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Mechanism underlying histamine-induced desensitization of amylase secretion in rat parotid glands

[1]Takafumi Eguchi, [1]Yasuko Ishikawa & [1,2]Hajime Ishida

Department of Pharmacology, Tokushima University School of Dentistry, 3-18-15 Kuramotocho, Tokushima City, Tokushima 770-8504, Japan

1 Histamine acted on H_2 receptors in rat parotid tissues and induced the amylase secretion. Immunoblot analysis by using anti-H_2 receptor protein antiserum demonstrated that histamine induced the increase and decrease in the amounts of H_2 receptor proteins in basolateral and intracellular membranes, respectively.

2 Short-term treatment with histamine resulted in decreases in amylase secretion, the density of H_2 receptors and their affinity for the agonists during further incubation with histamine, but showed an unaltered secretory response to isoproterenol, indicating that the histamine-induced desensitization was confined to H_2 receptors.

3 This treatment triggered a 20% decrease in the histamine-stimulated adenylate cyclase activity and a 40% decrease in the phosphorylation level of Gi2α protein in the tissues, resulting in an increase in pertussis toxin (IAP)-catalyzed ADP-ribosylation of the protein. An enhancement of cholera toxin-catalyzed ADP-ribosylation of Gs protein was observed only during the first incubation with histamine.

4 This treatment triggered a 30% decrease and a 60% increase in the histamine-stimulated activities of protein kinase A and protein phosphatase 2A in the tissues, respectively.

5 Pretreatment with okadaic acid completely blocked the histamine-induced decrease in amylase secretion and increase in IAP-catalyzed ADP-ribosylation of Gi protein. The levels of Gi2α and Gsα proteins in the tissues were not modified by histamine treatment and the level of Gi2α protein was not affected by pretreatment with okadaic acid, as assessed by immunoblot analyses with anti-Gi2α and anti-Gsα protein antiserum.

6 The regulation of Gi2α protein phosphorylation in parotid tissues plays an important role in the histamine-induced desensitization of amylase secretion.

Keywords: Histamine H_2 receptor; parotid gland; desensitization; amylase secretion; guanine-nucleotide-binding protein; ADP-ribosylation; phosphorylation; protein kinase A; dephosphorylation; protein phosphatase 2A

Introduction

It is known that the short-term exposure of plasma membrane receptors coupled with guanine-nucleotide-binding (G) proteins to an agonist results in a reduction of the physiological response (Wojcikiewicz *et al*., 1993). This phenomenon, termed desensitization, has been extensively studied using the β_2-adrenoceptors as a model. Regarding the mechanisms underlying the rapid β_2-adrenoceptor desensitization, the sequestration of the β_2-adrenoceptors quickly follows the rapid uncoupling of the β_2-adrenoceptors from the stimulatory G (Gs) proteins (Yu *et al*., 1993). This uncoupling phenomenon involves the phosphorylation of β-adrenoceptors by protein kinase A (PKA) and β-adrenoceptor kinase, which are activated under different desensitizing conditions. Receptor phosphorylation, which would presumably be caused by any hormone or drug that raises the level of cyclic AMP in the cell, reduces the ability of the receptor which is occupied by the agonist to stimulate the guanosine triphosphatase activity of the Gs proteins with which it couples, resulting in the induction of heterologous desensitization. The mechanisms underlying the homologous desensitization of β_2-adrenoceptors have also been shown to involve the receptor phosphorylation by β-adrenoceptor kinase (Sibley *et al*., 1986).

Attention has recently been focused on postreceptor mechanisms involved in the levels of G proteins coupled with desensitization and supersensitivity. We reported previously that the enhancement of inhibitory G (Gi) protein function was coupled with the isoproterenol (IPR)-induced desensitization of the mucin and amylase secretions from rat submandibular (Ishikawa *et al*., 1995) and parotid (Amano *et al*., 1996) glands, respectively, and that conversely the decline of Gi protein function was coupled with the IPR-induced supersensitivity of the amylase secretion from rat parotid glands. Our findings also indicated that the function of Gi proteins was controlled by their phosphorylation level.

Histamine, one of the autacoids, is primarily stored in mast cells in mammalian tissues and is released from these cells, and produces its effect via interactions with at least three different histamine receptor subtypes, the H_1, H_2 and H_3 receptors. In general, the interaction of histamine with H_1 receptors causes the contraction of smooth muscle, and the interaction with H_2 receptors causes the secretion of gastric acid from parietal cells in the stomach and of saliva from acinar cells in the salivary glands. H_3 receptors are localized as presynaptic receptors present on histaminergic nerve terminals in the central nervous system and a wide variety of tissues; they regulate histamine synthesis and release by a feedback mechanism.

The histamine-induced desensitization of the H_1 receptor has been studied in various tissues (Anderson *et al*., 1979; Bielkiewicz & Cook, 1984) and isolated cell systems (Baenzinger *et al*., 1981; McDonough *et al*., 1988). H_1 receptor desensitization appears to occur at the level of the receptor

[2] Author for correspondence.

itself, rather than through a postreceptor mechanism. There are, however, few studies examining whether histamine induces the desensitization of H_2 receptors or whether the changes in the function of the G proteins with which these receptors couple are involved in the mechanisms underlying the H_2 receptor desensitization.

We used rat parotid tissues as an experimental model system to study both the changes in the response of secretory cells induced by short-term exposure to histamine and the mechanisms responsible for changes in the secretory response. In this study, the brief exposure of the rat parotid tissues to histamine was found to induce the desensitization of the amylase secretion in the tissues in response to the stimulation by histamine. Coupled with this desensitization, we found decreases in the number of H_2 receptors in the tissues and in the affinity of the receptors for the agonists, with a concomitant enhancement of Gi protein function. The Gi2α protein function was controlled by the phosphorylation level, regulated by PKA and protein phosphatase 2A (PP2A).

Methods

Preparation and incubation of rat parotid tissues

Parotid glands were obtained from male Wistar rats (8 weeks old) killed by being stunned. Before the experiments, they were given laboratory chow (MF, Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*, they were maintained in a temperature-controlled environment ($22 \pm 2^{\circ}C$) with a 12 h light/dark cycle (lights on from 6:00 a.m.). The parotid glands were transferred into ice-cold Krebs-Ringer Tris (KRT) solution consisting of 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 3.0 mM $CaCl_2$, 16 mM Tris-HCl buffer (pH 7.4) and 5 mM glucose which was aerated with O_2 gas. The tissue slices (0.4 mm thick) prepared from the parotid glands with a McIlwain Tissue Chopper (The Mickle Laboratory Engineering Co., Surrey, U.K.) were equilibrated with the solution for 15 min at 37°C with shaking. The slices were treated with 1 mM histamine under the conditions described previously (Hata *et al*., 1983; 1986). In brief, the slices (50 mg wet weight) were incubated for 10 min at 37°C in 10 ml of KRT solution with 1 mM histamine(hereinafter referred to as the first incubation or histamine treatment). After being washed well with KRT solution, the slices were transferred into KRT solution for 10 min at 37°C (rest period) and then reincubated with 1 mM histamine(second incubation). In some experiments, the tissue slices were preincubated for 10 min at 37°C with okadaic acid at concentrations from 0.01 to 0.1 μM and then treated with 1 mM histamine in the presence of okadaic acid at the same concentration used in the preincubation. In other experiments, the tissue slices were preincubated with 0.2 mM cycloheximide for 30 min at 37°C and then treated for 10 min with 1 mM histamine in the presence of 0.2 mM cycloheximide. At time zero and at the end of the incubation of the slices, aliquots of the medium were used to determine the amylase activity.

Binding of radiolabelled ligands

After the first and second incubations, tissue slices were immediately suspended in ice-cold 50 mM Tris-HCl buffer (pH 7.4) with 10 mM $MgCl_2$ and homogenized in a Biotron (SM 4, Biotrona, Switzerland), operated for 15 s at a dial setting of 5, followed by centrifugation twice to remove the cell supernatant. The suspension was filtered through a single layer of nylon bolting cloth (150 mesh) and then used as the tissue membrane fraction.

H_2 receptors were quantitatively measured with [^{3}H]-tiotidine. The reaction mixture for the assay of receptors contained 850 μl of the tissue suspension (300 μg protein), 100 μl of various concentrations of the labelled ligand, and 50 μl of 25 mM histamine to displace the binding of the labelled ligand when necessary. The mixture was incubated for 30 min at 20°C and then passed through a filter (GF/B, Whatman, Maidstone, U.K.), which was then washed three times with 10 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$. The radioactivity remaining on the filter was counted, and the specific binding of [^{3}H]tiotidine was expressed as the difference between the radioactivity in the presence and absence of 5 mM histamine.

The maximal binding sites (Bmax) and the dissociation constant (Kd) in the binding studies were determined by Scatchard analysis (Scatchard, 1949) and by the method of least squares.

