The role of pannexin 1 hemichannels in ATP release and cell–cell communication in mouse taste buds

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ATP has been shown to be a taste bud afferent transmitter, but the cells responsible for, and the mechanism of, its release have not been identified. Using CHO cells expressing high-affinity neurotransmitter receptors as biosensors, we show that gustatory stimuli cause receptor cells to secrete ATP through pannexin 1 hemichannels in mouse taste buds. ATP further stimulates other taste cells to release a second transmitter, serotonin. These results provide a mechanism to link intracellular Ca^{2+} release during taste transduction to secretion of afferent transmitter, ATP, from receptor cells. They also indicate a route for cell–cell communication and signal processing within the taste bud.

afferent | gustation | serotonin | synapses

Gustatory receptor cells within taste buds detect sweet, bitter, and umami tastants via G protein-coupled taste receptors. Although detailed transduction mechanisms downstream of such receptors have been elucidated (1), our understanding of the signaling from taste cells to the afferent nerve is still limited. ATP has emerged as a key afferent neurotransmitter for taste buds (2). Gustatory stimulation of taste buds also results in release of serotonin (5-HT) (3). Yet, which cells release each neurotransmitter and the mechanisms of such release are unknown. These problems are particularly enigmatic, because in taste buds, the cells that express taste receptors (i.e., "receptor cells") comprise a separate population from the cells that possess synapses, express synaptic proteins, and exhibit depolarization-dependent calcium influx ("presynaptic cells") (4–6). We have used cellular biosensors (3) to measure taste-evoked transmitter release and, particularly, to identify which cells secrete ATP and 5-HT. Our results show that only receptor cells release ATP and only presynaptic cells release 5-HT. Further, we demonstrate an unexpected mechanism for nonexocytotic ATP secretion and present evidence for cell–cell signaling between receptor and presynaptic cells upon taste stimulation.

Results

We isolated taste cells from mouse circumvallate papillae, loaded them with the Ca^{2+} indicator Fura2-AM, and measured responses to taste stimulation and to KCl depolarization. Concurrently, we also measured transmitter release from individual taste cells using cellular biosensors (see below). Taste cells were unambiguously identified either as receptor cells or presynaptic cells by whether they responded to taste stimulation (receptor cells) or to KCl depolarization (presynaptic cells) (4). Isolated receptor and presynaptic cells were present in sufficiently low density in the recording chamber that there were no interactions (e.g., diffusible signals) between individual taste cells. The only interactions measured were between an isolated taste cell and its apposed biosensor.

Taste Receptor Cells Secrete ATP via Gap Junction Hemichannels. When a Fura2-loaded CHO cell stably expressing P2×2/P2×3 receptors (hereafter, "ATP biosensor") was positioned in close proximity to a receptor cell (Fig. 1*A*), we consistently recorded robust biosensor signals in response to a tastant mix (10 μM cycloheximide, 1 mM denatonium, 2 mM saccharin, and 100 μM SC45647), demonstrating taste-evoked ATP release (Fig. 1 *B* and *D*; 13 of 13 cells). We confirmed that ATP biosensors were not directly stimulated by tastants. In marked contrast, no ATP release was detected after KCl (50 mM) depolarization of receptor cells (0 of 11 cells) or of presynaptic cells (0 of 9 cells) (Fig. 1 *C* and *D*). Applying tastants to presynaptic cells did not evoke Ca^{2+} responses (4), or evoke ATP release.

Conversely, when an isolated taste bud presynaptic cell was apposed with a CHO cell stably expressing 5-HT2c receptors (hereafter, "5-HT biosensor"), KCl depolarization reliably evoked 5-HT biosensor responses (15 of 15 cells; Fig. 1 *E* and *G*). As above, stimuli did not directly affect the 5-HT biosensor. In no case did we observe 5-HT release from isolated receptor cells (0 of 9 cells; Fig. 1 *F* and *G*). Lastly, ATP (1 μM) dependably evoked Fura2 signals in presynaptic cells and stimulated them to release 5-HT (Fig. 1 *E* and *G*). Collectively, the data indicate that receptor cells secrete ATP and presynaptic cells secrete 5-HT.

Because receptor cells lack identifiable synapses, we explored nonvesicular ATP release mechanisms. Glial cells and erythrocytes have been shown to release ATP via gap junction hemichannels (7, 8). Hence, we investigated whether hemichannels might underlie ATP secretion from taste receptor cells. Indeed, carbenoxolone (5 μM), a potent gap junction channel blocker (9), reversibly and significantly reduced taste-evoked ATP secretion from isolated receptor cells (Fig. 1*H*). Carbenoxolone had no effect on the ATP biosensor itself [see supporting information (SI) Fig. 6) or on taste-evoked excitation of receptor cells (Fig. 1*H*). These data are consistent with hemichannel-mediated ATP release, which we next explore in greater detail.

