Serotonin 5-HT$_{1A}$ Receptor-mediated Erk Activation Requires Calcium/Calmodulin-dependent Receptor Endocytosis*

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Many receptors that couple to heterotrimeric guanine nucleotide-binding (G) proteins mediate rapid activation of the mitogen-activated protein kinases, Erk1 and Erk2. The G$_i$-coupled serotonin (5-hydroxytryptamine (5-HT)) 5-HT$_{1A}$ receptor, heterologously expressed in Chinese hamster ovary or human embryonic kidney 293 cells, mediated rapid activation of Erk1/2 via a mechanism dependent upon both Ras activation and clathrin-mediated endocytosis. This activation was attenuated by chelation of intracellular Ca$^{2+}$ and Ca$^{2+}$/calmodulin (CAM) inhibitors or the CAM sequestrant protein calpeptin. The CAM-dependent step in the Erk1/2 activation cascade is downstream of Ras activation, because inhibitors of CAM antagonize Erk1/2 activation induced by constitutively activated mutants of Ras and c-Src but not by constitutively activated mutants of Raf and MEK (mitogen and extracellular signal-regulated kinase). Inhibitors of the classical CAM effectors myosin light chain kinase, CAM-dependent protein kinases II and IV, PP2B, and CAM-sensitive phosphodiesterase had no effect upon 5-HT$_{1A}$ receptor-mediated Erk1/2 activation. Because clathrin-mediated endocytosis was required for 5-HT$_{1A}$ receptor-mediated Erk1/2 activation, we postulated a role for CAM in receptor endocytosis. Inhibition of receptor endocytosis by use of sequestration-defective mutants of $\beta$-arrestin, and dynamin attenuated 5-HT$_{1A}$ receptor-stimulated Erk1/2 activation. Inhibition of CAM prevented agonist-dependent endocytosis of epitope-tagged 5-HT$_{1A}$ receptors. We conclude that CAM-dependent activation of Erk1/2 through the 5-HT$_{1A}$ receptor reflects its role in endocytosis of the receptor, which is a required step in the activation of MEK and subsequently Erk1/2.

Receptors coupled to heterotrimeric guanine nucleotide-binding (G) proteins, the largest known family of cell surface receptors, mediate cellular responses to many extracellular stimuli, such as neurotransmitters, peptide hormones, odontants, and photons (1). In addition to regulating the generation of soluble second messengers, many G protein-coupled receptors mediate proliferative or differentiative signals in various cultured cell lines and tissues via mitogen-activated protein (MAP) kinases (2, 3). Erk1/2 MAP kinases are serine/threonine kinases involved in the activation of nuclear transcription factors. Erk1/2 activity is regulated by threonine/tyrosine phosphorylation, which is controlled by a highly conserved phosphorylation cascade. Phosphorylation of Erk1/2 is catalyzed by the MAP/Erk kinases 1 and 2 (MEK1 and MEK2) that are themselves phosphorylated and activated by MEK kinases such as raf-1 oncogene family proteins. Activation of raf-1 occurs as a consequence of membrane translocation, which can be mediated by the GTP-bound form of the small G protein, Ras.

Like the epidermal growth factor (EGF) receptor and other receptor tyrosine kinases, many G protein-coupled receptors regulate Ras function via tyrosine phosphorylation. Several G$_i$-coupled receptors stimulate pertussis toxin-sensitive, Ras-dependent Erk activation through tyrosine phosphorylation of adapter proteins, such as Shc and Gab1, and membrane recruitment of the Ras guanine nucleotide exchange factor, mSos (2, 4). Shc tyrosine phosphorylation and Erk activation by G$_i$-coupled receptors are sensitive to inhibitors of src family nonreceptor tyrosine kinases (4, 5), suggesting a role for src kinases in mitogenic signaling by G protein-coupled receptors. In some systems, Ca$^{2+}$/CAM has also been implicated in G$_i$- and G$_{\alpha}$-coupled receptor-mediated Erk activation (6–8).

