Expression and Trans-synaptic Regulation of P$_{2x4}$ and P$_{2z}$ Receptors for Extracellular ATP in Parotid Acinar Cells

EFFECTS OF PARASYMPATHETIC DENERVATION*

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Trans-synaptic regulation of muscarinic, peptidergic, and purinergic responses after denervation has been reported previously in rat parotid acinar cells (McMillan, M. K., Soltoff, S. P., Cantley, L. C., Rudel, R., and Talamo, B. R. (1993) Br. J. Pharmacol. 108, 453–461). Characteristics of the ATP-mediated responses and the effects of parasympathetic denervation were further analyzed through assay of Ca$^{2+}$ influx, using fluorescence ratio imaging methods, and by analysis of P$_{2z}$ receptor expression. ATP activates both a high affinity and a low affinity response with properties corresponding to the expression. ATP activates both a high affinity and a low affinity response with properties corresponding to the recently described P$_{2x4}$ and the P$_{2z}$ (P$_{2x7}$)-type purinoceptors, respectively. Reverse transcription-polymerase chain reaction analysis reveals mRNA for P$_{2x4}$ as well as P$_{2x7}$ subtypes but not P$_{2x1}$, P$_{2x2}$, P$_{2x3}$, P$_{2x5}$, or P$_{2x6}$. P$_{2x4}$ protein also is detected by Western blotting. Distribution of the two types of ATP receptor responses on individual cells was stochastic, with both high and low affinity responses on some cells, and only a single type of response on others. Sensitivity to P$_{2x4}$-type activation also varied even among cells responsive to low concentrations of ATP. Parasympathetic denervation greatly enhanced responses, tripling the proportion of acinar cells with a P$_{2x4}$-type response and increasing the fraction of highly sensitive cells by 7-fold. Moreover, P$_{2x4}$ mRNA is significantly increased following parasympathetic denervation. These data indicate that sensitivity to ATP is modulated by neurotransmission at parasympathetic synapses, as well as in the nervous system where P$_{2x4}$ receptors are widely expressed.

Extracellular ATP acts as a signaling molecule through the interaction of ATP with ligand-gated ion channels (P$_{2x}$) as well as metabotropic receptors (P$_{2z}$) (for reviews see Refs. 2–4). These receptors are distributed throughout the body, including synapses where ATP seems to function as a neurotransmitter (5, 6). The pharmacology and physiology of ATP-activated ion fluxes indicate the existence of multiple subtypes of P$_{2x}$ receptors, confirmed by molecular cloning of seven genes for P$_{2x}$ receptor subunits (3, 7–17).

Relatively little is known about the physiological regulation of P$_{2z}$ receptors. We have shown that removal of the autonomic innervation alters ATP responses in parotid acinar cells, which therefore provide a good model for investigating trans-synaptic regulation of receptor-mediated signaling. We previously described two ATP responses in rat parotid acinar cells, a P$_{2x}$/P$_{2z}$ type and a high affinity ATP receptor with distinctly different P$_{2z}$-like pharmacology (1).

Expression of the P$_{2z}$-type proteins in heterologous systems shows that each can form functional, homomeric, ligand-gated ion channels with a significant permeability to Ca$^{2+}$ ions as well as other inorganic cations, including Na$^+$ and K$^+$. Properties of P$_{2x7}$ receptors differ substantially from the other subtypes of P$_{2z}$ receptors and are similar to the P$_{2z}$ receptor response that has been extensively described in macrophages and related cells. P$_{2z}$ receptors not only function as cation-permeable channels but also are capable of mediating the flux of larger organic molecules of sizes ranging up to 900 Da without regard to the charge on the molecule (17, 18). Although P$_{2z}$ receptors are present on various cells of the immune lineage (17, 19–24), only parotid acinar cells are directly innervated and regulated by neuronal activity.

Sympathetic and parasympathetic nerves control the secretion and composition of parotid saliva (25). Physiologically mediated changes in tonic patterns of nerve activity elicited by surgical denervation or by dietary manipulation alter the rat parotid gland, modifying acinar cell proliferation, cell size, and sensitivity of parotid secretion to neurotransmitters (26–30). Parasympathetic denervation enhances secretory responses elicited in vivo by activation of calcium-mobilizing neurotransmitter receptors linked to phospholipase C (26–28). Our previous denervation studies also established that trans-synaptic regulation of sensitivity to these neurotransmitters can be demonstrated in vitro in dissociated cell suspensions and showed for the first time that sensitivity to ATP is increased very dramatically (1, 31). This suggests that P$_{2x}$ purinoceptors are modulated by changes in synaptic activity and that ATP plays a role in the physiologically important regulation of food intake and metabolism.

Here, we further characterize and identify the high and low affinity ATP receptors in parotid acinar cells and determine whether the receptors are co-expressed or independently distributed. P$_{2x}$ receptor types were classified in individual cells by pharmacology of the Ca$^{2+}$ response to ATP, as analyzed by fluorescence ratio imaging of cells loaded with Fura-2. The properties of the responses are consistent with those observed for homomeric P$_{2x4}$ receptors and P$_{2x7}$ receptors in expression sys-
tems. RT-PCR\textsuperscript{1} amplification provided evidence for expression of the cognate mRNAs. Imaging experiments established that 1) the high and low affinity responses are independently expressed among acinar cells and likely mediated by P2\textsubscript{x4} and P2\textsubscript{x7} receptors, respectively; 2) denervation produces an increase in sensitivity to ATP (decrease in threshold) in individual cells; and 3) denervation leads to an increase in the number (proportion) of cells with high affinity ATP responses. Both responses to denervation could be explained, at least in part, by quantitative RT-PCR data showing that P2\textsubscript{x4} receptor mRNA increases following denervation. These data suggest that elevated P2\textsubscript{x4} receptor protein contributes to the observed increases in the sensitivity and total magnitude of the glandular response.