Hemichannel Expression in Taste Buds. Pannexins (Pxs) and certain connexins (Cxs), including Cx30, -30.2, -32, -43, -45, -46, and -50 and Px1 and -2, form hemichannels on the cell surface (10–13). We tested the expression of each of these subunits in taste buds and in nontaste lingual epithelium by RT-PCR. mRNAs for Cx30.2, Cx32, Cx45, Cx46, Cx50, and Px2 were undetectable or expressed at insignificant levels in taste samples. In contrast, we found mRNAs for Cx30, Cx43, and Px1 in RNA in from taste epithelium (Fig. 2*A*). To evaluate the significance of each of these potential hemichannel-forming subunits, we used quantitative RT-PCR. Only Px1 was preferentially enriched in taste

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Abbreviations: Cx, connexin; Px, pannexin.

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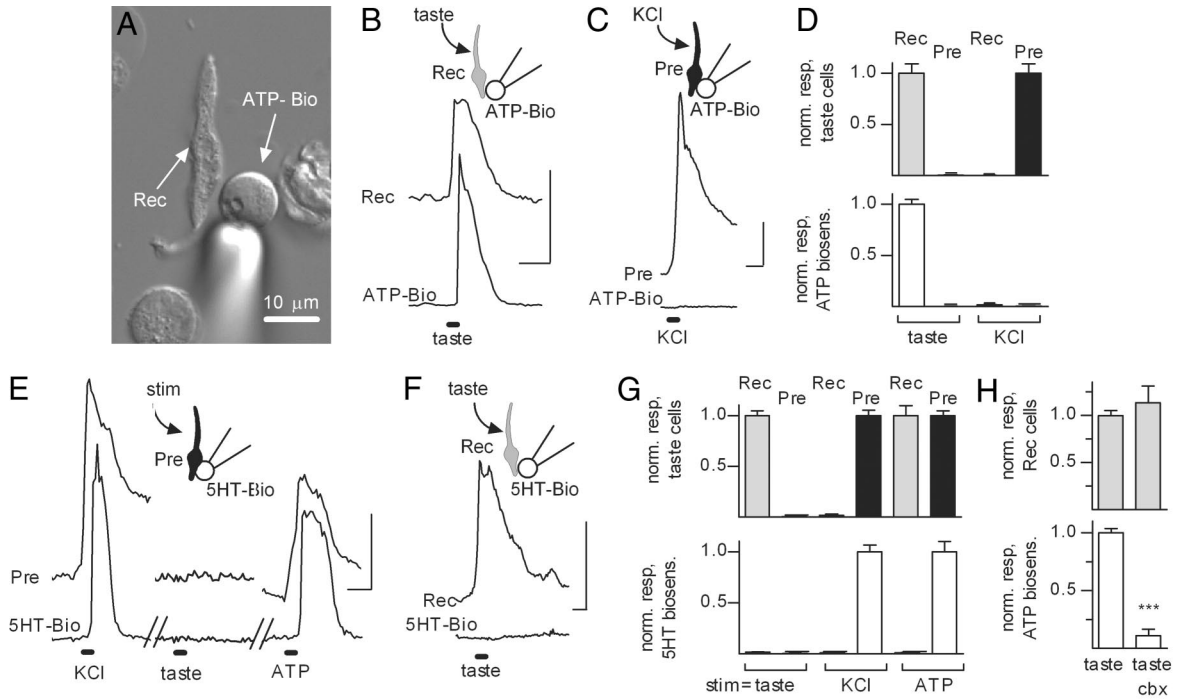

Fig. 1. Use of cellular biosensors to examine neurotransmitter secretion from receptor (Type II) and presynaptic (Type III) cells in the mouse taste bud. (*A*) Example of a pipette-held biosensor (ATP-Bio) apposed to an isolated taste receptor cell (Rec). Both cells were loaded with Fura-2. (*B*, *C*, *E*, and *F* insets) The recording arrangement with identified taste cell apposed to a biosensor cell. Paired traces show concurrent Ca^{2+} responses in a taste cell (*Upper*) and its apposed biosensor (*Lower*). Calibration bars for all traces: 20 sec and 0.5 F340/F380. (*B*) Taste receptor cells secrete ATP. Example of concurrent recordings of Ca^{2+} responses in a receptor cell (Rec) and an apposed ATP biosensor cell. Bath-applied tastants (bar at bottom of traces: a mix of 10 μM cycloheximide, 1 mM denatonium, 2 mM saccharin, and 100 μM SC45647) evoked responses in the receptor cell (upper trace) and after a brief delay, in the ATP biosensor (lower trace). (*C*) Presynaptic cells do not secrete ATP. Example of concurrent recordings from a presynaptic cell (Pre) and an ATP biosensor cell. KCl depolarization (50 mM) evoked a robust Ca^{2+} response in the presynaptic cell but did not stimulate ATP secretion. (*D*) Summary of recordings exemplified in *B* and *C*. Only receptor cells (*Upper*, gray bar) secrete ATP (*Lower*, white bar) and only in response to tastants, not KCl. In contrast, presynaptic cells (*Upper*, black bar) respond to KCl depolarization, not to taste stimulation and do not secrete ATP (*Lower*, white bar). Bars show means ± SEM. of Ca^{2+} responses, normalized to the maximum response for each stimulus (*Upper*), or for maximum ATP biosensor response (*Lower*). (*E*) Presynaptic cells release serotonin (5-HT) when stimulated. Example of concurrent Ca^{2+} responses in a presynaptic cell (upper trace) and an apposed 5-HT biosensor (lower trace) during sequential stimulation with KCl (50 mM), taste mix (as in *B*), and ATP (1 μM). KCl depolarization and ATP both triggered Ca^{2+} responses and 5-HT release. (*F*) Receptor cells do not release 5-HT. Example of a Ca^{2+} response in a receptor cell during taste stimulation (upper trace). There is no response in the apposed 5-HT biosensor cell (lower trace). (*G*) Summary of recordings exemplified in *E* and *F*. Only presynaptic cells (*Upper*, black bars) secrete 5-HT (*Lower*, white bars) and do so in response to KCl depolarization or ATP (1 μM) stimulation. Receptor cells (*Upper*, gray bars) also produce a Ca^{2+} response to ATP stimulation but do not secrete 5-HT (*Lower*). (*H*) Receptor cells (*Upper*, gray bars), stimulated with a taste mix (as in *B*) release ATP (*Lower*, white bars). In the presence of 5 μM carbenoxolone (cbx), a blocker of Px hemichannels, tastant-evoked ATP release (right) was severely diminished. ***, $P < 0.001$.

