Activation of a Mitogen-activated Protein Kinase (ERK2) by the 5-Hydroxytryptamine$_{1A}$ Receptor Is Sensitive Not Only to Inhibitors of Phosphatidylinositol 3-Kinase, but to an Inhibitor of Phosphatidylcholine Hydrolysis*

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A variety of receptors coupled to GTP-binding regulatory proteins (G proteins) initiate signals that culminate in activation of the mitogen-activated protein kinases ERK1 and ERK2. We demonstrate here that the human 5-HT$_{1A}$ receptor expressed in Chinese hamster ovary cells similarly promotes activation of ERK1 and ERK2, but that the pathway used does not conform entirely to those proposed previously for G protein-coupled receptors. Activation of ERK2 by the 5-HT$_{1A}$ receptor-selective agonist 8-hydroxy-N,N-dipropyl-2-aminotetralin hydrobromide (8-OH-DPAT) was inhibited completely by pertussis toxin and substantially by prolonged treatment of cells with phorbol 12-myristate 13-acetate. The implied requirement for protein kinase C, however, was negated in studies with bisindolylmaleimide and Ro-31-8220, which, although completely inhibiting activation of ERK2 by phorbol ester, had no impact on activation by 8-OH-DPAT. The anticipated inhibition by the tyrosine kinase inhibitors genistein and herbimycin A, moreover, was marginal at best. As expected for a Gi-coupled receptor, the inhibitors of phosphatidylinositol 3-kinase wortmannin and LY294002 inhibited activation of ERK2, albeit only partly (70%). Of significance, an inhibitor of a phosphatidylcholine-specific phospholipase C, tricyclodecan-9-yl-xanthogenate (D609), caused a similar degree of inhibition. When the two types of inhibitors were combined, an almost complete inhibition was achieved. Our data suggest that phosphatidylinositol 3-kinase and phosphatidylcholine-specific phospholipase C represent components of different, but partly overlapping pathways that can account almost entirely for the activation of ERK2 by the 5-HT$_{1A}$ receptor.

Perhaps the most detailed models of activation of mitogen-activated protein kinases (MAP kinases) such as ERK1 and ERK2 by receptors coupled to GTP-binding regulatory proteins (G proteins) are those provided by Lefkowitz and colleagues, in which the scheme of events depends on the G protein utilized. For agonists proceeding through Gi, release of $\beta\gamma$ leads to phosphorylation of Shc and consequent engagement of Grb2 and Sos through steps thought to involve phosphatidylinositol 3-kinase (PI 3-kinase) and a Src-like tyrosine kinase (1, 2). For agonists employing Go, the sequence of events appears to entail stimulation of phosphoinositide metabolism by $\alpha_q$ and subsequent activation of protein kinase C (PKC) and thus Raf (3). A similar pathway has been proposed for an agonist using Go, wherein $\alpha_q$ is the subunit implied to stimulate phosphoinositide metabolism (4).

Despite the compelling nature of these models, a greater degree of complexity clearly exists. In many instances, the pharmacological interventions used to identify the requisite steps do not completely attenuate activation of the MAP kinases. Conflicts also exist as to whether inhibitors of tyrosine kinases necessarily inhibit activation of Ras and/or MAP kinases achieved through Gi (5), an issue that may ultimately devolve to the nature of the cells and agonists employed. The extent to which PKC is involved in the activation of MAP kinases, too, is elusive. Phorbol ester-promoted down-regulation of PKC, the most often used means of assay, is selective for only certain forms of PKC (6, 7) and can additionally affect processes in which PKC need not be an upstream component (8, 9). Additional permutations in pathways leading to activation of MAP kinases, moreover, may exist. Daub et al. (10), for example, provide evidence for a scheme wherein the stimulation of MAP kinases in fibroblasts by endothelin, lysophosphatidic acid, and thrombin occurs through G protein-coupled receptors, but with epidermal growth factor (EGF) receptors used as an intermediate in the activation cascade.

