Activation of Extracellular-Regulated Kinase by 5-Hydroxytryptamine$_{2A}$ Receptors in PC12 Cells is Protein Kinase C-Independent and Requires Calmodulin and Tyrosine Kinases

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ABSTRACT

5-Hydroxytryptamine (5-HT)$_{2A}$ receptors have been implicated to play a role in both the treatment and pathophysiology of a number of psychiatric disorders. Therefore, the coupling of this receptor to signals, such as extracellular signal-regulated kinase (ERK), that elicit long-term neuronal changes may be relevant. In the present study we examined the coupling of the G$_i$-coupled receptor to ERK in PC12 cells, a cell line commonly used as a neuronal model system. Activation of ERK occurred through a pathway different than the protein kinase C-dependent pathways described previously in studies of non-neuronal cells. Activation of ERK, in PC12 cells, was inhibited by both chelation of extracellular Ca$^{2+}$ and by depletion of intracellular Ca$^{2+}$ stores. Surprisingly, activation was not inhibited, but actually potentiated, by a variety of protein kinase C inhibitors covering all known protein kinase C isoforms. In contrast, the coupling of receptor to activation of ERK was found to be sensitive to N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) and N-(4-aminobuty1)-5-chloro-1-naphthalenesulfonamide (W13), inhibitors of calmodulin, but not to 1-[(N,O-bis[5-isoquinolinesulfonyl]-N-methyl-L-tyrosyl)-4-phenylpiperezine (KN62) and 2-[(N-[2-hydroxyethyl])-N-4-methoxybenzenesulfonyl]amino-N-[4-(chlorocinnamyl)-N-methylbenzylamine) (KN93), inhibitors of calmodulin-dependent protein kinase. Additionally, the general tyrosine kinase inhibitor genistein, as well as the Src inhibitor PP1 and the epidermal growth factor receptor kinase inhibitor 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG 1478), inhibited receptor-mediated activation of ERK, suggesting a role for tyrosine kinases. In fact, 5-HT was found to stimulate tyrosine phosphorylation of a number of proteins, and this phosphorylation was inhibited by W7. 5-HT$_{2A}$ receptor-activation of ERK through a protein kinase C-independent pathway requiring Ca$^{2+}$/calmodulin/tyrosine kinases represents a pathway distinct from those described in studies of non-neuronal cells.

5-Hydroxytryptamine (5-HT)$_{2A}$ receptors are G$_i$-coupled receptors, expressed by a variety of cell types, that serve numerous functions. For example, receptors expressed on platelets play a role in stimulating aggregation. In contrast, on vascular smooth muscle cells, they stimulate contraction (Roth et al., 1986; Watts et al., 1996). In the central nervous system, 5-HT$_{2A}$ receptor expression is heterogeneous. Receptors are expressed at high density in areas such as the neo-cortex, claustrum, anterior cingulate cortex, mammillary nuclei, and basal ganglia. However, the receptors are expressed at low densities in the hippocampus, brainstem, and thalamus (Pazos et al., 1985). 5-HT$_{2A}$ receptors have been implicated to be involved in both the pathophysiology and treatment of a number of psychiatric disorders. A role for the receptor in psychotic disorders is consistent with the observation that activation of extracellular-regulated kinase by 5-HT$_{2A}$ is protein kinase C-independent and requires calmodulin and tyrosine kinases.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; H89, N-[2-([p-bromocinnamylamino]methyl)-5-isoquinolinesulfonamide; Pl, phosphoinositide; phorbol 12-myristate-13-acetate; CaM, calmodulin; PKA, protein kinase A; EGF, epidermal growth factor; AG 17, tyrphostin A9; W7, N-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamide hydrochloride; W13, N-(4-aminobutyl)-5-chloro-1-naphthalenesulfonamide; AG 1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline; KN62, 1-((N,O-bis[5-isoquinolinesulfon- 

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vations that hallucinogens, such as lysergic acid diethylamide, act as partial receptor agonists (Glenon et al., 1984; Ferry et al., 1993; Roth et al., 1999), whereas the newer, “atypical” antipsychotics are 5-HT<sub>2A</sub> receptor antagonists (Meltzer et al., 1989; Roth et al., 1999). A role for 5-HT<sub>2A</sub> receptors in the pathophysiology of depression has also been proposed. Receptor expression in prefrontal cortex has been shown, in post-mortem studies, to be increased in suicide victims (Mann et al., 1989).

