced no changes in PI hydrolysis (Fig. 1*D*). This observation is consistent with the lack of effect on basal $[Ca^{2+}]_i$, since generation of IP_3 via PI turnover is associated with increase in $[Ca^{2+}]_i$ in several cell types (Berridge and Irvine, 1989; Berridge, 1987). In contrast, 100 nM TRH, which induces characteristic spike and plateau phases of increase in $[Ca^{2+}]_i$ (Albert and Tashjian, 1984), induced a 3.8 ± 0.41-fold enhancement of total IP formation. Coaddition of 1 μM 5-HT during the 15-min incubation did not alter the TRH-induced enhancement of PI turnover. Thus, 5-HT was without effect on PI turnover in GH4ZD10 cells.

In summary, when expressed in GH4C1 pituitary cells, the 5-HT1A receptor is coupled to multiple intracellular signaling pathways (*i.e.* inhibition of adenylylcyclase, inhibition of calcium influx) characteristic of those found in differentiated neurons where the receptor is endogenously expressed.

5-HT1A Receptor Activation Increases $[Ca^{2+}]_i$ in Fibroblast Cells—A stably transfected clone of mouse Ltk$^-$ fibroblasts, named LZD-7, which expresses high levels (1.9 pmol/mg of protein) of specific rat 5-HT1A ligand binding sites (Albert *et al.*, 1990), provided a second cell type to examine the signaling phenotype of the 5-HT1A receptor. 5-HT (1 μM) had no effect on $[Ca^{2+}]_i$ in untransfected Ltk$^-$ cells (Fig. 2*A*), which lack endogenous 5-HT receptors (Albert *et al.*, 1990). By contrast, 5-HT induced an immediate increase in $[Ca^{2+}]_i$ in transfected LZD-7 cells, which was comprised of two phases: an acute "spike" phase and a sustained "plateau" phase. During the initial spike phase, $[Ca^{2+}]_i$ was increased by 2.67 ± 0.32 ($n = 8$) times the basal level (Table I), was maximal within 10–15 s, and lasted 30–40 s. During the following plateau phase, $[Ca^{2+}]_i$ remained increased by 1.45-fold basal level (Table I) for over 10 min. The selective 5-HT1A receptor agonist, 8-OH-DPAT (Albert *et al.*, 1990; Frazer *et al.*, 1990), mimicked the action of 5-HT to increase $[Ca^{2+}]_i$ (not shown). The 5-HT1A receptor antagonist spiperone (2 μM) (Albert *et al.*, 1990; Frazer *et al.*, 1990) abolished the increase in $[Ca^{2+}]_i$ induced by 100 nM 5-HT (Fig. 2*B*). Spiperone did not alter basal $[Ca^{2+}]_i$ and the vehicle (0.1% dimethyl sulfoxide) did not alter basal $[Ca^{2+}]_i$ or the 5-HT effect on $[Ca^{2+}]_i$. These results indicate that the change in intracellular calcium induced by 5-HT is indeed due to stimulation of the transfected 5-HT1A receptors.

Since the 5-HT1A receptor is thought to act by coupling to PTx-sensitive G proteins (Frazer *et al.*, 1990), the effect of PTx on 5-HT-induced increase in $[Ca^{2+}]_i$ was examined. As shown in Fig. 2*C*, actions of 5-HT on $[Ca^{2+}]_i$ were abolished completely by 24-h pretreatment of LZD-7 cells with 10 ng/ml PTx. The inhibitory effect of PTx on LZD-7 cells lasted 4–5 days, indicating that G protein turnover in this cell line is less rapid than in GH4C1 cells (48).

The effect of 5-HT to increase $[Ca^{2+}]_i$ in LZD-7 cells was dose-dependent, with an EC_{50} of 3 nM (Fig. 2, *D* and *E*), identical to the K_D of 5-HT at the rat 5-HT1A binding site (Albert *et al.*, 1990; Fargin *et al.*, 1986). Similar EC_{50} values were observed for both the spike and plateau phases of 5-HT-induced change in $[Ca^{2+}]_i$ (Fig. 2*E*), suggesting that the same receptor-mediated pathway mediates both phases. Taken together, these results indicate that 5-HT1A receptor activation mediates the 5-HT-induced increase in $[Ca^{2+}]_i$ in LZD-7 cells

