



Identification of the adenylyl cyclase-activating 5-hydroxytryptamine receptor subtypes expressed in the rat submandibular gland

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1 Serotonin (5-hydroxytryptamine, 5-HT) has been shown to increase cyclic AMP production in dispersed cell aggregates from the major salivary glands of the rat. The goal of the present study was to identify the 5-HT receptor subtypes that mediate these effects in rat submandibular glands (SMG).

2 Among the 5-HT receptor subtypes identified in the rat, 5-HT_{4(a,b)}, 5-HT₆ and 5-HT_{7(a,b,c)} activate adenylyl cyclase (AC). We used subtype specific primers to screen rat SMG by reverse transcription-PCR. Results indicate the presence of mRNA for 5-HT_{4(b)} and 5-HT_{7(a)} but not for 5-HT_{4(a)}, 5-HT₆ and 5-HT_{7(b,c)}.

3 In dispersed SMG cells, 5-carboxyamidotryptamine (5-CT), a 5-HT₇ receptor agonist, stimulated cyclic AMP synthesis with higher potency ($EC_{50} = 27 \pm 5$ nM) but lower efficacy than 5-HT, suggesting a 5-HT₇ component and an additional component in the response to 5-HT. The 5-HT₇ contribution was further supported by antagonism of the 5-CT effect by metergoline, a 5-HT₇ antagonist, which exhibited an affinity ($K_i = 50$ nM) similar to that obtained at the cloned 5-HT₇ receptor.

4 In the presence of a maximally effective concentration of 5-CT, 5-HT produced an additional increase in cyclic AMP production that was inhibited by the 5-HT₄ receptor antagonist, GR113808, suggesting that the second component of cyclic AMP production is mediated by 5-HT₄ receptors.

5 These findings indicate the presence in rat SMG of both 5-HT_{4(b)} and 5-HT_{7(a)} receptors positively coupled to AC.

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Abbreviations: 5-CT, 5-carboxyamidotryptamine; FSK, forskolin; SMG, submandibular gland

Introduction

Since the discovery of 5-HT more than 50 years ago, its actions have been well studied in many organ systems, including the gastrointestinal tract, the vasculature and the central nervous system. As currently understood, four effector systems are primarily associated with the known 5-HT receptor subtypes (Hoyer & Martin, 1996). 5-HT_{1A,B,D,E,F} receptors are negatively-coupled to AC (*via* G_i); 5-HT_{2A,B,C} are positively-coupled to phospholipase C (*via* G_q); 5-HT₃ is a ligand-gated ion channel; and 5-HT_{4,6,7} are positively coupled to AC (*via* G_s). The effector mechanisms for 5-HT_{5A,B} have yet to be established. Physiological effects in response to the activation of the 5-HT_{4,6,7} subtypes include smooth muscle relaxation (*via* stimulation of cyclic AMP production), smooth muscle contraction (*via* intracellular calcium mobilization or indirect release of acetylcholine), stimulation of chloride secretion in the rat distal colon, hormonal secretion from the adrenal cortex and modulation of cardiac function (Boess & Martin, 1994).

Our laboratory has reported that, in the isolated perfused rat SMG, 5-HT decreases acetylcholine-induced salivary flow and markedly increases saliva protein content (Turner *et al.*, 1996). These functional effects were accompanied by an increase in cyclic AMP production in dispersed cell aggregates

from the rat SMG in response to 5-HT, suggesting that 5-HT receptors positively coupled to AC are expressed in this organ. In this report, we describe results from molecular and pharmacological experiments that indicate the presence of two AC-activating 5-HT receptor subtypes in the rat SMG.

Methods

Reagents

5-HT, isobutylmethylxanthine, trichloroacetic acid, and buffer reagents were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). [³H]-adenine (29 Ci mmol⁻¹) was purchased from Dupont NEN (Wilmington, DE, U.S.A.). Metergoline phenylmethylester was obtained from Tocris (Ballwin, MO, U.S.A.). FSK and 5-CT were purchased from Research Biochemicals International (Natick, MA, U.S.A.). GR113808 was a generous gift provided by Glaxo Research Group (Middlesex, U.K.).

