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ACCELERATED COMMUNICATION

Amphetamine, 3,4-Methylenedioxymethamphetamine, Lysergic Acid Diethylamide, and Metabolites of the Catecholamine Neurotransmitters Are Agonists of a Rat Trace Amine Receptor

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ABSTRACT

The trace amine *para*-tyramine is structurally and functionally related to the amphetamines and the biogenic amine neurotransmitters. It is currently thought that the biological activities elicited by trace amines such as *p*-tyramine and the psychostimulant amphetamines are manifestations of their ability to inhibit the clearance of extracellular transmitter and/or stimulate the efflux of transmitter from intracellular stores. Here we report the discovery and pharmacological characterization of a rat G protein-coupled receptor that stimulates the production of cAMP when exposed to the trace amines *p*-tyramine, β-phenethylamine, tryptamine, and octopamine. An extensive pharmacological survey revealed that psychostimulant and hallucinogenic amphetamines, numerous ergoline derivatives, adrenergic ligands, and 3-methylated metabolites of the catecholamine neurotransmitters are also good agonists at the rat trace amine receptor 1 (rTAR1). These results suggest that the trace amines and catecholamine metabolites may serve as the endogenous ligands of a novel intercellular signaling system found widely throughout the vertebrate brain and periphery. Furthermore, the discovery that amphetamines, including 3,4-methylenedioxymethamphetamine (MDMA; "ecstasy"), are potent rTAR1 agonists suggests that the effects of these widely used drugs may be mediated in part by this receptor as well as their previously characterized targets, the neurotransmitter transporter proteins.

In vertebrates, the trace amines β-phenethylamine (β-PEA), *para*-tyramine, tryptamine, and octopamine are found in peripheral tissues as well as the central nervous system (Tallman et al., 1976; Paterson et al., 1990). In vivo, β-PEA and *p*-tyramine can be synthesized from phenylalanine or tyrosine by the enzyme amino acid decarboxylase. (Boulton and Dyck, 1974; Tallman et al., 1976). The trace amines are found in low amounts (accounting for less than 1% of the biogenic amines in most brain regions) and have been thought of as metabolic byproducts of catecholamine biosynthesis. Investigations into the effects of trace amines on smooth muscle and glandular preparations early in the twentieth century clearly demonstrated that amines produced by putrefaction and lacking the catechol nucleus were capable of producing robust sympathomimetic effects (Barger and Dale, 1910). Currently it is thought that *p*-tyramine and β-PEA manifest their peripheral effects by promoting the efflux of catecholamines from sympathetic neurons and adrenals (Schonfeld and Trendelenburg, 1989; Mundorf et al.,

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[1] Because the predicted amino acid sequences of the rat and human trace amine receptors described here are identical to the rTAR1 and hTAR1 sequences reported by Borowsky et al. (2001), we employ the same nomenclature.

ABBREVIATIONS: β-PEA, β–phenethylamine; MDMA, 3,4-methylenedioxymethamphetamine; GPCR, G protein-coupled receptor; 5-HT, 5-hydroxytryptamine (serotonin); 3-MT, 3-methoxytyramine (3-methyldopamine); TAR1, trace amine receptor 1; PCR, polymerase chain reaction; RT, reverse transcription; HEK, human embryonic kidney; KRH, Krebs-Ringer-HEPES; PBS, phosphate-buffered saline; BAC, bacterial artificial chromosome; DA, dopamine; NE, norepinephrine; Epi, epinephrine; COMT, catechol-*O*-methyltransferase; DOI, 2-amino,1-(2,5-dimethoxy-4-iodophenyl)propane.

1999) which results in the indirect stimulation of adrenergic receptors (Black et al., 1980). The abilities of *p*-tyramine and β-PEA to deplete neurotransmitter from storage vesicles, compete with neurotransmitters for uptake, and stimulate outward neurotransmitter flux through the plasma membrane carriers are similar to the actions of the β-PEA analog, α-methyl-β-phenethylamine, better known as amphetamine (Seiden et al., 1993; Amara and Sonders, 1998).

Amphetamines were originally marketed as stimulants and appetite suppressants, but their clinical use is now mostly limited to treating attention deficit hyperactivity disorder (Seiden et al., 1993). Although listed as controlled substances, amphetamines are widely consumed because of their ability to produce wakefulness and intense euphoria. Some substituted amphetamines, such as MDMA ("ecstasy") and DOI, are taken for their "empathogenic" and hallucinogenic effects. (Shulgin and Shulgin, 1991; Eisner, 1994). Numerous liabilities are associated with the use of amphetamines, including hyperthermia (Byard et al., 1998), neurotoxicity (Ricaurte and McCann, 1992), psychosis (Seiden et al., 1993), and psychological dependence (Murray, 1998). In addition to the actions of amphetamines at biogenic amine transporters, it is also clear that a subset of amphetamine analogs, especially those with hallucinogenic properties, can act directly on 5-HT receptors because they have much higher affinities for these sites than for the transporters (Marek and Aghajanian, 1998).

We report herein the discovery and functional expression of a rat G protein-coupled receptor with homology to members of the catecholamine receptor family. This receptor stimulates cAMP production when exposed to the trace amines *p*-tyramine and β-PEA. A pharmacological survey revealed that this rat trace amine receptor (rTAR1) is directly activated by a wide variety of clinically and socially important drugs, which include amphetamines, ergot derivatives, and adrenergic agents. Surprisingly, rTAR1 is more potently activated by the presumably "inactive" catecholamine metabolites 3-methoxytyramine (3-MT), normetanephrine, and metanephrine than by the neurotransmitters dopamine, norepinephrine, and epinephrine themselves.

