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Protein Tyrosine Kinase Inhibitors Promote Amylase Secretion and Inhibit Ornithine Decarboxylase Induction in Sialagogue-Stimulated Rat Parotid Explants

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Three sialagogues, isoproterenol (IPR), carbachol, and methoxamine, caused induction of ornithine decarboxylase (ODC) in cultured rat parotid explants. All the protein tyrosine kinase inhibitors tested suppressed this ODC induction but enhanced sialagogue-dependent amylase secretion. Sodium orthovanadate showed the reverse effects as the kinase inhibitors. Immunoblot analysis with anti-phosphotyrosine antibody revealed that herbimycin A depresses IPR-stimulated tyrosine phosphorylation of parotid proteins. Herbimycin A did not affect the IPR- or dibutyryl cAMP-induced surge of the parotid cAMP level but inhibited these agonist-dependent ODC inductions. These results suggest that sialagogue-induced ODC induction and amylase secretion are mediated by different signal transduction pathways and that protein tyrosine kinase participates in IPR-dependent ODC induction and amylase secretion in the process subsequent to the cAMP surge.

Diverse sialagogues such as isoproterenol (IPR, a β-adrenergic agonist), carbachol (CC, a muscarinic-cholinergic agonist), and methoxamine (MTX, an α-adrenergic agonist) are growth stimulants of murine parotid glands *in vivo* (1–3) and *in vitro* (4–7). These agonists cause increase in the steady-state level during the pre-replicative period of mRNA of ornithine decarboxylase (ODC, EC 4.1.1.17) followed by a marked increase in ODC activity, which is a prerequisite for parotid growth (2,8). ODC induction is preceded by phosphorylation of parotid proteins (9) and transient increases in mRNAs of several proto-oncogenes including c-*src*, which encodes a non-receptor type protein tyrosine kinase (PTK) (8). Nakagawa *et al.* showed that the administration of IPR to rats stimulates tyrosine phosphorylation of parotid proteins (10), but the relationship between this phosphorylation and IPR-induced cell proliferation is unknown.

Previously, opposite results were reported on the relationship between tyrosine phosphorylation and amylase secretion in pancreatic acinar cells; i.e., Ca^{2+}-induced amylase secretion was stimulated by the introduction of a recombinant tyrosine-specific phosphatase (11), and cholecystokinin (CCK)-induced amylase secretion was inhibited by tyrosine kinase inhibitors (12). The reason for these discrepant results is not clear.

In this study, we used a wide variety of PTK inhibitors and sodium orthovanadate, an inhibitor of protein tyrosine phosphatase (PTP), to investigate more precisely the relationships of protein tyrosine phosphorylation with ODC induction and amylase release stimulated by three sialagogues in rat parotid explants.

MATERIALS AND METHODS

Materials. Herbimycin A was a generous gift from Dr. Yoshimasa Uehara, National Institute of Health, Tokyo. Other inhibitors were purchased from the following sources: apigenin and genistein from Sigma; methyl 2,5-dihydroxycinnamate

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Abbreviations: IPR, DL-isoproterenol; ODC, ornithine decarboxylase; CC, carbachol; MTX, methoxamine; PTK, protein tyrosine kinase; CCK, cholecystokinin; PTP, protein tyrosine phosphatase; 2,5-MeC, methyl 2,5-dihydroxycinnamate; DBcAMP, dibutyryl cAMP; KDa, kilodalton; GAP, Ras·GTPase activating protein.

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(2,5-MeC) from Kyowa Medex, Tokyo, and lavendustin A from Research Biochemicals. All other reagents were obtained as described previously (8,9).

Culture of explants. Explants (approximately 1 mg each) prepared from the parotid glands of male Wistar rats (200–300 g) were incubated at 37°C under an atmosphere of 3% CO_2 in air on siliconized lens paper floating on Dulbecco's modified minimum essential medium (13) supplemented with antibiotics and hormones as reported previously (5,8).