Adenosine diphosphate-ribosylation of G proteins

The adenosine diphosphate (ADP)-ribosylation of Gs proteins was performed according to the method of Pyne *et al*. (1992a). The final suspension (5 mg protein), suspended in 600 μl of the reaction mixture consisting of 150 mM KH_2PO_4 (pH 7.6), 3 mM dithiothreitol (DTT), 5 μM $CaCl_2$, 1 mM $MgCl_2$, 7.5 mM thymidine and 6.7 μM [^{32}P]nicotinamide adenine dinucleotide (NAD) was incubated for 45 min at 37°C together with cholera toxin (CT: 66 μg) which had been preactivated for 10 min at 37°C with 50 mM DTT. The ADP-ribosylation of Gi proteins was performed according to the method of Ribeiro-Neto *et al*. (1985). The final suspension in 25 mM Tris-HCl buffer (pH 7.4), 50 mM KH_2PO_4, 2 mM DTT, 1 mM adenosine triphosphate (ATP), 1 mM ethylenediaminetetraacetic acid (EDTA), 100 μM guanosine triphosphate (GTP), 10 mM $MgCl_2$, 10 mM thymidine, 1 mM ADP-ribose and 6.7 μM [^{32}P]NAD was incubated for 1 h at 30°C together with pertussis toxin (IAP: 25 μg) which had been preactivated for 20 min at 30°C with 5 mM ATP and 20 mM DTT. The ADP-ribosylation reactions were stopped by the addition of trichloroacetic acid (TCA) at a final concentration of 6%. The mixtures were allowed to stand for 30 min on ice, after which they were centrifuged at 3,000 r.p.m. for 20 min at 4°C. The resulting pellets were washed with ethyl ether saturated with H_2O and collected by centrifugation. The remaining ether was allowed to evaporate. The samples were dissolved, without heating, in 960 μl of solubilizing buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol and 0.04% 3-(3-cholamido-propyl)dimethylammonio)-1-propanesulfonate (CHAPS), and then mixed with 50 μl of mercaptoethanol and 10 μl of 0.1% bromophenol blue. Aliquots (200 μl in each lane) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in 12.5% linear polyacrylamide gels. After electrophoresis, the location and intensity of the radioactive peptides were analysed and the areas of the radioactive intensity were measured with a Bio-Imaging Analyzer (model BAS-2000 II, Fuji Photo Film Co., Tokyo, Japan). The data obtained are expressed as photo-stimulated luminescence (PSL) units/mg of protein subjected to electrophoresis. In some cases, the bottom part of the gel containing [^{32}P]NAD was cut off and the remainder of the gel was exposed variably to Kodak X-ray film with an intensifying screen at $-80^{\circ}C$. The radioactive peptides were demonstrated in the autoradiograms.

Preparation of apical, basolateral and intracellular membranes

After the first and second incubations, the tissue slices were immediately transferred into ice-cold KRT solution to stop the reaction and were then homogenized in 20 vol of 5 mM HEPES buffer (pH 7.5) containing 50 mM mannitol and 0.25 mM $MgCl_2$ (Buffer 1) using a glass homogenizer with a teflon pestle. After the subsequent differential centrifugation of the homogenate which was filtered through a single layer of nylon bolting cloth (150 mesh), the final precipitate obtained at 35,000 g for 30 min at 4°C was used to separate apical membranes (APM) and basolateral membranes (BLM) by the method of Longbottom and Van Heyningen (1989) and the final supernatant was used to separate intracellular membranes (ICM) by the method of Nielsen *et al.* (1955). Briefly, the final precipitate was suspended in Buffer 1, followed by the addition of 1 M $MgCl_2$ to the final concentration of 10 mM and then left on ice for 30 min. The suspension was centrifuged at 3000 *g* for 15 min and the resultant precipitate was used as the fraction-1 (F-1) The resultant supernatant was again centrifuged at 35,000 *g* for 30 min to precipitate the fraction-2 (F-2). The final supernatant was centrifuged at 200,000 *g* for 1 h to precipitate the fraction-3 (F-3). Marker enzymes of membranes were measured to ascertain the cytoplasmic contamination in each fraction. Gamma-glutamyltranspeptidase (γ-GT), as a marker of APM, and K^+ stimulated p-nitrophenyl-phosphatase (KpNPPase), as a marker of BLM, were determined by the methods of Paul *et al.* (1992) and Turner *et al.* (1986), respectively. The specific activities of these enzymes were as follows: γ-GT activities were 36.9 ± 4.8, 359.8 ± 4.8 and 19.4 ± 1.4 nmol/min/mg protein in F-1, F-2 and F-3, respectively. KpNPPase activities were 59.8 ± 1.3, 48.3 ± 3.5 and 12.1 ± 2.1 nmol/min/mg protein in F-1, F-2 and F-3, respectively. These data suggest that F-1, F-2 and F-3 are the fractions enriched for BLM, APM and ICM, respectively, as supported by the results of Vayro *et al.* (1991) and Paul *et al.* (1992).

Preparation of anti-H_2 receptor protein rabbit anti serum

Anti-H_2 receptor protein rabbit antiserum raised against a synthetic peptide (HNSHKTSLRLNNSLLPRSQS) corresponding to deduced C-terminal amino acid sequence of rat H_2 receptor (Ruat *et al.*, 1991).

Immunoblot analysis

Membranes from rat parotid tissues after the first and second incubations with or without 1 mM histamine were separated by SDS–PAGE in 12.5% linea polyacrylamide gels, and then electrophoretically transferred to nitrocellulose transfer membranes (Hybond ECL, Amersham, Buckinghamshire, U.K.) in the transfer buffer containing 25 mM Tris-HCl buffer (pH 8.3), 192 mM glycine and 20% methanol for 30 min at 15 V using a Trans Blot apparatus (Bio-Rad, Hercules, CA, U.S.A.). After transfer, the nitrocellulose transfer membranes were quenched for 60 min in TBS-T (20 mM Tris-HCl buffer (pH 7.6), 137 mM NaCl and 0.1% Tween-20) containing 5% low-fat dried milk. The membranes were then incubated with anti-Gi2α protein rabbit antiserum (AS/7), diluted 1 : 1,000, anti-Gsα protein rabbit antiserum (RM/1), diluted 1 : 500, or anti-H_2 receptor protein rabbit antiserum, diluted 1 : 1,000, in the same buffer for 18 h at 4°C, followed by incubation with peroxidase-conjugated goat anti-rabbit secondary antibody for 1 h at room temperature. Immunodetection was performed according to the enhanced chemiluminescence (ECL) method (Amersham).

Phosphorylation of Gs and Gi proteins

Parotid glands were removed from rats described above that had been given [^{32}P]orthophosphate (158 TBq/kg body weight) intraperitoneally 1 h before the experiment; the glands were used for the preparation of tissue slices. The membranes prepared from the tissue slices after the first and second incubations with or without 1 mM histamine as described above were solubilized in 1% Triton X-100, 20 mM Tris-HCl buffer (pH 7.4), 125 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 μg/ml aprotinin and 10 μg/ml leupeptin at 4°C for 1 h with gentle stirring by the method of Der *et al.* (1986), and then centrifuged at 9,000 *g* for 10 min at 4°C to remove non-solubilized materials. The resulting supernatants were incubated with AS/7 or RM/1, diluted 1 : 20 in the same buffer for 12 h and applied to a Protein A-Sepharose CL-4B column (1.5 cm × 2.0 cm. Pharmacia Fine Chemicals, Uppsala, Sweden) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0). The immune complex was eluted at the flow rate of 0.2 ml/min with 100 mM glycine-HCl buffer (pH 2.7). After dialysis against H_2O, the eluate was concentrated and dissolved, without heating, in solubilizing buffer and subjected to SDS–PAGE in 12.5% linear polyacrylamide gels. After electrophoresis, the location and the intensity of the radioactive peptides were demonstrated in the autoradiograms and analysed with a Bio-Imaging Analyzer.

Determinations of PKA and PP

After the first and second incubations, the tissues were homogenized in the solution consisting of 50 mM Tris-HCl buffer (pH 7.5) and 5 mM EDTA to determine the PKA activity, and in the solution consisting of 50 mM Tris-HCl buffer (pH 7.4), 0.1 mM EDTA, 0.1 mM ethylene glycol bis (2-amino-ethylether) tetraacetic acid (EGTA), 0.1% β-mercaptoethanol, 25 μg/ml leupeptin and 25 μg/ml aprotinin to determine the PP activity. The homogenates were passed through a single layer of nylon bolting cloth (150 mesh) and then centrifuged at 100,000 *g* for 30 min at 4°C. The resultant supernatants were used to assay these enzyme activities.

PKA assay: The PKA activity in the tissues was measured with a PKA assay system (Life Technologies, Gaithersburg, MD, U.S.A.). In brief, 5 μg protein of the supernatant was added to a reaction mixture of final volume of 30 μl containing 50 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$ 100 μM [γ-^{32}P]-ATP ($1 \sim 2 \times 10^5$ d.p.m./nmol), 7.5 μg of bovine serum albumin (BSA), 50 μM Kemptide with or without 10 μM cyclic AMP and 1 μM 17-amino acid peptide derived from the heat-stable inhibitor protein of PKA, and incubated at 30°C. After 5 min of the reaction, 20 μl of the mixture was removed and spotted onto a phosphocellulose disc. The disc was washed three times with 10 ml of 1% phosphoric acid and then with H_2O, and the amount of radioactivity on it was counted in a vial containing 10 ml scintillation fluid in a scintillation spectrometer (LSC-5000, Alka, Tokyo, Japan).