In addition to enabling studies on its pharmacology and distribution, knowledge of rTAR1's DNA sequence led to the isolation of the rat and human genes. The human gene is located on chromosome 6 at q23, which falls within a region that has been identified by several schizophrenia linkage studies (Cao et al., 1997; Martinez et al., 1999; Levinson et al., 2000; Mowry and Nancarrow, 2001). Recently, Borowsky et al. (2001) reported the cloning of a family of GPCRs that includes the rTAR1[1] and the human ortholog that we describe here. Considering the broad spectrum of endogenous and exogenous molecules that activate rTAR1 and the multiplicity of related receptor genes, this family of trace amine receptors seems likely to mediate a variety of physiological functions that have yet to be fully understood.

Experimental Procedures

Materials. Oligonucleotide primers were synthesized by Invitrogen (Carlsbad, CA). *S*(+)- and *R*(−)-4-OH-amphetamine were kindly provided by the National Institute on Drug Abuse Drug Supply System (Bethesda, MD). 5-Carboxamidotryptamine and sumatriptan were gifts from Dr. John T. Williams (Vollum Institute for Advanced Biomedical Research, Oregon Health and Science University). All other drugs were purchased from Sigma Chemical Co. (St. Louis, MO), Aldrich (Milwaukee, WI), Tocris Cookson Inc. (Ballwin, MO), or Alltech Associates (State College, PA).

Cloning and Expression of Nucleic Acids. First strand cDNA was synthesized from the rat pancreatic tumor cell line AR42J (American Type Culture Collection, Manassas, VA) and was used as a template for the original polymerase chain reactions (PCRs) employing a pair of degenerate oligonucleotide primers based on a derived consensus sequence of the third and sixth transmembrane domains of known members of the catecholamine receptor family: primer TM III, 5′-GAGTCGACCTGTG(C/T)G(C/T)(C/G)AT(C/T)(A/G)CIIT(G/T)GAC(C/A)G(C/G)TAC-3′; primer TM VI, 5′-CAGAATTCAG(T/A)AGGGCAICCAGCAGAI(G/C)(G/A)(T/C)GAA-3′ (where I = inosine).

The conditions used were: 94°C for 90 s, 50°C for 90 s, and 72°C for 120 s, for 35 cycles. Products from 400 to 750 base pairs were purified from a 1.0% agarose gel using Prep-A-Gene (Bio-Rad, Hercules, CA), digested with *Eco*RI and *Sal*I, and subcloned into the vector pBluescript (Stratagene, La Jolla, CA). Plasmid DNA from these clones was purifed, and the nucleotide sequence of the insert was determined by the dideoxynucleotide chain termination method. The deduced amino acid sequence of a 0.4-kilobase PCR fragment displayed regions of homology to the known catecholamine receptors. This clone was subsequently labeled with ^{32}P and used to probe a rat genomic library (CLONTECH, Palo Alto, CA) that had been transferred to nylon membranes (Gene Screen Plus; PerkinElmer Life Science Products, Boston, MA) resulting in the identification of several full-length clones. Full-length clones were also obtained by RT-PCR from the rat pancreatic tumor cell line RIN5 and rat cerebellum. The nucleotide sequence of the human TAR has been assigned GenBank accession number AF200627. The nucleotide sequence of the rat TAR has been assigned GenBank accession number AF421352.

For the purpose of expressing the putative receptor in tissue culture, a 16-amino-acid signal sequence from the influenza hemagglutinin virus followed by the 8-amino-acid M1-"Flag" epitope and a "MetGly" linker were added to the N terminus of the cerebellar cDNA before its insertion into the expression vector pcDNA3.1/V5/His-TOPO (Invitrogen). The resulting construct was transiently expressed in HEK293 and COS-7 cells after LipofectAMINE-assisted transfection (Invitrogen). A line of G418 resistant HEK293 cells stably expressing the rat receptor was eventually established and these cells were used in all of the cAMP assays. A construct of the hD1 dopamine receptor sequence cloned into the same expression vector (a gift from Dr. Mark von Zastrow, University of California, San Francisco) was expressed as a positive control in HEK293 cells. As a negative control, HEK293 cells were transfected with a pcDNA3.1/V5/His-TOPO plasmid that lacked a receptor sequence.

Tissue Distribution of Receptor mRNA by RT-PCR. Total RNA was extracted from freshly dissected rat (Sprague-Dawley) brain regions and peripheral tissues using the Absolutely RNA RT-PCR miniprep kit (Stratagene, La Jolla, CA). In the course of these studies, all animals were treated in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. Tissue fragments were mechanically homogenized with a Polytron homogenizer (Brinkman Instruments, Westbury, NY). The RNA was treated with DNase to remove all genomic DNA. For the synthesis of first strand cDNA, equal amounts of total RNA for each reaction (1.0 μg) were mixed with ProSTAR RT-PCR reagents (Stratagene, La, Jolla, CA). A PCR using oligonucleotide primers complementary to the rat receptor was then performed. All reactions for the samples were carried out in parallel using the same reaction mixtures for the cDNA synthesis and the PCR so that a semiquantitative measure of RNA quantity could be evaluated by ethidium bromide staining of the PCR products on an agarose gel.

cAMP Assays. HEK293 cells were harvested in Krebs-Ringer-HEPES buffer (KRH) and preincubated in KRH with 200 μM

3-isobutyl-1-methylxanthine. For drug treatments, cells were incubated in KRH with 100 μM 3-isobutyl-1-methylxanthine with the test compound (or 10 μM forskolin) for 1 h at 37°C. The cells were then boiled for 20 min after adding an equal volume of 0.5 mM sodium acetate buffer, centrifuged to remove cell debris, and the resulting extract was analyzed for cAMP content using competitive binding of [^{3}H]cAMP to a cAMP binding protein (Diagnostic Products Corp., Los Angeles, CA). Data were normalized according to protein content as determined using the Bradford reagent (Bio-Rad). Concentration-response curves were plotted and EC_{50} values calculated with Prism software (GraphPad, San Diego, CA).