buds relative to surrounding, nontaste epithelium (Fig. 2*B*). By *in situ* hybridization, we detected Px1 mRNA within taste buds and, at much lower levels, in adjacent nontaste epithelium, connective tissue, and muscle (Fig. 2*D*).

If Px hemichannels are conduits for taste-evoked ATP release, they should be expressed in receptor cells, a prediction we tested by two assays. First, we used gene expression profiling of isolated single taste cells (4). Each cell was typed by scoring its expression of markers for three defined cell types: NTPDase2 for glial-like ("Type I") taste bud cells (14); PLCβ2 for gustatory receptor ("Type II") cells; and SNAP25 for presynaptic ("Type III") cells (4, 5). Each cell displayed only one (or none) of the markers, as expected. We detected Px1 mRNA in every receptor cell analyzed (10 of 10 cells) and also in approximately half the glial-like and presynaptic cells (Fig. 2*C*). Second, we double-immunostained sections of vallate papillae and confirmed that taste cells expressing PLCβ2 (receptor cells) were immunopositive for Px1 (Fig. 3). Fig. 3*D* clearly demonstrates the overlap between Px1- and PLCβ2-expressing cells. Immunofluorescence for Cx30 and Cx43 was detected in lingual keratinocytes, as shown for skin (15). However, both Cx30 and Cx43 were absent from, or expressed at very low levels in, taste buds. Instead, they are expressed in cells immediately surrounding the taste buds (Fig. 3 *A* and *B*). This observation is consistent with our quantitative RT-PCR result (Fig. 2*B*) insofar as Cx30 and Cx43 expressing cells may contaminate the taste sample. In sum, the expression pattern of Px1, but not that of Cx subunits, is consistent with a role in tastant-evoked ATP release from receptor cells.

Functional Px1 Hemichannels in Taste Buds. Human Px1 hemichannels, when opened by elevated cytosolic Ca^{2+}, pass carboxyfluorescein, a fluorescent dye (8). We expressed mouse Px1 in CHO cells (mPx1-CHO) and tested a number of dyes, including carboxyfluorescein. Upon stimulation of intracellular Ca^{2+} release, this dye permeated into mPx1-CHO cells (Fig. 4*A*). As reported for human erythrocytes (8), we also found that carboxyfluorescein could be loaded into mouse erythrocytes upon elevation of intracellular Ca^{2+}.

Next, we tested whether carboxyfluorescein penetrated taste cells when intracellular Ca^{2+} ($[Ca^{2+}]_i$) was raised. Because many taste cells express P2Y receptors (16), we elevated $[Ca^{2+}]_i$ by bath-applying ATP (5 μM). Repeated exposures to carboxyfluorescein in the absence of ATP did not progressively raise fluorescence in isolated taste cells (SI Fig. 8). Fluorescence after a single treatment (i.e., resting state) was thus defined as background. In contrast, exposure to carboxyfluorescein along