PP assay: The PP activity in the tissues was measured with a PP assay system (Life Technologies). To prepare the ^{32}P-labelled glycogen phosphorylase-a as the substrate for the enzyme, 500 μl of the reaction mixture containing 11 mg of phosphorylase-b, 10 μl of phosphorylase kinase, 1 mM ATP containing 185 MBq of [γ-^{32}P]ATP, 10 mM $MgCl_2$, 0.5 mM

$CaCl_2$, 80 mM β-glycerophosphate and 150 mM Tris-HCl buffer (pH 8.2) was incubated for 1 h at 30°C, and 500 μl of a 90% saturated ammonium sulfate solution was added to precipitate the protein. After being washed with a 45% saturated ammonium sulfate solution, the precipitate was dissolved in 1 ml of the solution containing 50 mM Tris-HCl (pH 7.0), 0.1 mM EDTA, 15 mM caffeine and 0.1% β-mercaptoethanol, and concentrated by ultrafiltration with an ultra filter (UK-10, Advantec, Tokyo, Japan). The PP assay was conducted for 5 min at 30°C in a reaction mixture of final volume of 60 μl consisting of 0.1 mM EDTA, 20 μg of BSA, 20 mM imidazole-HCl buffer (pH 7.6), 0.1% β-mercaptoethanol, 5 μg protein of the tissue supernatant and 60 μg protein of the ^{32}P-labelled substrate. The reaction was stopped by adding 180 μl of 20% TCA. After the centrifugation of the reaction mixture, the resultant supernatant was used to determine the amount of ^{32}P released. Two and 200 nM okadaic acid were used to distinguish between PP1 and PP2A activities. In our experimental conditions, these PP activities showed linearity with respect to the protein concentration of the supernatant and the reaction time.

Other methods

Cyclic AMP in the tissues was measured by a radioimmunoassay with a Yamasa cyclic AMP assay kit (Yamasa Shoyu Co., Tokyo, Japan). The amylase activity was measured as described by Bernfeld (1955) with amylose as the substrate, and the activity is expressed as maltose liberated into the medium in mg/5 min at 20°C. Protein was measured by the method of Lowry *et al*. (1951).

Statistical analysis

All data are expressed as mean values ± standard error (s.e.) and were tested for statistical significance using Student's *t*-test. Values of $P<0.05$ were considered significant.

Materials

[^{32}P]Orthophosphate(315 ~ 337 TBq/mmol), [adenylate-^{32}P]-NAD (1.11 TBq/mmol), [methyl-^{3}H]tiotidine (3.0 TBq/mmol) and [γ-^{32}P]ATP (111 TBq/mmol) were obtained from Du Pont/New England Nuclear Co., Boston, MA, U.S.A. Histamine, cimetidine, ranitidine, diphenhydramine, aprotinin, leupeptin and CT were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), dimaprit from Tocris Cookson (St. Louis, MO, U.S.A.), tioperamide and IAP from Funakoshi (Tokyo, Japan) and okadaic acid from Katayama (Osaka, Japan). AS/7 and RM/1 were obtained from NEN™ Life Science Products, Inc (Boston, MA, U.S.A.).

Results

Effect of histamine on amylase secretion from rat parotid tissues

Amylase secretion from rat parotid tissues was clearly caused by histamine at the concentrations higher than 10 μM, as shown in Figure 1. This secretory response of the tissues to histamine was rapid and dose-dependent. The EC_{50} value of histamine calculated from the values shown in Figure 1 was 60.3 ± 1.3 μM. Cimetidine (1.0 mM) and ranitidine (1.0 mM) completely inhibited the amylase secretion induced by

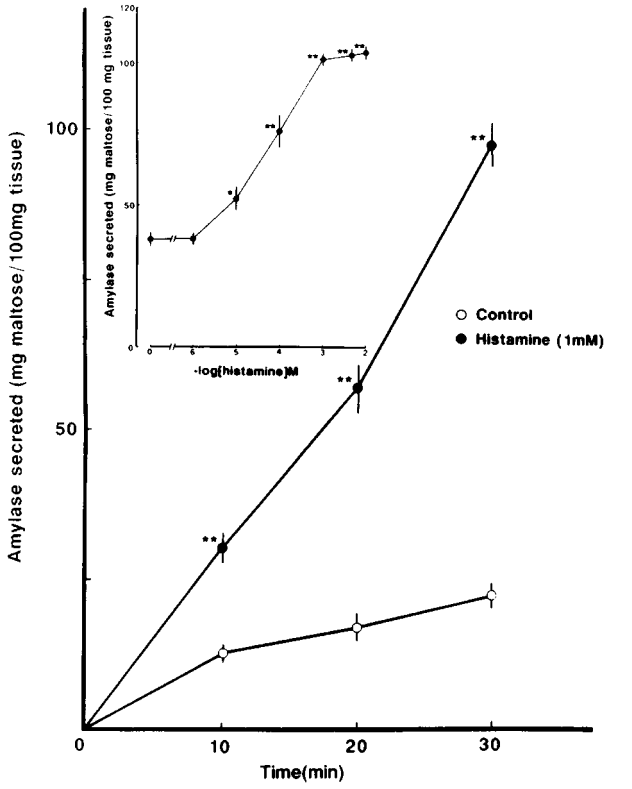

Figure 1 Effect of histamine on amylase secretion from rat parotid tissues. Rat parotid tissues were incubated with or without 1 mM histamine. The cumulative amylase secretion into the medium was measured at intervals during incubation. Inset: Concentration-response curve of rat parotid tissues to histamine. Rat parotid tissues were incubated for 30 min with the indicated concentrations of histamine. Each point represents the mean of four experiments ± s.e. Significantly different from the control values at $*P<0.05$, $**P<0.01$.

histamine (1.0 mM), but diphenhydramine (1.0 mM) and tioperamide (1.0 mM) did not (Table 1). Propranolol (1.0 mM) and atropine (1.0 mM) had no effect on the histamine-induced amylase secretion (data not shown). These results show that histamine acts on H_2 receptors on the tissues and induces amylase secretion from the tissues.

Effect of treatment with histamine on the secretory response to the agonist in rat parotid tissues

The treatment of rat parotid tissues with 1 mM histamine for 10 min (first incubation) significantly influenced the amylase secretion during further incubation with the same concentration of histamine (second incubation). During the second incubation, the secretory response to histamine was significantly suppressed, and the amount of amylase secreted during the second incubation with 1 mM histamine (19.0 ± 0.8 mg maltose/100 mg tissue) was decreased by 40% in comparison with that during the first incubation with the same concentration of histamine (31.4 ± 1.1 mg maltose/100 mg tissue) (Figure 2). The initial rate of amylase secretion induced by histamine was also reduced from 2.4 ± 0.1 to 1.6 ± 0.1 mg maltose/min/100 mg tissue in the first and second incubations, respectively. A period of less than 10 min for the first incubation with histamine did not enhance the secretory response of the tissue to histamine, in contrast to the results obtained previously in rat parotid tissues treated with IPR (Hata *et al*., 1983; Amano *et al*., 1996). The total amounts of

Table 1 Effect of H_1-, H_2- and H_3-antagonists on the amylase secretion from rat parotid tissues induced by histamine

Agonist	*Antagonist*	*Amylase activity* (mg maltose/ 100 mg tissue)
None	None	16.6±1.1
Histamine (1.0 mM)	None	*30.5±0.9
None	Diphenhydramine (0.1 mM)	17.0±1.6
None	Diphenhydramine (1.0 mM)	17.6±1.1
Histamine (1.0 mM)	Diphenhydramine (0.1 mM)	*28.7±2.5
Histamine (1.0 mM)	Diphenhydramine (1.0 mM)	*30.4±0.5
None	Cimetidine (0.1 mM)	18.6±0.9
None	Cimetidine (1.0 mM)	17.2±1.4
Histamine (1.0 mM)	Cimetidine (0.1 mM)	20.4±1.5
Histamine (1.0 mM)	Cimetidine (1.0 mM)	17.1±0.1
None	Ranitidine (0.1 mM)	14.9±1.2
None	Ranitidine (1.0 mM)	15.0±1.2
Histamine (1.0 mM)	Ranitidine (0.1 mM)	20.6±2.0
Histamine (1.0 mM)	Ranitidine (1.0 mM)	14.9±1.3
None	Tioperamide (0.1 mM)	16.7±1.1
None	Tioperamide (1.0 mM)	17.7±0.6
Histamine (1.0 mM)	Tioperamide (0.1 mM)	*31.8±1.0
Histamine (1.0 mM)	Tioperamide (1.0 mM)	*32.4±1.1

Rat parotid tissues were incubated for 10 min with 1 mM histamine and H_1-, H_2-, or H_3-antagonists at the concentrations described in this table. The amylase activity was determined in aliquots of the medium. Value is mean value of four experiments±s.e. Significantly different from the control value at $*P<0.01$.

amylase did not change during the first and second incubations with or without 1 mM histamine (data not shown).

In order to establish whether the histamine-induced desensitization of the secretory response in rat parotid tissues was confined to the H_2 receptor, we investigated the effect of treatment with histamine on the β-adrenoceptor, which is also present on the tissues. The treatment with 1 mM histamine showed an unaltered amylase secretory response to 1 μM IPR (58.5±0.5 and 56.1±1.0 mg maltose/100 mg tissue from the tissues treated with and without 1 mM histamine during further incubation, respectively), indicating that the histamine-induced desensitization is restricted to the H_2 receptor.