Although 5-HT<sub>2A</sub> receptors seem to couple to signaling pathways that exert long-term neuronal changes, the pathway for receptor-coupling to ERK mitogen-activated protein kinases has not been delineated in neurons. These kinases have been shown to phosphorylate a number of transcription factors, including c-Jun, p62TCF/Elk-1, c-Fos, and c-Myc, and also seem to regulate translation of mRNA (Denton and Pizzuti, 1999; Pang et al., 1995; Hetman et al., 1999). Coupling of 5-HT<sub>2A</sub> receptors to activation of differentiation and neuroprotection (Pang et al., 1995; Hetman et al., 1999). In fact, it seems survival and is required for normal neuronal functioning (Encinas et al., 1999; Erhardt et al., 1999). In neurons, the phosphorylation of ERK has not been delineated in neurons. These kinases have been shown to phosphorylate a number of transcription factors, including c-Jun, p62TCF/Elk-1, c-Fos, and c-Myc, and also seem to regulate translation of mRNA (Denton and Pizzuti, 1999; Pang et al., 1995; Hetman et al., 1999). Coupling of 5-HT<sub>2A</sub> receptors to activation of ERK has been demonstrated to occur in vascular and trabecular smooth muscle cells (Hershenson et al., 1995; Watts et al., 1996), as well as in mesangial cells (Greene et al., 2000). In both smooth muscle cells and mesangial cells, 5-HT<sub>2A</sub> receptors were found to stimulate activation of ERK through the G protein G<sub>i</sub> and consequent activation of protein kinase C (PKC) (Hershenson et al., 1995; Greene et al., 2000). Interestingly, findings from two studies by Zwiller and colleagues suggest that PC12 cells may serve as a useful model for studying the coupling of 5-HT<sub>2</sub> receptors to activation of ERK in neuronal cell types. Treatment of PC12 cells with 5-HT was reported to stimulate ERK (Estève et al., 2001). In a separate study, a receptor with pharmacology consistent with 5-HT<sub>2A</sub>/5-HT<sub>2C</sub> receptors was found to mediate PC12 cell induction of TIS8/eGr-1 and c-fos expression (Humbolt et al., 1997). Together, these findings suggest the possibility that endogenous 5-HT<sub>2</sub> receptors couple to activation of ERK in PC12 cells. In the current studies we directly demonstrate that the activation of ERK is mediated through 5-HT<sub>2A</sub> receptors. Unlike the findings reported for non-neuronal types of cells, we found that the activation of ERK, in PC12 cells, occurs through a PKC-independent pathway requiring Ca<sup>2+</sup>/calmodulin and tyrosine kinases.

Materials and Methods

Materials. MDL 100907 was kindly provided by Aventis (Bridgewater, NJ). G66983, G66976, HS9, KN93, W13, bisindolylmaleimide I, Ro-31-8220, thapsigargin, ionomycin, PP1, AG 1478, and tyrphostin A9 (AG 17) were obtained from Calbiochem (San Diego, CA). 5-HT, ketanserin, R(-)-2,5-dimethoxy-4-iodoamphetamine hydrochloride, KN62, SB206553, and W7 were obtained from Sigma-Aldrich (St. Louis, MO). Genistein was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA).

Cell Culture. PC12 cells were obtained from American Type Culture Collection (Manassas, VA) and were routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with L-glutamine, minimal essential medium nonessential amino acids, 15% dialyzed fetal bovine serum (dialyzed in membranes with 1000-Da molecular weight cutoffs against a 100-fold greater volume of 150 mM NaCl to remove endogenous 5-HT), and 100 units of penicillin-100 μg of streptomycin/ml (95% air/5% CO<sub>2</sub>). A stable, tightly adherent cell population was obtained after several cycles of washing off loosely adherent cells.