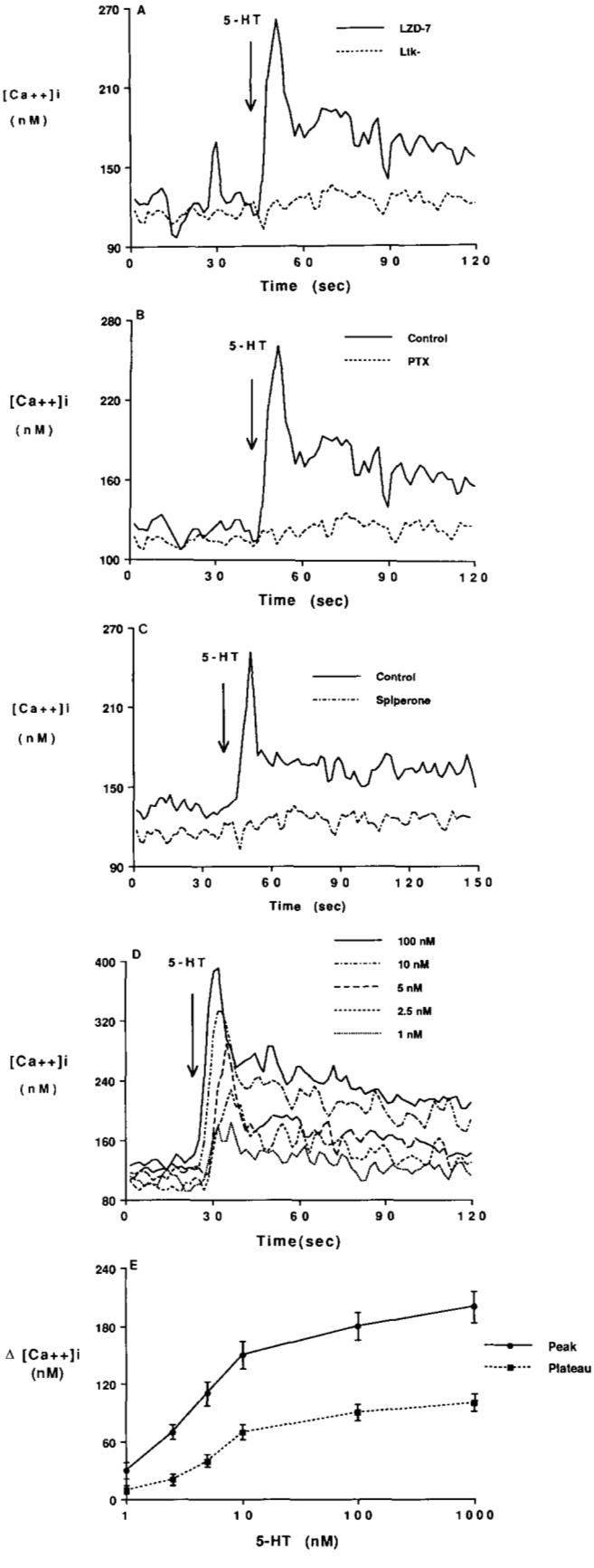

FIG. 2. **5-HT1A receptor activation increases in $[Ca^{2+}]_i$ in LZD-7 cells.** Changes in fluorescence intensity were monitored and converted to values of $[Ca^{2+}]_i$ as described under "Experimental Procedures." Curves generated by computer represent one of at least three independent experiments. *A*, 5-HT increases $[Ca^{2+}]_i$ in LZD-7 but not in Ltk$^-$ cell. 5-HT (10 nM) was added to the cuvette as indicated. *B*, spiperone abolishes 5-HT-induced increase in $[Ca^{2+}]_i$ (control) in LZD-7 cells. 5 μM spiperone was added to the cell suspension as indicated. *C*, PTx blocks 5-HT-induced increase $[Ca^{2+}]_i$ in LZD-7 cells. PTx (10 ng/ml) was added to growth medium 24 h prior to assay. *D*, concentration dependence of 5-HT induced increase of $[Ca^{2+}]_i$ in LZD-7 cells. All curves were generated by a computer and were from a single experiment which has been repeated three times. *E*, dose-response curves for 5-HT-induced spike and plateau phases in increase of $[Ca^{2+}]_i$. Data were averaged from experiments in *D* and represent mean ± S.D. of at least five determinations.

TABLE I

Effect of protein kinases C and A on 5-HT-induced changes in $[Ca^{2+}]_i$

The influence of various acute pretreatments in LZD-7 cells on 100 nM 5-HT-induced changes in $[Ca^{2+}]_i$ are indicated as -fold basal level of $[Ca^{2+}]_i$ and represent mean ± S.D. of at least three independent determinations. A value of unity (1) indicates no change in the basal level of $[Ca^{2+}]_i$.

Treatment	5-HT-induced Increase in $[Ca^{2+}]_i$	
	Peak	Plateau
	-fold basal	
Control	2.67 ± 0.32	1.45 ± 0.21
TPA (1 nM)	2.42 ± 0.25[a]	1
TPA (10 nM)	1	1
Forskolin (10 μM)	2.13 ± 0.26[a]	1.34 ± 0.10[a]
8-Br-cAMP (1 mM)	2.21 ± 0.28[a]	1.28 ± 0.11[a]
Forskolin + 1 nM TPA	1	1
8-Br-cAMP + 1 nM TPA	1	1

[a] Indicates $p > 0.05$ compared with control values, *i.e.* no significant change.

via coupling to PTx-sensitive G proteins.

Sources of Calcium Mobilized by 5-HT—To characterize the sources of calcium for the 5-HT-induced increase in $[Ca^{2+}]_i$, LZD-7 cells were treated acutely with ionomycin, a calcium ionophore which releases cellular calcium stores (Albert and Tashjian, 1984, 1986). As shown in Fig. 3*A*, 100 nM ionomycin induced a 1.5-fold increase in $[Ca^{2+}]_i$ and nearly abolished the action of 5-HT added 2 min later. At a 10-fold higher concentration, ionomycin induced a 6-fold increase in $[Ca^{2+}]_i$, which returned to a near-basal plateau level (Fig. 3*B*). This concentration of ionomycin completely blocked 5-HT action. Since ionomycin did not cause a large sustained elevation in $[Ca^{2+}]_i$, it is likely that ionomycin preferentially depleted cellular calcium stores (Albert and Tashjian, 1984) and hence blocked 5-HT-induced calcium mobilization. Prior addition of 5-HT reduced by 30% the ionomycin-induced release of calcium (Fig. 3*C*), indicating that 5-HT partially releases ionomycin-sensitive calcium stores (Albert and Tashjian, 1986). Taken together, these data indicate that the major source of calcium for the 5-HT-induced changes is from release of ionomycin-sensitive intracellular stores, although a small component of calcium influx may be present. Since L cells lack voltage-dependent calcium channels (Liao *et al.*, 1990; Perez-Reyes *et al.*, 1990), it was not possible to test these cells for inhibition by 5-HT of (±)-Bay K8644-induced calcium influx.