Assay of enzyme activities and cAMP. α-Amylase activity in the culture medium and ODC activity in the cytosol of parotid explants were measured 1 and 6h, respectively, after addition of a sialagogue as reported previously (6,9). cAMP was determined with a cAMP [^{125}I] assay system (Amersham) according to the manufacturer's protocol under conditions in which DBcAMP was not measured.

Immunoblot analysis of phosphotyrosine-containing proteins. Explants precultured with or without 2 μM herbimycin A for 30 min were stimulated by 10 μM IPR for 15 min and then homogenized in RIPA buffer (14). Parotid protein (50 μg) measured with a Bio-Rad protein assay kit was then subjected to electrophoresis in SDS-8% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (8,15) and probed with mouse antiphosphotyrosine antibody PY-20 (ICN) and [^{125}I] goat anti-mouse IgG antibody (NEN) (16). Autoradiography was done with Fuji medical X-ray film at −70°C for 6h or 15h.

RESULTS

First we examined the effects of the PTK inhibitors listed in Table 1 on the ODC activity and amylase release stimulated by sialagogues of cultured rat-parotid explants. A variety of inhibitors with different chemical features were tested; namely, herbimycin A (an ansamycin antibiotic) (13), genistein (an isoflavonoid) (17), methyl 2,5-dihydroxycinnamate (an erbstatin derivative) (18), apigenin (a flavonoid) (17), and lavendustin A (19). All these inhibitors suppressed ODC induction stimulated by IPR, CC, or MTX by 24 to 80%. The inhibition was dose-dependent (data not shown) and herbimycin A was the most effective. In contrast, amylase secretion was significantly augmented by all these PTK inhibitors (Table 1). All inhibitors alone showed no effect on ODC activity and amylase secretion (data not shown), except that genistein alone stimulated amylase secretion at 100 μM but not at 20 μM (Table 1).

Incubation of the explants with a protein tyrosine phosphatase inhibitor, sodium orthovanadate, increased their ODC activity dose-dependently even in the absence of the sialagogues. Vanadate caused maximal stimulation (333% of the control) at a concentration of 50 μM which did not affect amylase secretion appreciably (Table 2). This increase in ODC activity was inhibited by herbimycin A (data not shown), suggesting that vanadate-dependent increase in ODC activity is medi-

TABLE 1
Effects of Tyrosine Kinase Inhibitors on Sialagogue-Dependent ODC Induction and Amylase Secretion of Cultured Rat Parotid Explants

	IPR (10 μM)		CC (10 μM)		MTX (20 μM)	
Inhibitor	ODC	Amylase	ODC	Amylase	ODC	Amylase
None (control)	100[a)]	100[b)]	100[c)]	100[d)]	100[e)]	100[f)]
Herbimycin A (2 μM)	20 ± 2	126 ± 8	43 ± 5	108 ± 2	25 ± 5	126 ± 7
Genistein (100 μM)	38 ± 9	132 ± 9[g)]	45 ± 8	124 ± 11[g)]	34 ± 6	147 ± 15[g)]
2,5-MeC (10 μM)	42 ± 5	113 ± 2	48 ± 2	111 ± 3	36 ± 3	121 ± 6
Apigenin (50 μM)	33 ± 2	111 ± 4	65 ± 9	118 ± 3	60 ± 2	120 ± 2
Lavendustin A (50 μM)	51 ± 2	112 ± 3	76 ± 2	112 ± 3	65 ± 6	119 ± 2

Note. Inhibitors were added to the culture medium 30 min before addition of sialagogues. ODC and amylase secretion were determined 6h and 1h, respectively, after adding sialagogues. Values (means ± SD) are shown as percentages of the control values (sialagogue alone) in 3–6 separate experiments. The control values were as follows: a) 13.46 ± 0.65 nmol/g explant/h, b) 10.80 ± 1.00 μmol/mg explant/min, c) 5.94 ± 0.45 nmol/g explant/h, d) 3.29 ± 0.55 μmol/mg explant/min, e) 2.38 ± 0.25 nmol/g explant/h, f) 2.28 ± 0.24 μmol/mg explant/min. The values obtained without agonists were 0.81 ± 0.11 nmol/g explant/h (ODC) and 1.04 ± 0.25 μmol/mg explant/min (amylase), g) 20 μM genistein was used for amylase secretion experiments because genistein alone slightly stimulates amylase secretion at 100 μM but not at 20 μM. Data were analyzed by Student's *t*-test and all values with inhibitors were significantly different from those of the corresponding controls ($p < 0.05$).