RT-PCR

Total RNA was isolated from rat SMG and whole rat brain to investigate 5-HT receptor subtype expression *via* RT-PCR. Rat brain was used as the positive control tissue for 5-HT₄, 5-

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HT₆ and 5-HT₇. Total RNA was prepared from 30 mg of fresh SMG or brain tissue using an RNeasy kit (Qiagen Inc., Chatsworth, CA, U.S.A.) according to the manufacturer's protocol. Purified total RNA was treated with DNase (Boehringer Mannheim, Indianapolis, IN, U.S.A.) for 15 min at 37°C, followed by RNeasy cleanup. cDNA was synthesized from 1 µg of DNase-treated total RNA using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, U.S.A.) according to the manufacturer's protocol. Twenty per cent of the cDNA reaction product was used for PCR as described previously (Park *et al.*, 1997). Receptor-specific oligonucleotide primers were synthesized at the University of Missouri DNA Core Facility according to the published 5-HT_{4(a)} and 5-HT_{4(b)} (Gerald *et al.*, 1995), 5-HT₆ (Ruat *et al.*, 1993a), and 5-HT_{7(a,b,c)} (Heidmann *et al.*, 1998; Ruat *et al.*, 1993b) sequences (GenBank accession numbers U20906, U20907, L19656, L19654, U68489, and U68490 respectively). Primer sequences (5'-to-3'), nucleotide positions relative to the initiation codon and predicted PCR product sizes (base pairs, bp) are described in Table 1.

Due to the nature of the splice variations of the 5-HT₇ receptor gene, the primer set shown in Table 1 would be expected to yield one or more of three different products that differ in size and carboxyterminal sequence. PCR products obtained with all primer sets were resolved by electrophoresis on 2% agarose gels, visualized with UV light, and purified using a Wizard PCR Prep DNA kit (Promega, Madison, WI, U.S.A.). The purified products were sequenced by the University of Missouri DNA Core Facility using an ABI Prism automated DNA sequencer. Oligonucleotide primers for sequencing were the same as those used in PCR. Sequence comparisons were performed using the Wisconsin Package software (Genetics Computer Group, Madison, WI, U.S.A.).

Cyclic AMP production

SMG dispersed cell aggregates were prepared from anaesthetized (sodium pentobarbitone) adult male Sprague-Dawley rats (175–225 g) as described previously (Turner & Camden, 1990). The animal use protocol for these studies has been reviewed and approved by the University of Missouri Animal Care and Use Committee. Briefly, SMG were minced and placed in 20 ml of Dulbecco's modified Eagle's medium-Ham's F12 (DMEM-F12) containing 0.2 mM CaCl₂, 50 units ml⁻¹ collagenase, 400 units ml⁻¹ hyaluronidase and 1% (w v⁻¹) BSA, for 40 min at 37°C. The cells were further dispersed by passage through a 10 ml transfer pipette at 20, 30 and 40 min. Dispersed cell aggregates were then washed (centrifuged at 200 × g for 2 s) with buffer A [in mM: NaCl 120, KCl 4, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 1, glucose 10, and HEPES 15 (pH 7.4)] containing 1% (w v⁻¹) BSA, then resuspended in 20 ml of

buffer A containing 0.1% BSA (Buffer B). Cyclic AMP production in the cell aggregates was assessed by the [³H]-adenine pre-loading procedure, as described previously (Turner *et al.*, 1996). Dispersed cell aggregates were incubated with 5 µCi ml⁻¹ [³H]-adenine for 30 min at 37°C, washed, pelleted and resuspended in 3 ml of ice cold buffer B. The 5 min stimulation step was initiated by the addition of 50 µl of the cell aggregate suspension to test tubes containing agonist (± antagonist) and 1 µM FSK in a final volume of 500 µl of buffer B. FSK was included throughout because the magnitude of the cyclic AMP responses to 5-HT receptor agonists is synergized by inclusion of 1 µM FSK without affecting the potency of the agonists (Turner *et al.*, 1996). The stimulation was terminated by addition of 25 µl of 100% trichloroacetic acid. The tubes were centrifuged (5 min at 1800 × g) and the supernatants were passed over Dowex and alumina columns, as described previously (Turner & Camden, 1990). The collected [³H]-ATP and [³H]-cyclic AMP fractions were quantitated on a Beckman LS5000TD liquid scintillation counter. Data are expressed as per cent [³H]-ATP to [³H]-cyclic AMP conversion:

$$\frac{[\text{^3H}]\text{-cyclic AMP}}{([\text{^3H}]\text{-cyclic AMP}) + ([\text{^3H}]\text{-ATP})} \times 100 \quad (1)$$

Data analysis

Data from the cyclic AMP experiments are reported as the mean ± s.e. mean. Agonist potencies (expressed as EC₅₀ values) were calculated using a nonlinear curve fitting procedure (GraphPad Prism, San Diego, CA, U.S.A.) based on the sigmoidal concentration-response relationship with an unconstrained slope. Antagonist affinity (K_i) was determined *via* Schild analysis.