5-HT Increases Hydrolysis of Phosphatidylinositol—The above results suggested that the change in $[Ca^{2+}]_i$ represented an IP_3-induced release of cellular calcium due to 5-HT1A receptor-mediated PI turnover, as seen for other receptors (Albert and Tashjian, 1984; Berridge and Irvine, 1989; Berridge, 1987). The actions of 5-HT on PI turnover in LZD-7 cells pre-equilibrated with [^{3}H]*myo*-inositol were examined. As shown in Fig. 4*A*, 5-HT increased total [^{3}H]inositol phosphate accumulation in a dose-dependent manner (EC_{50} = 5 nM). 5-HT-induced enhancement of PI turnover was mim-

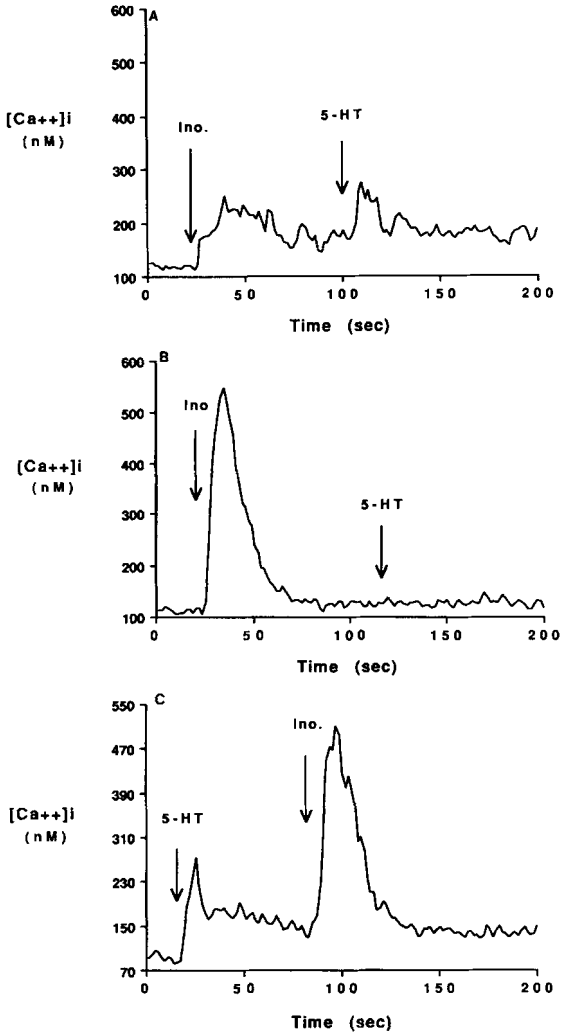

FIG. 3. **Sources of calcium for 5-HT-induced change in $[Ca^{2+}]_i$.** *A*, Ionomycin-induced increase in $[Ca^{2+}]_i$ and block of 5-HT induced changes. Ionomycin (*Ino.*, 100 nM) and 5-HT (100 nM) were added as indicated. Ionomycin (100 nM) induced an increase of $[Ca^{2+}]_i$ of 1.48 ± 0.10-fold basal level (mean ± S.D., n = 3). *B*, complete block of 5-HT-induced change in $[Ca^{2+}]_i$ by ionomycin (1 μM). Ionomycin (1 μM) induced a 6.3 ± 0.4-fold increase in basal $[Ca^{2+}]_i$ (mean ± S.D., n = 3). *C*, 5-HT was added prior to addition of ionomycin (1 μM). Following 5-HT, 1 μM ionomycin induced a 3.9 ± 0.2 (n = 2)-fold increase in $[Ca^{2+}]_i$. 10 nM 5-HT was added as indicated.

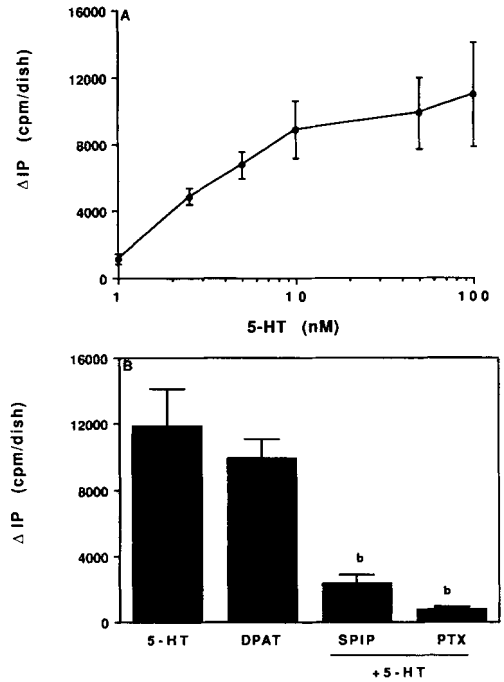

FIG. 4. **5-HT1A receptor activation enhances PI turnover in LZD-7 cells.** The *ordinate* values in each part represent (mean treated-mean untreated) values ± S.D. and are from at least three separate experiments. *A*, dose dependence of 5-HT-induced increase in inositol phosphate accumulation. Accumulation of $[^3H]IP$, $-IP_2$, and $-IP_3$ was measured as described (see "Experimental Procedures"). The basal $[^3H]IP$ production in untreated cells was 29,000 ± 3,000 cpm/dish. *B*, effect of various compounds on 5-HT-induced increase total PI turnover. The basal level of PI turnover was 30,000 ± 2,700 cpm/dish. PTx (10 ng/ml) was added 24 h before experimentation. The final concentrations of 5-HT and 8-OH-DPAT were 100 nM. Spiperone (*SPIP*, 5 μM) was added 5 min prior to addition of 5-HT and was present throughout the assay. "*b*" denotes a significant ($p < 0.05$) difference compared with 5-HT-treated controls.

icked by 1 μM 8-OH-DPAT and was blocked by 10 μM spiperone or by pretreatment with 10 ng/ml PTx (Fig. 4*B*), indicating that 5-HT1A receptor stimulates a phosphatidylinositol-specific phospholipase C via coupling to PTx-sensitive G proteins.