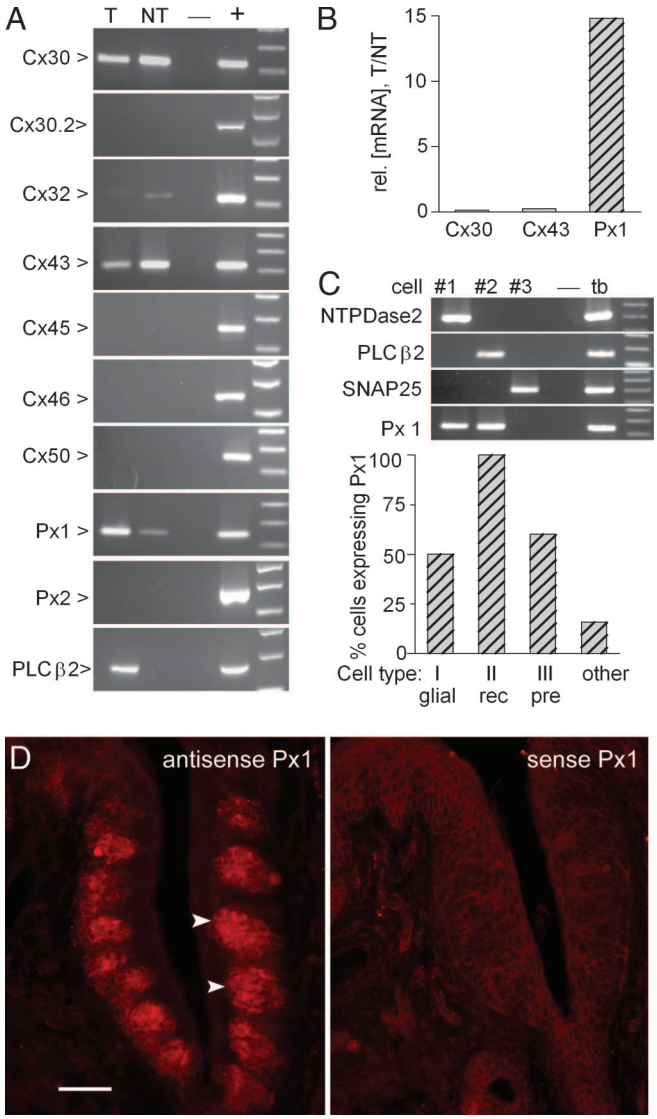

Fig. 2. Taste cells express hemichannel-forming Px 1 subunits. (*A*) RT-PCR for hemichannel-forming subunits expressed in taste bud-enriched (T) or non-taste (NT) lingual epithelium, a negative control lacking template (−), and a positive control tissue (+; lens for Cx46 and Cx50, vallate papilla for PLCβ2, or brain for all others). Only Cx30, Cx43, and Px1 were prominent in the taste samples. (*B*) Real-time RT-PCR shows that, of the abundantly expressed hemichannel-forming subunits, only Px1 is preferentially expressed in taste buds. Copy number of mRNA for each gene was normalized to β-actin mRNA copy for each of three independent samples. (*C*) Expression profiling of isolated taste cells shows that Px1 is expressed in all receptor cells. Linear-amplified RNA from 51 individual cells was tested by RT-PCR for Px1 and for markers diagnostic of the three taste cell types. Gels show RT-PCRs for one representative cell of each type: a glial-like (Type I), a receptor (rec) (Type II), and a presynaptic (pre) (Type III) cell. The bar graph summarizes data on the prevalence of Px1 in each taste cell type and in cells lacking all three markers ("other") (n = 12, 10, 10, and 19 respectively). See SI Table 1 for complete data on all cells. (*D*) *In situ* hybridization using antisense probe (*Left*) on cryosections of vallate taste papilla shows a strong signal for Px1 in taste buds (arrowheads) but not when the control sense probe (*Right*) was used. (Scale bar, 100 μm.)