Immunofluorescence Microscopy. HEK293 cells stably expressing the rTAR1sequence or the hD1R were maintained in Dulbecco's minimal essential medium containing 10% fetal calf serum and 700 μg/ml G418 (Invitrogen). Confluent cells were detached with a PBS solution containing 0.05% trypsin and 0.53 mM EDTA, harvested, and after diluting 1:10, plated on glass microscope coverslips coated with poly(D-lysine) and left to grow in the incubator for 48 h. Cells were washed twice with PBS, fixed with 2.5% paraformaldehyde in PBS for another 20 min, then incubated with anti-FLAG monoclonal antibody (1:500; Sigma Chemical Co., St. Louis MO) in blocking solution (3% dry milk, 1 mM $CaCl_2$, 50 mM Tris HCl, pH 7.5) with or with out 0.1% Triton X-100, for 30 min. After 3 washes with Tris-BS containing 1 mM $CaCl_2$, cells were incubated in blocking solution containing goat anti-mouse IgG conjugated to Cy5 (1:200; Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 30 min. Cells were then washed three times before being mounted onto microscope slides with Mowiol (Sigma/Aldrich, Milwaukee, WI). Confocal microscopy was performed using MRC-1000 laser scanning confocal imaging system (Bio-Rad) equipped with an Optiphot II Nikon microscope (Nikon, Tokyo, Japan) and a Plan Apo 60 $\times$ 1.4 oil immersion objective.

Human Chromosomal Localization. The complete coding region of the rTAR1 was used to screen a human genomic DNA library (CLONTECH). This attempt yielded a 600-bp fragment of the receptor's amino terminal sequence. A full-length genomic clone was eventually identified in a human bacterial artificial chromosome (BAC) after PCR screening of a BAC library (Research Genetics Inc., Huntsville, AL) using primers based on the original partial human sequence. DNA from the hTAR-containing BAC was nick-translated using digoxigenin-11-UTP and used to probe spreads of metaphase chromosomes, as described previously (Grandy et al., 1990). Localization of a human trace amine receptor (hTAR) gene was determined by fluorescent in situ hybridization. Chromosome identification was accomplished by sequential G-banding, as described previously (Grandy et al., 1990).

Results and Discussion

Cloning of Rat and Human Trace Amine Receptors. In an effort to identify novel GPCRs that are activated by dopamine and other related biogenic amines, RT-PCRs were performed on RNA isolated from cell lines whose tissue of origin was known to receive sympathetic innervation. These RT-PCRs employed degenerate oligonucleotide primers that incorporated conserved sequences present in the putative transmembrane domains III and VI of the G protein-coupled catecholamine receptor gene family. One of the cell lines examined in this way was the rat pancreatic tumor cell line ARJ42. DNA sequence analysis of the RT-PCR products produced from the ARJ42 cell line revealed the presence of a cDNA fragment, r2-3, that predicted a novel amino acid sequence related to several known biogenic amine-recognizing GPCRs.

Although attempts to synthesize a full-length r2-3 cDNA from the ARJ42 RNA were unsuccessful, 5′ and 3′ rapid amplification of cDNA ends using RNA prepared from another rat pancreatic tumor cell line, RIN5, as well as RNA isolated from rat cerebellum, provided the complete coding region. The cDNA sequences were confirmed by the cloning and sequence analysis of a rat genomic clone that encoded the complete r2-3 coding region in a single exon. Analysis of the deduced amino acid sequence (Fig. 1) predicted a novel 332 amino acid protein having seven putative transmembrane domains and significant amino acid identity with the catecholamine receptors and with three orphan GPCRs whose ligand(s) are presently unknown (Zeng et al., 1998; Lee et al., 2000). The human homolog of the r2-3 gene was subsequently cloned from a human BAC library using the full-length rat clone as a probe and was found to code for a protein of 340 amino acid residues.

Analysis of the rat and human 2-3 sequences revealed that they share two putative *N*-linked glycosylation sites in the putative amino terminal domain of the receptor and a potential protein kinase C site in the C terminus (Kennelly and Krebs, 1991). Interestingly, there are two Ser residues in the very short C-terminal domain of the putative human receptor that are not present in the rat sequence and that could be potential targets for receptor kinases (Premont et al., 1995).

To aid in the search for an agonist, the distribution of the putative receptor's mRNA was analyzed. Both Northern blotting of total RNA prepared from different brain regions and peripheral tissues as well as an initial attempt at in situ hybridization of rat brain sections revealed no detectable signal, suggesting that the message is of low abundance. Using a more sensitive RT-PCR amplification method, however, signals were detected from oligo-dT–primed RNA prepared from different rat brain regions and peripheral tissues. By this semiquantitative method, the message seemed to be widely distributed throughout the brain, with the highest levels of expression detected in the olfactory bulb, nucleus accumbens/olfactory tubercle, prefrontal cortex and other cortical regions, midbrain regions consisting of substantia nigra and ventral tegmentum, cerebellum, and pons/medulla. Among peripheral tissues, the highest level was observed in the liver, with lesser expression detected in kidney, gastrointestinal tract, spleen, pancreas, and heart.