Recent data have also suggested that clathrin-mediated endocytosis plays a crucial, if poorly understood, role in Erk activation via EGF, insulin-like growth factor-1 receptors (9, 10), lysophosphatidic acid (LPA), and $\beta$$_2$-adrenergic receptors (11, 12). Inhibition of $\beta$$_2$-adrenergic receptor sequestration with dominant inhibitory mutants of $\beta$-arrestin1, or dynamin impairs Erk activation with no effect on receptor-G protein coupling (12).

5-HT mediates mitogenic effects in many cell types (13, 14). In CHO-K1 cells, the 5-HT$_{1A}$ receptor rapidly activates Erk via a pathway that involves pertussis toxin-sensitive G protein $\beta$γ subunits, phosphatidylinositol 3′-kinase and src kinase, the Shc and Grb2 adapter proteins, mSos, Ras, and Raf (13, 15). Moreover, 5-HT$_{1A}$ receptors have been colocalized with adenylate...
lyl cyclase and G proteins in clathrin-rich brain vesicles (16). In this report, we show that 5-HT1A receptor-mediated Erk activation is sensitive to inhibitors of Ca2+/CAM and clathrin-mediated endocytosis but not to inhibitors of the known CAM effectors myosin light chain kinase, CAM-dependent protein kinases II and IV, PP2B, and CAM-sensitive phosphodiesterases. Rather, the Ca2+/CAM dependence of Erk activation derives from a requirement for CAM in 5-HT1A receptor endocytosis. These data suggest a previously unappreciated role for CAM in the sequestration of G protein-coupled receptors and provide a mechanism for cooperativity between Ca2+/CAM and tyrosine phosphorylation in mitogen signal transduction.

**Experimental Procedures**

**Materials**—1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA), phenol red 12-myrystate 13-acetate (PMA), and LPA were from Sigma. Fluphenazine, W-7, EGF, cyclopamine A, PK506, A20587, and ionomycin were from Calbiochem. Basic fibroblast growth factor (bFGF) was from Upstate Biotechnology (Lake Placid, NY). ML-7, ML-9, HA1077, KN-62, KN-92, vinpocetine, and 8-methoxymethyl isobutylmethylxanthine were from Biomol (Plymouth Meeting, PA).

**DNA Constructs**—The cDNAs for FLAG-tagged β2-adrenergic receptor and activated Y530F-Src, in which the regulatory carboxyl-terminal tyrosine residue has been mutated, were prepared as described (20–22). cDNAs encoding the dominant-interfering mutants V530D-β-arrestin1 and K44A-dynamin were the kind gift of M. Caron. The cDNA encoding the CAM-sequestering protein calpermin was the gift of A. Means. Calpermin cDNA was subcloned into the pcDNA3 vector via a NcoI/XbaI digest of pcaMPL-calsp followed by blunt-end treatment with the Klenow fragment of DNA polymerase I. The insert was placed into EcoRV-digested, calf intestinal alkaline phosphatase-treated pcDNA3. Correct orientation was verified by diagnostic restriction digests.

**Cell Culture and Transfection**—HEK-293 cells were maintained in minimum essential medium with Earle’s salts (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Inc.) and 50 μg/ml gentamicin (Life Technologies, Inc.) at 37 °C in a humidified 5% CO2 atmosphere. CHO-1A-27 cells (18) were maintained in Ham’s F-12 nutrient mixture (Life Technologies, Inc.) supplemented with 10% FBS, 50 μg/ml gentamicin, and 400 μg/ml G418. Transfections were performed on 80–90% confluent monolayers in 100-mm dishes, using calcium phosphate coprecipitation for HEK-293 cells (23) and LipofectAMINE for CHO cells (9 μg of DNA, Life Technologies, Inc.) (15). Empty pcDNA3 vector was added to transfections to keep the total mass of DNA added per dish constant within experiments. Prior to stimulation, cells were incubated overnight in serum-free medium containing 0.1% bovine serum albumin.