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Results

5-HT<sub>2A</sub> Receptors Couple to Activation of ERK in PC12 Cells. Similar to a previous report by Esteve et al. (2001), we found that 5-HT stimulated activation of ERK mitogen-activated protein kinases in PC12 cells. Treatment of cells with 5-HT caused large increases in the level of activated, double-phosphorylated ERK1 and ERK2 (Fig. 1). Maximal activation by 5-HT occurred at 10 μM and could be seen at concentrations greater than 10 nM (Fig. 1A). Phosphorylation of ERK occurred within 2 min of treatment and was maximal at 5 min (Fig. 1B). By 15 min, the level of activated ERK approached, but did not entirely reach, basal levels. Ketanserin (100 nM), an antagonist for 5-HT<sub>2A</sub>/5-HT<sub>2C</sub> receptors, inhibited the activation of ERK (Fig. 1C). To determine whether 5-HT<sub>2A</sub> or 5-HT<sub>2C</sub> receptors were the relevant receptors, MDL 100907 and SB206553, antagonists selective for 5-HT<sub>2A</sub> and 5-HT<sub>2B/2C</sub> receptors, respectively, were tested. Pretreatment with 10 nM MDL 100907 (K<sub>i</sub> = 0.9 nM for 5-HT<sub>2A</sub> and 90 nM for 5-HT<sub>2C</sub>; Kehne et al., 1996) completely inhibited the actions of 5-HT. In contrast, pretreatment with 10 nM SB206553 (K<sub>i</sub> = 1.6 μM for 5-HT<sub>2A</sub>, 1 nM for 5-HT<sub>2B</sub>, and 13 nM for 5-HT<sub>2C</sub>; Kennett et al., 1996) caused no inhibition, demonstrating that activation of ERK was mediated by 5-HT<sub>2A</sub> receptors. R(-)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (1 μM), an agonist for 5-HT<sub>2A</sub>/5-HT<sub>2C</sub> receptors, stimulated activation of ERK, but consistent with it being a partial agonist, stimulated an increase in phosphorylated ERK only 50% of that stimulated by 1 μM 5-HT<sub>1</sub> (data not shown). PC12 cells have been reported to express 5-HT<sub>3</sub> receptors (Hanna et al., 2000). However, 10 μM LY-278,584, a potent, selective 5-HT<sub>3</sub> receptor antagonist, did not inhibit 5-HT-stimulated activation of ERK, nor did 10 μM 1-(m-chlorophenyl)-biguanide, a potent, selective 5-HT<sub>3</sub> receptor agonist cause activation (data not shown).
Activation of ERK Requires an Increase in Intracellular \([\text{Ca}^{2+}]\). Agonists for 5-HT\(_{2A}\) receptors have been shown to use G\(_{q/11}\) type G proteins to stimulate phosphoinositide (PI) turnover and consequently increase the level of intracellular \([\text{Ca}^{2+}]\) (Roth et al., 1986; Pritchett et al., 1988; Ferry et al., 1993). We therefore examined the role of \([\text{Ca}^{2+}]\) in mediating 5-HT\(_{2A}\) receptor-stimulated activation of ERK. Activation of ERK was found to be inhibited by 50% when the extracellular \([\text{Ca}^{2+}]\) was reduced from 2 mM to 200 nM (a concentration similar to the intracellular \([\text{Ca}^{2+}]\) seen in many types of resting cells) by pretreatment with 2 mM EGTA (Fig. 2A). A similar magnitude of reduction in 5-HT-stimulated activation of ERK was seen when extracellular \([\text{Ca}^{2+}]\) was reduced further to 100 nM (data not shown). Pretreatment of cells with 30 nM thapsigargin, to slowly deplete intracellular stores of \([\text{Ca}^{2+}]\) before treatment with 5-HT, caused a 63% reduction in activated ERK. Therefore, maximal 5-HT-stimulated activation of ERK requires an increase in intracellular \([\text{Ca}^{2+}]\) originating both from influx of extracellular \([\text{Ca}^{2+}]\) and release from intracellular stores.