Results

Molecular identification of 5-HT receptor subtype-specific mRNA

Among the identified 5-HT receptor subtypes, 5-HT₄, 5-HT₆ and 5-HT₇ have been shown to be positively coupled to AC (Hoyer & Martin, 1996; Lucas & Hen, 1995). We thus made the initial assumption that 5-HT-induced cyclic AMP formation in the rat SMG is through one or more of these known receptor subtypes, with a novel 5-HT receptor being an alternative explanation. Our first approach therefore was to use RT-PCR with subtype specific oligonucleotide primers to screen rat SMG mRNA for expression of 5-HT₄, 5-HT₆ and 5-HT₇ receptor messages. The results shown in Figure 1 indicate that rat SMG expresses mRNA for the 5-HT_{4(b)} and 5-HT_{7(a)}

Table 1

Gene	Primer	Position	Oligonucleotide sequence	Size (bp)
5-HT _{4(a)}	forward	730–352	GACCAGCACAGCACACATCGCAT	417
	reverse	1146–1123	GGACTCTGGGTCATTGTGTATGGG	
5-HT _{4(b)}	forward	730–752	GACCAGCACAGCACACATCGCAT	469
	reverse	1198–1174	TACAGCTGTCTTCTAGGTCATTGTC	
5-HT ₆	forward	501–526	CGAAGTGGGCAAAGCTCGAACACCTG	495
	reverse	995–971	CCCAGGGCCCTCTGAAGTCCCGCA	
5-HT _{7(a)}	forward	957–978	CTCCATCTTCAAGCGGGAACAG CCCTTTTTCCACAGTGGTCAG	388
	reverse	1313–1334		
5-HT _{7(b)}	forward	957–978		383
	reverse	1318–1339		
5-HT _{7(c)}	forward	957–978	475	
	reverse	1431–1410		

receptors. No cDNA product was obtained when primers for the short form of 5-HT₄, 5-HT_{4(a)} and 5-HT₆ were used, although these latter two primer sets yielded products from the positive control tissues. No product of the appropriate size for the 5-HT_{7(c)} receptor mRNA was obtained and sequencing of the 5-HT₇ PCR product gave no indication of the presence of the 5-HT_{7(b)} splice variant. Nucleotide sequence comparisons between the RT-PCR products generated from SMG mRNA and published nucleotide cDNA sequences demonstrated 100% identity with both the 5-HT_{4(b)} and 5-HT_{7(a)} receptors, suggesting that these two 5-HT receptor subtypes are expressed in rat SMG.

Pharmacological characterization of 5-HT induced cyclic AMP production

Functional studies in SMG dispersed cell aggregates were performed to confirm the molecular data. As shown in Figure 2, cyclic AMP production increased in dispersed cell aggregates upon treatment with both 5-HT, as reported previously (Turner *et al.*, 1996), and 5-CT, a 5-HT₇ receptor-selective (with respect to 5-HT₄) full agonist with nM affinity (Markstein *et al.*, 1999; Tsou *et al.*, 1994; Plassat *et al.*, 1993; Ruat *et al.*, 1993b). 5-CT ($EC_{50} = 27 \pm 5$ nM) was more potent

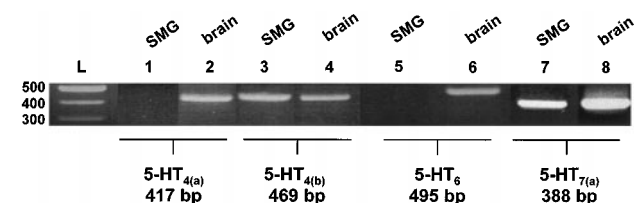


Figure 1 RT-PCR analysis of 5-HT receptor subtype mRNA expression in the rat SMG. 5-HT receptor subtype-specific oligonucleotide primers were designed and used in the RT-PCR as described in Methods. Migration of 500, 400 and 300 base pair (bp) ladder bands (L) in 2% agarose gel electrophoresis are shown on the left and labels above each lane indicate the cDNA source. Primers used and size of product obtained are indicated below each band. Absence of genomic DNA contamination in the cDNA samples was verified by PCR without prior incubation with reverse transcriptase.