**Erk1/2 Phosphorylation and Kinase Assay**—Stimulations were carried out at 37 °C in serum-starving medium. Monolayers were then lysed directly using 200 μl/well Laemmli sample buffer. Phosphorylation of Erk1/2 was detected by immunoblotting using rabbit phospho-specific Erk IgG (New England Biolabs) as described previously, except that the bands were visualized with Vistra ECF reagent (Amer-}

**RESULTS AND DISCUSSION**

**Inhibitors of Ca2+/CAM Impair 5-HT1A Receptor-mediated Erk Activation in CHO-1A-27 Cells**—To examine the role of Ca2+/CAM in Gβγ-coupled receptor-mediated Erk activation, we employed CHO cells stably expressing the 5-HT1A receptor (CHO-1A-27 cells) (18). In this system, we have previously shown that 5-HT stimulation results in rapid, Ras-dependent, activation of Erk2, which is mediated via Gβγ subunits derived from pertussis toxin-sensitive G proteins (13). As shown in Fig. IA, stimulation of CHO-1A-27 cells with 5-HT resulted in a 7- to 9-fold stimulation of Erk2 kinase activity compared with unstimulated cells. Stimulation of endogenous fibroblast growth factor receptors and acute protein kinase C activation with PMA also induced Erk2 activation. Pretreatment with BAPTA, a cell-permeable Ca2+ sequestrant, in Ca2+-free medium inhibited 5-HT1A receptor- and bFGF-stimulated Erk2 activation, with no effect on the protein kinase C-mediated signal.

To determine whether CAM activity is required for receptor-mediated Erk activation, CHO-1A-27 cells were pretreated with the pharmacologically distinct CAM antagonists, fluphenazine and W-7. As shown in Fig. 1B, both agents impaired 5-HT- and bFGF-stimulated Erk2 activation. To confirm the specificity of the fluphenazine and W-7 effects, the experiments were repeated in HEK-293 cells transiently coexpressing the 5-HT1A receptor with the CAM-binding protein calpermin (26), which has been shown to function as an intracellular CAM sequestrant. As shown in Fig. 1C, calpermin expression attenuated Erk1/2 phosphorylation induced by 5-HT1A or endogenous EGF receptors. Neither isoproterenol-stimulated intracellular cAMP production nor 5-HT- and LPA-induced inhibition of forskolin-stimulated cAMP production was affected by W-7 or fluphenazine, indicating that the CAM antagonists had no effect on receptor-G protein coupling (data not shown).

**Co2+/CAM Antagonists Impair 5-HT1A Receptor-mediated Erk Activation at a Point in the Pathway Downstream of Ras Activation**—In many systems, the pathways of Gβγ-coupled receptor- and receptor tyrosine kinase-mediated Erk activation converge upstream of Ras activation (27). The finding that CAM antagonists affected both receptor tyrosine kinase- and 5-HT1A receptor-mediated Erk activation suggested that Ca2+/CAM was involved in regulating a common component of the pathway. To determine the point in the signaling cascade at which Ca2+/CAM is required, we assayed the effects of fluphenazine and W-7 on Erk activation via constitutively active mutants of Src, Ras, Raf, and MEK. As shown in Fig. 2, the constitutively activated proteins Y530F-Src, V12-Ras, Raf-CAAX, or DD-MEK were sufficient to induce chronic Erk activation in transiently transfected CHO-1A-27 cells. Fluphenazine or W-7 significantly inhibited Y530F-Src- and V12-Ras-induced Erk2 activation, without affecting Raf-CAAX or DD-MEK. Because the Ca2+/CAM inhibitor-induced blockade can be “bypassed” by activated Raf and MEK but not by activated

2 A. R. Means, personal communication.
Clathrin-mediated Endocytosis Is Required for 5-HT\textsubscript{1A} Receptor-mediated Erk Activation—A requirement for clathrin-mediated endocytosis has recently been demonstrated for activation of the Erk cascade via several receptors, including the EGF receptor (1) and insulin-like growth factor 1 (10) receptor tyrosine kinases, and the LPA (11) and β\textsubscript{2}-adrenergic (12) G protein-coupled receptors. Specific inhibitors of G protein-coupled receptor endocytosis, including dominant negative mutants of β-arrestin, and dynamin, inhibit β\textsubscript{2}-adrenergic receptor-mediated Erk activation. β\textsubscript{2}-Adrenergic receptor stimulation results in the appearance of both receptor and Raf kinase in a clathrin-rich endocytic vesicle compartment (12). These data suggest that an endocytic process may be required to achieve activation of MEK and Erk kinases following the formation of an activated Ras-Raf complex on the plasma membrane.