Although the increase (1.5-fold basal) in PI turnover induced by 5-HT in L cells was less pronounced than that induced by TRH in GH cells (Fig. 1*D*), the basal level of PI turnover under identical conditions was over 50-fold higher (*i.e.* 32,000 cpm *versus* 500 cpm). Thus the 5-HT-induced increment in PI turnover was actually over five times greater than with TRH in GH cells.

5-HT Inhibits Forskolin-stimulated cAMP Accumulation—Since the 5-HT1A receptor is coupled to inhibition of adenylylcyclase in other systems (Albert *et al.*, 1990; Clark *et al.*, 1987; Dumuis *et al.*, 1988; Fargin *et al.*, 1989; Okada *et al.*, 1989; Zgombick *et al.*, 1989), the effect of 5-HT on cAMP accumulation in media from LZD-7 cells was determined. As shown in Fig. 5*A*, 5-HT had no effect on basal cAMP accumulation under the present experimental conditions. However, the increase in cAMP accumulation (*i.e.* 4-fold above basal) induced by forskolin, which directly stimulates adenylylcyclase, was decreased by up to 60% in the presence of 0.01–1 μM 5-HT (EC_{50} = 10 nM, Fig. 5*B*). The effect of 5-HT to decrease forskolin-stimulated cAMP accumulation was mimicked by 8-OH-DPAT and blocked by spiperone or by pretreatment with 10 ng/ml PTx (Fig. 5*A*).

Selective Blockade of 5-HT Actions by Activation of Protein Kinases C and A—Receptor-mediated activation of phospholipase C produces DAG, an endogenous stimulator of protein kinase C (Nishizuka, 1986, 1988). Activation of protein kinase C exerts feedback inhibition of phospholipase C in several systems (Bell *et al.*, 1985; Brock *et al.*, 1985; Daniel-Issakani *et al.*, 1989; Rink *et al.*, 1983; Smith *et al.*, 1987; Ueda *et al.*, 1989, Van Corven *et al.*, 1989), including fibroblast cells (Brown *et al.*, 1987; Mendoza *et al.*, 1986). TPA, which mimics the action of DAG to stimulate protein kinase C activity (Castagna *et al.*, 1982; Nishizuka, 1986), was used to examine the importance of protein kinase C in 5-HT1A receptor signaling. LZD-7 cells were pretreated with various concentrations of TPA 1 min prior to addition of 5-HT (Fig. 6). Although TPA alone did not alter $[Ca^{2+}]_i$, the 5-HT-induced increase in $[Ca^{2+}]_i$ was abolished in a dose-dependent manner by acute pretreatment with 1–10 nM TPA, in multiple experiments (Table I). Interestingly, the two phases of change in $[Ca^{2+}]_i$ induced by 5-HT had different sensitivities to TPA. Following addition of 1 nM TPA, the plateau phase was almost

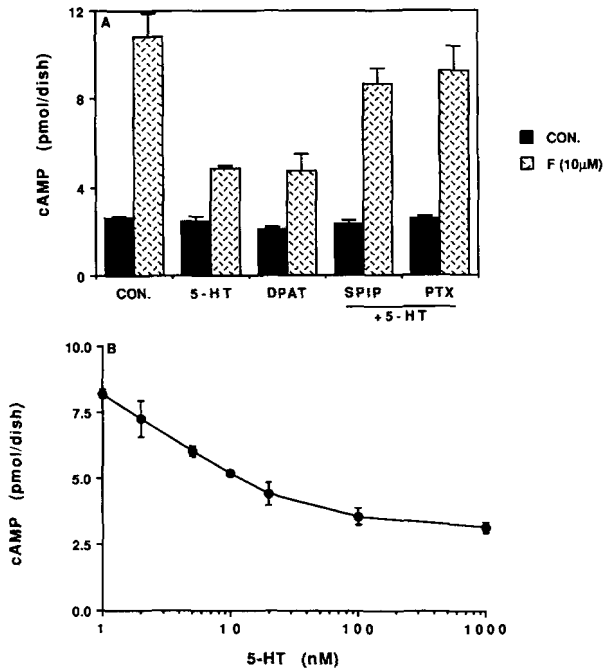

FIG. 5. **5-HT1A receptor activation inhibits adenylylcyclase activation in LZD-7 cells.** *A*, effects of various compounds on 5-HT actions on cAMP accumulation. PTx (10 ng/ml) was added 24 h before experimentation. Drug concentrations were as in Fig. 4. *B*, dose dependence of 5-HT-induced inhibition of forskolin (*F*)-stimulated cAMP levels in LZD-7 cells. Values in each part are the mean ± S.D. of results from three separate experiments.

totally blocked; the spike phase was less affected. However, in the presence of 10 nM TPA, the spike phase was also blocked (Fig. 6*B*). These results indicates that the 5-HT-induced increase in $[Ca^{2+}]_i$ consists of two distinct phases with different sensitivities to protein kinase C.