Pharmacological Characterization of the Rat Trace Amine Receptor. Although the distribution of r2-3 mRNA did not immediately suggest a potential agonist for the putative receptor, the striking similarity in amino acid sequence between r2-3 and biogenic amine GPCRs (Fig. 1) indicated that catecholamines or related compounds might activate the receptor. When tested in functional assays of cAMP production using HEK293 cells stably expressing r2-3, DA did stimulate synthesis of the second messenger. However, *p*-tyramine and β-PEA were considerably more potent. Nanomolar concentrations of the trace amines generated responses comparable with that of 10 μM forskolin in the cells expressing r2-3 (Fig. 2B) but not in HEK293 cells that were stably transfected with the empty expression vector. Subsequently, other closely related trace amines, including tryptamine, octopamine, and synephrine, were tested and found to stimulate cAMP production (Fig. 2A). These molecules had EC_{50} values (Table 1) in the following rank order (lowest to highest): *p*-tyramine $<$ β-PEA $<$ tryptamine $<$ synephrine $<$ octopamine $<$ *meta*-tyramine $\leq$ dopamine $<$ 5-HT $\ll$ NE, Epi. Based on this preference for the trace

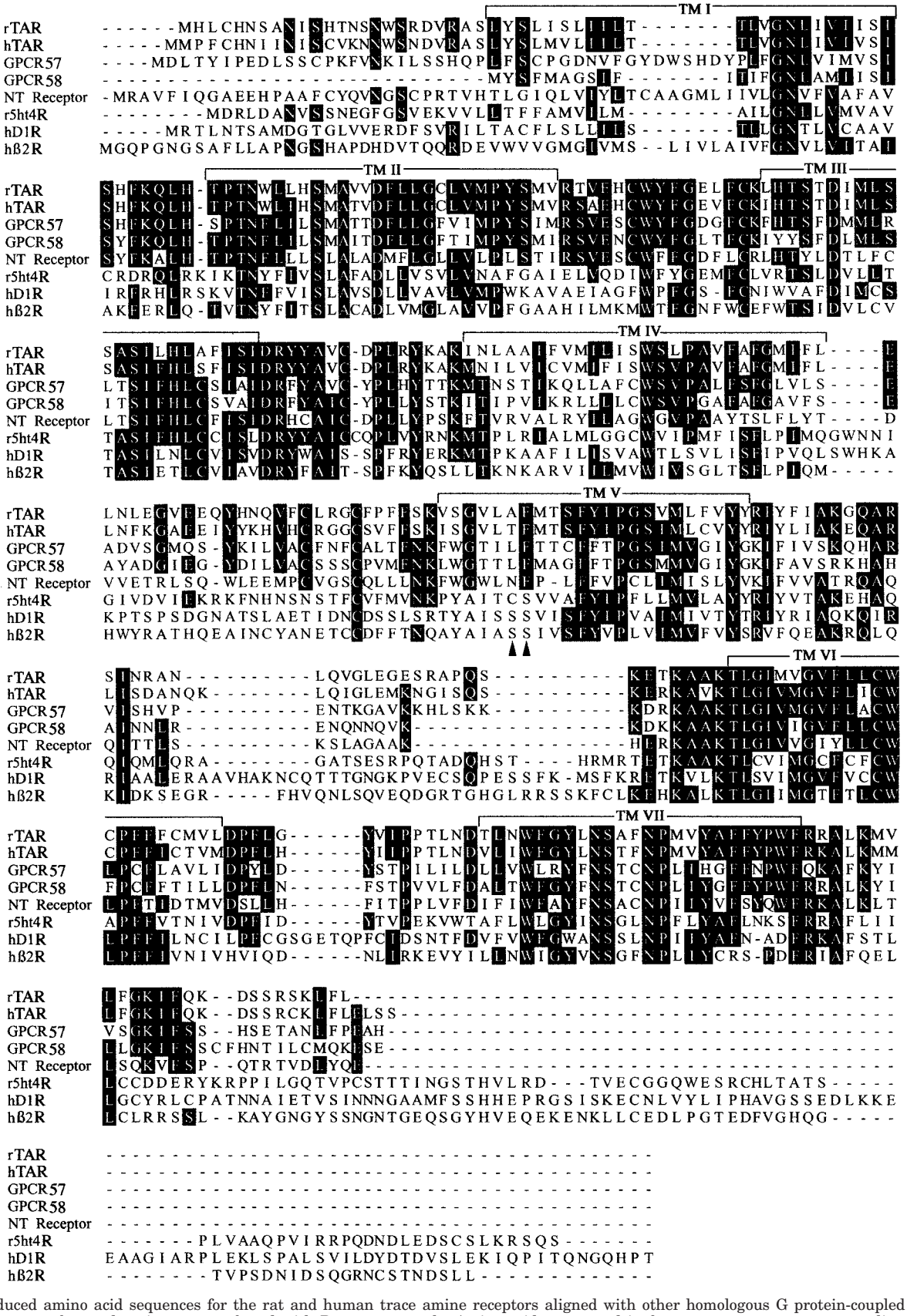

Fig. 1. Deduced amino acid sequences for the rat and human trace amine receptors aligned with other homologous G protein-coupled receptors. Putative transmembrane domains are numbered with Roman numerals. Amino acids conserved in four or more sequences are outlined in black. Abbreviations used: rTAR, rat trace amine receptor1; hTAR, human trace amine receptor1; NT Receptor, orphan neurotransmitter receptor (Zeng et al., 1998); orphan receptors GPCR57 and GPCR58 (Lee et al., 2000); hD1R, human dopamine D1 receptor; hβ2R, human β_2 adrenergic receptor; r5ht4R, rat serotonin 5HT$_4$ receptor. Arrowheads indicate positions designated as 5.42 and 5.43.

amines over the catecholamines and 5HT, the rat orphan receptor coded for by r2-3 was renamed the rTAR1.