**EXPERIMENTAL PROCEDURES**

**Dissociated Acinar Cell Preparation**—Dissociated parotid acinar cells were prepared by trypsin and collagenase treatment as described previously (32). The cell pellet was suspended in oxygenated HEPES/Ringer buffer of the following composition (in mM): 120 NaCl, 5 KCl, 2.2 MgCl\textsubscript{2}, 1 CaCl\textsubscript{2}, 20 HEPES, 5 β-hydroxybutyrate, 10 glucose, and 0.1% bovine serum albumin, pH 7.4. This preparation is composed of single cells and small clumps containing up to five cells; acinar cells comprise 85% or more of the dissociated cell preparation.

**Parasympathetic Denervation**—Unilateral deafferentation of one parotid gland of Sprague-Dawley rats (50–100 g) was carried out surgically by avulsion of the right auriculotemporal nerve, which carries post-ganglionic parasympathetic nerve fibers. Tetracycline HCl (Polygram Line, Englewood, NJ) was administered intraperitoneally for 4 days ad libitum for 4 days before the experiment. The parotid glands were denervated as described above, removed 2–3 weeks later, and immediately immersed in liquid nitrogen.

**Acinar Cell Culture**—Parotid glands of Sprague-Dawley rats (50–100 g) were dissected and pooled, crushed to a fine powder. The powdered tissue was washed three times with Fura-2 acetoxyethyl ester (1.5 μM) for 60 min at room temperature as described previously (32). Washed cells were suspended in Mg\textsuperscript{2+}-free HEPES/Ringer buffer, and 150 μl of Fura-2-loaded cells were plated on an acid-washed glass coverslip coated with concanavalin A (1 mg/ml) 5–10 min before mounting in the perfusion chamber for Ca\textsuperscript{2+} measurements. Ca\textsuperscript{2+} was estimated by the ratio method, using a K\textsubscript{0} of 2 mM for Ca\textsuperscript{2+} binding to Fura-2 (33). Maximum and minimum fluorescence values were obtained by the addition of ionomycin and EGTA, respectively, to the perfusion buffer.

**Fluorescence Ratio Imaging**—Cells were imaged using a Nikon Diaphot inverted fluorescence microscope equipped with a Xenon light source, Fura-2 barrier and emission filter sets, and a CCD camera. A computerized filter wheel (Sutter Instruments) equipped with an electronic shutter regulated excitation at 340 or 380 ± 10 nm. Data were collected on a 486 computer equipped with an Iteh 100 frame-grabbing board. Software was developed to collect data simultaneously from as many as 50 cells, by placing circles of about 50 pixels in area over intracellular regions of fluorescent cells imaged on a 512 × 512 video monitor. Fluorescent cells were visualized using a Nikon fluorescent oil immersion objective and excitation light at 380 nm. Cells with the morphology of polarized acinar cells were selected for analysis, including both fully dissociated individual cells as well as polarized cells organized in clusters of three to five cells around a lumen. Strings of duct cells were not analyzed. Ratio-pair data from all selected areas were usually collected every 5–7 s during the experiment. Data from a single frame were saved with no averaging. Background fluorescence was determined by placing a data collection circle in an area without cells; this value was subtracted for each data point before calculating the ratio of 340:380 measurements.

**TABLE I**

| P2\textsubscript{x4} | GGGTGGGTTTGGTCATGAA | GCTGATGCTGTGTTTGTGGA | 447 |
|----------------------|----------------------|-----------------------|-----|
| P2\textsubscript{x3} | TGGGACTCAGAGACCCTAAG | ATGGTGGAGGATGACTGAT | 953 |
| P2\textsubscript{x3} (up/lo) | TTTTGGGGTTGTTTCTTCTG | TAATGGGCGGATGTGTGGA | 867 |
| P2\textsubscript{x4} (A/V) | AACACTCTCAGTTGGAAT | AGGTGAGGCTGTGATGTTG | 418 |
| P2\textsubscript{x4} (A/Par) | AACACTCTCAGTTGGAAT | AGGTGAGGCTGTGATGTTG | 418 |
| P2\textsubscript{x5} | AGCCCTCACTGAAACACAC | AGGTGAGGCTGTGATGTTG | 927 |
| P2\textsubscript{x6} | GCTGGGATTTGCTTACCTACA | GCTGATGCTGTGTTTGTGGA | 613 |
| P2\textsubscript{x7} (Trunc) | TTTTACAGGGTGGCGGTCTCA | TGGTGAGGAGAGGGAGGAGAGGAC | 578 |
| P2\textsubscript{x7} (C-term) | TGGTGGATTTGCTTACCTACA | GCTGATGCTGTGTTTGTGGA | 529 |
| P2\textsubscript{x7} (3'-UTR) | GTTTTGACATCCTGGTTTTTG | GGGGCTTTTAGTGGTTTCTG | 1677 |
| GAPDH | CATCATCCATCATCTGGGAGG | CCGTTTCTCCCACTCCCTGGT | 505 |

\textsuperscript{1} The abbreviations used are: RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s); GDP\textsubscript{S}, guanosine 5'-β-thio)diphosphate; PPAD, pyridoxal phosphate-6-azophenyl-2',4'-difulorid acid; H\textsubscript{2}DIDS, dihydro-4,4'-disothio-2,2'-stilbenedisulfonate.
the internal standard is shorter than the product from the cDNA, and they can be separated by gel electrophoresis (see Fig. 7a). The ratio of the intensities of the two bands is derived from densitometric measurements of the ethidium bromide-stained gel. Ratios from data of Fig. 7a are plotted in Fig. 7b. At a ratio of 1.0, the intensity of the two bands is the same, and the quantity of mRNA in the original sample may be read on the x axis.