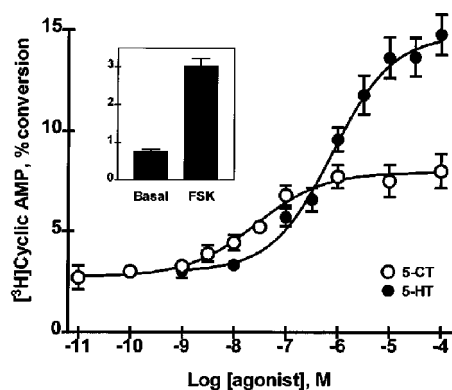


Figure 2 Concentration-dependent effects of 5-CT and 5-HT on cyclic AMP production in rat SMG dispersed cell aggregates. Production of cyclic AMP in response to 5-HT receptor agonists (5 min incubation) was measured by the [³H]-adenine loading procedure as outlined in Methods. EC_{50} values for the 5-CT and 5-HT concentration-response curves were 27 ± 5 and 700 ± 100 nM, respectively. Per cent conversion values of basal and $1 \mu\text{M}$ FSK-treated alone (inset) were 0.8 ± 0.1 and 3.1 ± 0.2 respectively ($n = 22$). Each concentration-response curve is the mean \pm s.e. mean of at least four experiments in the presence of $1 \mu\text{M}$ FSK.

than 5-HT ($EC_{50} = 700 \pm 100$ nM) but was less efficacious, producing a maximal response that was only about 50% of that elicited by 5-HT. This pharmacological profile, indicative of co-expression of the 5-HT₄ and 5-HT₇ receptors, has been observed recently in studies with rat brain (Markstein *et al.*, 1999). These data suggest two components of the cyclic AMP response to 5-HT in rat SMG: one component that is mediated by 5-HT_{7(a)} receptors and mimicked with 5-CT and a second, non-5-HT_{7(a)} component that is not sensitive to 5-CT and which, based on the RT-PCR results, would be predicted to be mediated by 5-HT_{4(b)} receptors.

To support the contention that 5-HT_{7(a)} receptors are responsible for the cyclic AMP response to 5-CT, we performed a Schild analysis on 5-CT-stimulated cyclic AMP production using the 5-HT₇ antagonist, metergoline phenylmethylester (Plassat *et al.*, 1993; Ruat *et al.*, 1993b). As shown in Figure 3A, metergoline, present at fixed concentrations of 50, 100, 250 and 500 nM, shifted the 5-CT concentration-response curve ($EC_{50} = 20 \pm 4$ nM) dextrally to yield EC_{50} values of 44 ± 17 nM, 99 ± 21 nM, $1.1 \pm 0.2 \mu\text{M}$ and $5 \pm 2 \mu\text{M}$, respectively. Metergoline had no effect on FSK-stimulated cyclic AMP production (Figure 3B). The calculated K_i value for metergoline was 50 nM (Figure 3C), in agreement with published values for this antagonist at the cloned and expressed 5-HT₇ receptor (Plassat *et al.*, 1993; Ruat *et al.*, 1993b). Methiothepin, another 5-HT₇-selective (relative to 5-HT₄) antagonist, inhibited the 5-HT receptor-stimulated response with a potency (data not shown) in agreement with earlier reports (Tsou *et al.*, 1994). We also observed that the 5-HT₄-selective antagonist, GR113808, when used at concentrations up to $10 \mu\text{M}$, did not inhibit the 5-CT effect (data not shown), whereas GR113808 was found to be effective against the 5-CT-independent component of the 5-HT effect (see below).

As demonstrated in Figure 2, the 5-CT concentration-dependent increase in cyclic AMP production exhibited a maximal plateau at a concentration of $1 \mu\text{M}$. An additional increase in cyclic AMP production was observed in response to 5-HT ($EC_{50} = 730 \pm 90$ nM) in the presence of $1 \mu\text{M}$ 5-CT, (Figure 4A), further supporting the presence of two