Evaluation of H_2 receptors in rat parotid tissues pretreated with histamine

Changes in the amount of H_2 receptors : The H_2 receptor antagonist [^{3}H]tiotidine bound specifically to rat parotid membranes prepared after the first and second incubations with or without 1 mM histamine. The binding became saturated with the increase in the concentration of [^{3}H]-tiotidine (Figure 3, Exp. A). A Scatchard analysis gave a single straight line, indicating a single population of binding sites (Figure 3, Exp. B). The maximal binding sites (Bmax values) in the tissues increased from 177±7 to 237±13 fmol/mg protein after the first incubation with 1 mM histamine for 10 min. The treatment with histamine of the tissues which were treated with 0.2 mM cycloheximide during both the preincubation and incubation as described in the methods had no effect on the increase in the Bmax values (226±10 fmol/mg protein). These findings suggested that this increase was not caused by a *de novo* production of the receptor protein. The treatment of the tissues with 1 mM histamine for 10 min decreased the Bmax values from 237±13 to 111±8 fmol/mg protein after the second incubation with the agonist.

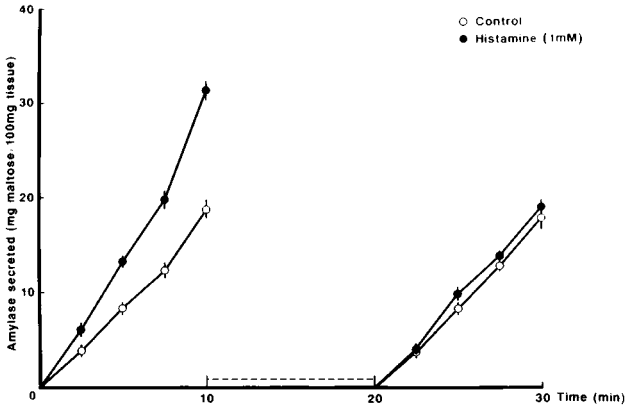

Figure 2 Effect of the first incubation with histamine on amylase secretion during the second incubation with histamine. Rat parotid tissues were incubated with or without 1 mM histamine in the first and second incubations. The dashed line represents the rest period of the tissues in KRT solution. The cumulative amylase secretion into the medium was measured at intervals during the first and second incubations. Each point represents the mean of four experiments±s.e.

Changes in the distribution of H_2 receptors in APM, BLM and ICM: APM, BLM and ICM prepared from rat parotid tissues were separated by SDS–PAGE and transferred to nitrocellulose membranes. The blots were then probed with specific anti-H_2 receptor protein antiserum. The antibody recognized a clear solitary band with a mobility corresponding to predicted molecular mass of 58 kDa in BLM and ICM (Figure 4, Exp. A and B). The bands were fully ablated by the anti-H_2 receptor protein antibody preadsorbed with the excess immunizing peptide (data not shown). However, the antibody did not recognize the expression of H_2 receptor protein in APM. To investigate whether histamine affected the distribution of H_2 receptors in rat parotid gland cells, the tissue slices were incubated with 1 mM histamine. In some experiments, the tissue slices which had been preincubated with 0.2 mM cycloheximide for 30 min were incubated with 1 mM histamine in the presence of 0.2 mM cycloheximide. APM, BLM and ICM were then prepared from the tissues. Immunoblot analysis showed that histamine markedly increased the amount of H_2 receptor protein in BLM, and conversely decreased it in ICM (Figure 4, Exp. A). The histamine-induced increase in the amounts of H_2 receptor protein in BLM was also observed in the tissues treated with cycloheximide as described above (Figure 4, Exp. B). The treatment of the tissues with histamine did not alter the yields of APM, BLM and ICM, and did not affect the immunoblot analysis (data not shown). These findings suggested histamine-induced movement of H_2 receptor protein from ICM to BLM.

Changes in the affinity of H_2 receptors: The changes in the affinity of H_2 receptors for agonists and antagonists after histamine treatment were examined by measuring the ability of agonists and antagonists to displace bound [^{3}H]tiotidine in membranes that had been treated with or without 1 mM histamine. The treatment of the tissues with 1 mM histamine for 10 min increased the IC_{50} values (the concentration for 50% inhibition of specific [^{3}H]tiotidine binding) of histamine from 0.27±0.02 to 1.49±0.11 nM, and those of dimaprit from 0.39±0.09 to 1.43±0.05 nM after the second incubation with the agonist. In contrast to the H_2 agonists, ranitidine and cimetidine, the H_2 antagonists caused no significant changes in the IC_{50} values (Table 2).

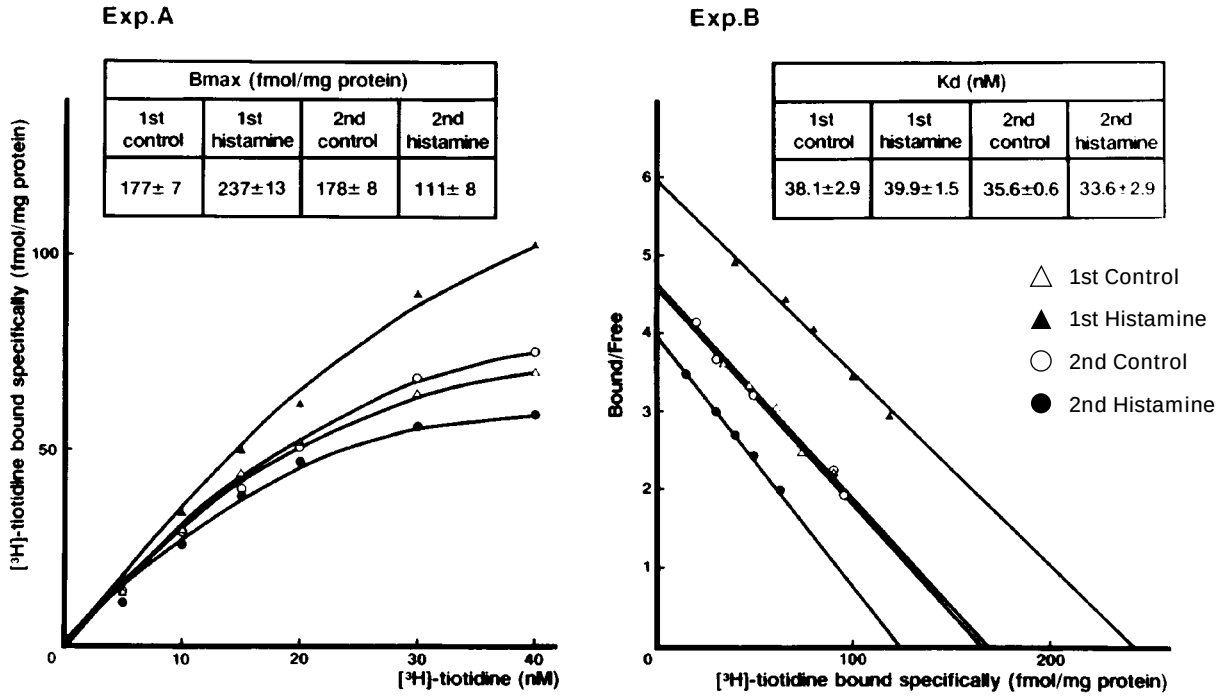

Figure 3 Binding of [^{3}H]tiotidine to preparations obtained from control and histamine-treated rat parotid tissues. Rat parotid tissues were incubated with or without 1 mM histamine for 10 min during the first and second incubations. Tissue membrane suspensions were used for the [^{3}H]tiotidine binding experiments. A typical pattern of the effects of histamine treatment on the binding of [^{3}H]tiotidine is shown. (Exp. A) saturation curves. (Exp. B) Scatchard plots of data in (Exp. A) Insets: Bmax and Kd values (the mean value of four experiments $\pm$ s.e.) in the tissues after the first (1st) and second (2nd) incubations with (Histamine) or without (Control) 1 mM histamine.

Effect of histamine treatment on cyclic AMP content in rat parotid tissues

The cyclic AMP content in the tissues was measured after the first and second incubations with or without 1 mM histamine. A marked increase in the content occurred during the first incubation with 1 mM histamine (44.4 ± 2.0 and 136.9 ± 9.6 pmol/mg protein in the control and histamine-treated tissues, respectively). However, after the second incubation with histamine, the content was lower than that after the first incubation with 1 mM histamine (47.2 ± 2.4 and 116.8 ± 8.3 pmol/mg protein in the control and histamine-treated tissues, respectively). This finding shows that the cyclic AMP content in the tissues was decreased by 20% after the second incubation with histamine.

