Ractopamine, a Livestock Feed Additive, Is a Full Agonist at Trace Amine–Associated Receptor 1

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ABSTRACT

Ractopamine (RAC) is fed to an estimated 80% of all beef, swine, and turkey raised in the United States. It promotes muscle mass development, limits fat deposition, and reduces feed consumption. However, it has several undesirable behavioral side effects in livestock, especially pigs, including restlessness, agitation, excessive oral-facial movements, and aggressive behavior. Numerous in vitro and in vivo studies suggest RAC's physiological actions begin with its stimulation of β_1 - and β_2 -adrenergic receptor-mediated signaling in skeletal muscle and adipose tissue; however, the molecular pharmacology of RAC's psychoactive effects is poorly understood. Using human cystic fibrosis transmembrane conductance regulator (hCFTR) chloride channels as a sensor for intracellular cAMP, we found that RAC and *p*-tyramine (TYR) produced concentration-dependent increases in chloride conductance in oocytes coexpressing hCFTR and mouse trace amine–associated receptor 1

(mTAAR1), which was completely reversed by the trace amine–associated receptor 1 (TAAR1)-selective antagonist EPPTB [*N*-(3-ethoxyphenyl)-4-pyrrolidin-1-yl-3-trifluoromethylbenzamide]. Oocytes coexpressing hCFTR and the human β_2 -adrenergic receptor showed no response to RAC or TYR. These studies demonstrate that, contrary to expectations, RAC is not an agonist of the human β_2 -adrenergic receptor but rather a full agonist for mTAAR1. Since TAAR1-mediated signaling can influence cardiovascular tone and behavior in several animal models, our finding that RAC is a full mTAAR1 agonist supports the idea that this novel mechanism of action influences the physiology and behavior of pigs and other species. These findings should stimulate future studies to characterize the pharmacological, physiological, and behavioral actions of RAC in humans and other species exposed to this drug.

Introduction

In the United States and 21 other countries, ractopamine hydrochloride (RAC; *EL737*, *LY031537*), a synthetic, racemic biogenic amine, is approved as a livestock feed additive to promote weight gain, increase muscle mass, limit fat deposition, reduce feed consumption, and lessen waste production (Yen, 1984; Yen et al., 1990, 1991; Gu et al., 1991; Rikard-Bell et al., 2009). The presence of RAC in swine has had an impact on the international trade of farm products, and there are reports of food poisonings from other β -receptor agonists (Wu et al., 2013). Due to human health concerns, the use of RAC as a feed additive is banned in 122 countries, including China, Russia, and members of the European Union. Although the physiologic effects of racemic RAC and its active R/R stereoisomer butopamine (BUT; *LY131126*) have

been studied in several mammalian species, surprisingly only four human studies—involving a total of 20 male volunteers—have been reported (Marchant-Forde et al., 2003; Poletto et al., 2009, 2010a,b). The focus of these studies was on the cardiovascular effects of RAC and BUT. No study examining the pulmonary or psychological effects of RAC or BUT in humans has been published. Although statistically underpowered, the four published human studies continue to guide regulatory policies establishing safe limits of human exposure despite the serious adverse reactions to RAC, particularly in swine, that have been documented (Marchant-Forde et al., 2003; Poletto et al., 2009, 2010a,b).

The structure of RAC resembles adrenergic agonists but also agonists of the recently discovered $G_{\alpha s}$ -coupled trace amine–associated receptor 1 (TAAR1) (Bunzow et al., 2001), including amphetamine, methamphetamine, *p*-tyramine (TYR), β -phenylethylamine (PEA), synephrine, octopamine, and 4-ethylphenol-substituted iodothyronamines (Scanlan et al., 2004) (Fig. 1). RAC and BUT are reported to exert their physiological effects by stimulating β_1 -adrenergic receptor (β_1 AR) and β_2 -adrenergic receptor (β_2 AR) expressed in skeletal muscle and adipose tissue (Mills et al., 1990). However, the molecular

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ABBREVIATIONS: β_1 AR, β_1 -adrenergic receptor; β_2 AR, β_2 -adrenergic receptor; BUT, butopamine; EPPTB, *N*-(3-ethoxyphenyl)-4-pyrrolidin-1-yl-3-trifluoromethylbenzamide; h β_2 AR, human β_2 AR; hCFTR, human cystic fibrosis transmembrane conductance regulator; IBMX, 3-isobutyl-1-methylxanthine; mTAAR1, mouse trace amine–associated receptor 1; PEA, β -phenylethylamine; RAC, ractopamine; TAAR1, trace amine–associated receptor 1; TYR, *p*-tyramine.