The rank order of potencies observed for rTAR1 leads to the inference that a hydroxyl group at the *meta* position on β-PEA analogs or at the 5-position on tryptamine has deleterious effects on agonist potency, a trend that is contrary to that observed for catecholamine receptors. Comparison of the rTAR1 amino acid sequence with those of catecholamine and 5-HT receptors suggests a structural basis for this change in selectivity. It has been proposed from mutagenesis studies of the β-adrenergic receptor and the 5-HT_{1A} receptor (Ho et al., 1992) that serine residues in transmembrane domain V contribute to the binding affinity of agonists, and that $Ser^{5.42}$ and/or $Ser^{5.43}$ form a hydrogen bond network with the catecholamine *meta*-hydroxyl groups (Liapakis et al., 2000). The Ser residue in position 5.42 is conserved in every catecholamine receptor. Curiously, the corresponding residues in rTAR1 are instead $Ala^{5.42}$ and $Phe^{5.43}$ (Fig. 1) whereas the more deeply positioned $Ser^{5.46}$, proposed to interact with the *para*-hydroxyl group, is found in rTAR1 and in the catecholamine receptors alike (Liapakis et al., 2000). We propose that the absence of Ser residues in positions 5.42 and 5.43 of rTAR1 diminishes the potencies of phenethylamine agonists that have *meta*-hydroxyl groups (e.g., catecholamines, *m*-tyramine) compared with those that do not.

Another trend observed in the pharmacological survey also differentiates rTAR1 from known biogenic amine receptors and, furthermore, may hint at a physiological role of the rTAR1. Namely, we found that the *meta*-*O*-methyl metabolites of the catecholamines—3-MT, normetanephrine, and metanephrine—are efficacious activators of rTAR1 and are significantly more potent than their precursors DA, NE, and Epi (Fig. 3B). This finding is unusual because at other known catecholamine receptors, these *meta*-*O*-methyl metabolites generated by COMT have vastly diminished affinities and/or intrinsic efficacies compared with their parent catecholamines (Langer and Rubio, 1973; Seeman, 1980). Our data indicate that increasing lipophilicity of catecholamine *meta*-substituents by *O*-methylation actually increases their affinity for rTAR1. These data are consistent with the finding of Liapakis et al. (2000) that replacement of $Ser^{5.42}$ in the β-adrenergic receptor with Ala or Val residues decreased the affinities of β-PEA analogs containing *meta*-hydroxyl groups but increased the potencies of analogs lacking them. Accordingly, it seems reasonable to hypothesize that endogenous agonists of rTAR1 may include some "inactive" catecholamine metabolites such as 3-MT, the principal extracellular metabolite of DA (Wood and Altar, 1988). It is important to note that 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid), the oxidized metabolite of 3-MT lacking the amine group, displayed no detectible activity at rTAR1. A possible functional linkage between the *meta*-O-methylated catecholamines and rTAR1 is also circumstantially supported by the anatomical correlation between the tissues that contain the highest levels of rTAR1 mRNA and those reported to express high levels of COMT—liver, kidney, gastrointestinal tract, and brain (reviewed in Männistö and Kaakkola, 1999).

Given the structural similarity of amphetamine to β-PEA and *p*-tyramine, it was of obvious interest to determine whether amphetamine analogs including methamphetamine and its congener MDMA could activate rTAR1. Experiments revealed that these and several other amphetamine analogs potently stimulated cAMP production (Table 1 and Fig. 3C). Amphetamines act directly on rTAR1 because these drugs (1 μM concentrations) produced no cAMP stimulation in control cells transfected either with an empty vector or with the human D1 receptor (data not shown). Amphetamine analogs that activate rTAR1 include both classic neurotransmitter transporter substrates as well as a prototypical hallucinogenic amphetamine DOI, which has poor affinity for transporters but high affinity for 5-HT_2 receptors (Marek and Aghajanian, 1998). Some structural modifications of amphetamine significantly changed their potencies at rTAR1: *p*-OH-amphetamine (α-methyl-*p*-tyramine), the major amphetamine metabolite (Cho and Kumagai, 1994), proved to be the most potent agonist of rTAR1 yet identified (Table 1, Fig. 3C). In contrast, two *N*-ethyl analogs, (±)-fenfluramine and (±)-*N*-ethylamphetamine, had substantially lower activities than the *N*-methyl congeners methamphetamine and MDMA or than the primary amine congeners.

The ability of tryptamine to activate the rTAR1 suggested that some ergot alkaloids might act as agonists (Fig. 3D). A variety of widely used ergot alkaloids and ergoline derivatives, including ergometrine, dihydroergotamine, *d*-lysergic acid diethylamide, and the antiparkinsonian agents bromocriptine and lisuride, potently activate rTAR1. The addition of rTAR1 to their multiple, previously described sites of action may help to elucidate the complex in vivo pharmacology of the ergolines.