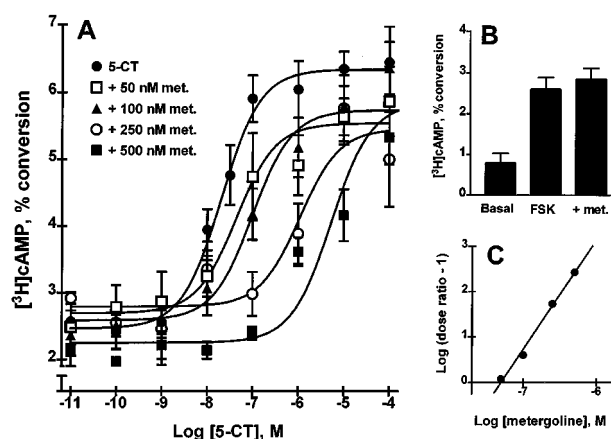


Figure 3 Effect of 5-HT₇ antagonist, metergoline, on 5-CT-stimulated cyclic AMP production. (A) 5-CT concentration-responses displayed a rightward shift in EC_{50} when metergoline, a 5-HT antagonist selective for 5-HT₇ vs 5-HT₄ receptors, was added to SMG dispersed cell aggregates at concentrations of 50, 100, 250 or 500 nM. (B) Treatment of the dispersed cell aggregates with 500 nM metergoline alone had no effect on the $1 \mu\text{M}$ FSK-stimulated response. (C) A Schild plot of the data in Figure 3A yielded a K_i value for metergoline of 50 nM. Data are the mean \pm s.e. mean of at least four replicate experiments.

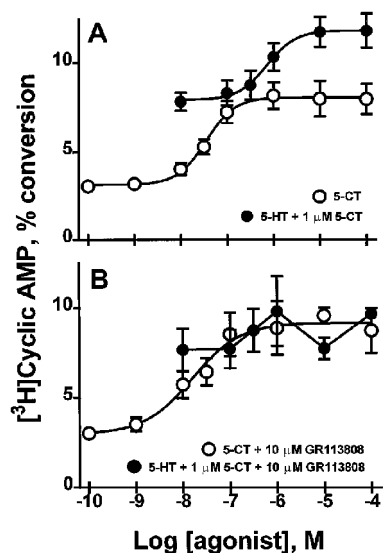


Figure 4 Two components of 5-HT-stimulated cyclic AMP production. 5-HT-stimulated cyclic AMP production was characterized as either 5-CT-dependent or 5-CT-independent. (A) The high potency component of this two-component response was induced by 5-CT (0.1 nM–100 μM , EC₅₀ = 35 ± 3 nM) and reached a maximum at 1 μM . With 1 μM 5-CT present, the low potency component was elicited with 5-HT (0.01–100 μM , EC₅₀ = 730 ± 90 nM). (B) This two component concentration-response study was repeated in the presence of a 5-HT₄-selective antagonist, GR113808 (10 μM), in which the 5-CT-dependent component (EC₅₀ = 16 ± 5 nM) was insensitive to GR113808, while the 5-CT-independent response was attenuated by GR113808. (A) and (B) are the mean ± s.e. mean of at least four replicate experiments each.

components of cyclic AMP production. This second, 5-HT-induced component of cyclic AMP production was inhibited by GR113808 (Figure 4B), a 5-HT₄-selective antagonist (Saxena, 1995; Grossman *et al.*, 1993). Taken together, these data indicate that the cyclic AMP response to 5-HT in rat SMG is due to activation of both 5-HT_{4(b)} and 5-HT_{7(a)} receptors.

Discussion

The key finding of this study is that the rat SMG expresses two 5-HT receptor subtypes, 5-HT_{4(b)} and 5-HT_{7(a)}, both of which are coupled to increased cyclic AMP production. This finding extends our previous observations (Turner *et al.*, 1996) of 5-HT-induced elevations of cyclic AMP levels in the submandibular, sublingual and parotid salivary glands of the rat and of modulation by 5-HT of SMG saliva flow and protein content.

Both of the 5-HT receptor subtypes identified in rat SMG have been characterized (Eglen *et al.*, 1997; Hegde & Eglen, 1996). The 5-HT₇ receptor cDNA was cloned from rat brain (Ruat *et al.*, 1993b) as well as the rat proximal convoluted tubule (Shen *et al.*, 1993). In addition, splice variants of the rat and human receptors have been identified (Heidmann *et al.*, 1997; 1998; Eglen *et al.*, 1997; Stam *et al.*, 1997; Jasper *et al.*, 1997). The pharmacological profile of this receptor subtype includes the observation by several groups that 5-CT is a full agonist with high potency and selectivity with respect to 5-HT₄ (Plassat *et al.*, 1993; Ruat *et al.*, 1993b). In addition, 5-HT antagonists (e.g., metergoline and methiothepin) with selectivity for 5-HT₇ receptors vs 5-HT₄ receptors, have been used to study the tissue distribution of the 5-HT₇ subtype (Eglen *et al.*,

1997). The observation that 5-CT stimulates cyclic AMP production with high potency (Figure 2) and that this stimulation is blocked by metergoline (Figure 3) complements the RT-PCR data (Figure 1) to indicate that functional 5-HT_{7(a)} receptors are expressed in the rat SMG.