Fig. 1. Structures of RAC, EPPTB, and other TAAR1 agonists: TYR, synephrine, octopamine, iodothyronamines, amphetamine, and methamphetamine.

pharmacology of their effects has not been systematically investigated, even though it is structurally similar to the amphetamines, and elevated dopamine tone in the brains of RAC-fed swine has been reported (Marchant-Forde et al., 2003; Poletto et al., 2010b, 2011). Given the similarities between RAC and the pharmacophores recognized by TAAR1, we hypothesized RAC would be a TAAR1 agonist. To test this hypothesis, we monitored cAMP-dependent chloride conductances produced by *Xenopus* oocytes expressing the human cystic fibrosis transmembrane conductance regulator (hCFTR) alone or together with either mouse TAAR1 (mTAAR1) or the human β_2 AR (h β_2 AR). Our results indicate that RAC, contrary to expectations, is not an agonist of the h β_2 AR but rather is a full agonist of the closely related mTAAR1. Since TAAR1-mediated signaling can influence cardiovascular tone (Fellows, 1947; Frascarelli et al., 2008; Herbert et al., 2008; Broadley, 2010; Fehler et al., 2010; Broadley et al., 2013) and behavior in several animal models (Miller, 2011; Revel et al., 2011; Achat-Mendes et al., 2012), our finding that RAC is a full mTAAR1 agonist constitutes a novel mechanism by which RAC can influence the physiology and behavior of pigs (Marchant-Forde et al., 2003; Poletto et al., 2009, 2010a,b) and other species. These findings should stimulate future studies to characterize the pharmacological, physiological, and behavioral actions of RAC in humans and other species exposed to this drug.

Materials and Methods

Reagents. IBMX (3-isobutyl-1-methylxanthine), forskolin, isoproterenol, TYR, and racemic RAC were purchased from Sigma-Aldrich (St. Louis, MO). EPPTB [N-(3-ethoxyphenyl)-4-pyrrolidin-1-yl-3-trifluoromethylbenzamide]

was synthesized in the Grandy laboratory according to previously published procedures (Bradaia et al., 2009), and its identity was verified using NMR. Purity was greater than 95%.

In Vitro Transcription. hCFTR cDNA cloned into pBQ4.7 plasmid was a gift from Dr. D. C. Dawson (Department of Physiology and Pharmacology, Oregon Health and Science University, Portland, OR). cRNAs for hCFTR and mTAAR1 (Bunzow et al., 2001) were generated using Ambion Message Machine T7 Ultra Transcription Kit (Ambion, Carlsbad, CA).

Preparation and Microinjection of Oocytes. *Xenopus laevis* oocytes were prepared using our previously reported methods (Liu et al., 2012). In brief, oocyte follicular membranes were removed using 0.2 Wünsch units/ml Liberase TL (Roche Molecular Biochemicals, Indianapolis, IN) for 1–2 hours in a Ca^{2+} -free solution containing (in mM): 82.5 NaCl, 2 KCl, 1 MgCl_2 , and 5 HEPES (pH 7.5). Defolliculated oocytes were washed and maintained in a modified Barth's solution containing (mM) 88 NaCl, 1 KCl, 0.82 MgSO_4 , 0.33 $\text{Ca}(\text{NO}_3)_2$, 0.41 CaCl_2 , 2.4 NaHCO_3 , 10 HEPES (hemisodium salt), and 250 mg/l Amikacin plus 150 mg/l gentamicin at pH 7.5. Stage V–VI oocytes were injected with 50 nl cRNA. The concentrations of cRNAs were as follows: 3.4 ng/ μl for hCFTR, 1.2 ng/ μl for h β_2 AR, and 170 or 340 ng/ μl for mTAAR1. RNA concentrations were adjusted so that the maximum steady-state stimulated conductance was less than 200 μS .