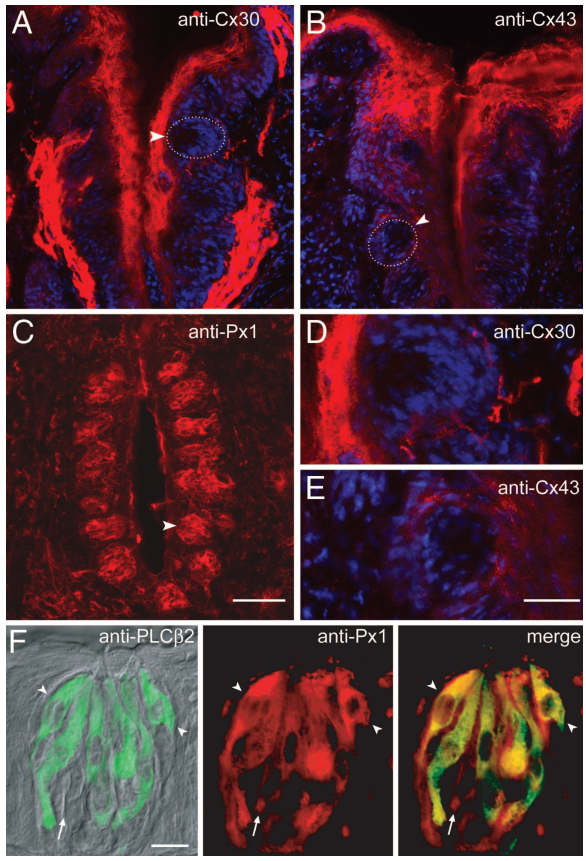

Fig. 3. Immunostaining for Cx and Px in taste buds. (*A*) Cx30 immunofluorescence (red) is prominent in keratinocytes of nontaste epithelium and in nerve fibers or glia in the lamina propria. It is undetectable in taste buds (e.g., arrowhead). Nuclei stained with DAPI reveal immunonegative cells. (*B*) Similarly, Cx43 immunostaining is prominently found in keratinocytes and minimally, if at all, within taste buds (e.g., arrowhead). (*C*) In contrast, Px1-immunofluorescence is seen in most vallate taste buds (arrowhead) and, at lower intensity, in other cells in the surrounding tissue. (*D*) A taste bud (from *A*) at higher magnification, shows no immunoreactivity for Cx30, although surface keratinocytes and a nerve plexus around the taste bud are stained. (*E*) Higher magnification (from *B*) demonstrates that taste buds lack Cx43 signal, although surrounding epithelial cells are immunopositive. (*F*) Higher magnification of Px1 immunostaining in one taste bud. Double immunofluorescence shows that receptor cells, identified with anti-PLCβ2 (*Left*, green; merged with Nomarski optics) also are immunostained with anti-Px1 (*Center*, red). Two representative double-immunostained cells are indicated (yellow, arrowheads) in the merged image (*Right*). Px1 is also detected in some taste bud cells that lack PLCβ2 (arrow), consistent with cDNA profiling results in Fig. 2*C*. [Scale bars, 100 μm (*A–C*); 50 μm (*D* and *E*); 10 μm (*F*).]

with ATP (5 μM) resulted in a pronounced increase in fluorescence in many taste cells (Fig. 4 *B* and *C*). Approximately 2/3 of the cells showed ATP-stimulated uptake above background (Fig. 4*B*). This is consistent with the broad incidence of Px1 expression in taste cells (Figs. 2 *C* and *D* and 3*C*) and the fact that not all taste cells are stimulated by ATP. More important, when the gap junction antagonist carbenoxolone (5 μM) was included during ATP-stimulation, dye uptake was significantly reduced (Fig. 4 *E* and *G*). At this low concentration, carbenoxolone is a selective blocker of Px1 hemichannels (12, 17). ATP-stimulated dye uptake recovered upon washout of carbenoxolone(Fig. 4 *F* and *G*). As a control for nonspecific membrane permeabilization after ATP, we tested two additional dyes. Neither resorufin nor fluorescein permeated into ATP-stimulated mPx1-CHO cells, mouse erythrocytes, or mouse taste cells. Taken together, these results support the presence of functional Px1 hemichannels in taste buds.

ATP-Mediated Cell–Cell Communication in Taste Buds. We postulate (Fig. 5*G*) that taste stimulation of gustatory receptor cells leads to ATP secretion via Px1 hemichannels and that this transmitter

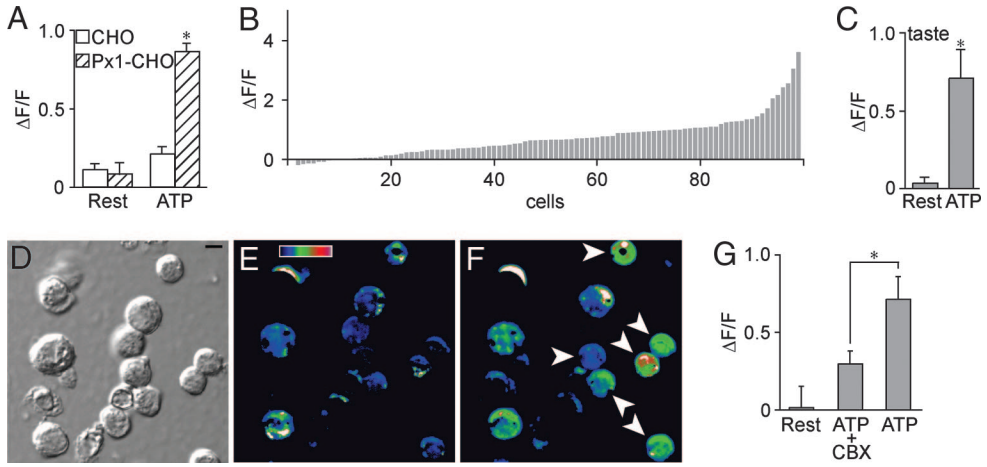

Fig. 4. Carboxyfluorescein uptake demonstrates functional Px1-like hemichannels in taste cells. (*A*) Validation of dye-uptake assay for Px1 hemichannels. CHO cells, control (open bars) or transfected with mouse Px1 (hatched bars), were incubated with carboxyfluorescein (5 mM) to estimate uptake at rest. Cells were stimulated with 5 μM ATP to increase $[Ca^{2+}]_i$ and open Px1 hemichannels while dye was still present. Bars represent mean fluorescence ± SEM., normalized to maximum, ATP-stimulated fluorescence for each cell ($n = 113$ from three independent experiments; $P \leq 0.05$). (*B*) Isolated mouse vallate taste cells, incubated in dye and stimulated with 5 μM ATP as in *A* show a broad range of ΔF/F (increase in relative fluorescence over background level) upon stimulation with ATP. (*C*) Mean ± SEM. ($n = 52$ cells) of the relative increase of fluorescence. (*D–F*) Confocal micrographs of isolated taste cells in the dye assay. (*D*) Bright-field (Nomarski optics) image of taste cells. (*E*) Fluorescence intensity (represented by pseudocolor) after ATP-stimulated dye uptake in the presence of 5 μM carbenoxolone (cbx) to block Px1 hemichannels. (*F*) Fluorescence levels in the same field of cells after cbx was washed out and cells were restimulated with ATP in presence of dye, as in *E*. Basal level of fluorescence (i.e., unstimulated dye uptake) was subtracted from images *E* and *F*. Examples of cells that took up dye are indicated with arrowheads. (Scale bar, 5 μm.) (*G*) Summary of effects of cbx on dye uptake in isolated mouse taste cells (mean ± SEM.; $n = 99$ cells; *, $P \leq 0.05$).