The 5-HT₄ receptor, initially characterized pharmacologically (Dumuis *et al.*, 1988), was subsequently cloned, expressed and further characterized (Gerald *et al.*, 1995). Two 5-HT₄ receptor splice variants, 5-HT_{4(a)} and 5-HT_{4(b)} for short and long forms, respectively, were found (Gerald *et al.*, 1995). Pharmacological characteristics of the 5-HT₄ receptor include a relatively low potency of 5-HT compared with other subtypes (Clarke *et al.*, 1989) and high selectivity of the antagonist, GR113808 (Hegde & Eglen, 1996). The 5-CT-independent component of 5-HT-stimulated cyclic AMP production in rat SMG exhibits both of these characteristics and, combined with the RT-PCR results (Figure 1), strongly suggests that the 5-CT-independent component of cyclic AMP production is mediated by 5-HT_{4(b)} receptors. Together, these results indicate that the activity of these two receptor subtypes can account for the entire increase in cyclic AMP production in response to 5-HT.

As reviewed previously (Eglen *et al.*, 1997; Hegde & Eglen, 1996; Boess & Martin, 1994), the 5-HT₇ receptor is expressed in various regions of the brain (Heidmann *et al.*, 1998). In the guinea-pig, 5-HT₇ receptors are coupled to ileal relaxation (Carter *et al.*, 1995). In addition, evidence suggests that this subtype may be involved in the regulation of vascular tone (Terron, 1997), γ -aminobutyric acid-mediated neurotransmission (Kawahara *et al.*, 1994) and tachycardia (Villalon *et al.*, 1997). 5-HT₄ receptors are abundantly expressed in the central nervous system and in the periphery (Hegde & Eglen, 1996). In smooth muscle, 5-HT₄ receptor activation has been shown to either induce relaxation or constriction in the rat oesophagus or guinea-pig ileum, respectively. In addition, 5-HT₄ receptors stimulate chloride secretion in the rat colon, enhance hormone release from frog adrenocortical cells and produce tachycardia in a porcine model (Boess & Martin, 1994).

Definitive *in vivo* studies demonstrating a physiological role for 5-HT on mammalian salivary gland function have not been performed, although previous reports have suggested roles for 5-HT in the regulation of the salivary glands of the blowfly (*Calliphora erythrocephala*, Berridge & Heslop, 1981) and the rat (Turner *et al.*, 1996; Chernick *et al.*, 1989). There is abundant evidence that cyclic AMP-mobilizing agonists have key roles in modulating saliva volume and content, particularly protein content. Norepinephrine, through the activation of β -adrenergic receptors, has the predominant role in modulating salivary secretion through regulation of cyclic AMP levels (Turner & Camden, 1990). However, agonists at other AC-coupled receptors, including vasoactive intestinal peptide (VIP, Turner & Camden, 1990) and prostanoids (Martinez *et al.*, 1987), are also important in the regulation of salivary gland function. It is worth noting that, while less effective than β -adrenergic receptor agonists in stimulating cyclic AMP production in the salivary glands (Turner *et al.*, 1996), 5-HT (Figure 2) is as effective as VIP (Turner & Camden, 1990) and the prostanoids (Martinez *et al.*, 1987), supporting the idea that 5-HT may also be important in salivary gland regulation. A key extension of the current studies is to define the precise, and perhaps distinct, roles that 5-HT_{4(b)} and 5-HT_{7(a)} receptors play in salivary gland physiology. Ongoing studies are focused on defining the distribution of the 5-HT_{4(b)} and 5-HT_{7(a)} receptor subtypes among the various cell types within the gland, identifying the endogenous sources of the 5-HT involved in the activation of

these receptors, and assessing the effects of subtype-specific antagonists on salivary function *in vivo*. The list of agents shown to be involved in the regulation of salivary gland function has increased steadily in recent years. The identification of two AC-coupled 5-HT receptor subtypes in rat SMG suggests that this list may eventually include 5-HT.

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