In the studies described here, the human 5-hydroxytryptophan$_{1A}$ (5-HT$_{1A}$) receptor was employed to investigate further the pathways leading to activation of MAP kinases by G protein-coupled receptors. We report that the 5-HT$_{1A}$ receptor is coupled through Gi to the activation of ERK1 and ERK2 in CHO cells, but that the pathway(s) used does not conform entirely to those proposed previously for G proteins. While the activation is sensitive to inhibitors of PI 3-kinase, as expected for a Gi-coupled process, a similar degree of inhibition can also be achieved with tricyclodecan-9-yl-xanthogenate (D609), an inhibitor of phosphatidylinositol-specific phospholipase C (PC-PLC). Our data suggest that PI 3-kinase and PC-PLC represent components of different but interacting pathways that can account almost entirely for agonist-induced activation of MAP kinase.

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1 The abbreviations used are: MAP kinase, mitogen-activated protein kinase; 8-OH-DPAT, 8-hydroxy-N,N-dipropyl-2-aminotetralin hydrobromide; 5-HT, 5-hydroxytryptamine(serotonin); CHO, Chinese hamster ovary; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase, also referred to as MAP kinase; MPP1, 4-(2'-methoxyphenyl)-1'-2'-(N'-(2'-pyridyl)-p-indolbenzamidoethyl)pyrazine; PC, phosphatidylinolcholine; PI, phosphatidylinositol; PKC, protein kinase C; PLC, phospholipase C; PMN, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PI 3-kinase, phosphatidylinositol 3-kinase; PTX, pertussis toxin.
Activation of MAP Kinase by 5-Hydroxytryptamine<sub>IA</sub> Receptors

EXPERIMENTAL PROCEDURES

Materials—(±)-8-Hydroxy-N,N-dipropyl-2-aminotetralin hydrobromide (8-OH-DPAT) was purchased from Research Biochemicals (Natick, MA). Rabbit polyclonal IgGs recognizing ERK1 (C-16) and ERK2 (C-14) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The 9-amino acid MAP kinase substrate peptide, containing residues 95–98 of myelin basic protein, was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Wortmannin, phorbol 12-myristate 13-acetate (PMA), herbimycin A, Ro-31–8220, LY294002, bisindylmaleimide, and PTX were purchased from Calbiochem (San Diego, CA). Tricyclodecan-9-yl-xanthogenate, D609, was purchased from Calbiochem or Biomol (Plymouth Meeting, PA). 4-[2′-Methoxyphenyl]-1-[2′-N-2-pyridyl]-p-iodobenzamidoethylpiperazine (MPPI) was kindly provided by Dr. H.F. Kung.

Cell Culture—CHO cells expressing the human 5-HT<sub>IA</sub> receptor (by means of a XbaI-BamHI restriction fragment of G21 (11) in pCDNA1/neo) were provided as a gift from Dr. Perry B. Molinoff. The cells were maintained in monolayer culture in Ham’s F-12 nutrient mixture with l-glutamine, 10% charcoal-treated fetal bovine serum, 400 µg/ml Geneticin, and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere (5% CO<sub>2</sub>). The B<sub>max</sub> for [<sup>22</sup>Na]MPPI was 2.5 pmol/mg of membrane protein.

Assay of MAP Kinase Activity—MAP kinase activity was measured following immunoprecipitation of ERK1 or ERK2 essentially as described by Flordellis et al. (12). Cells were first cultured overnight in serum- and Geneticin-free medium, then treated with agonists and/or other reagents as described in the text. Incorporation of [γ-<sup>32</sup>P] ATP into substrate peptide was measured by scintillation spectrometry after extensive washing of filters with 0.75% phosphoric acid and acetone.

Miscellaneous—Inositol phosphate accumulation was measured as [<sup>3</sup>H]inositol phosphate following labeling of cells with [<sup>3</sup>H] myo-inositol (13). Water-soluble metabolites of phosphatidylinositol were measured 2–3 days following labeling of cells with [methyl-<sup>3</sup>H]choline as release of radioactivity into extracellular medium (14); the period of treatment with agonists was 40 min. Immunoprecipitation of G protein subunits labeled in CHO cells with [sup35S] methionine (150 µCi/ml) was achieved with the peptide-directed antibodies 8730 (recognizing α<sub>i1</sub> and α<sub>i2</sub> equally, and α<sub FileAccesso</sub>, less well), 9072 (directed toward α<sub>i2</sub> also), and 2921 (α<sub>i2</sub>) as described previously (15).