acts on both afferent terminals and presynaptic cells. In turn, presynaptic cells release 5-HT. We tested this model by conducting biosensor experiments on whole taste buds isolated from mouse vallate papillae (Fig. 5). First, we confirmed that taste stimulation evoked 5-HT release from intact taste buds, as previously shown (3). We then bathed taste buds in 10 μM suramin, a broad purinoceptor antagonist. Tastant-evoked release of 5-HT was strongly and reversibly reduced (Fig. 5*B*). Suramin had no effect on the 5-HT biosensor itself, nor did it affect KCl-triggered release of 5-HT (Fig. 5*B*), confirming that direct depolarization of presynaptic cells by-passes cell–cell communication. To confirm that ATP is the signal transmitted

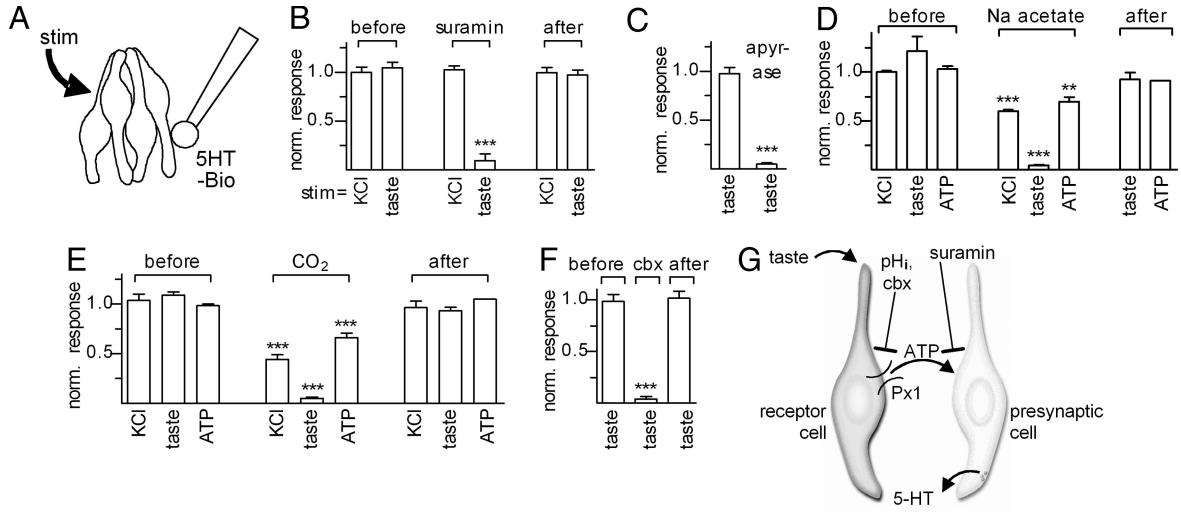

Fig. 5. Taste-evoked release of 5-HT from taste buds is abolished by treatments that interrupt ATP transmission within the taste bud. (*A*) Diagram showing stimulus/recording arrangement for the experiments in *B–F*. A 5-HT biosensor cell is apposed to an isolated taste bud and evoked release of 5-HT is measured under varying treatments. The biosensor cell itself was unaffected by these treatments (SI Fig. 6). (*B*) Suramin, a broad-spectrum P2 receptor antagonist, blocked 5-HT release evoked by taste stimulation (taste mixture as in Fig. 1) but not by KCl depolarization. (*C*) bathing taste buds in exogenous ATPase (apyrase, 10 units/ml) strongly reduced taste-evoked 5-HT release, consistent with ATP mediating cell–cell signaling. (*D–F*) treatments that block gap junction hemichannels eliminate taste-evoked 5-HT release. (*D*) Intracellular acidification produced by 20 mM Na acetate eliminated taste-evoked 5-HT release. [The 5-HT release evoked by direct stimulation of presynaptic cells by KCl depolarization or by ATP (1 μM) was also reduced, possibly because of actions of decreased cytosolic pH on voltage-gated Ca channels and second messenger cascades, respectively.] (*E*) Similarly, buffer equilibrated with 5% CO_2 eliminated taste-evoked 5-HT release and decreased 5-HT release evoked by direct stimulation of presynaptic cells. (*F*) Carbenoxolone (5 μM), a potent and selective blocker of Px1 gap junction channels at this concentration, eliminated taste-evoked 5-HT release. (*G*) proposed model for cell–cell communication in mouse vallate taste buds. Taste stimulation of receptor cells opens Px1 hemichannels, leading to ATP secretion. Secreted ATP acts on nearby serotonergic presynaptic (Type III) cells to stimulate 5-HT release. (Because afferent fibers are absent from our experiments, we have omitted the role of ATP in stimulating sensory afferents.)

between receptor cells and presynaptic cells, we bathed isolated taste buds in an ATPase, apyrase. This treatment significantly decreased tastant-evoked 5-HT release (Fig. 5*C*), similar to the reported effect of apyrase on quenching ATP-mediated paracrine communication in neurons and glia (18). Finally, ATP itself also stimulated 5-HT release (Fig. 5 *D* and *E*).