Effect of histamine treatment on the ADP-ribosylation of G proteins in rat parotid tissues

The ADP-ribosylation of Gs and Gi proteins in the tissues after the first and second incubations with or without histamine was catalyzed by CT and IAP, respectively. No specific labelling, either in the control or histamine-treated membranes, was observed in the absence of these bacterial toxins (data not shown). CT catalyzed the ADP-ribosylation of two protein bands with molecular masses of 42 and 47 kDa (Figure 5, Exp. A). Histamine produced a 40% increase in the ability of CT to catalyze the ADP-ribosylation of the 42 kDa protein (Gs proteins) in the tissues after the first incubation, but had no effect after the second incubation (Figure 5, Exp. B). The treatment with histamine did not affect the CT-catalyzed ADP-ribosylation of the 47 kDa protein. In contrast, IAP catalyzed the ADP-ribosylation of one protein band with a molecular mass of 41 kDa (Figure 6, Exp. A), and

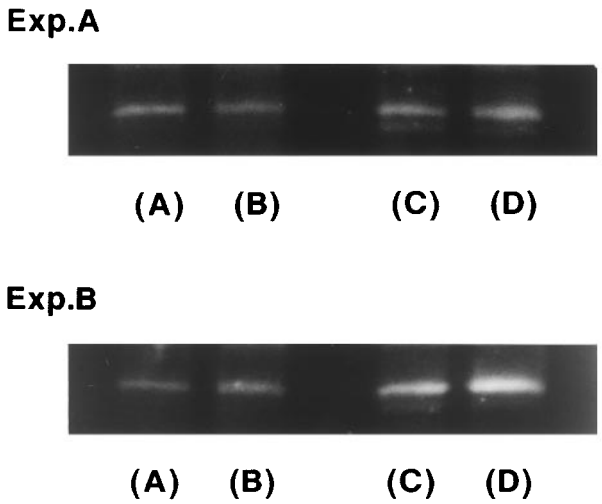

Figure 4 Effect of histamine treatment on the distribution of H_2 receptors in BLM and ICM of rat parotid tissues. Rat parotid tissues which had been preincubated for 30 min in the presence of 0.2 mM cycloheximide were treated for 10 min with (B and D) or without (A and C) 1 mM histamine in the presence of 0.2 mM cycloheximide as described in the text (Exp. B). In (Exp. A) the tissues which had been preincubated without cycloheximide were treated with (B and D) or without (A and C) 1 mM histamine for 10 min in the absence of cycloheximide. BLM (C and D) and ICM (A and B) prepared from the tissues were separated by SDS–PAGE, and immunoblot analyses by using anti-H_2 receptor protein antiserum were then performed as described in the text. The figure is representative of at least four independent experiments.

the treatment of the tissues with histamine triggered a 40% increase in the ability of IAP to catalyze the ADP-ribosylation of this 41 kDa protein (Gi proteins) after the second

incubation with histamine (Figure 6, Exp. B). There was no change in the ability of IAP to catalyze the ADP-ribosylation of Gi proteins in the tissues after the first incubation with or without histamine.

Effect of histamine treatment on the amounts of Gi2α and Gsα proteins in rat parotid tissues

AS/7 recognized a band with the molecular mass of 41 kDa, consistent with the estimated molecular weight of Gi2α protein, in rat parotid tissue membranes (Figure 7, Exp. A).

Table 2 Effect of H_2-agonists and -antagonists on IC_{50} values of [^{3}H]tiotidine binding to rat parotid membranes

	IC_{50} (nM)			
	1st incubation		*2nd incubation*	
Drugs	*Control*	*Histamine*	*Control*	*Histamine*
Histamine	0.38±0.04	0.27±0.02	0.41±0.01	*1.49±0.11
Dimaprit	0.45±0.04	0.39±0.09	0.45±0.05	*1.43±0.05
Ranitidine	0.43±0.03	0.42±0.02	0.37±0.03	0.41±0.01
Cimetidine	0.40±0.02	0.36±0.01	0.36±0.01	0.40±0.03

Rat parotid tissue membranes prepared from the tissues after the first (1st) and second (2nd) incubations with (Histamine) or without (Control) 1 mM histamine were used for the experiment examining the displacement of bound [^{3}H]tiotidine by H_2-agonists and H_2-antagonists at various concentrations. The concentration of [^{3}H]tiotidine was 10 nM. Value is a mean value of four experiments±s.e. Significantly different from the control value at $*P<0.01$.

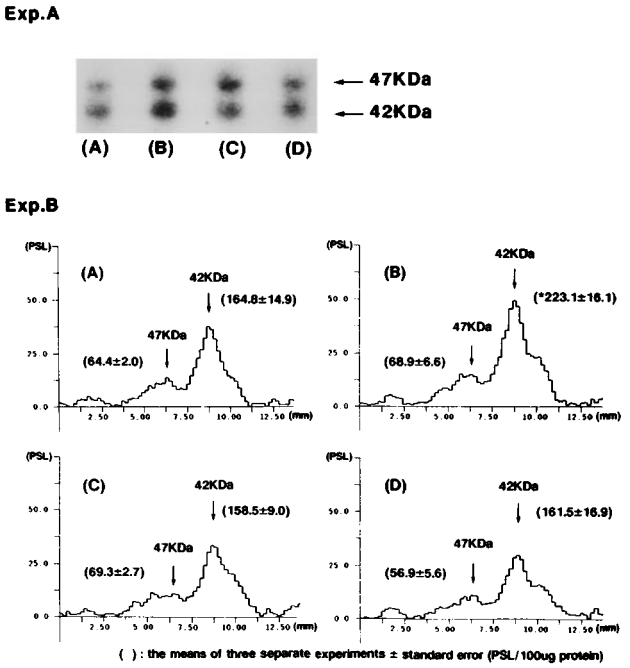

Figure 5 Typical patterns of the effect of histamine treatment on the ADP-ribosylation of Gs proteins in rat parotid tissues. Membranes from rat parotid tissues prepared after the first (A and B) and second (C and D) incubations with (B and D) or without (A and C) 1 mM histamine as described in the text were incubated in the reaction mixture for ADP-ribosylation by CT. After SDS–PAGE of the CT-catalyzed ADP-ribosylation products, the gel was autoradiographed (Exp. A). The intensities of the radioactive peptides on the gel were analysed with a Bio-Imaging Analyzer (Exp. B). The figure is representative of at least three independent experiments. The values are shown as PSL value per 100 μg of protein subjected to electrophoresis. The number in parenthesis is a mean value of three experiments±s.e. Significantly different from the control values at $*P<0.01$.

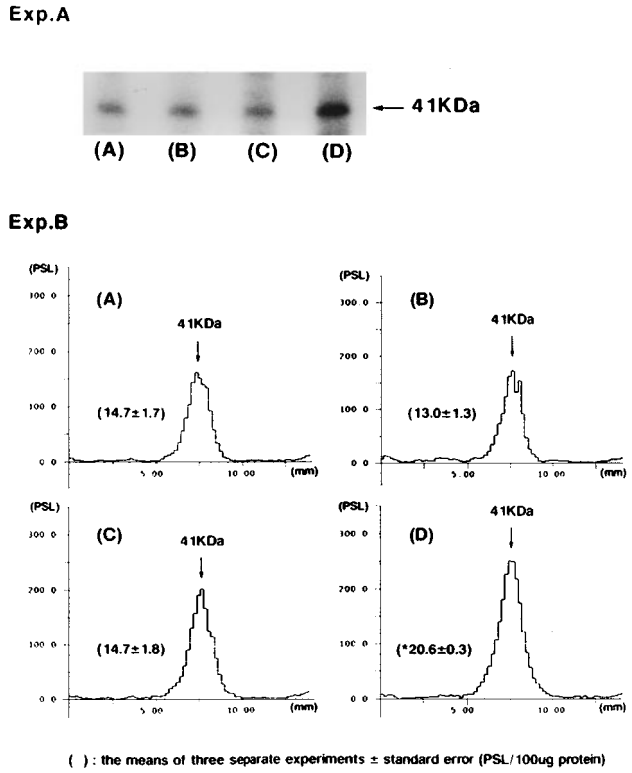

Figure 6 Typical patterns of the effect of histamine treatment on the ADP-ribosylation of Gi proteins in rat parotid tissues. Membranes prepared after the first (A and B) and second (C and D) incubations with (B and D) or without (A and C) 1 mM histamine as described in the text were incubated in the reaction mixture for ADP-ribosylation by IAP. After SDS–PAGE of the ADP-ribosylation products, the gel was autoradiographed (Exp. A). The intensities of the radioactive peptide on the gel were then analysed with a Bio-Imaging Analyser (Exp. B). The figure is representative of at least three independent experiments. The values are shown as PSL value per 100 μg of protein subjected to electrophoresis. The number in parenthesis is a mean value of three experiments±s.e. Significantly different from the control values at $*P<0.01$.

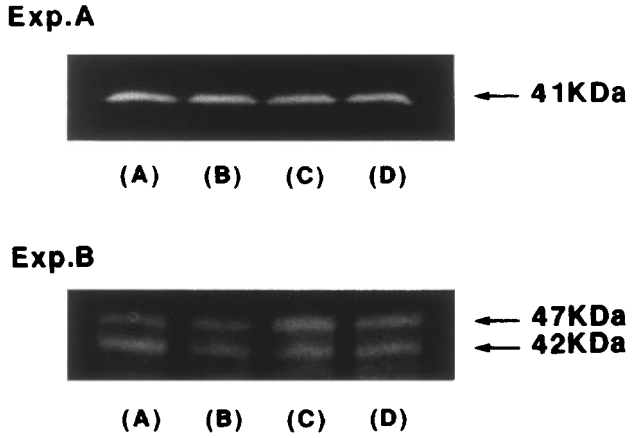

Figure 7 Effect of histamine treatment on the amounts of Gi2α and Gsα proteins in rat parotid tissue. Membranes prepared after the first (A and B) and second (C and D) incubations with (B and D) or without (A and C) 1 mM histamine as described in the text. After SDS–PAGE, immunoblot analyses by using AS/7 (Exp. A) or RM/1 (Exp. B) were performed as described in the text. This figure is representative of at least four independent experiments.