In order to investigate the role of cAMP in the 5-HT-induced increase of $[Ca^{2+}]_i$, and the interrelationship between protein kinase C and protein kinase A, the actions of forskolin on the 5-HT-induced increase in $[Ca^{2+}]_i$ and on the inhibitory actions of protein kinase C were examined. As shown in Fig. 6*C*, pretreatment with 10 μM forskolin had no significant effect on the 5-HT-induced increase in $[Ca^{2+}]_i$ and only partially (20–30%) inhibited the spike phase (Table I). Although increase in cAMP levels did not greatly alter the 5-HT-induced change in $[Ca^{2+}]_i$, it did synergize with TPA to block the change in $[Ca^{2+}]_i$. After pretreatment of the cells with 10 μM forskolin for 5 min, the 5-HT-induced increase in $[Ca^{2+}]_i$ was completely abolished by 1 nM TPA (Fig. 6*C*), whereas 1 nM TPA alone only partially-blocked the 5-HT response. The above actions of forskolin were mimicked by treatment with 10 mM 8-Br-cAMP (Fig. 6*D*, Table I), a nonhydrolyzable analog of cAMP, indicating that activation of protein kinase A mediates the forskolin-induced potentiation of protein kinase C actions on $[Ca^{2+}]_i$. Prior down-regulation of protein kinase C using 24–48-h pretreatment with 500 nM TPA blocked acute inhibition of 5-HT-induced increase in $[Ca^{2+}]_i$ by TPA alone or TPA plus forskolin (not shown), suggesting that protein kinase C is essential for these responses.

To study whether action of protein kinase C on calcium mobilization could be mediated via inhibition of phospholipase C, the effect of TPA on total PI turnover in LZD-7 cells was tested. As shown in Fig. 7*A*, at 1 and 10 nM concentrations, TPA had a small (less than 10% above basal level) but not significant effect on basal IP production in LZD-7 cells. However, TPA attenuated 5-HT-induced PI turnover by 80–

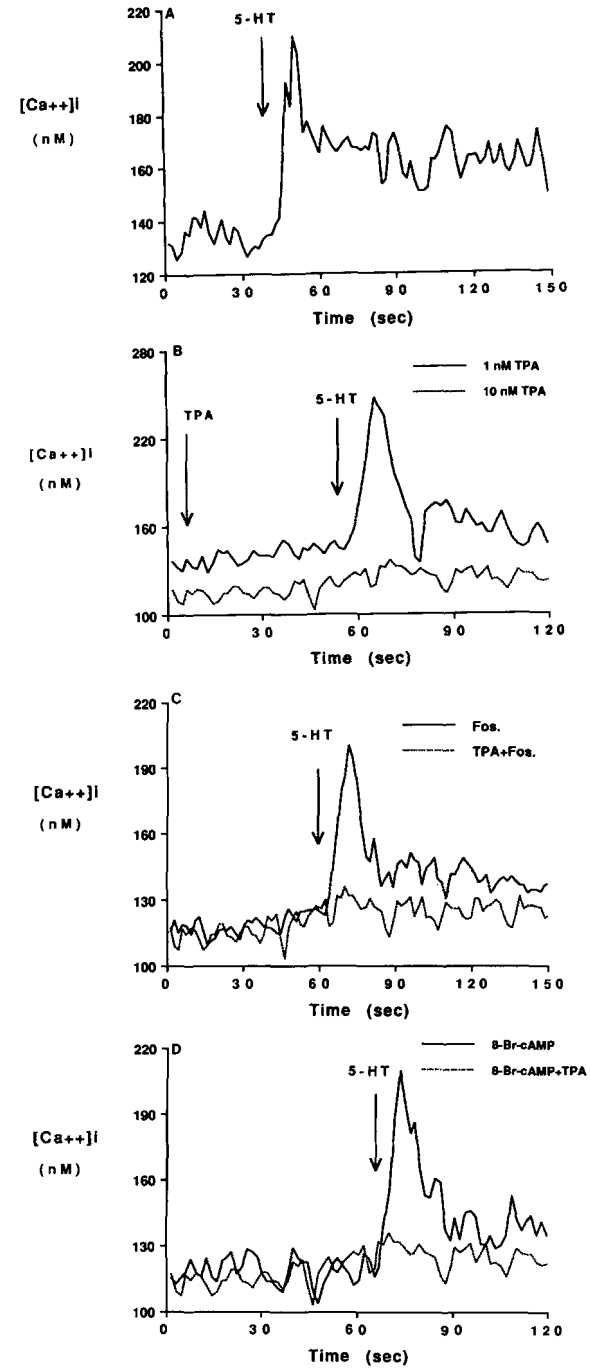

FIG. 6. **TPA blocks 5-HT-induced increase $[Ca^{2+}]_i$ in LZD-7 cells.** Curves represent a typical experiment which has been repeated at least five times with similar results. *A*, control: 10 nM 5-HT-induced increase in $[Ca^{2+}]_i$. *B*, blockade by 1 and 10 nM TPA of 5-HT-induced increases $[Ca^{2+}]_i$ in LZD-7 cells. TPA was added as indicated. *C*, forskolin (*Fos.*) potentiated the action of TPA on 5-HT-induced increase $[Ca^{2+}]_i$ in LZD-7 cells. LZD-7 cells were pretreated 10 μM forskolin for 5 min. 1 nM TPA and 10 nM 5-HT were added as indicated. *D*, 8-Br-cAMP (10 mM) potentiates the action of TPA (1 nM) to block the 5-HT-induced increase $[Ca^{2+}]_i$ in LZD-7 cells. 8-Br-cAMP was added 6 min prior to 5-HT.