The competitive template for P2x4 was constructed using a plasmid (pcDNA3, Invitrogen) containing the P2x4 receptor cDNA (courtesy of G. Buell, Glaxo (Ref. 12)). Two HaeIII restriction endonuclease sites within the sequence amplified by PCR with primer set P2x4 (up/lo) were employed to remove 200 bp from the cDNA. The fragments were then ligated back into the empty vector to create the template, P2x4*HaeIII. A similar template was generated from a plasmid containing the human GAPDH cDNA (pHCAP; ATCC, Rockville, MD) by restriction digest with XbaI and BstXI to remove 95 bp. The resulting product was then blunted with T4 DNA polymerase (New England Biolabs) and ligated to form GAPDH X/B. The nucleotide sequence recognized by the primers is the same for both the human and rat GAPDH cDNA. Identical primers were used for competitive templates and the cDNA samples, and products differed only slightly in size, so both were amplified with the same efficiency. In some experiments, annealing between the heterologous strands of the competitor and cDNA products was observed. The third band generated in this manner was not included in the analysis because it contains equal quantities of both products and did not affect the quantification.

**Heterologous Expression and Immunoblotting of P2x4 Receptor Protein—**Immunoblotting of P2x4 receptor protein was carried out using samples from rat parotid gland and, as a control, human embryonic kidney 293 cells (293 cells), which heterologously expressed the receptor. A plasmid containing the full-length cDNA sequence for the rat P2x4 receptor (p484) was transfected into the 293 cells using the calcium phosphate precipitation method of Okayama and Chen, as modified by Pritchett et al. (35). Protein samples from parotid gland samples were prepared by freezing and pulverizing the tissue in liquid nitrogen followed by direct transfer into SDS sample buffer containing 62.5 mM Tris-HCl, 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate, 5% (v/v) 2-mercaptoethanol, 0.1 mg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 2 mM EDTA, pH 6.8. The sample was then homogenized for 30 s using a Brinkman polytron and boiled for 5 min. Extracts from 293 cells were prepared by scraping the cells from 100-mm tissue culture dishes in SDS sample buffer and homogenizing the sample as described above. Total protein was separated by 10 or 12% SDS-polyacrylamide gel electrophoresis, and the P2x4 receptor was detected by immunoblotting with an antibody (final concentration, 2 µg/ml; kindly donated by Gary Buell, Glaxo, Geneva) against the COOH-terminal of the rat protein. Crude anti-serum and affinity-purified antibody were used in these experiments. Some blots were also reacted with antibody preabsorbed for 2 h at 20°C with the antigenic peptide (20 µg/ml). Protein concentrations were determined by the Coomassie protein reagent assay (Pierce). P2x4 receptor-like immunoreactivity was visualized by the enhanced chemiluminescence method (Pierce).

**RESULTS**

The basis for increased sensitivity of denervated parotid acinar cells to ATP was investigated by characterizing Ca2+i responses in individual cells and by molecular analysis.

The properties of the Ca2+i responses have been partially determined in previous studies and can be evaluated through Fura-2 analysis. Although muscarinic, α-adrenergic, and substance P receptors in parotid acinar cells elevate Ca2+i through G protein-coupled activation of phospholipase C and subsequent production of inositol 1,4,5, trisphosphate (32, 36, 37), our results show that the ATP responses do not seem to be of the metabotropic type. Both ATP responses required extracellular Ca2+i. ATP is not effective in stimulating inositol phosphate formation in this preparation (however, see also Ref. 38), and whole cell patch clamp recordings demonstrate that activation of whole cell currents by ATP does not involve a GDPβS-sensitive step (36).

P2x3 responses in parotid cells resemble those in other cell types, requiring relatively high concentrations of ATP (100 µM or greater) to stimulate influx of both Na+ and Ca2+ (39). 3’-O-(4-benzoyl)benzoyl-ATP is the most potent and effective agonist, and the response is inhibited by high concentrations of divalent cations such as Mg2+, Brilliant Blue G, reactive blue 2, and H2DIDS (36, 40). The second type of response to ATP is of higher affinity (EC50 less than 10 µM), insensitive to nucleotides UTP, GTP, adenosine, and α,β-methylene ATP and weakly responsive to 2-methylthio-ATP. Further, it is neither activated by 3’-O-(4-benzoyl)benzoyl-ATP nor inhibited by high concentrations of divalent cations, Brilliant Blue G, reactive blue 2, and H2DIDS (1, 36, 40). An additional distinguishing feature of the two responses to ATP is that they are differentially modulated by protein kinase inhibitors (39).

**Characterization of Calcium Mobilization by Multiple Neurotransmitters in Individual Parotid Acinar Cells**

Ca2+i is elevated in parotid acinar cells by at least four different neurotransmitters, including agonists of the metabotropic G protein-coupled receptors and P2x purinoceptors for extracellular ATP. To determine whether the muscarinic and P2x receptors are differentially distributed across acinar cells, Fura-2 loaded cells were perfused sequentially with maximally effective concentrations of the agonists carbachol (30 or 100 µM) and ATP (30 or 300 µM). Between agonist doses, cells were washed with buffer until Ca2+i values returned to basal levels. Almost all cells responded to maximal concentrations of carbachol, and cells that did not respond to carbachol rarely showed responses to ATP. Only the cells responding to carbachol were analyzed. Among the carbachol-responsive cells, 93% (75/81) also responded to 300 µM ATP (Fig. 1), a concentration that would activate both high and low affinity receptors. This indicated that the majority of cells had P2x receptors. Addition of a maximal dose of carbachol rapidly elevated Ca2+i to a peak value that then declined to a slightly lower steady-state level followed by recovery to a basal value when agonist was washed out.