**Activation of ERK Is Independent of Protein Kinase C.** PKC has been shown in studies of smooth muscle cells and mesangial cells to be required for coupling of 5-HT\(_{2A}\) receptors to activation of ERK (Hershenson et al., 1995; Greene et al., 2000). We therefore studied the role of PKC in mediating activation of ERK by 5-HT\(_{2A}\) receptors in these cells. Our finding that activation of ERK required \([\text{Ca}^{2+}]\) suggested that the conventional \([\text{Ca}^{2+}]\)/diacylglycerol-dependent PKCs (\(\alpha, \beta_1/\beta_2, \gamma\)) could be the potentially relevant kinases. However, pretreatment with 1 \(\mu\)M Go6976, an inhibitor of the \([\text{Ca}^{2+}]\)/diacylglycerol-dependent PKCs, as well as...
the Ca\(^{2+}\)-insensitive PKC \(\mu\) (Martiny-Baron et al., 1993; Zang et al., 1994; Gschwendt et al., 1996) caused no inhibition of 5-HT-stimulated activation of ERK (Fig. 2C). To the contrary, an increase in 5-HT-stimulated activation was observed. Because PC12 cells express multiple isoforms of PKC, including Ca\(^{2+}\)/diacylglycerol-dependent \(\alpha, \beta/\beta_2, \gamma, \), Ca\(^{2+}\)-insensitive/diacylglycerol-dependent \(\delta, \epsilon, \) and \(\mu\) (protein kinase D); and Ca\(^{2+}\)/diacylglycerol-independent \(\zeta\) and \(\delta/\lambda\) (Wooten et al., 1997), we also tested the effects of protein kinase C inhibitors with broader spectrums of inhibition. Concentrations (1 \(\mu\)M) of Ro-31-8220 that inhibit \(\alpha, \beta/\beta_2, \gamma, \delta, \epsilon, \), and \(\zeta\) and \(\delta/\lambda\) isoforms of PKC (Standeart et al., 1997; Anthonsen et al., 2001) and bisindolylmaleimide I, which inhibits \(\alpha, \beta/\beta_2, \gamma, \delta, \epsilon, \) and \(\zeta\) (Martiny-Baron et al., 1993), caused no inhibition of 5-HT-stimulated activation of ERK. To the contrary, each PKC inhibitor caused a 1.6-fold potentiation. Basal levels of activated ERK were not altered by treatment with the PKC inhibitors (data not shown). The 1 \(\mu\)M concentrations of inhibitors used were relatively high and were sufficient to inhibit PKC, as demonstrated in studies in which phorbol 12-myristate 13-acetate (PMA) was used to directly activate PKC. Although the activation of ERK stimulated by PMA was significantly greater than that stimulated by 5-HT, it was almost completely inhibited by 1 \(\mu\)M concentrations of the broad-spectrum PKC inhibitors Ro-31-8220 and bisindolylmaleimide I (Fig. 3A). As might be expected, G66976, with a profile of inhibition limited to the Ca\(^{2+}\)/diacylglycerol-dependent PKCs and PKC \(\mu\), caused only partial inhibition. In contrast, even when the concentration of Ro-31-8220 was increased to 10 \(\mu\)M, no inhibition of 5-HT-stimulated ERK activation was observed (Fig. 3B). Similarly, 10 \(\mu\)M G66983, which inhibits all isoforms of PKC (including the atypical PKC \(\zeta\)) except PKC \(\mu\) (Gschwendt et al., 1996), caused complete inhibition of PMA-stimulated activation of ERK, but caused no inhibition of 5-HT-stimulated activation (Fig. 3C). Therefore, the activation of ERK stimulated by 5-HT\(_{2A}\) receptors was not mediated by any of the known isoforms of PKC.

**Activation of ERK Requires Calmodulin, but Is Independent of Calmodulin (CaM)-Dependent Protein Kinase**. In the next set of studies, a role for the Ca\(^{2+}\) binding protein calmodulin was examined. Pretreatment of PC12 cells with 50 \(\mu\)M W7 or W13, two selective inhibitors of calmodulin, caused a 70% inhibition of 5-HT-stimulated activation of ERK (Fig. 4A). At this concentration, W7 and W13 have been shown to not alter the kinetics of Ca\(^{2+}\) entry into PC12 cells (Egea et al., 1999). Demonstrating that isolated increases in intracellular [Ca\(^{2+}\)] would be sufficient to explain the actions of 5-HT, we found that directly increasing intracellular [Ca\(^{2+}\)] with the Ca\(^{2+}\) ionophore ionomycin similarly stimulated activation of ERK (Fig. 4B). Pretreatment of cells with 50 \(\mu\)M W7 inhibited ionomycin-stimulated activation by approximately 60%. Increasing the concentration of W7 to 100 \(\mu\)M caused complete inhibition of both 5-HT- and ionomycin-stimulated ERK activation (data not shown) but also seemed to cause nonspecific effects in that the cells became more loosely attached to the culture dishes. The requirement for calmodulin suggested a possible role for CaM-dependent protein kinase. However, pretreatment of PC12 cells with 10 \(\mu\)M KN62 or KN93, two selective CaM kinase inhibitors with \(K_i\) values of 0.9 and 0.37 \(\mu\)M, respectively (Tokumitsu et al., 1990; Sumi et al., 1991), caused no inhibition of ERK activity (Fig. 4C).