The immunoblot analysis with AS/7 demonstrated that rat parotid membranes from the tissues which had been treated with 1 mM histamine expressed no changes in the levels of Gi2α protein during further incubation with the agonist.

The levels of the 42 and 47 kDa forms of Gsα proteins in these membranes were also assessed by using RM/1 (Figure 7, Exp. B). Such studies demonstrated that the histamine treatment elicited no changes in the level of the 42 and 47 kDa form of Gsα protein in rat parotid membranes. These results suggested the importance of functional modifications of Gi2α and Gsα proteins in the histamine-induced changes in the ADP-ribosylation of these proteins.

Effect of pretreatment of rat parotid tissues with okadaic acid on the amylase secretion and ADP-ribosylation of G proteins in rat parotid tissues

Parotid tissues which had been pretreated with 0.01 to 0.1 μM okadaic acid were reacted with 1 mM histamine. The amounts of amylase secreted by 1 mM histamine during the first and second incubations in the tissues pretreated with 0.05 μM okadaic acid were 29.8±2.5 and 28.0±3.4 mg maltose/100 mg tissue, respectively, and those pretreated with 0.1 μM okadaic acids were 31.8±3.4 and 32.3±3.1 mg maltose/100 mg tissue, respectively, indicating that okadaic acid completely abolished the histamine-induced desensitization of amylase secretion in the tissues (Table 3). These effects of pretreatment with okadaic acid on the amylase secretory response to histamine during the second incubation were concentration-dependent between 0.025 and 0.1 μM. The pretreatment of the tissues with 0.01 to 0.1 μM okadaic acid did not produce any changes in the amylase secretion during the first and second incubations without 1 mM histamine. The pretreatment of the tissues with 0.1 μM okadaic acid completely blocked the histamine-induced increase in the IAP-catalyzed ADP-ribosylation of Giα protein (Figure 8, Exp. B). In contrast, this okadaic acid pretreatment did not have any effects on the CT-catalyzed ADP-ribosylation of Gsα protein during the second incubation with histamine (data not shown). The immunoblot analysis demonstrated that the okadaic acid pretreatment did not affect on the levels of Gi2α protein in the tissues, indicating that the pretreatment altered the ADP-ribosyltransferase capacity of the Gi proteins, but did not alter the activity of IAP (Figure 8, Exp. A). These findings indicated that the histamine-induced desensitization of amylase secretion from rat parotid tissues and the histamine-induced enhancement of the function of Gi2α protein were completely abolished by the treatment of the tissues with okadaic acid.

Table 3 Effect of okadaic acid histamine-induced desensitization of amylase secretion

	Amylase activity (mg maltose/100 mg tissue)			
Okadaic acid	*Ist incubation*		*2nd incubation*	
conc. (μM)	*Control*	*Histamine*	*Control*	*Histamine*
None	16.1±1.3	**25.6±2.2	16.2±1.2	15.5±1.7
0.01	15.0±1.0	**26.6±2.6	13.5±0.7	17.3±2.2
0.025	17.1±1.2	*28.0±2.0	15.7±1.8	20.9±2.4
0.05	17.6±0.8	**29.8±2.5	16.5±2.1	*28.0±3.4
0.1	17.5±0.1	**31.8±3.4	15.8±0.8	**32.3±3.1

Rat parotid tissue which had been pretreated with or without (None) various concentrations of okadaic acid for 10 min were incubated in the presence (Histamine) or the absence (Control) of 1 mM histamine during the first (1st) and second (2nd) incubations. The amylase activity was determined in aliquots of the medium. Value is a mean value of four experiments±s.e. Significantly different from the control values at $*P<0.05$, $**P<0.01$.

Effect of histamine treatment on the stoichiometry of the phosphorylation of Gi2α and Gsα proteins

The effect of histamine treatment on the stoichiometry of the labelling of Gi2α and Gsα proteins in the parotid tissues of rats which had been given [^{32}P]orthophosphate was examined to determine whether the phosphorylation sites on Gi2α and Gsα proteins in the tissues were affected by histamine treatment.

After the SDS–PAGE of the immunoprecipitate with AS/7 from rat parotid tissues incubated with or without 1 mM histamine during the first and second incubations, the radioactivity on the gel was localized on a single protein band with a molecular mass of 41 kDa, indicating that there were active sites for phosphorylation on Gi2α protein in the tissues (Figure 9, Exp. A). The analysis of the intensity of radiolabelled Gi2α protein in the rat parotid tissues treated with 1 mM histamine showed a 40% decrease during the second incubation with 1 mM histamine in the tissues, but did not change during the first incubation with this agonist (Figure 9, Exp. B).

In contrast, the immunoprecipitate with RM/1 from the tissues with or without 1 mM histamine during the first and second incubations showed a single protein band with a molecular mass of 42 kDa, indicating that Gsα protein was also phosphorylated markedly and rapidly. The analysis of the intensity of radiolabelled 42 kDa protein in the rat parotid tissues treated with 1 mM histamine showed a 70% increase during the first incubation with 1 mM histamine in the tissues (327±3 and 562±26 PSL/mg protein in the control and histamine-treated tissues, respectively) and showed no change during the second incubation with histamine (314±20 and 287±4 PSL/mg protein in the control and histamine-treated tissues, respectively).

These findings indicate that histamine causes an increase in the phosphorylation level of Gsα protein during the first incubation and a decrease in that of Gi2α protein during the second incubation in parotid tissues. The increases and decreases in the phosphorylation levels of Gsα and Gi2α proteins then induce the alteration of the ADP-ribosylation of the proteins, as reported previously (Amano *et al*., 1996).

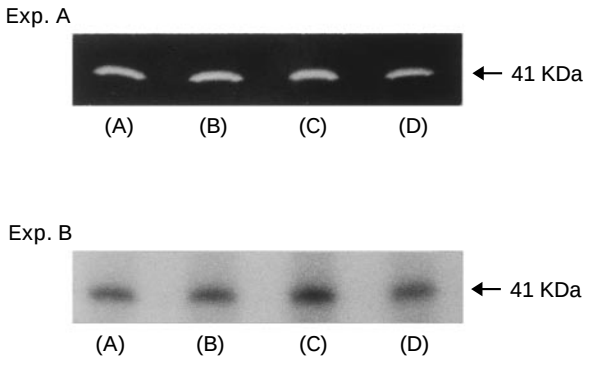

Figure 8 The effect of okadaic acid on the level and the ADP-ribosylation of Gi proteins in rat parotid tissues after histamine treatment. Membranes prepared from the tissues which had been preincubated with 0.1 μM okadaic acid were incubated with (B and D) or without (A and C) 1 mM histamine during the first (A and B) and second (C and D) incubation in the presence of okadaic acid as described in the text. After SDS–PAGE, the gel was autoradiographed (Exp. B) and then immunoblot analysis was performed as described in the text (Exp. A). Each figure is representative of at least four independent experiments.

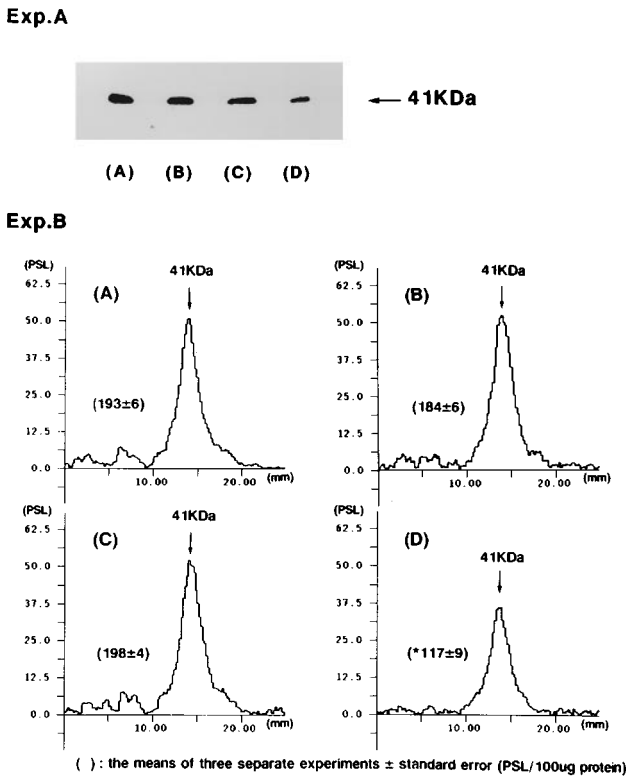

Figure 9 Effect of histamine treatment on the stoichiometry of phosphorylation of Gi protein in rat parotid tissues. Parotid tissues from rats which had been given [^{32}P]orthophosphate were incubated with (B and D) or without (A and C) 1 mM histamine during the first (A and B) and second (C and D) incubations as described in the text. After SDS–PAGE of the immunoprecipitates with AS/7 from the tissues, the gel was autoradiographed (Exp. A). The intensities of the radioactive peptide on the gel were then analysed with a Bio-Imaging Analyzer (Exp. B). The figure is representative of at least three independent experiments. The values are shown as PSL value per 100 μg of protein subjected to electrophoresis. The number in parenthesis is a mean value of three experiments ± s.e. Significantly different from the control values at $^*P<0.01$.