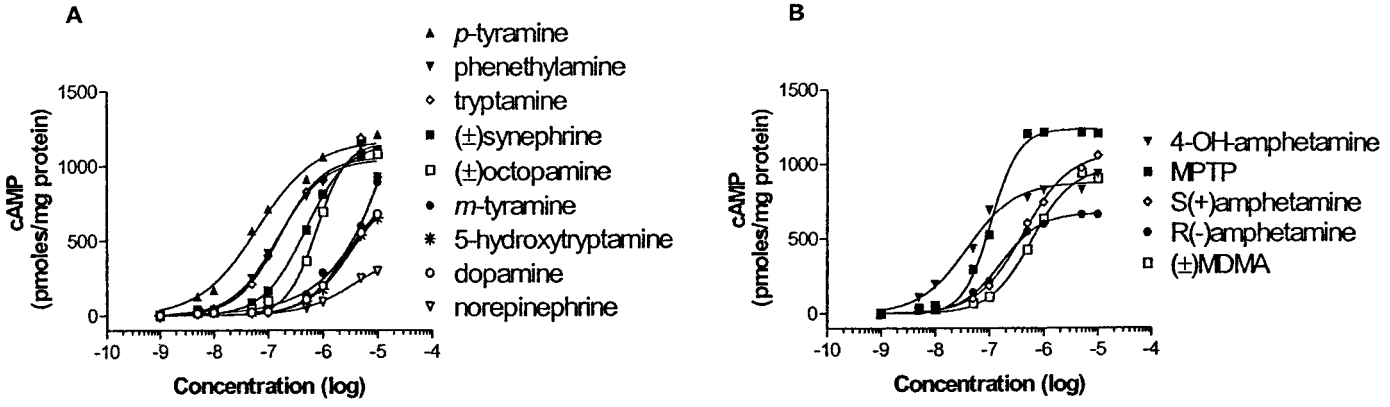

Fig. 2. Agonist-induced cAMP production in HEK293 cells expressing rTAR1. A, concentration-response for naturally occurring compounds. B, concentration-response for synthetic compounds. The curves depicted are from a single representative experiment performed in duplicate. Concentrations units are log (M). Results from all concentration-response experiments are summarized in Table 1.

TABLE 1
Potencies and efficacies of compounds stimulating the receptor

	EC_{50}	Maximal Stimulation[a]
	nM	%
Neurotransmitters		
p-Tyramine	69 ± 9	100
β-Phenethylamine	240 ± 71	76 ± 16
Tryptamine	310 ± 76	90 ± 6
(±)-Synephrine	580 ± 100	90 ± 2
(±)-Octopamine	1,300 ± 350	100 ± 3
m-Tyramine	5,400 ± 1,200	74 ± 3
Dopamine	5,900 ± 2,600	48 ± 5
5-Hydroxytryptamine	>10,000	47 ± 9
Drugs		
4-Hydroxyamphetamine	51 ± 12	79 ± 2
MPTP	99 ± 11	93 ± 7
R(−)-amphetamine	210 ± 44	48 ± 7
S(+)-amphetamine	440 ± 10	84 ± 3
(±)-MDMA	1,700 ± 1,200	65 ± 13

[a] Values are expressed as percent of stimulation by p-tyramine.
n = 2 to 4 experiments, each performed in duplicate.

Another unexpected aspect of rTAR1 pharmacology was revealed by testing antagonists of biogenic amine receptors and transporters, where it was observed that several of these efficiently stimulated cAMP production (Fig. 3, E-H). Such compounds include the adrenergic antagonists phentolamine and tolazoline; the serotonergic antagonists cyproheptadine, dihydroergotamine, and metergoline; and the nonsubstrate inhibitors of DA transporter protein, nomifensine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. The antipsychotic drug chlorpromazine, typically considered a dopamine receptor antagonist, also acted as a weak agonist at the rTAR1. None of the biogenic amine receptor antagonists shown in Fig. 3 were able to antagonize rTAR1 when coincubated (at 1 or 10 μM concentrations) with EC_{50} concentrations of β-PEA or *p*-tyramine (data not shown). Although rTAR1 displays broad ligand selectivity when expressed in HEK293 cells, many compounds, including acetylcholine,