Whole-Cell Recordings. The data were acquired using an Oocyte 725 amplifier (Warner Instruments, Hamden, CT) and the pClamp 8 data acquisition program (Molecular Devices, Sunnyvale, CA). Individual oocytes were placed in a recording chamber (RC-1Z; Warner Instruments) and continuously perfused with frog Ringer's solution containing (in mM): 98 NaCl, 2 KCl, 1 MgCl_2 , 1.8 CaCl_2 , and 5 HEPES-hemi-Na (at pH 7.4) and at indicated times were exposed to various reagents dissolved in frog Ringer's solution for lengths of time as indicated in each graph. Oocytes were maintained in the open circuit condition, and the membrane potential was periodically ramped from -120 to $+60$ mV over 1.8 seconds.

Statistical Analysis. Data are presented as the mean $\pm$ S.E.M. Significance was determined by Student's *t* test with $P \leq 0.05$ considered significant.

Results

Effects of RAC, TYR, and EPPTB Are Specific to mTAAR1. To assess the selectivity of RAC, TYR, and the TAAR1 antagonist EPPTB (Bradaia et al., 2009) for mTAAR1, we first determined no chloride conductance was evoked in uninjected oocytes, oocytes expressing hCFTR alone, mTAAR1 alone, or the combination of hCFTR and h β_2 AR even at micromolar concentrations (Fig. 2). In contrast, oocytes expressing only hCFTR (Fig. 2C) responded to 10 μ M forskolin by producing a robust chloride conductance, whereas oocytes coexpressing hCFTR and h β_2 AR (Fig. 2D) responded to 10 μ M β_2 AR agonist isoproterenol (Al-Nakkash and Hwang, 1999; Liu et al., 2005) by producing a chloride conductance that was insensitive to EPPTB (100 nM).

mTAAR1 Exhibits Constitutive Activity. Given that mTAAR1 has been reported to display constitutive activity in other in vitro assays (Bradaia et al., 2009), we explored this possibility in oocytes by inhibiting the oocyte's endogenous phosphodiesterase activity with IBMX. Oocytes expressing hCFTR alone or together with mTAAR1 were exposed to IBMX at concentrations ranging from 10 to 200 μ M (Al-Nakkash and Hwang, 1999; Liu et al., 2005). As expected, increasing the concentration of IBMX resulted in small but significant increases in the chloride conductance produced by oocytes expressing only hCFTR that were insensitive to TYR at its EC₈₀ concentration (76 $\pm$ 15 nM; Fig. 3, A and D). In contrast, exposing oocytes coexpressing hCFTR and mTAAR1 to either stepwise increases in IBMX (10, 20, 50, 100, and 200 μ M) or TYR (76 nM) resulted in chloride conductances approximately 10 times those produced by oocytes expressing hCFTR alone (Fig. 3, B and D). To determine whether the robust chloride currents produced by oocytes coexpressing hCFTR and mTAAR1 were a function of mTAAR1-mediated increases in cAMP production, oocytes expressing both proteins were exposed to IBMX (50 and 200 μ M) in the absence and presence of the TAAR1-selective antagonist EPPTB. We tested the effect of EPPTB over a wide range of CFTR conductance (the conductance stimulated by 200 μ M IBMX ranged from 37 to 118 μ S; $n = 5$). Within each oocyte, 100 nM EPPTB attenuated the response to 50 μ M IBMX (Fig. 3C). The conductance averaged 18 $\pm$ 8 μ S ($n = 5$) and 5 $\pm$ 1 μ S ($n = 5$) in the absence and presence of 100 nM EPPTB (Fig. 3D). However, we are detecting a very small change in a data set with some variation, and so this difference was not statistically significant ($P = 0.14$; $n = 5$). Nevertheless, the fact that EPPTB attenuated the response to 50 μ M IBMX in every oocyte that we tested suggests that mTAAR1 is constitutively active.

RAC Is a Full mTAAR1 Agonist. Since the structure of RAC resembles the trace amine receptor agonists PEA and TYR (Fig. 1), and given RAC had no effect on oocytes coexpressing h β_2 AR and hCFTR, we examined the effects of RAC on oocytes coexpressing mTAAR1 and hCFTR. Increasing concentrations of RAC (50 and 500 nM, and 2, 5, 10, 20, 50, and 100 μ M) led to reversible stepwise increases in chloride conductance in oocytes coexpressing hCFTR and mTAAR1 (Fig. 4A), suggesting RAC binding to mTAAR1 resulted in an elevation of intracellular cAMP, the subsequent opening of hCFTR, and a concomitant