Effect of histamine treatment on PKA activity in rat parotid tissues

The PKA activity in the rat parotid tissues was measured after the first and second incubations with or without 1 mM histamine. The enzyme activity increased from 70.7 ± 2.3 to 117.9 ± 8.7 pmol/min/mg protein during the first incubation with 1 mM histamine and then decreased to 92.7 ± 1.3 pmol/min/mg protein during the second incubation with the agonist. These results, therefore, indicate that PKA is activated by cyclic AMP increased in the tissues during adenylate cyclase activation with histamine, and suggest that the enzyme phosphorylates Gsα protein and Gi2α protein during the first and second incubations with 1 mM histamine, respectively.

Effect of histamine treatment on PP1 and 2A activities in rat parotid tissues

The PP1 and 2A activities in the rat parotid tissues were measured after the first and second incubations with or without 1 mM histamine. The PP2A activity in the tissues did not change during the first incubation with 1 mM histamine, but increased by 60% during the second incubation with this agonist (Table 4). The PP1 activity in the tissues did not change during the first and second incubations with 1 mM histamine. These findings indicate that the decrease in the state of the phosphorylation of Gi2α protein in the tissues pretreated with 1 mM histamine during the second incubation with the agonist is caused by PP2A activated in the tissues and the increase in the ADP-ribosylation of Gi2α protein is then triggered.

Table 4 Effect of histamine treatment on PP activities in rat parotid tissues

Type of PP	*PP activity* (nmol/min/mg protein) *1st incubation* *Control*	*Histamine*	*2nd incubation* *Control*	*Histamine*
Total PP	4.24 ± 0.02	4.20 ± 0.01	4.85 ± 0.47	5.09 ± 0.03
PP1	2.72 ± 0.02	2.60 ± 0.01	2.66 ± 0.11	2.83 ± 0.13
PP2A	0.87 ± 0.05	1.00 ± 0.04	1.00 ± 0.05	*1.56 ± 0.18

After the first (1st) and second (2nd) incubations with (Histamine) or without (Control) 1 mM histamine, the tissues were homogenized in a solution consisting of 50 mM Tris-HCl buffer (pH 7.4), 0.1 mM EDTA, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 25 μg/ml leupeptin and 25 μg/ml aprotinin, and then centrifuged. The supernatant (5 μg protein) was used to assay PP activities with a PP assay system using ^{32}P-labelled glycogen phosphorylase-a as the substrate for the enzyme. To determine the PP1 and 2A activities, 2 nM okadaic acid was used to inhibit PP2A, and 200 nM to inhibit both PP1 and 2A. Value is a mean value of four experiments ± s.e. Significantly different from the control value at $^*P<0.01$.

Discussion

In this study, we investigated the mechanisms underlying the histamine-induced desensitization of amylase secretion in rat parotid glands following short-term exposure to histamine. Histamine activated adenylate cyclase in the rat parotid tissues, and amylase was secreted from the tissues. This effect appeared to be mediated through the H_2 receptor, because the H_2 selective antagonists, ranitidine (1.0 mM) and cimetidine (1.0 mM), completely inhibited the histamine-induced amylase secretion, whereas appropriate concentrations of an H_1 antagonist and an H_3 antagonist did not have any inhibitory effect on this response (Table 1).

The treatment of rat parotid tissues with 1 mM histamine for 10 min resulted in a marked attenuation of the secretory response of the tissues to a subsequent stimulation by the same concentration of histamine (Figure 2).

We reported previously that the brief exposure of rat parotid tissues to IPR for 10 or 30 min resulted in the supersensitivity or the desensitization, respectively, of the amylase secretion from the tissues during further incubation with IPR (Hata *et al*., 1983; Amano *et al*., 1996). The supersensitivity of the amylase secretion induced by IPR was strictly dependent on the reaction duration in the first incubation with IPR. However, the desensitization of the amylase secretion induced by histamine was not dependent on the duration of the reaction during the first incubation of the tissues with the agonist, as in the case of rat submandibular tissues treated with IPR (Ishikawa *et al*., 1995). The present treatment of rat parotid tissues with 1 mM histamine for 10 min resulted in an unaltered amylase secretory response to 1 μM IPR during further incubation, indicating that the histamine-induced desensitization of secretory response in rat parotid tissues was confined to H_2 receptors on the tissues, as reported in the human monocytic cell line U937 (Smit *et al*., 1994).

The number of H_2 receptors in the rat parotid tissues treated with 1 mM histamine was increased from 177 ± 7 to 237 ± 13 fmol/mg protein after the first incubation with 1 mM histamine and then decreased to 111 ± 8 fmol/mg protein after the second incubation with histamine and this treatment with histamine markedly increased the IC_{50} values of the H_2 receptor for histamine and dimaprit in the tissues (Figure 3 and Table 2), indicating that the treatment of the tissues with histamine induced a short-term down-regulation of H_2 receptors. In the experiments using cycloheximide, a protein synthesis inhibitor, this increase in the number of H_2 receptor during the first incubation with histamin was not affected. The experiments demonstrated by using a specific anti-H_2 receptor protein antiserum that the antibody recognized the expression of H_2 receptor protein in BLM and ICM and that histamine caused the increase in the amount of H_2 receptor protein in BLM and the decrease in ICM, respectively. The histamine-induced increase in the amounts of H_2 receptor protein in BLM was also observed in the tissues which had been treated with cycloheximide. These findings suggested that histamine induced a movement of H_2 receptor protein from ICM to BLM in rat parotid tissues. Further investigations are, however, necessary to clarify the regulatory mechanisms underlying a movement of H_2 receptor protein in rat parotid tissues exposed to histamine. These findings were supported by the results that the addition of cycloheximide to astrocytoma cells treated with IPR to down-regulate the β-adrenoceptors did not prevent the recovery of the receptors (Doss *et al*., 1981), that the recycling of insulin receptors was demonstrated in isolated rat adipocytes (Marshall *et al*., 1981) and that sequestration of β_2-adrenoceptor was suggested to be a mechanism involved in reactivating and recycling desensitized receptors (Lohse, 1993; Yu *et al*., 1993).

Histamine produced 40 and 70% increases in the ability of CT to catalyze the ADP-ribosylation and in the phosphorylation, respectively, of Gs proteins in rat parotid tissues during the first incubation, but had no effects on these events during the second incubation (Figure 5). This finding was supported by our previous observation that the CT-catalyzed ADP-ribosylation of Gs proteins increased during the first incubations of rat submandibular (Ishikawa *et al*., 1995) and parotid (Amano *et al*., 1996) tissues with IPR, but was not altered during the second incubations of these tissues with IPR. It has been known that there are phosphorylation sites on Gsα protein for protein kinase C (Pyne *et al*., 1992b) and PKA (Pyne *et al*., 1992c). However, no direct evidence of the modulation of Gs protein function by the phosphorylation of this protein has been found. Further investigations are required to establish the effect of the phosphorylation of Gs proteins on the ability of CT to catalyze the ADP-ribosylation of the proteins.

We observed in the present study that the impaired ADP-ribosylation of Giα protein was involved in the mechanism underlying the histamine-induced desensitization of amylase secretion from rat parotid tissues (Figure 6). The increase in the apparent level of Giα protein has been identified in the glucagon-induced desensitization of MDCK cell adenylate cyclase (Rich *et al*., 1984), the noradrenaline-induced desensitization of adenylate cyclase stimulation in rat heart muscle cells (Reithman *et al*., 1989) and the IPR-induced desensitization of mucin and amylase secretion in rat submandibular (Ishikawa *et al*., 1995) and parotid (Amano *et al*., 1996) tissues, respectively.

The present immunoblot analysis with AS/7 demonstrated clearly that the rat parotid membrane from the tissues which had been treated with 1 mM histamine did not express increases in the amount of Gi2α protein during further incubation with the same concentration of histamine (Figure 7, Exp. A). These results indicated that the histamine treatment did not induce the increase in the amount of Giα protein in rat parotid tissues, but enhanced the function of the protein. The phosphorylation levels of Gi2α protein immunoprecipitated with AS/7 from the parotid tissues of rats which had been given [^{32}P]orthophosphate showed a 40% decrease during the second incubation with histamine, but showed no changes during the first incubation with the agonist (Figure 9). This result is supported by our previous findings that Gi2α protein in rat parotid tissues was phosphorylated by PKA and that the phosphorylated Gi2α protein in the tissues resulted in a decrease in the IAP-catalyzed ADP-ribosylation of the protein, and that conversely the dephosphorylated Gi2α protein resulted in an increase in the IAP-catalyzed ADP-ribosylation of the protein (Ishikawa *et al*., 1995; Amano *et al*., 1996). In addition, a histamine-induced desensitization of amylase secretion was not observed in the tissues pretreated with okadaic acid at concentrations from 0.025 to 0.10 μM (Table 3). This result is also supported by our previous findings that IPR-induced increases in IAP-catalyzed ADP-ribosylation were not observed in rat submandibular and parotid tissues pretreated with $0.1 \sim 0.25$ μM okadaic acid (Ishikawa *et al*., 1995; Amano *et al*., 1996).