TABLE 2
Effects of Vanadate on Sialagogue-Dependent ODC Induction and Amylase Secretion of Cultured Rat Parotid Explants

Addition	ODC		Amylase	
	nmol/g explant/h	%	μmol/mg explant/min	%
None	0.78 ± 0.08	100	1.09 ± 0.16	100
Vanadate (50 μM)	2.60 ± 0.41**	333	1.10 ± 0.18	100
IPR (1 μM)	1.69 ± 0.13	100	5.44 ± 0.38	100
IPR (1 μM) + vanadate	4.00 ± 0.42**	236	3.81 ± 0.19**	70
CC (1 μM)	2.01 ± 0.08	100	2.14 ± 0.11	100
CC (1 μM) + vanadate	3.28 ± 0.51**	163	1.88 ± 0.09*	88
MTX (20 μM)	2.74 ± 0.28	100	2.63 ± 0.11	100
MTX (20 μM) + vanadate	4.49 ± 0.82**	164	2.24 ± 0.26*	85

Note. ODC and amylase secretion were determined 6h and 1h, respectively, after addition of sodium orthovanadate (50 μM) and/or sialagogues. Values are means ± SD for 3–6 separate experiments. Data were analyzed by Student's *t*-test. *$p < 0.05$, **$p < 0.01$ compared with the corresponding control value.

ated by protein tyrosine phosphorylation. Table 2 also shows the effects of vanadate on ODC activity and amylase secretion stimulated by a suboptimal dose of IPR or CC or the optimal dose of MTX. Orthovanadate enhanced the sialagogue-stimulated ODC induction but depressed the sialagogue-dependent amylase secretion.

Dibutyryl cAMP (DBcAMP, 200 μM) also increased ODC activity (6.77 nmol/g explant/h) and amylase exocytosis (3.54 μmol/mg explant/min), its effects being comparable to those of IPR, since the action of IPR is mediated via cAMP (5). Thus, the effect of herbimycin A on the cAMP level in IPR- or DBcAMP-stimulated explants was investigated (Fig. 1). Herbimycin A did not affect increase of intracellular cAMP induced by IPR or DBcAMP at all. We next examined the effect of herbimycin A on the action of DBcAMP. Herbimycin A (2 μM) inhibited the DBcAMP-induced increase in ODC activity by 70% (specific activity, 2.03 nmol/g explant/h), but enhanced DBcAMP-induced amylase secretion to 4.50 μmol/mg explant/min. These results indicate that

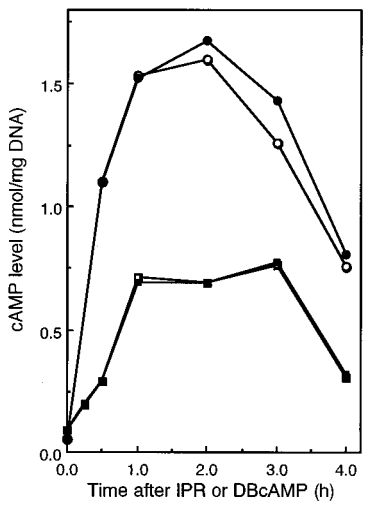

FIG. 1. Effects of herbimycin A on cAMP levels in IPR- or DBcAMP-stimulated rat parotid explants. Herbimycin A was added to the culture medium 30 min before addition of a stimulant (10 μM IPR or 500 μM DBcAMP). cAMP levels were determined at the indicated times after addition of the stimulants, as described under Materials and Methods. Data are means for two separate experiments. ○, DBcAMP; ●, DBcAMP+Herbimycin A (4 μM); □, IPR; ■, IPR+Herbimycin A (2 μM).

herbimycin A altered IPR-dependent ODC induction and amylase secretion without change in cAMP level.