90% only at 10 nM and had no significant effect at 1 nM (Fig. 7, legend). Forskolin alone altered neither basal nor 5-HT-induced increase in total PI turnover, indicating that activation of protein kinase A had no direct effect on phospholipase C activity. In the presence of 10 μM forskolin, 1 nM TPA now also inhibited 5-HT action effectively. These results correlated well with actions of these compounds to inhibit 5-HT-

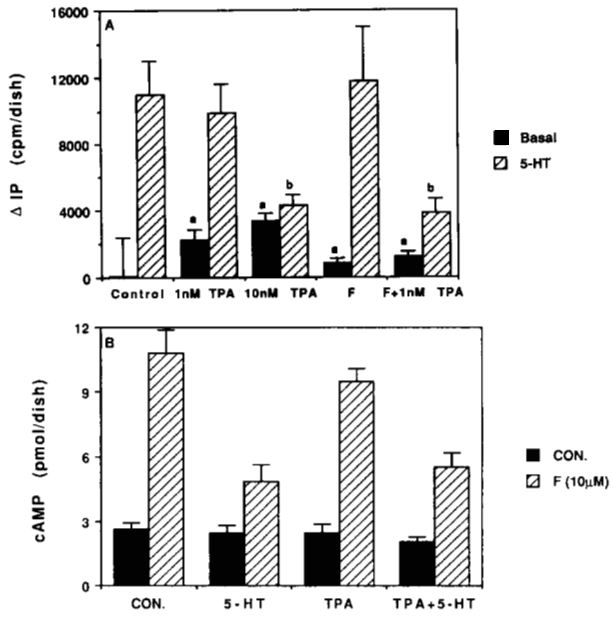

FIG. 7. **Actions of TPA on 5-HT-induced changes in cAMP and PI turnover.** *A*, TPA blocks the 5-HT-induced increase of total PI turnover. Concentrations used were: 5-HT, 10 nM; 10 μM forskolin (*F*); TPA, as indicated. The data for accumulation of [^{3}H]IP accumulation are presented as means ± S.D. ($n = 3$), with the basal level (32,000 ± 2270 cpm/dish) subtracted. "*a*" denotes $p > 0.05$ compared with control basal level; "*b*" denotes a significant difference ($p < 0.05$) compared with control 5-HT-treated samples. *B*, TPA does not alter 5-HT-induced decrease of forskolin (*F*)-induced cAMP levels. Concentrations used were: forskolin (*F*), 10 μM; 5-HT, 10 nM; and TPA, 10 nM. In both parts, the indicated compounds (*e.g.* 5-HT, TPA, or forskolin) were added acutely during the assay. Values in each part are the mean ± S.D. from three separate experiments.

induced enhancement of $[Ca^{2+}]_i$, indicating that activation of protein kinase C inhibits the 5-HT action at or prior to the activation of phospholipase C. The action of protein kinase A is indirect and requires simultaneous activation of protein kinase C.

We also tested whether activation of protein kinase C could block 5-HT-induced inhibition of cAMP accumulation to indirectly modulate protein kinase A. 10 nM TPA, which completely blocked 5-HT-induced increase in $[Ca^{2+}]_i$ (Fig. 6*B*), altered neither the basal level of cAMP nor forskolin-stimulated cAMP accumulation (Fig. 7*B*). Similarly, TPA had no effect on inhibition of forskolin-induced cAMP accumulation by 5-HT, indicating that this pathway is insensitive to protein kinase C activation, in contrast to the 5-HT-induced increase in $[Ca^{2+}]_i$, which is modulated by protein kinase C. Since 5-HT had no effect on the basal level of cAMP, the 5-HT-induced increase in $[Ca^{2+}]_i$ was not due to alteration of protein kinase A activity. Thus, TPA induced a rapid and differential uncoupling of 5-HT1A receptor action, inhibiting phospholipase C and calcium mobilization without altering 5-HT-induced inhibition of cAMP.

DISCUSSION

Phenotypic Switch of 5-HT1A Signal Transduction Pathway—Previous studies have shown that the 5-HT1A receptor is a member of the conserved family of receptors that interact with PTx-sensitive G proteins to inhibit adenylylcyclase activity (Frazer *et al.*, 1990). The 5-HT1A receptor is prominently expressed in neuronal cells (*e.g.* hippocampal CA-1, dorsal raphé nuclei) where it also opens potassium channels via activation of a PTx-sensitive G protein (Colino and Halliwell, 1987; Hoyer *et al.*, 1986; Ropert, 1988; Sprouse and Aghajanian, 1988; Zgombick *et al.*, 1989) and closes calcium channels (Pennington and Kelly, 1990; Ropert, 1988). This results in hyperpolarization of the membrane potential, closing of voltage-dependent calcium channels, and decrease in $[Ca^{2+}]_i$. A variety of other neurotransmitter receptors direct the opening of the G protein-gated potassium channel (including dopamine-D_2), each by activating a PTx-sensitive G protein (Birnbaumer *et al.*, 1990). As illustrated in Fig. 8, expression of the rat 5-HT1A receptor in pituitary GH4C1 cells (GH4ZD10 cells) resulted in a 5-HT-induced inhibition of $[Ca^{2+}]_i$ and cAMP accumulation similar to that observed in neurons. However, when expressed in Ltk$^-$ fibroblast cells (LZD-7 cells), activation of the 5-HT1A receptor caused a striking increase in $[Ca^{2+}]_i$, characteristic of receptors that induce PI turnover. In the same cells, 5-HT inhibited forskolin-stimulated cAMP accumulation in LZD-7 cells by over 50%. However, unlike in GH cells, 5-HT did not inhibit basal cAMP levels in LZD-7 cells. Thus, in L cells, the 5-HT1A receptor mediates a reversed intracellular signaling phenotype to induce both a dominant phospholipase C-mediated stimulatory pathway, as well as inhibitory intracellular actions.