**Two Distinct ATP Responses (Receptors) in Individual Parotid Acinar Cells**

*Heterogeneity Shown by Dose-dependent Responses—*Previous studies using cell suspensions demonstrated that the response to ATP is biphasic and that there are two pharmacologically distinct responses. However, under those assay conditions, the signal is a composite of responses from a large population of cells, and it is difficult to determine whether the
two responses arise from subsets of cells with different receptors or whether the two receptors are homogeneously distributed across all responding cells. In the present studies, the distribution of the two distinct receptor types was characterized on individual cells. Cells were assayed in buffer without added Mg$^{2+}$ to optimize the $P_{2z}$ response. Perfusion with both low and high concentrations of ATP identified some cells that displayed only the low affinity or only the high affinity response as defined below, but many cells showed both responses (Fig. 1). In 73% of the cells with responses to 300 μM ATP (164/225 cells), a small, rapid elevation in Ca$^{2+}$ was detected at low concentrations of ATP (1–30 μM, high affinity response), and Ca$^{2+}$ usually reversed to basal level when agonist was washed out (Fig. 1). At higher concentrations of ATP (300 μM), Ca$^{2+}$ increased more slowly and reached higher levels (low affinity response). After exposure to 300 μM ATP, the elevated Ca$^{2+}$ did not always reverse to basal level after washing out the ATP (Fig. 1).

**Effects of Mg$^{2+}$ and High Concentrations of Divalent Cations on ATP Responses in Single Cells**—In parotid acinar cell suspensions, the large increase in Ca$^{2+}$ in response to a high concentration of ATP (300 μM) is primarily mediated by $P_{2z}$ receptors. Thus, Mg$^{2+}$ or high concentrations of Ca$^{2+}$ would be expected to reverse or block this response in cells with low affinity receptors (see for example, Ref. 41). To test this, single cells were first exposed to 300 μM ATP, and Mg$^{2+}$ was then added to the perfusion chamber (final concentration = 1 mM). The effect of 300 μM ATP was reversed by Mg$^{2+}$ (n = 22). When 1 mM Mg$^{2+}$ was present together with ATP in solution, the low affinity response to 300 μM ATP was blocked (Fig. 3b). The effect of a high concentration of divalent cation also could be demonstrated by stimulating cells with various concentrations of ATP, first at low concentrations of CaCl$_2$ (3 mM) and then at high concentrations of CaCl$_2$ (10 mM) (Fig. 2). When cells suspended in Mg$^{2+}$-free KRH containing 3 mM CaCl$_2$ were exposed to 30 and 60 μM ATP, Ca$^{2+}$ rose rapidly and then recovered to basal when ATP was removed by washout. Under identical conditions, exposure of the same cells to 300 μM ATP led to a larger and less rapid increase in Ca$^{2+}$. Raising the extracellular Ca$^{2+}$ concentration from 3 to 10 mM (Mg$^{2+}$-free conditions, Fig. 2) did not significantly alter the response of the cells to 300 μM ATP but the large response to 300 μM ATP was reduced to a value comparable with that elicited by 30 μM ATP (n = 10 cells). This depression by high Ca$^{2+}$ was not nonspecific, as the response to carbachol at the end of the experiment was normal. Mg$^{2+}$ also did not alter the response to low concentrations of ATP, indicating that divalent cations do not interfere with the high affinity response/receptor, consistent with our previous findings in cell suspensions. Inhibition of the low affinity response by the addition of 1 mM Mg$^{2+}$ (at 1 mM Ca$^{2+}$) or an increase of the calcium concentration from 3 to 10 mM might be due to alterations in the amounts of different species of ATP (such as ATP$^3-$, Mg$^{2+}$ ATP, Ca$^{2+}$ ATP), which vary in agonist efficacy. However, the effect of divalent cations on the low affinity response does not directly correlate with ATP$^4+$ concentration, and the response can still be detected at 3 mM Ca$^{2+}$ but not in 1 mM Ca$^{2+}$ plus 1 mM Mg$^{2+}$, even though the ATP$^4+$ concentration is calculated to be the same (for 300 μM ATP total, ATP$^4+$ = 15 μM in 1 mM Ca$^{2+}$ plus 1 mM Mg$^{2+}$ versus 15.3 μM ATP$^4+$ in 3 mM Ca$^{2+}$) (42). These data are consistent with a recent report that divalent cations inhibit P$_{2z}$ receptors principally by a direct effect on the receptor protein (41).

**ADP Blocks Only the High Affinity ATP Responses**—We have shown previously that the low affinity calcium response to >300 μM ATP in a suspension of parotid acinar cells is activated only weakly if at all by ADP but that ADP blocked the more sensitive (high affinity) ATP response (1). In the present studies, we examined the effects of ADP on individual cells shown to have a high affinity response. Perfusion with 500 μM ADP alone had variable effects on Ca$^{2+}$, with little or no effect on Ca$^{2+}$, in most cells examined. However, in some cells a small response similar to the high affinity ATP (30 μM ATP) response was observed. In these cells responding to 30 μM ATP (n = 18), addition of ADP reversed the Ca$^{2+}$ response mediated by the high affinity ATP receptors even in the continued presence of ATP (Fig. 3a). However, ADP neither blocked (Fig. 3b) nor reversed (data not shown) the low affinity response mediated by high concentrations (300 μM) of ATP. Under conditions where only the high affinity receptor is activated (in the presence of Mg$^{2+}$), no response to ATP was observed when 500 μM ADP also was present (Fig. 3b). The response to 300 μM ATP subsequently recovered after ADP was removed by washout (Fig. 3b). These results indicate that ADP is a weak partial agonist at the high affinity ATP receptor, inhibiting further responses to 30 μM ATP but with no inhibitory effect on the low affinity receptor.