RESULTS AND DISCUSSION

Activation of the human 5-HT<sub>IA</sub> receptor by the selective agonist 8-OH-DPAT promotes activation of ERK1 (data not shown) and ERK2 (Fig. 1) in CHO cells. As shown for ERK2, the activation is dose-dependent, with an EC<sub>50</sub> of 4 nM, and transient. The activity achieved with 8-OH-DPAT is almost identical to that observed with serotonin (data not shown). The activation is blocked by the 5-HT<sub>IA</sub> receptor-selective antagonist MPPI, and does not occur for CHO cells that were not transfected with the plasmid encoding the 5-HT<sub>IA</sub> receptor.

In most types of cells, the 5-HT<sub>IA</sub> receptor utilizes G proteins sensitive to PTX, i.e. G<sub>i</sub> and/or G<sub>o</sub> (16, 17). G<sub>i</sub>, which is not PTX-sensitive, may also be used (15). When the CHO cells were treated overnight with PTX, activation of ERK2 by 8-OH-DPAT was completely suppressed (Fig. 2). Thus, the receptor uses G<sub>i</sub> or G<sub>o</sub>. CHO cells contain G<sub>i</sub>, but data for the existence of G<sub>o</sub> are inconsistent (4, 18, 19). We therefore investigated the complement of potentially relevant G proteins using immunoprecipitation of α<sub>i</sub> subunits labeled with [sup35S]methionine. The CHO cells employed here contain one or more forms of α<sub>i</sub>. They do not contain detectable levels of α<sub>o</sub> or α<sub>z</sub>.

8-OH-DPAT causes a greater than 80% inhibition of forskolin-stimulated cAMP accumulation in CHO cells expressing the 5-HT<sub>IA</sub> receptor (data not shown). However, inhibition of adenylyl cyclase cannot account for the activation of MAP kinase. Treatment of the CHO cells with the cAMP analogs 8-bromo-adenosine-3′,5′-monophosphate or N<sub>6</sub>,2′-O-dibutylryl-adenosine-3′,5′-monophosphate (1 mM each) had no effect on agonist activation of Erk and no effect on forskolin or 3-isobutyl-1-methylxanthine.

Herbimycin A and genistein, inhibitors of tyrosine kinases, had little impact. Herbimycin A at a concentration of 1 µg/ml had no effect on 8-OH-DPAT-stimulated MAP kinase activity. Genistein at 75 µM similarly had no effect, although at 100 µM caused an approximately 35% inhibition. Concentrations beyond 100 µM appeared to be toxic. We also tested whether EGF had any effect, given the report by Daub et al. (10) that the EGF receptor was a necessary link to activation of MAP kinases by G protein-coupled receptors. EGF did not stimulate ERK2 in the CHO cells, implying the absence of EGF receptors capable of serving as intermediates for EGF-DPAT. Like other investigators, we found that exposure of cells to PMA for a short period of time results in activation of MAP kinase (Fig. 3). This activation can be completely prevented by...
obtained with the PKC inhibitor Ro-31–8220, also used at 1 M. We therefore further tested the involvement of PKC using the PKC inhibitor bisindolylmaleimide at a concentration of 1 μM (Fig. 3). As anticipated, activation of MAP kinase by PKC was inhibited by this reagent. However, bisindolylmaleimide had no effect on activation of MAP kinase by 8-OH-DPAT. Similar results were obtained with the PKC inhibitor Ro-31–8220, also used at 1 μM. We conclude that while prolonged treatment with PMA inhibits activation of MAP kinase by 8-OH-DPAT, possibly indirectly through some form of cellular accommodation (8, 9), 8-OH-DPAT utilizes a pathway distinct from traditional forms of PKC.