The evidence that the 5-HT1A receptor mediated both stimulatory and inhibitory responses in LZD-7 cells is summarized here. 5-HT-induced increases in PI hydrolysis, in $[Ca^{2+}]_i$, and inhibition of cAMP accumulation in LZD-7 cells were: 1) mimicked by 8-OH-DPAT, a selective 5-HT1A receptor agonist, and blocked by spiperone, a 5-HT1A receptor antagonist; 2) dose-dependent, with potencies (EC_{50} = 3, 5, and 10 nM, respectively) that correlated well with receptor affinity of the 5-HT1A receptor for 5-HT (K_D = 1.7 nM) (Albert *et al.*, 1990; Frazer *et al.*, 1990); 3) abolished by pretreatment with PTx, which uncouples the receptor from G proteins, shifting the receptor from a high affinity state to a low affinity state (Albert *et al.*, 1990). This last-mentioned result indicates that all actions of the 5-HT1A receptor expressed in L cells were mediated via coupling to PTx-sensitive G proteins. Furthermore, both 5-HT-induced PI turnover and increase in $[Ca^{2+}]_i$ (but not inhibition of cAMP accumulation) were blocked by TPA-induced activation of protein kinase C. Thus, the 5-HT1A receptor appears to activate a phosphatidylinositol-specific phospholipase C to increase IP3 and $[Ca^{2+}]_i$ in LZD-7 cells via coupling to a PTx-sensitive signal transduction pathway that is distinct from the inhibitory neuroendocrine pathway and is subject to feedback inhibition by activation of protein kinase C.

The difference in 5-HT1A receptor signaling does not appear to be an artifact of overexpression of the receptor, whereby the 5-HT1A receptor is expressed at such high levels

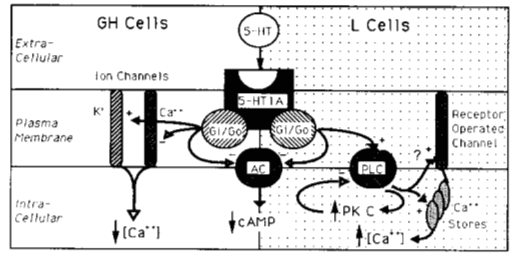

FIG. 8. **Model of the signal transduction pathways of the 5-HT1A receptor.** 5-HT binds to the 5-HT1A receptor and inhibits adenylylcyclase in both GH and L cells. In GH cells, 5-HT inhibits activation of Ca^{2+} channels and may (by analogy with neurons) open K^+ channels, leading to decrease in $[Ca^{2+}]_i$. In L cells, 5-HT activates PLC to release calcium stores and possibly activate receptor operated channels, to increase $[Ca^{2+}]_i$, and activate protein kinase C (*PK C*) via release of DAG. Protein kinase C negatively regulates PLC activation. Abbreviations are as in text except G_i/G_o, PTx-sensitive G proteins; *AC*, adenylylcyclase; *PK C*, protein kinase C.

in LZD-7 cells that an abnormal coupling to PLC results. The level of expression of 5-HT1A receptors in LZD-7 cells (1.9 pmol/mg of protein) is comparable with the expression in GH4ZD10 cells (1.1 pmol/mg) and is similar to the estimated level for expression in the CA1 region of the hippocampus (0.5 pmol/mg) (Albert *et al.*, 1990; Hoyer *et al.*, 1986). At a half-maximal concentration, which would activate 1.0 pmol/mg protein of 5-HT1A receptors, 5-HT induced a clear increase in PI turnover and in $[Ca^{2+}]_i$ in LZD-7 cells, but no increase in these parameters was observed in GH4ZD10 cells at maximal concentrations of 5-HT (Figs. 1 and 4). Thus, the activation of similar numbers of 5-HT1A receptors in different cell types produces different responses.

Recently, using a cloned human 5-HT1A receptor, it was shown that this receptor stimulates phospholipase C in HeLa cells, but not in COS-7 kidney cells (Fargin *et al.*, 1989; Raymond *et al.*, 1989). In HeLa cells, activation of PI turnover by 5-HT was regarded as a "weaker accessory signal pathway" to stimulate sodium-dependent phosphate uptake (Raymond *et al.*, 1989), since the EC_{50} for PI turnover was 10-fold higher than for inhibition of adenylylcyclase. In L cells, the actions of 5-HT to increase PI turnover and $[Ca^{2+}]_i$ appear to be primary actions of the 5-HT1A receptor, since the EC_{50} values for these actions range near to 5-HT binding affinity. Closely analogous observations have been reported for the dopamine-D_2 receptor, which is also coupled to PTx-sensitive G proteins to inhibition of $[Ca^{2+}]_i$ and cAMP in neurons. When expressed in L cells, the dopamine-D_2 receptor increases PI turnover and $[Ca^{2+}]_i$ that are blocked by pretreatment with PTx (Vallar *et al.*, 1990). Thus, the phenotypic switch from inhibition to excitation, which we observe with the 5-HT1A receptor, is not peculiar to this receptor alone but may be a general property of receptors which couple to PTx-sensitive G proteins. Expression of the stimulatory pathway seems to correlate with the differentiation state, in that rapidly growing tumor cell lines (*e.g.* L or HeLa cells) express the stimulatory fibroblast pathway, whereas cells (*e.g.* GH4C1 or neurons) which express differentiated properties (hormone production, excitability) express the inhibitory neuroendocrine pathway. Switching of receptor signaling phenotype would alter cellular regulation radically and may trigger changes in the differentiation state of the cell.