**Brilliant Blue G Blocks Only the Low Affinity ATP Responses**—Brilliant Blue G blocks the P$_{2z}$ response as well as binding of [α-32P]ATP in rat parotid acinar cell suspensions (1, 40). Consistent with these results, addition of Brilliant Blue G (1 μM final concentration) reversed the low affinity response (in 33/34 cells), even in the continued presence of ATP (Fig. 3c), but had no effect on the high affinity ATP response (detected in Mg$^{2+}$-containing solution; data not shown). The effects of Brilliant Blue G were irreversible (Fig. 3c).

**Heterogeneous Distribution of the Two Distinct ATP Responses among Individual Cells**

The effects of Mg$^{2+}$, high concentrations of Ca$^{2+}$, Brilliant Blue G, and ADP on single cell responses to ATP clearly dem-
onstrate the presence of two distinct responses, likely to correspond to P2x4 and P2x7 receptors (Table II). Evaluation of these characteristics across individual cells showed that the P2x4- and P2x7-type ATP responses were differentially distributed (Table III). Among the cells that responded to ATP, 47% (106/225) showed both the P2x4- and P2x7-type responses; 27% (61/225) had only the P2x4-type response, and 26% (58/225) showed only the P2x7-type response. This distribution was not an artifact of experimental design, as the diversity of response was observed on cells in the same experimental run, with some cells having both responses while other cells within the same acinar cluster had only one type or the other.

**Molecular Characterization of P2x Receptor Expression by RT-PCR and Western Blotting**

Identification of the High and Low Affinity Receptors as the P2x4 and P2x7 Subtypes, Respectively—The low affinity/P2x2 response to ATP in rat parotid acinar cells has the properties of heterologously expressed P2x7 receptors. The physiological properties of the high affinity response are those of P2x receptors, but previous data did not allow us to clearly identify which of the known cDNA clones is likely to mediate the response. To determine which P2x receptor mRNA subtypes are expressed, we carried out RT-PCR on total RNA from rat parotid gland using oligonucleotide primers which discriminate between the seven identified P2x receptor clones (Fig. 4). The major transcripts amplified from parotid gland cDNA were P2x4 and P2x7. Fig. 4a shows the results obtained using the P2x4 (up/lo) and P2x7 (trunc) primers (see Table I); as predicted, the sizes of these transcripts were 618 and 578 bp, respectively, and their identity was confirmed by sequencing (very low levels of PCR products were detected with P2x1 and P2x3 primers, but identification was not supported by sequencing results). No product was amplified using primers specific for P2x2, P2x5, and P2x6, consistent with reports that P2x receptor subtypes 1, 2, 3, 5, and 6 are not present in the rat salivary gland (15). We verified the functionality of primers that did not amplify a product from rat parotid cDNA by using rat genomic DNA (Fig. 4b) or cDNA from rat brain and dorsal root ganglia (Fig. 4c) as templates. With genomic DNA as a template for P2x4, P2x2, and P2x4 primers, the products were all larger than those obtained using cDNA, indicating that the primers amplify across intron/exon boundaries in the P2x receptor genes. This established that we do not have any genomic DNA contamination in our cDNA preparations. In the case of the P2x2 receptor gene, whose sequence is known, the size of the product obtained using genomic DNA as a template (2218 bp) corresponds to that predicted (43) (Fig. 4b). Controls for P2x3, P2x5, and P2x6 recep-
Parasympathetic Denervation Regulates P2 Purinoceptors

Properties of ATP responses

| Agonist concentration | High affinity response | Low affinity response |
|-----------------------|------------------------|----------------------|
| Time course           | Activated at low dose; 0.3–30 μM | Activated at high dose; 300–600 μM |
| Agonist               | ATP/MgATP              | ATP α4 |
| Mg2+                 | No effect              | Blocked or reversed |
| High concentrations of divalent cations | No effect | Blocked |
| Brilliant Blue G      | No effect              | Blocked and/or reversed |
| ADP                   | Blocked                 | No effect |

**Table III**

Heterogeneous distribution of the high and the low affinity ATP responses in individual cells

Cells that responded to ATP (n = 225) were further analyzed, and responses to ATP were classified as high affinity or low affinity based on the effects of Mg2+, high Ca2+, ADP, and brilliant blue G. Cells showing either low or high affinity responses were indicated as “+” and “−”, and cells that did not show high or low affinity responses were indicated as “−”. Percentages in parentheses indicate the fraction of total cells that had each of the response distributions noted. Cells that did not respond to ATP were not included in this table. xx, no cells in this category.

| High affinity ATP response | Low affinity |
|---------------------------|--------------|
| Response                  | Response    |
| (+)                       | (+)          |
| (+)                       | (+)          |

**Table II**

Properties of the enhanced response to ATP in Individual Cells—The threshold concentration of ATP for activation of individual cells had P2x4-type responses (defined by response to 30 μM ATP) was determined by perfusing the cells with concentrations of ATP ranging from 0.3 to 30 μM. Ca2+ responses of 20 nm or more above basal were scored as positive. Cells responding to 0.3–1.0 μM ATP were classified as supersensitive. Sensitivity of control cells was variable (Fig. 6). Activation by 0.3 μM ATP was infrequent (3% of cells) in control preparations. At higher concentrations of ATP (1 and 3 μM), the proportion of responsive cells increased to 31 and to 72%, respectively (the number of cells responding to 30 μM ATP was set at 100%).