Given previous work with G i coupling to Shc phosphorylation (2) and receptor coupling to MAP kinase (20, 21), a possibly key component in the activation of MAP kinase induced by 8-OH-DPAT is PI 3-kinase. Indeed, we find the activation of MAP kinase to be inhibited by wortmannin (Fig. 4). The inhibition is notable at 10 nM and achieves a maximum of 70% by 30 nM. The concentrations causing inhibition are consistent with those required for inhibition of PI 3-kinase (22). However, because the specificity of wortmannin for PI 3-kinase alone has been questioned (23), we also examined the actions of the structurally different inhibitor LY294002 (24). The inhibition of MAP kinase achieved with LY294002 was similar to that achieved by wortmannin, again at concentrations consistent with inhibition of PI 3-kinase. These observations suggest that PI 3-kinase forms an important link between the 5-HT 1A receptor and the activation of MAP kinase. However, PI 3-kinase does not form the only link, as a significant level of activation occurs even in the presence of saturating concentrations of wortmannin or LY294002.

A critical question, therefore, is what comprises the wortmannin/LY294002-insensitive pathway of activation. We turned our attention to the phosphatidylincholine-specific phospholipase C (PC-PLC). While PC has long been viewed as a source of diacylglycerol for sustained activation of PKC (25), products of its hydrolysis (perhaps diacylglycerol itself) are also critical to the activation of Raf by Ras in response to receptor tyrosine kinase activation (26). We therefore tested the possibility that PC hydrolysis is required for activation of MAP kinase by a G protein-coupled receptor, and that it forms a pathway in part distinct from that sensitive to wortmannin and LY294002. Tricyclodecan-9-yl-xanthogenate (D609) is a specific inhibitor of PC-PLC (27, 28). Remarkably, D609 inhibits 8-OH-DPAT-stimulated MAP kinase activity to an extent comparable to that of wortmannin or LY294002 (Fig. 4, bottom panel). The concentration used was 50 μg/ml, a concentration comparable to those used previously to inhibit PC-PLC (26–31). Increasing the concentration of D609 to 100 μg/ml caused no further inhibition (data not shown). When CHO cells were treated simultaneously with both D609 and a PI 3-kinase inhibitor (either wortmannin or LY294002), 8-OH-DPAT-stimulated activity was almost completely inhibited. Thus, the pathways leading to activation of MAP kinase can be accounted for almost entirely by those inhibited by wortmannin and D609.

Identification of PC-PLC using D609, of course, is subject to issues of selectivity. As noted above, D609 inhibits PC-PLC in a variety of cells. In separate experiments here, 8-OH-DPAT and PMA elicited small but significant increases PC metabolism as measured by release of radioactivity from cells labeled with [3H]choline (60 ± 12% and 88 ± 12%, respectively (n = 4)). The increases were reversed by D609. The effects of D609 on the actions of PMA were anticipated from previous studies examining PMA-stimulated PC-PLC or sequellae (26, 27). As reported elsewhere, D609 does not have an effect on phospho-
lipase A₂, phospholipase D, PI-PLC, or sphingomyelinase activities in U937 cells (28), nor on PI-PLC or PI 3-kinase activities in NIH 3T3 cells (26). These data notwithstanding, the conclusive demonstration of a role for PI-PLC will require other inhibitors and/or means to eliminate enzymic activity.

The magnitude of inhibition achieved with wortmannin and D609 alone and together suggests that the pathways presumably involving PI 3-kinase and PC-PLC are not entirely redundant nor completely independent. PC-PLC may well be interposed between Ras and Raf. Cai et al. (26, 32) report that the activation of PC-PLC in NIH 3T3 cells by serum and EGF is attenuated by the dominant negative form of Ras, RasN17, as is cell replication, and that introduction of a bacterial form of PC-PLC bypasses the block to cell replication. Introduction of the enzyme does not bypass the block to replication achieved with dominant negative forms of Raf. Moreover, PC-PLC activates Raf, and the activation of Raf by serum or EGF is completely inhibited by D609 (26). Thus, one or more products of PC hydrolysis likely support the activation of Raf either following its recruitment to membrane by Ras or in an independent pathway. With respect to 8-OH-DPAT-stimulated MAP kinase activity, we hypothesize that the wortmannin-sensitive pathway is relevant to the activation of Ras, e.g. through Grb2/Sos, and that activation of Raf by Ras in turn is enhanced by PC hydrolysis. PC hydrolysis itself can nevertheless represent an independent input to the activation of Raf or downstream components of the MAP kinase activation cascade. Our data imply two convergent pathways relevant to the activation of MAP kinases by a single G protein-coupled receptor, both of which are required for optimal activation.

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