Mechanism of Change in Calcium Levels—As in other systems (Clark *et al.*, 1987; Dumuis *et al.*, 1988; Fargin *et al.*, 1989; Zgombick *et al.*, 1989), the 5-HT1A receptor in LZD-7 cells inhibited forskolin-stimulated adenylylcyclase activity, but had no effect on basal activity. We found no evidence for stimulation of adenylylcyclase by 5-HT1A receptor activation in L cells or in GH4C1 cells, as reported in some neurons (Dumuis *et al.*, 1988). Since 5-HT had no effect on the basal accumulation of cAMP, we conclude that cAMP is not the second messenger which mediates 5-HT-induced mobilization of intracellular calcium stores. Instead, 5-HT-induced increase of $[Ca^{2+}]_i$ were correlated with the 5-HT-induced enhancement of PI turnover.

The phosphoinositide signal transduction mechanism leads to production of two messengers, DAG and IP_3, each of which independently or together modulate many cellular responses in the number of cell types (Berridge and Irvine, 1989; Berridge, 1987). In many signaling systems, evidence has implicated 1,4,5-IP_3 as the molecule which links receptor-activated PI turnover to transient mobilization of calcium from intracellular stores (Prentki *et al.*, 1984; Schulz *et al.*, 1989; Smith *et al.*, 1987; Walker *et al.*, 1987), resulting in a transient spike in $[Ca^{2+}]_i$ (Berridge and Irvine, 1989; Berridge, 1987) as observed with 5-HT in LZD-7 cells. The results with ionomycin (Fig. 3) suggest that ionomycin-releasable calcium stores may account for both acute and sustained actions of 5-HT on $[Ca^{2+}]_i$, since ionomycin blocked both phases of 5-HT action.

Modulation by Protein Kinases C and A—We found that 5-HT-induced changes in PI turnover and $[Ca^{2+}]_i$ in LZD-7 cells, but not changes in cAMP accumulation, were blocked by 1–2-min pretreatment with TPA, an activator of protein kinase C. This indicates that signaling of PI turnover by the 5-HT1A receptor is under negative feedback regulation, since the product, DAG, can inhibit its own production via activation of protein kinase C. Other cellular systems which display this sort of feedback inhibition include 3T3 (Brown *et al.*, 1987; Mendoza *et al.*, 1986) and human fibroblast cells (Van Corven *et al.*, 1989), smooth muscle cells (Brock *et al.*, 1985), and platelets (Bell *et al.*, 1985; Rittenhouse and Sasson, 1985). In each case, the receptor is coupled via a PTx-sensitive G protein to PI turnover.

There is evidence that TPA acts by blocking the coupling of PTx-sensitive G proteins to PLC (Ryu *et al.*, 1990; Smith *et al.*, 1987), either by phosphorylation of the G protein (Daniel-Issakani *et al.*, 1989) or of phospholipase C (Ryu *et al.*, 1990). Reconstitution experiments utilizing purified G_i proteins have demonstrated that these proteins do couple to phospholipase C (Ueda *et al.*, 1989). Activation of protein kinase C by TPA leads to phosphorylation of a specific PTx-sensitive G protein, G_i2, to inhibit insulin-like growth factor I receptor-mediated PIP_2 turnover (Drummond, 1985). A similar mechanism may mediate the action of TPA to block 5-HT1A receptor-induced PI turnover. By transfecting antisense G_i2, we have evidence that the 5-HT1A receptor is coupled via G_i2 to enhance $[Ca^{2+}]_i$ in LZD-7 cells.[2] Recently, it has become apparent that different PTx-sensitive G proteins may link to different signal transduction pathways (Birnbaumer *et al.*, 1990; Ross, 1989). For example, G_i3 couples the somatostatin receptor to potassium channels (Yatani *et al.*, 1987), G_i2 and G_o couple couple to activation of PI turnover in *Xenopus* oocytes (Moriarty *et al.*, 1990). Understanding the specificity of coupling of receptors to G protein in different cellular models will be a key step to understanding why receptors possess different signal transduction pathway in different cell types.

A novel finding was the potentiation of TPA action by activators of protein kinase A. Inhibition (Sibley *et al.*, 1984) or enhancement (Bell *et al.*, 1985; Cronin and Canonico, 1985) of cAMP generation by activation of protein kinase C has been reported, but little is known about actions of protein kinase A on protein kinase C (*e.g.* McAtee and Dawson, 1989), particularly in modulating signal transduction. The present results indicate that activators of the adenylylcyclase pathway can enhance C kinase-mediated homologous uncoupling of receptors in the phospholipase C pathway.

Conclusion—We have shown that the signal transduction pathway of the 5-HT1A receptor depends on the cell type in which it is expressed. In fibroblast L cells, the receptor couples efficiently to phospholipase C to increase $[Ca^{2+}]_i$, due to both influx of extracellular calcium and release of stored intracellular calcium. This represents an altered phenotype of 5-HT1A receptor signaling, where inhibition of $[Ca^{2+}]_i$ is changed to stimulation. We have also shown that stimulation of $[Ca^{2+}]_i$ by 5-HT is rapidly and potently blocked by activation of protein kinase C (potentiated by protein kinase A), whereas the inhibitory branch (inhibition of adenylylcyclase) is not. Thus, the phenotypic switch of the receptor is accompanied by an altered regulation of receptor coupling. The role of a switch in signaling phenotype of this and other receptors

[2] Y. F. Liu and P. R. Albert, unpublished observations.

in vivo remains an open question. However, evidence relating activation of phospholipase C and protein kinase C to the growth-promoting actions of oncogenes and growth factors (Berridge, 1987; Mendoza *et al.*, 1986; Weinstein, 1988) point to a possible role of the phenotypic switch in regulation of precursor cell growth and differentiation.

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