To test whether gap junction hemichannels mediated ATP-mediated cell–cell communication, we used three independent methods that close these channels (9, 19, 20): carbenoxolone, sodium acetate, and CO_2-equilibrated buffer (the latter two acidify the cytosol). All these conditions completely and reversibly blocked taste-evoked 5-HT release (Fig. 5 *D–F*). (Intracellular acidification by CO_2 or sodium acetate also reduced KCl- and ATP-stimulated 5-HT release, possibly because of the effects of H^+ on voltage-gated Ca channels and on second-messenger cascades downstream of ATP receptors). The findings are consistent with carbenoxolone and intracellular acidification closing hemichannels, blocking taste-stimulated release of ATP (Fig. 1*H*) and thus interrupting cell–cell signaling between receptor cells and serotonergic presynaptic cells.

Discussion

Gustatory stimulation evokes ATP release from taste epithelium (2). Yet, bitter-, sweet-, and umami-sensitive cells within taste buds (i.e., sensory receptor cells) lack recognizable synapses and other hallmarks of vesicle exocytosis such as SNAP25 and voltage-gated Ca channels (4–6, 21). Synaptic specializations are found only in taste bud presynaptic cells, which do not directly detect tastants. Our demonstration that Px1 hemichannels underlie ATP secretion from taste receptor cells explains the lack of conventional synaptic features in those cells. Nonvesicular release of ATP through hemichannels is well documented for glia, endothelial cells, and erythrocytes (7, 8, 10).

Although Px1 also makes intercellular gap junctions when overexpressed in juxtaposed *Xenopus* oocytes, there is no evidence for Px1 gap junctions endogenously or *in vivo* (22, 23). Instead, Px1, expressed in many neuronal and epithelial tissues, is thought to form hemichannels (17, 22, 23). Importantly, Px1 hemichannels underlie stretch-induced ATP release from erythrocytes (8) and ATP secretion from glial cells during Ca^{2+} waves (17). Px1-mediated (nonvesicular) release of ATP as a neurotransmitter has not been reported.

Phospholipase C (PLCβ2)-mediated intracellular Ca^{2+} release is a canonical feature of taste transduction for sweet-, bitter-, and umami-tasting compounds (1, 24). Additionally, TRPM5, a nonselective cation channel expressed in receptor cells (25) is a critical component of taste transduction (24, 26), but its exact role remains undefined. Our findings provide a link, missing until now, between the initial steps of taste transduction and the subsequent release of neurotransmitters. TRPM5, activated by released Ca^{2+}, allows cation influx and consequently, membrane depolarization (27). Px1 hemichannels are activated both by cytoplasmic Ca^{2+} and by membrane depolarization (8, 11, 17, 28). Thus, tastant-evoked ATP secretion via Px1 hemichannels may be triggered by the concurrent elevation of Ca^{2+} (PLCβ2-mediated) and membrane depolarization (TRPM5-mediated). Other cells in the taste bud that express Px1 channels (without TrpM5) may not exhibit this combination of tastant-evoked Ca^{2+} and depolarization. This may explain the restriction of ATP release to receptor cells.

A recent report confirmed Cx and Px expression in taste tissue and speculated that either or both may be involved in ATP release (29). Although some Cxs also are reported to produce hemichannels, it is unlikely that they contribute to ATP release from taste buds because most Cx hemichannels open only under conditions of extremely low (nonphysiological) extracellular Ca^{2+} (17, 20). Possible exceptions to this generalization are Cx30.2 and Cx32 (13, 30). We saw no evidence for expression of either of these Cxs in taste buds.

Our findings are consistent with both ATP and 5-HT secretion being important in taste buds. Although the specific role of 5-HT remains illusive, it has been proposed as a paracrine or afferent neurotransmitter (31, 32). Human taste thresholds were recently shown to be affected by altered levels of 5-HT in taste buds (33). Finger *et al.* (2) showed that gustatory epithelium secretes ATP upon taste stimulation and that the ATP acts on sensory afferent fibers. Our present results extend those findings by specifying which cells release ATP (receptor cells), and how ATP is secreted, i.e., via Px1 hemichannels. We also show that ATP plays a key role in cell–cell communication within the taste bud. ATP secretion via hemichannels, as opposed to exocytosis at discrete synapses, may explain the curiously broad tuning (sweet, bitter, etc.) of taste afferent fibers despite the narrow "molecular tuning" of receptor cells (24). Taste-evoked ATP secretion from gustatory receptor cells challenges the existence of highly selective synaptic interactions between specific receptor cells and individual sensory afferent fibers. There may instead be a more diffuse activation of one or more afferent terminals in the vicinity of the stimulated receptor cell(s). Furthermore, transmitter secretion via hemichannels would be more compatible with a continually renewing population of taste receptor cells, rather than making and breaking highly specialized synaptic connections.