Fig. 2. The effects of RAC, TYR, and EPPTB are specific to mTAAR1. (A) Shown is the response of a representative uninjected oocyte exposed sequentially, with washing in between, to 36 μ M RAC (EC₈₀), 76 nM TYR (EC₈₀), and 100 nM EPPTB. (B) The response of a representative oocyte expressing mTAAR1 exposed sequentially, with washing in between, to 36 μ M RAC, 76 nM TYR, and 100 nM EPPTB is shown. (C) The response of an oocyte expressing hCFTR exposed sequentially, with washing in between, to 10 μ M forskolin, 36 μ M RAC, 76 nM TYR, 100 nM EPPTB, and then 10 μ M forskolin in the presence of 100 nM EPPTB is shown. The conductances stimulated by forskolin in the presence and absence of EPPTB were not significantly different (18 $\pm$ 5 μ S, $n = 4$ versus 21 $\pm$ 6 μ S, $n = 4$; $P = 0.73$). (D) The response of a representative oocyte coexpressing hCFTR and h β_2 AR exposed sequentially, with washing in between, to 10 μ M isoproterenol (ISOP), 36 μ M RAC, 76 nM TYR, 100 nM EPPTB, and then 10 μ M ISOP in the presence of EPPTB is shown. The conductances stimulated by ISOP in the presence and absence of EPPTB were not significantly different (12 $\pm$ 2 μ S, $n = 4$ versus 13 $\pm$ 2 μ S, $n = 4$; $P = 0.66$).

increase in chloride current. The concentration-response profile for RAC was fit to a single exponential function from which an EC₅₀ value of 16 $\pm$ 4 μ M was calculated (Fig. 4B). TYR exerted a similar concentration-dependent response with an EC₅₀ of 34 $\pm$ 6 nM (Fig. 4C). EPPTB blocked the effect of RAC (EC₈₀ = 36 μ M) in a concentration-dependent manner (Fig. 4D), with an IC₅₀ of 20 $\pm$ 6 nM (Fig. 4E). EPPTB blocked TYR's effect in a similar fashion with an IC₅₀ of 12 $\pm$ 2 nM (Fig. 4F). These results demonstrate RAC is a full agonist for mTAAR1.

RAC and TYR Stimulate hCFTR via a mTAAR1 cAMP-Dependent Pathway. To explore whether RAC exerts its effect on hCFTR via a cAMP-dependent pathway, we exposed oocytes coexpressing hCFTR and mTAAR1 to RAC (Fig. 5A) or TYR (Fig. 5B) at their respective EC₈₀ values. After washout, oocytes exposed to a combination of RAC plus IBMX or TYR plus IBMX produced larger conductances than those produced by either agonist alone. Furthermore, IBMX treatment prevented the rapid drop in conductance that typically follows the removal of agonist from the perfusate. These results

Fig. 3. mTAAR1 exhibits constitutive activity in *Xenopus* oocytes. (A) The response of a representative oocyte expressing hCFTR exposed to increasing concentrations of IBMX (10, 20, 50, 100, and 200 μ M). After washout, TYR was added to the perfusate at its EC₅₀ (76 nM) without effect. (B) The response of a representative oocyte coexpressing hCFTR and mTAAR1 exposed to increasing concentrations of IBMX (10, 20, 50, 100, and 200 μ M). After washout, TYR (76 nM) produced a rapid and robust chloride current. (C) The response of a representative oocyte coexpressing hCFTR and mTAAR1 exposed to IBMX (50 and 200 μ M) in the presence and absence of the TAAR1 antagonist EPPTB (100 nM). (D) A summary of the chloride conductances recorded from nine oocytes exposed to 50 and 200 μ M IBMX.

support the interpretation that both RAC and TYR exert their actions via a cAMP-dependent pathway coupled to the activation of mTAAR1.

Discussion

Contrary to expectations based on the literature (Mills et al., 1990, 2003), our *in vitro* results failed to confirm RAC as an agonist of the h β_2 AR coexpressed with hCFTR in *Xenopus* oocytes. Importantly, whether the other documented target of RAC's actions, human β_1 AR, responds to RAC when coexpressed with hCFTR in *Xenopus* oocytes remains to be investigated. Using an *in vitro* cAMP assay, Mills et al. (2003) reported RAC-stimulated cAMP accumulation in Chinese hamster ovary cells expressing cloned porcine β_1 AR and β_2 AR. Consequently, the differences between their results and ours could be due to the fact that the two studies used receptors from different species, different expression systems, and different methods of monitoring changes in intracellular cAMP levels. Therefore, a systematic re-examination of RAC's pharmacology *in vitro* and *in vivo* seems warranted.