Histamine markedly increased the PKA activities in rat parotid tissues during the first incubation and the PP2A activities in the tissues during the second incubation (Table 4). These findings suggest that increases in the dephosphorylation of Gi2α protein by PP2A in rat parotid tissues during the second incubation with histamine cause an increase in the ability of IAP to catalyze the ADP-ribosylation of the protein, and support the changes in the phosphorylation levels of Gsα and Gi2α proteins in rat parotid tissues during the first and second incubations with the agonist. We also showed that the increase in the IAP-catalyzed ADP-ribosylation of Giα protein in the tissues treated with histamine was abolished by pretreatment with 0.1 μM okadaic acid. These findings show the importance of the role of PP, especially PP2A, in the regulation of Gi protein phosphorylation. Despite the progress that has been made in the study of the mechanisms of the action of PKA, little is known about the regulatory mechanism of PP for reversing the phosphorylation events. The regulatory mechanisms of PP2A, which dephosphorylates Gi2α protein, require further investigation.

In summary, the regulation of Gi2α protein phosphorylation by PKA and PP2A in rat parotid tissues plays an important role in the histamine-induced desensitization of amylase secretion from the tissues.

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References

AMANO, I., ISHIKAWA, Y., EGUCHI, T. & ISHIDA, H. (1996). Regulation of phosphorylation of Gi2α protein controls the secretory response to isoproterenol in rat parotid tissues. *Biochim. Biophys. Acta.,* **1313,** 146–156.

ANDERSON, W.H., KRZANOWSKI, J.J., POLSON, J.B. & SZENTIVANYL, A. (1979). Characteristics of histamine tachyphylaxis in canine tracheal smooth muscle. *Arch. Pharmacol.,* **308,** 117–125.

BAENZINGER, N.L., FOGERTY, F.J., MERTZ, L.F. & CHERNUTA, L.F. (1981). Regulation of histamine mediated prostacycline synthesis in cultured human vascular endothelial cells. *Cell,* **24,** 915–923.

BERNFELD, P. (1955). Amylase, α and β. In *Methods in Enzymology*. eds Colowick, S.P. & Kaplan, N.O. vol.1, pp. 149–150. New York: Academic Press.

BIELKIEWICZ, B. & COOK, D.A. (1984). The mechanism of the histamine-induced desensitization of guinea-pig ileum. *Gen. Pharmacol.,* **15,** 51–54.

DER, C.J., FINKEL, T. & COOPER, G.M. (1986). Biological and biochemical properties of human rasH genes mutated at codon 61. *Cell,* **44,** 167–176.

DOSS, R.C., PERKINS, J.P. & HARDEN, T.K. (1981). Recovery of β-adrenergic receptors following long term exposure of astrocytoma cells to catecholamine. *J. Biol. Chem.,* **256,** 12281–12286.

HATA, F., ISHIDA, H., KAGAWA, K., KONDO, E., KONDO, S. & NOGUCHI, Y. (1983). β-Adrenoceptor alterations coupled with secretory response in rat parotid tissues. *J. Physiol.,* **341,** 185–196.

HATA, F., ISHIDA, H. & KONDO, E. (1986). Dopamine-induced amylase secretion from rat parotid salivary gland in vitro: an effect mediated via noradrenergic and cholinergic nerves. *Br. J. Pharmacol.,* **89,** 473–483.

ISHIKAWA, Y., AMANO, I., EGUCHI, T. & ISHIDA, H. (1995). Mechanism of isoproterenol-induced heterologous desensitization of mucin secretion from rat submandibular glands: Regulation of phosphorylation of Gi proteins controls the cell response to the subsequent stimulation. *Biochim. Biophys. Acta,* **1265,** 173–180.

LOHSE, M.J. (1993). Molecular mechanisms of membrane receptor desensitization. *Biochim. Biophys. Acta,* **1179,** 171–188.

LONGBOTTOM, D. & VAN HEYNINGEN, S. (1989). The activation of rabbit intestinal adenylate cyclase by cholera toxin. *Biochim. Biophys. Acta,* **1014,** 289–297.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* **193,** 265–275.

MARSHALL, S., GREEN, A. & OLEFSKY, J.M. (1981). Evidence for recycling of insulin receptors in isolated rat adipocytes. *J. Biol. Chem.,* **256,** 11464–11470.

MCDONOUGH, P.M., EUBANKS, J.H. & BROWN, J.H. (1988). Desensitization and recovery of muscarinic and histaminergic Ca^{2+} mobilization in 1321N1 astrocytoma cells. *Biochem. J.,* **249,** 135–141.

NIELSEN, S., MARPLES, D., BIRN, H., MOHTASHAMI, M., DALBY, N.O., TRIMBLE, W. & KNEPPER, M. (1955). Vasopressin increases water permeability of kidney collecting duct by inducing translocation of aquaporin-CD water channels to plasma membrane. *J. Clin. Invest.,* **96,** 1834–1844.

PAUL, E., HURTUBISE, Y. & LEBEL, D. (1992). Purification and characterization of the apical plasma membrane of the rat pancreatic acinar cell. *J. Membrane Biol.,* **127,** 129–137.

PYNE, N.J., FREISSMUTH, M. & PALMER, S. (1992b). Phosphorylation of the spliced variant forms of the recombinant stimulatory guanine-nucleotide-binding regulatory protein(Gsα) by protein kinase C. *Biochem. J.,* **285,** 333–338.

PYNE, N.J., FREISSMUTH, M. & PALMER, S. (1992c). Phosphorylation of the recombinant spliced variants of the α-sub-unit of the stimulatory guanine-nucleotide binding regulatory protein(Gs) by the catalytic sub-unit of protein kinase. *A. Biochem. Biophys. Res. Commun.,* **186,** 1081–1086.

PYNE, N.J., GRADY, M.W., SHEHNAZ, D., STEVENS, P.A., PYNE, S. & RODGER, I.W. (1992a). Muscarinic blockade of beta-adrenoceptor-stimulated adenylyl cyclase: the role of stimulatory and inhibitory guanine-nucleotide binding regulatory proteins(Gs and Gi). *Br. J. Pharmacol.,* **107,** 881–887.

REITHMANN, C., GIERSCHIK, P., SIDIROPOULOS, D., WERDAN, K. & JAKOBS, K.H. (1989). Mechanism of noradrenaline-induced heterologous desensitization of adenylate cyclase stimulation in rat heart muscle cells: increase in the level of inhibitory G-protein alpha-subunits. *Eur. J. Pharmacol.,* **172,** 211–221.

RIBEIRO-NETO, F.A.P., MATTERA, R., HILDERBRANDT, J.D., CODINA, J., FIELD, J.B., BIRNBAUMER, L. & SEKURA, R.D. (1985). ADP-ribosylation of membrane components by pertussis and cholera toxin. In *Methods in Enzymology*. eds. Colowick, S.P. & Kaplan, N.O. vol. 109, pp. 566–572. New York: Academic Press.

RICH, K.A., CODINA, J., FLOYD, G., SEKURA, R., HILDEBRANDT, J.D. & IYENGAR, R. (1984). Glucagon-induced heterologous desensitization of the MDCK cell adenylyl cyclase. Increases in the apparent levels of the inhibitory regulator(Ni). *J. Biol. Chem.,* **259,** 7893–7901.

RUAT, M., TRAIFFORT, E., ARRANG, J.M., LEURS, R. & SCHWARTZ, J.C. (1991). Cloning and tissue expression of a rat histamine H_2-receptor gene. *Biochem. Biophys. Res. Commun.,* **179,** 1470–1478.

SCATCHARD, G. (1949). The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.,* **52,** 660–672.

SIBLEY, D.R., STRASSER, R.H., BENOVIC, J.L., DANIEL, K. & LEFKOWITZ, R.J. (1986). Phosphorylation/dephosphorylation of the beta-adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular distribution. *Proc. Natl. Acad. Sci. U.S.A.,* **83,** 9408–9412.

SMIT, M.J., LEURS, R., SHUKRULA, S.R., BAST, A. & TIMMERMAN, H. (1994). Rapid desensitization of the histamine H_2 receptor on the human monocytic cell line U937. *Eur. J. Pharmacol.,* **288,** 17–25.

TURNER, R.J., GEORGE, J.N. & BAUM, B.J. (1986). Evidence for a $Na^+/K^+/Cl^-$ cotransport system in basolateral membrane vesicles from the rabbit parotid. *J. Membrane Biol.,* **94,** 143–152.

VAYRO, S., KEMP, R., BEECHEY, B. & SHIRAZI-BEECHEY, S. (1991). Preparation and characterization of basolateral plasma-membrane vesicles from sheep parotid glands. *Biochem. J.,* **279,** 843–848.

WOJCIKIEWICZ, R.J.H., TOBIN, A.B. & NAHORSKI, S.R. (1993). Desensitization of cell signalling mediated by phosphoinositidase C. *Trends Pharmacol. Sci.,* **14,** 279–289.

YU, S.S., LEFKOWITZ, R.J. & HAUSDORF, W.P. (1993). β-adrenergic receptor sequestration: A potential mechanism of receptor desensitization. *J.Biol. Chem.,* **268,** 337–341.

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