**Fig. 4.** PCR of P2x-receptor DNA. Agarose gels stained with ethidium bromide show the products amplified by PCR using specific primers for the seven identified P2x-receptor sequences (see Table I). a, the predominant transcripts in rat parotid gland cDNA are P2x7 (X7, using P2x7 (trunc) primers) and P2x4 (X4, using P2x4 (up/lo) primers) receptors. A faint band is detected with primers for P2x3 receptor (X1), confirming that the P2x7-receptor subunit in rat parotid gland is probably identical to the protein in other tissues. These data support the conclusion that the low affinity response to ATP is mediated by the P2x7 receptor and that the high affinity response is probably due to P2x4 receptors.

**Fig. 5a.** The antibody does not react with protein from 293 cells transfected with a plasmid containing the P2x4-receptor cDNA (Fig. 5a). The antibody does not react with protein from 293 cells transfected with empty vector. Binding of the antibody is blocked by pre-incubation with the peptide to which the antibody was raised. The estimated size of the immunoreactive protein is substantially larger than the size predicted from the amino acid sequence (approximately 43 kDa), possibly due to post-translational glycosylation. Having confirmed that the antibody is specific for P2x4 receptors in a heterologous expression system, we tested for the presence of this receptor in total protein extracts from rat parotid gland. Immunoblotting with crude antisera detects a protein in parotid samples that is the same size as the protein recognized in 293 cells expressing the P2x4 receptor, and binding is blocked by pre-incubation with the antigenic peptide (Fig. 5b). This protein was very susceptible to degradation, and it was necessary to flash-freeze and crush the tissue in liquid N2 prior to adding sample buffer to obtain an intact sample.

**Sensitivity**

Receptor Sensitivity to ATP in Individual Cells—The threshold concentration of ATP for activation of individual cells that had P2x4-type responses (defined by response to 30 μM ATP) was determined by perfusing the cells with concentrations of ATP ranging from 0.3 to 30 μM. Ca2+ responses of 20 nm or more above basal were scored as positive. Cells responding to 0.3–1.0 μM ATP were classified as supersensitive. Sensitivity of control cells was variable (Fig. 6). Activation by 0.3 μM ATP was infrequent (3% of cells) in control preparations. At higher concentrations of ATP (1 and 3 μM), the proportion of responsive cells increased to 31 and to 72%, respectively (the number of cells responding to 30 μM ATP was set at 100%).

**Parasympathetic Denervation Super sensitivity to ATP—** Properties of the enhanced response to ATP were examined in individual cells after parasympathetic denervation. 2–3 weeks following parasympathetic denervation, the wet weight of parotid glands was reduced to 56.6 ± 2.5% (n = 7) of that of the contralateral glands. The fraction of cells with P2x4-type responses (to 30 μM ATP) increased dramatically in denervated glands. For reasons that are not clear, in these experiments and all subsequent experiments, fewer control

Acids for the large pore-forming function of the P2x7/P2x4 receptor (17). Each of these sets of primers yielded a single product, indicating that the P2x7-receptor subunit in rat parotid gland is probably identical to the protein in other tissues. These data support the conclusion that the low affinity response to ATP is mediated by the P2x7 receptor and that the high affinity response is probably due to P2x4 receptors.

P2x4-Receptor Protein Is Expressed in Rat Parotid Gland—We have further examined the expression of P2x4 receptors in the rat parotid gland by immunoblotting of proteins separated on denaturing SDS gels. Western blotting was carried out with a specific antibody raised against 16 amino acids in the COOH terminus of the receptor protein. The affinity-purified antibody recognizes a single protein in extracts from 293 cells transfected with a plasmid containing the P2x4-receptor cDNA (Fig. 5a). The antibody does not react with protein from 293 cells transfected with empty vector. Binding of the antibody is blocked by pre-incubation with the peptide to which the antibody was raised. The estimated size of the immunoreactive protein is substantially larger than the size predicted from the amino acid sequence (approximately 43 kDa), possibly due to post-translational glycosylation. Having confirmed that the antibody is specific for P2x4 receptors in a heterologous expression system, we tested for the presence of this receptor in total protein extracts from rat parotid gland. Immunoblotting with crude antisera detects a protein in parotid samples that is the same size as the protein recognized in 293 cells expressing the P2x4 receptor, and binding is blocked by pre-incubation with the antigenic peptide (Fig. 5b). This protein was very susceptible to degradation, and it was necessary to flash-freeze and crush the tissue in liquid N2 prior to adding sample buffer to obtain an intact sample.

Sensitivity

Receptor Sensitivity to ATP in Individual Cells—The threshold concentration of ATP for activation of individual cells that had P2x4-type responses (defined by response to 30 μM ATP) was determined by perfusing the cells with concentrations of ATP ranging from 0.3 to 30 μM. Ca2+ responses of 20 nm or more above basal were scored as positive. Cells responding to 0.3–1.0 μM ATP were classified as supersensitive. Sensitivity of control cells was variable (Fig. 6). Activation by 0.3 μM ATP was infrequent (3% of cells) in control preparations. At higher concentrations of ATP (1 and 3 μM), the proportion of responsive cells increased to 31 and to 72%, respectively (the number of cells responding to 30 μM ATP was set at 100%).
cells had high affinity receptors compared with the original series of experiments evaluating the pharmacology of the low and high affinity ATP receptors. We found that among control cells that responded well to carbachol, only 20% also responded to 30 μM ATP (46/233 cells). This does not seem to be a function of the age of the animal nor to be related to the method of preparing dissociated cells and was observed for cells prepared from unoperated animals as well as from control glands in operated animals. P2x4-type responses to 30 μM ATP were much more frequent in cells from denervated glands and were observed in 67% of the cells that responded to carbachol (222/330, n = 10 cell preparations).