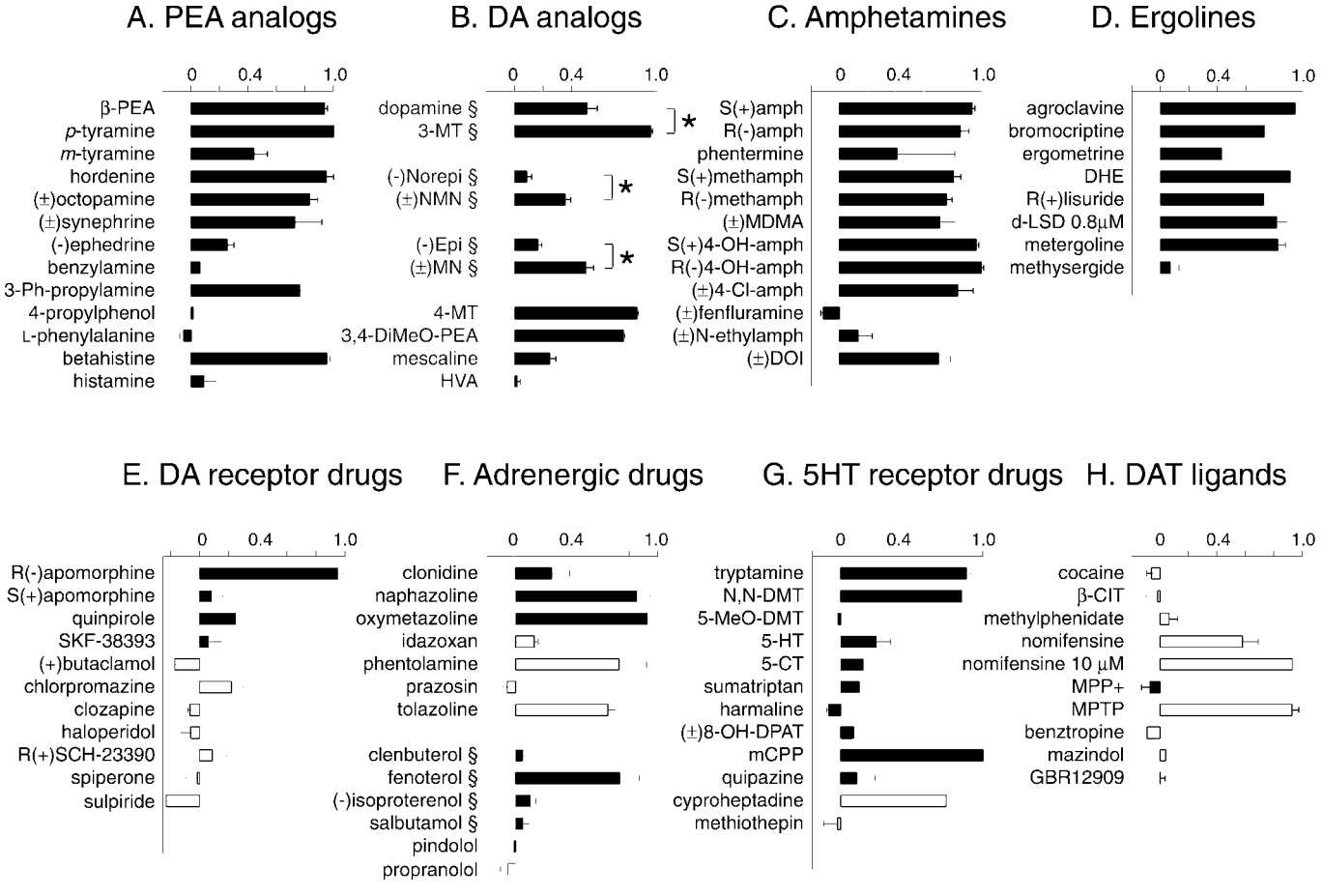

Fig. 3. Pharmacological survey of drugs eliciting cAMP production through the rTAR1 stably expressed in HEK293 cells. Bars indicate the activity of test compounds administered alone (1 μM concentrations except where specified) as a fraction of the cAMP produced in the same assay by 1 μM *p*-tyramine. Forskolin (10 μM) was tested in all experiments and elicited comparable stimulation. Error bars indicate standard error of n = 2 to 8 different experiments. Open bars designate known antagonists of biogenic amine receptors or nontransported inhibitors of DA transporter protein. 3-Ph-propylamine, 3-phenyl-1-propylamine; Norepi, norepinephrine; NMN, β-4-dihydroxy-3-methoxy-β-phenethylamine (normetanephrine); MN, β-4-dihydroxy-3-methoxy-*N*-methyl-β-phenethylamine (metanephrine); 4-MT, 4-methoxytyramine; 3,4-DiMeO-PEA, 3,4-dimethoxy-β-phenethylamine; HVA, 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid); DOI, 2-amino,1-[2,5-dimethoxy-4-iodophenyl]propane; DHE, dihydroergotamine; *d*-LSD, *d*-lysergic acid diethylamide; *N*,*N*-DMT, *N*,*N*-dimethyltryptamine; 5-MeO-DMT, 5-methoxy-*N*,*N*-dimethyltryptamine; 5-CT, 5-carboxamidotryptamine; 8-OH-DPAT, 8-hydroxy-*N*,*N*-dipropylamino-2-amino-1,2,3,4-tetrahydronapthalene; mCPP, 1-(3-chlorophenyl)piperazine; β-CIT, (−)-2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane; MPP^+, 1-methyl-4-phenyltetrahydropyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. * $P < 0.01$, two-tailed t test, $n \geq 3$ experiments. §, Drugs tested in the presence of 0.3 μM propranolol to eliminate a modest component of cAMP production attributable to endogenous β-adrenergic receptors in the HEK293 cells that could be activated by Epi, NE, and the β-agonists. Responses to DA, 3-MT, MN, NMN, *p*-tyramine, β-PEA, and amphetamines were unaffected by inclusion of propranolol or pindolol and were not observed in cells transfected with an empty vector or sequence encoding the hD1 receptor (with the exception of DA).

nicotine, GABA, glutamate, morphine (data not shown), and histamine, do not activate it.

The pharmacological profile that has emerged to date for rTAR1 is noteworthy for several reasons. First, it begins to define a previously unknown yet widely distributed neurotransmitter/neuromodulatory system that can be activated by several endogenous compounds, including β-PEA and *p*-tyramine. Because these are potent stimulators of cAMP production at rTAR1, they may be capable of activating the receptor even at "trace" concentrations in vivo. Second, some of the compounds that seem to be potent and efficacious TAR agonists, such as 3-MT, were previously considered simply to be "inactive" metabolic byproducts of the catecholaminergic neurotransmitters. Third, several TAR agonists have been identified that were previously thought of as selective agonists (or antagonists) at other GPCRs. Fourth, numerous psychostimulant and hallucinogenic amphetamines including "ecstasy" directly activate rTAR1, in addition to their better recognized roles as substrates of plasma membrane and vesicular biogenic amine transporters.