Furthermore, the results presented herein are consistent with the interpretation that RAC is a full agonist of mTAAR1. This G_{as} protein-coupled receptor, the best-studied member of a 15-gene family in mice (16 in rats and 6 in humans; Lindemann and Hoener, 2005), is homologous to other biogenic amine receptors, including those that recognize norepinephrine, dopamine, and serotonin (Borowsky et al., 2001; Bunzow et al., 2001). In fact, mouse, rat, and human TAAR1 are all activated by these three important neurotransmitters, as well as by PEA, TYR, octopamine, synephrine, 3-iodothyronamine, amphetamine, methamphetamine, and methylenedioxymethamphetamine (ecstasy; Bunzow et al., 2001; Scanlan et al., 2004; Reese et al., 2007, 2014; Wainwright et al., 2007; Lewin et al., 2009). The conservation of TAAR1's pharmacological profile across these three species supports the hypothesis that RAC is also a full agonist of bovine, porcine, and turkey species of TAAR1. Of course, additional experiments with the TAAR1 of those species will have to be performed to confirm this prediction. However, if RAC is confirmed to be an agonist of hTAAR1, it will be important to explore the extent to which

Fig. 4. RAC and TYR elicit chloride conductances that are concentration-dependent and EPPTB-sensitive in oocytes coexpressing hCFTR and mTAAR1. (A) The response of a representative oocyte coexpressing hCFTR and mTAAR1 exposed sequentially to increasing concentrations of RAC from 50 nM to 100 μ M is shown. (B) RAC produces concentration-dependent increases in chloride conductance. (C) TYR produces a concentration-dependent increase only in oocytes coexpressing hCFTR and mTAAR1. (D) The response of a representative oocyte coexpressing hCFTR and mTAAR1 exposed to 36 μ M RAC. At steady-state stimulation, it was then exposed sequentially to increasing concentrations of EPPTB from 1 nM to 2 μ M. (E) The concentration-response curve for EPPTB in the presence of RAC. (F) The concentration-response profile for EPPTB in the presence of TYR. The y-axis represents normalized conductance with the concentrations plotted on a log scale along the x-axis.

variation in the hTAAR1 gene contributes to RAC responsiveness and potentially constitutes a human health risk factor. Furthermore, it could be informative to determine whether RAC

Fig. 5. RAC and TYR stimulate hCFTR via a mTAAR1-mediated cAMP-dependent pathway. (A) The response of a representative oocyte coexpressing hCFTR and mTAAR1 exposed to 36 μ M RAC. After washout, it was then exposed to 36 μ M RAC and 1 mM IBMX. At steady state of stimulation, RAC was removed from the perfusate with no effect on conductance. Afterward, IBMX was removed from the perfusate, and the conductance gradually returned to background level. (B) The response of a representative oocyte coexpressing hCFTR and mTAAR1 exposed to 76 nM TYR (EC_{80}). After washout, it was then exposed to 76 nM TYR and 1 mM IBMX. At steady state of stimulation, TYR was removed from the perfusate with no effect on conductance. Afterward, IBMX was removed from the perfusate, and the conductance gradually returned to background level.

can influence the activity of other receptors coded for by this large gene family.

Although our results provide unequivocal evidence that RAC is a TAAR1 agonist, the relatively high EC_{50} value reported here (16 μ M) exceeds the estimated total concentration in livestock flesh of about 1.25 μ M (based on livestock feed of about 200 mg/head per day without consideration of metabolism). Further work will be needed to evaluate the potential impact on human health of the RAC residue present in meat prepared from RAC-fed livestock. Interestingly, two independent reports (Ungemach, 2004; Bories et al., 2009) conclude a minimum safe level for human exposure to RAC cannot be determined from the available evidence and recommend more extensive studies with larger sample sizes. Nevertheless, in light of our current findings, we suggest additional studies be undertaken to investigate the extent to which human, porcine, turkey, and bovine species of TAAR1 respond to RAC. The results of such an analysis are likely to be of interest to those concerned about animal welfare, the meat-eating public, the scientific community, and policy makers.

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Author Contributions

Participated in research design: Grandy, Janowsky, Liu.
Conducted experiments: Liu.
Performed data analysis: Liu.
Wrote or contributed to the writing of the manuscript: Grandy, Janowsky, Liu.

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