**Activation of ERK Is Mediated by Tyrosine Kinases but Is Independent of Protein Kinase A**. In PC12 cells, activation of Ca\(^{2+}\)/calmodulin-activated forms of adenylyl cyclase has been reported to cause activation of ERK (Grewal et al., 2000). Therefore, the role of protein kinase A (PKA) in mediating 5-HT\(_{2A}\) receptor-stimulated activation of ERK was assessed using the PKA inhibitor H89. At the high concentration of 10 \(\mu\)M, H89 (\(K_i\) for PKA = 48 nM; Chijiwa et al., 1990) caused minimal inhibition of 5-HT-stimulated activation of ERK (Fig. 5A). In contrast, pretreatment with the tyrosine kinase inhibitor genistein caused almost complete inhibition of 5-HT-stimulated activation of ERK, suggesting a role for one or more tyrosine kinases. In fact, treatment of PC12 cells with 5-HT was found to stimulate tyrosine phosphorylation of several proteins (Fig. 5B). A protein doublet with a weight slightly greater than the 40-kDa molecular weight marker likely represented activated tyrosine-phos-
phorylated ERK1 (p44) and ERK2 (p42) (full activation of ERK requires phosphorylation of threonine 202 and tyrosine 204). Pretreatment with W7, to inhibit calmodulin, caused inhibition of 5-HT-induced tyrosine phosphorylation of all discernible proteins. However, phosphorylation of a protein migrating with a molecular weight below the 199-kDa marker was inhibited the least. Interestingly, treatment with W7 actually caused increased phosphorylation of a protein migrating just below the 87-kDa marker. However, phosphorylation of this protein was not increased by treatment with 5-HT, alone, relative to control.

The effects of several tyrosine kinase inhibitors were tested to identify potential tyrosine kinases required for 5-HT2A receptor-mediated activation of ERK. Pretreatment of cells with the Src inhibitor PP1 (10 μM) was found to inhibit 5-HT-stimulated activation of ERK by 83% (Fig. 6). Similarly, the epidermal growth factor (EGF) receptor kinase inhibitor AG 1478 (100 nM) caused a 63% inhibition. However, 10 μM AG 17, a platelet-derived growth factor receptor kinase inhibitor that has been reported to also inhibit activation of the tyrosine kinase PYK2 (Avdi et al., 2001; Fuortes et al., 1999), caused no inhibition.

**Discussion**

Our finding that coupling of 5-HT2A receptors to activation of ERK was independent of PKC was somewhat surprising. In tracheal smooth muscle cells and mesangial cells, 5-HT2A receptors have been reported to stimulate activation of ERK through pathways sensitive to PKC inhibitors (Hershenson et al., 1995; Greene et al., 2000). Our finding of an absence of a role for PKC was not the result of lack of expression of PKC in PC12 cells. The cells have been shown to express multiple isoforms of PKC, including conventional isoforms (α, β1/β2, and γ), novel isoforms (δ, ε, but not θ), atypical isoforms (ζ...
and v/α), and PKC μ (also referred to as protein kinase D) (Wooten et al., 1997). In fact, we found that 5-HT-stimulated activation of one or more PKC isoforms. A time-dependent autophosphorylation of PKC was observed using an antibody detecting phosphorylation at Ser 660 of PKC αII and the homologous serines on other PKC isoforms. Interestingly, in contrast to the inhibition of PMA-stimulated ERK activation induced by pretreatment with all of the tested PKC inhibitors, a potentiation of 5-HT-stimulated activation was observed. This increase may have been the result of an attenuation of PKC-mediated inhibition of receptor-stimulated PI turnover, resulting in increased levels of intracellular Ca\(^{2+}\). Phorbol esters have been found to inhibit 5-HT\(_{2A}\) receptor-mediated PI turnover in rat aorta (Roth et al., 1986) and platelets (Kagaya et al., 1990), whereas the PKC inhibitors staurosporine (Berg et al., 1998) and mezerein (Kagaya et al., 1990) have been shown to enhance 5-HT-stimulated PI turnover. Because 5-HT was found to cause activation of PKC in PC12 cells (demonstrated by autophosphorylation), it is not clear why this activation did not contribute to activation of ERK. It is possible that the specific PKC isoforms responsible for PMA-stimulated activation of ERK were not activated by 5-HT. Future studies will be required to determine whether activation of ERK in PC12 cells is mediated by only specific isoforms of PKC and whether the increases in Ca\(^{2+}\) and diacylglycerol stimulated by 5-HT\(_{2A}\) receptor occupancy are sufficient to activate these relevant isoforms.

The results of our studies are consistent with previous reports that activation of calmodulin is sufficient to stimulate activation of ERK in PC12 cells. For example, cell depolarization (Egea et al., 1998, 1999; Grewal et al., 2001) and agonists for nicotinic acetylcholine receptors (Nakayama et al., 2001) have been shown to induce activation of ERK through calmodulin-dependent pathways. However, the steps subsequent to calmodulin seem to vary, depending on the stimulus. For example, the activation of ERK resulting from depolarization has been reported to be inhibited by the protein kinase A inhibitors H89 and KT5720 (Grewal et al., 2000), suggesting a requirement for calcium/calmodulin-ac-


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