In the fruit fly *Drosophila melanogaster*, tyramine has been shown to play an essential role in behavioral sensitization to cocaine (McClung and Hirsh, 1999). In the discussion of their findings, these authors speculated that behavioral sensitization to cocaine in vertebrates might also involve tyramine because of the number of developmental and functional similarities between invertebrate and vertebrate nervous systems. At the time, the existence of a vertebrate tyramine receptor had not been demonstrated conclusively. Now, given the molecular and pharmacological evidence for the existence of tyramine receptors in vertebrates, an effort to test this hypothesis seems timely.

Cellular Localization of the Rat Trace Amine Receptor. When considering the physiological role of rTAR1 activation the question arises as to its localization in the cell. HEK293 cells stably expressing the epitope-tagged rTAR1 (see *Experimental Procedures*) were examined in parallel with HEK293 cells stably expressing another G_s-coupled receptor, the human dopamine D1 receptor (hD1R), similarly tagged at its N terminus. The results of a confocal immunofluorescence study were somewhat surprising in that the distribution of rTAR1 in the HEK293 cells (Fig. 4, A and B) appeared as intracellular puncta, in marked contrast to the localization of hD1R at the plasma membrane (Fig. 4C). Whether this localization is representative of the protein's localization in vivo remains to be seen, but it does raise the interesting possibilities that 1) rTAR1 requires an accessory protein for proper trafficking to the plasma membrane that is lacking in HEK293 cells or 2) rTAR1 may reside primarily intracellularly, perhaps even in vesicular membranes. The notion that rTAR1 might function in an intracellular environment is supported in part by knowledge that such receptors would have access to several agonists: rTAR1 agonists could be synthesized in the cytoplasm (e.g., β-PEA, *p*-tyramine, DA) of biogenic amine-producing cells or could be imported into the cytoplasm and/or vesicular lumen because they serve as substrates of plasma membrane and vesicular transporters (e.g., amphetamines, DA).

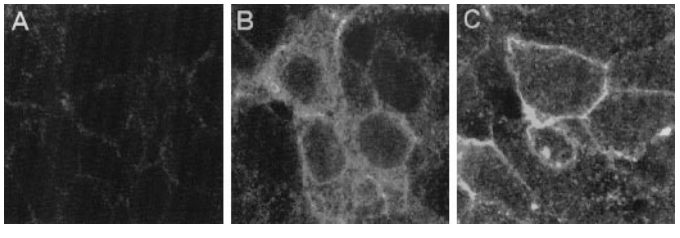

Fig. 4. Cellular distribution of the M1 flag-tagged rTAR1 stably expressed in HEK293 cells. Cells were treated with anti-FLAG monoclonal antibody in the absence (A) or presence (B) of 0.1% Triton X-100, followed by Cy5 goat anti-mouse IgG, and were visualized using a confocal microscope. According to this pattern of immunoreactivity, the rTAR1 seem to be almost exclusively retained within the cytoplasm. C, for comparison, HEK293 cells stably expressing hD1R carrying the M1 epitope tag were stained in parallel in the presence of Triton X-100. As expected, the hD1R was primarily localized around the surface of the cell.

Chromosomal Localization of a Human Trace Amine Receptor Gene. With the identification of GPCRs for trace amines and the evidence suggesting that these compounds are involved in monoamine release (Schonfeld and Trendelenburg, 1989; Mundorf et al., 1999), drug abuse (Shannon and Degregorio, 1982), hypertension (Brown et al., 1989), anxiety (Lapin, 1990), schizophrenia (Boulton, 1982), Parkinson's disease (Da Prada et al., 1984), and diabetes (Mosnaim et al., 1982), it was of interest to determine the chromosomal localization of the human TAR gene (hTAR1) to establish whether or not it lies within a region of interest based on linkage or association studies. For the chromosomal mapping of hTAR1, fluorescence in situ hybridization was performed on human metaphase chromosomes. Hybridization signals were visualized over the long arms of both chromosomes 6 (data not shown) in a position consistent with a chromosomal localization of 6q23.2, where at least two other orphan GPCRs related to hTAR1 reside (NT receptor, Zeng et al., 1998; GPCR57, GPCR58, Lee et al., 2000). This chromosomal localization is particularly noteworthy because it is one of the few regions that has been reproducibly associated with schizophrenia in linkage studies (Cao et al., 1997; Martinez et al., 1999; Levinson et al., 2000; Mowry and Nancarrow, 2001), suggesting the possibility that hTAR1 may be involved in the mechanism of psychosis. The relevance of this receptor to the etiology of psychosis is enhanced by the evidence that 3-MT is a potent and efficacious agonist. 3-MT is the major metabolite of dopamine produced by the enzyme COMT, a variant of which was recently found to be transmitted with greater frequency to schizophrenic offspring in a family-based association study (Egan et al., 2001).

In summary, we propose that the rat and human orphan GPCRs we have cloned are activated by endogenous trace amines as well as metabolites of the biogenic amine neurotransmitters. Consequently, some of the molecular, cellular, physiological, and behavioral effects manifested by these compounds are going to be mediated by these receptors. However, with respect to the function(s) of this putative neurotransmitter/neuromodulatory system in vivo, further progress must await the development of more selective reagents. These efforts will hopefully lead to the synthesis of highly selective TAR antagonists that may foster the development of therapeutic agents useful in the treatment of shock, hypertension, arrhythmia, asthma, migraine headache, psychosis, and anaphylactic reactions.

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