Materials and Methods

Physiological Buffers, Dyes, and Reagents. Taste bud isolation and recordings were conducted in Tyrode's: 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, 10 mM Na pyruvate, and 5 mM $NaHCO_3$, pH 7.2 (318–323 mOsm). Dyes, 5,6-carboxyfluorescein and TO-PRO-3 iodide, were from Invitrogen (Carlsbad, CA). All other reagents were from Sigma (St. Louis, MO).

Taste Bud Collection and Cell Isolation. Adult C57/BL6 mice were killed following National Institutes of Health guidelines, as approved by the University of Miami Animal Care and Use Committee. Tongues were injected with enzymes and taste buds were collected (3, 4). For single taste cell isolation, peeled epithelium was redigested in enzyme mix for 2 min, and taste buds were collected and gently dissociated. Individual taste cells were collected with a polished glass pipette and transferred to a recording chamber.

5-HT and ATP Biosensors. The 5-HT biosensors were described in ref. 3. ATP biosensors consisted of CHO cells transfected with rat P2×2 and P2×3 receptors (34). Biosensor cells were loaded with 4 μM Fura2-AM For each experiment, we selected a 5-HT or ATP biosensor cell by initially applying 3 nM 5-HT or 300 nM ATP, respectively, and identifying responsive cells. (5-HT or ATP at these concentrations does not stimulate taste cells, which were also in the recording chamber).

RNA Preparation and RT-PCR. Total RNA was extracted from taste bud-enriched (or equivalent-sized nontaste) epithelium (4). Contaminating genomic DNA was removed with DNase I, and RNA was reverse-transcribed with SuperScript III (Invitrogen). One taste bud equivalent of cDNA was used for each PCR. Primers are listed in SI Table 2. Amplification was for 30 (Cx30, Cx43 and Px1) or 35 cycles. Real-time RT-PCR was carried out on an iCycler with reagents from Bio-Rad (Hercules, CA). Melting analysis confirmed that each product was homogeneous and specific. Three samples of each tissue were independently dissected and processed; each cDNA was amplified in triplicate for every gene analyzed. Standard curves were run in parallel by using sequence-validated templates, and mRNA concentration

was estimated by using MyiQ Real-Time PCR Detection Software (v1.0). β-Actin mRNA served as a reference for normalization. Negative (no template) controls were run in parallel. Single-cell expression profiling through linear T7 RNA amplification was as described (4).

***In Situ* Hybridization.** Px1 cDNA (396–1575 bp on coding sequence, BC049074) was produced by RT-PCR from mouse brain, cloned into pSPT18/19, and confirmed by sequencing. Digoxigenin-labeled RNA probes were synthesized with T7 RNA polymerase. Mice were perfused with 4% paraformaldehyde, and taste papillae were postfixed for 1 h at 4°C and cryosectioned (20 μm). Sections were treated with proteinase K (5 μg/ml) for 5 min and 0.3% H_2O_2 for 20 min, prehybridized (in 50% formamide, 5× saline sodium citrate (SSC), 5× Denhardt's solution, 500 μg/ml herring sperm DNA) at 45°C for 5 h. Hybridization was with 200 ng/ml probe in the same buffer at 45°C for 16 h. Sections were washed sequentially in 5× SSC, 0.2× SSC at 45°C for 1 h, and 0.2× SSC for 5 min. Signals were detected with anti-digoxigenin-alkaline phosphatase conjugate and a Fast Red substrate (HNPP Fluorescent Detection Set; Roche, Indianapolis, IN).

Immunostaining. Primary antibodies used were 1:2,000 chicken anti-Px1 (ANT0027; Diatheva, Fano, Italy), 1:1,000 anti-His (#34698, Qiagen, Valencia, CA), 1:2,000 rabbit anti-PLCβ2 (Santa Cruz Biotechnology, Santo Cruz, CA). Immunostaining of cryosectioned tissues or cultured cells was as reported (4). Px1 signal was amplified with tyramide (Invitrogen). Negative controls were run in parallel and included omitting primary antibody and preabsorbing against antigen (see SI Fig. 10).

Dye-Uptake Assay. Full-length mouse Px1 cDNA was produced by RT-PCR and validated by sequencing. A His_6 tag was then introduced at the C terminus. Both tagged and untagged constructs were cloned into pcDNA3. The tagged construct was used for validating anti-Px1 antibody by immunoblot and immunofluorescence (SI Figs. 7 and 9). The untagged construct was used for functional experiments (Fig. 4).

Isolated taste cells were transferred to an imaging chamber. Unhealthy and dead cells were identified with a membrane-impermeant nuclear dye TO-PRO-3 (1 μM) and were omitted from analyses. Cells were viewed with a confocal microscope (Fluoview; Olympus, Melville, NY). To test for ATP-stimulated dye uptake, cells were perfused with Tyrode's solution containing 5 mM 5,6-carboxyfluorescein with or without 5 μM ATP for 10 min. Cells were then washed with Tyrode's solution for 15 min. Background uptake of the dye was defined as fluorescence achieved in the absence of ATP (i.e., "rest") (see SI Fig. 8). Signals in Fig. 4 are expressed as relative change of fluorescence, $\Delta F/F = (F - F_0)/F_0$, where F_0 denotes background fluorescence.

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