To determine whether receptor endocytosis was required for 5-HT\textsubscript{1A} receptor-mediated Erk activation, we examined the effects of dominant negative K44A-dynamin\textsubscript{1}, or V53D-β-arrestin\textsubscript{1}, a β-arrestin\textsubscript{1} mutant that disrupts homologous desensitization and internalization of adrenergic receptors (28), on 5-HT-stimulated Erk1/2 phosphorylation in transiently transfected HEK-293 cells. Fig. 3 shows that both V53D-β-arrestin\textsubscript{1} and K44A-dynamin\textsubscript{1} attenuated 5-HT-stimulated Erk1/2 activation by the 5-HT\textsubscript{1A} receptor. EGF receptor-induced Erk1/2 activation was sensitive to expression of K44A-dynamin\textsubscript{1}, in accordance with previously published findings (9), but not to expression of V53D-β-arrestin\textsubscript{1}.

Ca\textsuperscript{2+} /CAM Antagonists Impair 5-HT\textsubscript{1A} Receptor-mediated Erk Activation by Interfering with Receptor Endocytosis—Ca\textsuperscript{2+}/CAM regulates multiple enzymes, including CAM-dependent protein kinases, myosin light chain kinase, protein phosphatase 2B, and CAM-sensitive phosphodiesterases. In addition, CAM is known to bind to neuronal clathrin light chains (29), to interact with the actin microfilament attachment protein, fodrin, a member of the spectrin family of membrane proteins (30, 31), and to regulate the activity of the G protein receptor kinases GRK5 and -6 (32–34). CAM also plays an as yet undefined role in endocytic events (35–37) and is important for endocytosis in adrenal chromaffin cells (38).

To test whether known enzymatic effectors of CAM participated in 5-HT\textsubscript{1A} receptor-mediated Erk activation, pharmacologic inhibitors of several CAM effectors were assayed for the ability to antagonize 5-HT-stimulated Erk2 activation in CHO-1A-27 cells. Preincubation for 30 min with the myosin light chain kinase inhibitors ML-7 (20 μM), ML-9 (50 μM), and HA1077 (50 μM); the protein phosphatase PP2B inhibitors cyclosporin A (1 μM) and FK506 (1 μM); the CAM-dependent protein kinase II and IV inhibitors KN-62 (10 μM) and KN-92 (10 μM); and the CAM-sensitive phosphodiesterase type I inhibitors vinpocetine (50 μM) and 8-methoxymethyl isobutylmethylxanthine (20 μM) had no effect upon 5-HT\textsubscript{1A} receptor-stimulated Erk activation (data not shown).

Because 5-HT\textsubscript{1A} receptor endocytosis is an obligate step in the Erk activation cascade, we tested the hypothesis that Ca\textsuperscript{2+}/CAM is involved in receptor endocytosis. Agonist-induced se-

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**Fig. 1. Effect of Ca\textsuperscript{2+} sequestration and CAM antagonism on 5-HT\textsubscript{1A} receptor-mediated Erk activation.** A, CHO-1A-27 cells were treated either with vehicle (control) or with BAPTA (25 μM). Cells were then stimulated for 5 min with 5-HT (10 μM), bFGF (10 ng/ml), or PMA (1 μM) prior to determination of Erk activity. Values shown represent means ± S.E. from four separate experiments each performed in duplicate. E, CHO-1A-27 cells were pretreated for 15 min with vehicle (control), W-7 (50 μM), or fluphenazine (Flu, 10 μM). Cells were then stimulated with 5-HT (10 μM), bFGF (10 ng/ml), or PMA (1 μM) for 5 min prior to determination of Erk activity. Values shown represent means ± S.E. from eight separate experiments, each performed in duplicate. C, HEK-293 cells were cotransfected with cDNA encoding HA1077 (50 μg/dish) plus either calsperrin cDNA (5 μg/dish) or empty vector (control, 5 μg/dish). After 24 h of serum starvation, cells were stimulated with 5-HT (10 μM), EGF (10 ng/ml), or PMA (1 μM) prior to determination of Erk activity. Values shown represent means ± S.E. from two separate experiments, each performed in duplicate. Data for all three panels are expressed as –fold Erk activity in which the values in unstimulated cells †were defined as 1.0. NS, not stimulated. ‡, indicates p < 0.05; *, indicates p < 0.01 versus agonist alone (black bars). Reverse Bonferroni correction was used.
questration of HA-tagged 5-HT$_{1A}$ and FLAG-tagged $\beta_2$-adrenergic receptors was determined in the presence of fluophenazine, W-7, or hypertonic sucrose. As shown in Fig. 4, both antagonists of Ca$^{2+}$/CAM impaired the sequestration of 5-HT$_{1A}$ receptors in transiently transfected HEK-293 cells. These results were duplicated for the $\beta_2$-adrenergic receptor (data not shown). The effect of the CAM inhibitors was comparable with that of preincubation in hypertonic medium and expression of K44A-dynamin$_1$ (data not shown), both known inhibitors of clathrin-mediated endocytosis. Coexpression of calpermin with epitope-tagged 5-HT$_{1A}$ receptors also resulted in similar abrogation of agonist-dependent receptor internalization (data not shown).