Sensitivity to ATP also increased dramatically in cell preparations from parasympathetically denervated glands as shown by a drop in the threshold dose of ATP needed for activation. As noted above, in control preparations only 3% of cells with P2x4 responses were activated by 0.3 μM ATP (cells responding to 30 μM ATP set at 100%), whereas 21% of cells in denervated preparations were responsive. At 1.0 μM ATP, the proportion of responding cells increased from 31% in control cells to 54% in denervated cells. On the other hand, higher concentrations of ATP (3 μM) triggered a response in nearly the same proportion of P2x4-responsive cells in control (72%) and denervated (80%) preparations. These data are expressed in terms of the fraction of cells that are activated at different threshold concentrations of ATP in Fig. 6.

**Regulation of P2x4 Receptor mRNA Levels following Parasympathetic Denervation**—With supporting evidence that the high affinity response to ATP is mediated by the P2x4 receptors, we tested the hypothesis that up-regulation of P2x4 receptor expression underlies the supersensitivity of acinar cells to ATP following parasympathetic denervation. Competitive, quantitative PCR assays measured the relative levels of P2x4-receptor mRNA in control and denervated rat parotid glands as shown in Fig. 7. Rat parotid RNA preparations were obtained 2 weeks after parasympathectomy from control and denervated glands of eight animals. Fig. 7, a and b, show data obtained in a typical competitive PCR experiment using samples from a single animal. There was substantial variation in the yield of RNA from the glands, probably because of atrophy of the tissue and increased amounts of connective tissue in the denervated gland. We controlled for these changes by normalizing to the levels of mRNA for GAPDH determined by competitive PCR. The relative amount of P2x4-receptor mRNA, corrected for GAPDH mRNA content, was higher in the denervated gland than in the control tissue in every set of samples (n = 8). There was some variation in these values, but on average, 2.8 times more P2x4-receptor mRNA was present in the denervated tissue (Fig. 7c) compared with the control side (n = 7). In one animal, the level of P2x4 receptor mRNA in the control gland was below detectable limits even though the levels of GAPDH mRNA indicated that the yield of cDNA in this preparation was not lower than average. P2x4 receptor mRNA was up-regulated in the denervated gland from this animal, which is consistent with the data obtained from the other animals. The data from this one animal are not included in Fig. 7c.

Our results show that parasympathetic denervation causes up-regulation of P2x4 receptor mRNA and support the hypothesis that the increased sensitivity of rat parotid acinar cells to ATP can be attributed, at least in part, to increased expression of P2x4 receptor.

**DISCUSSION**

Electrolyte secretion in parotid acinar cells is mediated by mobilization of calcium from intracellular stores via receptors that are linked to phospholipase C. In contrast, extracellular ATP also increases Ca2+, but does so primarily by activating influx of extracellular Ca2+, independently of phospholipase C activation and without the participation of heterotrimeric guanine-nucleotide binding proteins (32, 36). Examination of the
Fig. 7. Competitive RT-PCR of rat parotid gland P2X4-receptor mRNA. a, ethidium bromide-stained agarose gels showing the results from control (left) and parasympathetic-denervated (right) parotid glands. In each lane, the top bands are the products from the target cDNA, and the
distribution of responsiveness to neurotransmitters on individual cells indicates that almost all cells with a muscarinic response also respond to maximally effective concentrations of ATP. However, closer examination reveals heterogeneity in purinergic receptor subtype, magnitude of the response, and sensitivity to particular agonists. Regulation of these factors determines the physiological set point and capacity of the organ to respond to a stimulus.

Previous observations showing two different responses to ATP in suspensions of parotid acinar cells could not establish whether there were two separate populations of cells, one with high and another with low affinity receptors, or whether both receptors co-existed on the same cells (1, 36, 40). The present studies on individual acinar cells clearly demonstrate a stochastic distribution of two distinct purinergic responses and show that the response pathways are independent. Properties of the low affinity response are characteristic of the P2x response and correspond to properties of the P2x7 receptor. Taken together with RT-PCR and in situ hybridization evidence for P2x7 mRNA, this suggests a functional role for P2x7 in the parotid gland.

A high affinity response corresponds to the properties of the cloned P2x4 receptor (11, 12, 14). P2x4 receptors are widely expressed in the rat central nervous system, in peripheral ganglia, and in serous and mucous acini of rat salivary gland as well as respiratory and laryngeal epithelia (12). Patch clamp recordings of P2x4 receptors transiently expressed in 293 cells are similar to antagonist-insensitive currents reported in dissociated rat submandibular gland acinar cells, indicating that expression of a single type of subunit is adequate to convey properties of the receptor seen in vivo (12). The parotid high affinity ATP receptor described here has similar properties; it desensitizes very little, responds to ATP concentrations between 0.3 and 30 μM, and is not activated by α,β-methylene ATP, UTP, GTP, or adenosine. Analysis by RT-PCR confirms in situ hybridization reports that P2x4 is expressed in rat parotid gland and that P2x1, 2, 3, 5, and 6 receptor subtypes are not present (15). Further, a protein immunoreactive with P2x4 antibodies is observed in Western blots of parotid gland extracts, corresponding to the band seen in HEK293 cells heterologously expressing P2x4. However, properties of the P2x4 receptor of parotid cells may differ somewhat from those reported in the 293 cell expression system and in submandibular cells, as parotid cells are not well activated by 2-methylthio-ATP. Further evaluation of the pharmacology using the antagonists PPADS and suramin, which distinguish P2x4 and P2x6 receptors from the others, could not be carried out in this analysis because these drugs absorb light in the wavelength range of the calcium-sensitive dyes.