Several authors have postulated a role for Ca$^{2+}$/CAM in the activation of the Erk cascade. Both a-adrenergic receptor-mediated Erk activation in HEK-293 cells (6) and angiotensin receptor-mediated Erk activation in vascular smooth muscle cells (8) are sensitive to CAM inhibitors. Simultaneous expression of activated mutants of CaM kinase IV and CaM kinase kinase is sufficient to induce weak activation of Erk in NG-108 cells (39). We find that antagonism of Ca$^{2+}$/CAM using either pharmacologic inhibitors or the calcium sequestrant protein calpermin disrupts both Erk1/2 activation and agonist-induced endocytosis of the 5-HT$_{1A}$ receptor. The effects of CAM inhibition are not mimicked by pharmacologic inhibition of CAM effectors, including CaM kinase IV. Rather, our data indicate that the effects of CAM antagonists on receptor endocytosis are sufficient to account for the observed inhibition of Erk activation. However, because CAM inhibitors were somewhat more effective in preventing endocytosis than in blocking Erk activation, it is possible that there is an additional endocytosis-independent Erk activation pathway.

The mechanism whereby endocytosis of G protein-coupled receptors contributes to activation of the Erk cascade is unclear. The appearance of both Raf and $\beta_2$-adrenergic receptors in a clathrin-rich light vesicle fraction following agonist exposure suggests that the endocytic process may be required to transduce signals between an activated Ras-Raf complex on the plasma membrane and the MEK and Erk kinases in the cytosol (12). Our finding that CAM antagonists inhibit Erk activation mediated by constitutively active mutants of Src and Ras, but not Raf and MEK, supports such a model by localizing the putative endocytosis-dependent step in the signaling cascade downstream of Ras activation. CAM has been shown to bind to clathrin light (29, 41) and heavy chains (42) and to regulate vesicle recycling by influencing vesicle tubulation (43) and recruitment of clathrin to ligand-receptor complexes (44). Thus, CAM inhibitors could prevent Erk activation by 1) preventing recruitment of receptor-ligand complexes to clathrin, and/or 2) preventing successful activation of Raf by Ras, and/or 3) preventing endocytosis-dependent step in the signaling cascade.

Fig. 4. Effect of CAM antagonists and hypertonic sucrose on agonist-dependent sequestration of the 5-HT$_{1A}$ receptor in HEK-293 cells. Cells were transiently transfected with cDNA encoding the HA-tagged 5-HT$_{1A}$ receptor (1 $\mu$g/dish) and cDNA encoding V53D-5-arrin-1, (5 $\mu$g/dish), K44A-dynamin$_1$, (5 $\mu$g/dish), or empty vector (control, 5 $\mu$g/dish). Serum-starved cells were stimulated with 5-HT (10 pm) or EGF (10 ng/ml) for 5 min before determination of Erk activity. Data are expressed as –fold Erk activity in which the values in unstimulated cells were defined as 1.0. Values shown represent means ± S.E. from three separate experiments performed in duplicate. NS, not stimulated. †, indicates $p < 0.05$; *, indicates $p < 0.01$ versus agonist alone (black bars). Reverse Bonferroni correction was used.

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