Not all parotid acinar cells showed P2x4 responses, similar to the limited distribution of P2x responses reported in a submandibular acinar cell preparation (12). Although almost all parotid acinar cells have the P2x2 response, as noted above, some have only the P2x5 or only the P2x6 response, supporting the idea that these receptors are distinct and independent of one another. Co-expression of two different ATP receptors may confer different temporal responses as well as the possibility of activating different second messenger pathways.

Sensitivity to ATP was variable in parotid cells with P2x4 responses. Few of the P2x4-responsive control cells were supersensitive to ATP, but the fraction of cells responding to ATP increased as the concentration of ATP was raised, consistent with the dose-dependent recruitment of cells with different sensitivities. Heterogeneity is also seen in pancreatic acinar cells, which show differential sensitivity of Ca2+ responses to cholecystokinin (44) and to stimulation of amylase secretion by acetylcholine (45). Coupling of acinar cells through gap junctions has been reported in mouse salivary gland acinar cells (46), but in the present investigation, cell coupling did not seem to contribute to sensitivity. Ca2+ measurements were recorded from individual parotid acinar cells present as singlets, as part of doublets or triplets, or within acinar formations consisting of five to six cells. However, we did not observe a spread of Ca2+ from cell to cell in acini in the dissociated preparations from either contralateral unoperated parotid glands or denervated glands (data not shown), nor did we note any systematic difference in the sensitivity of cells within acini versus isolated single cells. Further, we observed acinar cells with different sensitivities within the same acinus, indicating that they were not coupled.

Homeostatic regulation of physiological events traditionally is thought to provide accommodation to changing conditions and to compensate for altered activity or demands on a particular system. Nerve activity influences both sensitivity and functional responses of parotid acinar cells. Removal of the parasympathetic innervation, the primary regulator of fluid, and electrolyte secretion (25, 47–49) results in adaptive “supersensitivity” or enhanced sensitivity of the gland to muscarinic agonists in vivo (26, 50). Diet also tunes the animal’s response to food ingestion as well as its metabolism and modifies salivary gland physiology (51, 52). The development of supersensitivity to ATP following denervation suggests that parasympathetic activation patterns and signals modulate secretory activity through a pathway that is functionally mediated by ATP. The source of the ATP is not known, but it is likely to be the parasympathetic nerve terminals. ATP is stored at high concentrations and released along with acetylcholine from synaptic nerve endings (53) and with noradrenaline from sympathetic nerve terminals (54). However, there may be other non-synaptic sources of ATP. It has been suggested that ATP is transported through the cystic fibrosis transmembrane conductance regulator, for example, although this is still controversial (55). There is compelling evidence for the release of nucleotides from many tissues and cell types through non-lytic processes (56–63). Wounded or damaged cells also may release high concentrations of ATP that function in pathological situations, and ATP has been implicated in programmed cell death in lymphocytes (7).

Increased P2x4 signaling after denervation might be attributed to an increase in receptor number, to direct receptor modification, or through interaction with other proteins. Our data suggest that more cells respond to ATP because more cells express P2x4 mRNA and receptor protein. Increased sensitivity may be due to higher receptor density per cell. Advances in development of new specific agonist or antagonist compounds...
that can be used to assay receptor binding will be required to evaluate the role of receptor number or changes in binding affinity for ATP. Additionally, other factors may also contribute to the increase in functional receptors. The possibility that pre-existing receptors are activated following denervation cannot be ruled out. Recently, we reported that purinergic receptor responses in parotid acinar cell suspensions are greatly potentiated by treatment with protein kinase inhibitors, leading to the hypothesis that the response is modulated by protein kinases and that the dephosphorylated state of the receptor may be the most active form (39). A component of the sensitivity increase triggered by parasympathetic denervation could be mediated by alteration of the phosphorylation/dephosphorylation state of either the receptor or of another modulatory molecule that regulates receptor function.

The physiological role of extracellular ATP and the P2 receptor responses to parotid acinar cells is still unclear. Synaptic modulation via depolarization and calcium entry may modify parotid secretion. At other synapses, ATP acts as a modulatory cotransmitter, and ATP has been reported to inhibit muscarinic and tachykinin responses through the P2 receptor in submandibular or parotid cells (38, 64, 65), although relatively high concentrations of ATP are required for activation in the presence of divalent cations.

In conclusion, our results clearly indicate the presence and heterogeneous distribution of both P2x and P2p purinoceptor responses to extracellular ATP in parotid acinar cells. These data correlate with the robust expression of P2x4 and P2x7 mRNA in parotid glands, suggesting that ATP-gated channels containing P2x4 and P2x7 subunits mediate physiological responses. Parasympathetic denervation increases the number of cells with P2x4 ATP responses, the level of P2x4 mRNA, and the proportion of supersensitive cells, suggesting an important physiological role for these receptors in parotid cells. Trans-synaptic regulation is probably mediated by increased expression of receptors, but modulation by other mechanisms may also contribute. Cloning of the P2x7 receptor will now permit further studies of how this channel is regulated and how cell-specific interactions can generate differences in pore-forming abilities of the P2x receptor observed in parotid and other cell types (66–68). These studies provide the basis for developing molecular and protein probes for a better understanding of signal transduction mechanisms in the different ligand-gated channel and G protein-coupled receptors of this family and provide the foundation for structural and functional studies. Examination of regulation by neuronal signaling will give further insight into the physiological role and mechanisms of modulation of receptors in this important and widely distributed receptor family.

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