Next, the presence of phosphotyrosine-containing proteins in unstimulated and IPR-stimulated explants was studied. Typical results are shown in Fig. 2. Unstimulated explants gave several bands that reacted with anti-phosphotyrosine antibodies (lane 1). The explants stimulated by IPR for 15 min showed increased tyrosine phosphorylation of 220, 130, 120, 102, and 84 kDa proteins. In particular, IPR increased intensities of the 220, 130, and 120 kDa bands approximately three-fold (lane 2). The rapid increases in phosphorylation of these proteins may be correlated with the rapid secretory and/or growth-promoting responses observed on IPR stimulation (5,8). To evaluate the involvement of PTKs in the observed phenomena, we examined the effect of herbimycin A on IPR-dependent tyrosine phosphorylation in explants (lane 3). Herbimycin A (2 μM) added 30 min before IPR greatly attenuated the effect of IPR, and its effect was roughly proportional to its inhibition of ODC activity.

DISCUSSION

In this work we showed that ODC induction stimulated by IPR, CC, or MTX was suppressed by all five PTK inhibitors tested and activated by vanadate. Although the exact sites of action of PTK inhibitors and vanadate are unknown, the data indicate that protein tyrosine phosphorylation plays a regulatory role in ODC induction in rat parotid glands. Intracellular mediation of ODC induction by protein tyrosine phosphorylation has also been observed in gastrin-treated colonic mucosa (20) and prolactin-stimulated mammary tissues (21), and so protein tyrosine phosphorylation may be a prerequisite for ODC induction in many mammalian tissues.

The involvement of tyrosine phosphorylation in the regulation of amylase secretion in pancreatic acini have been suggested by several groups. Jena *et al.* (11) reported stimulation of Ca^{2+}-induced amylase secretion by the introduction of a recombinant tyrosine-specific phosphatase from rat brain into permeabilized pancreatic acini. Moreover, Piiper *et al.* (22) stated that tyrosine phosphorylation probably has inhibitory effects on the secretory apparatus in pancreatic acini. In contrast, Lutz *et al.* (12) observed that CCK, a major physiological pancreatic secretagogue, stimulates protein tyrosine phosphorylation in pancreatic acinar cells and that inhibition of tyrosine kinases reduces CCK-stimulated amylase release. In the present study, amylase secretion induced by sialagogues was suppressed by vanadate and augmented by PTK inhibitors, suggesting that amylase secretion decreases in proportion to the level of tyrosine phosphorylation. This conclusion is consistent with

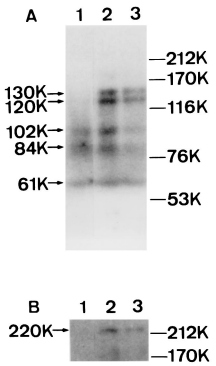

FIG. 2. Immunoblot analysis of phosphotyrosine-containing proteins in cultured rat parotid explants. Explants incubated with or without 2 μM herbimycin A for 30 min were stimulated with 10 μM IPR for 15 min. Subsequent procedures are described under Materials and Methods. Panel A shows a 6h autoradiogram and panel B shows the 170–230K region of a 15h autoradiogram of the same membrane. Lane 1, no addition; lane 2, 10 μM IPR; lane 3, 2 μM herbimycin A+10 μM IPR. The positions of molecular weight markers are shown on the right: 212K (myosin), 170K (α_2-macroglobulin), 116K (β-galactosidase), 76K (transferrin), 53K (glutamic dehydrogenase).

a previous report that protein phosphorylation has an inhibitory effect on amylase release in pancreatic acini (22). However, as shown in Tables 1 and 2, the changes in amylase secretion induced by herbimycin A were markedly smaller than those in ODC activity. These results suggest that protein tyrosine phosphorylation is a minor, not a main signaling pathway for regulating amylase secretion in rat parotid glands.

We also found that herbimycin A did not inhibit the IPR-induced surge of parotid cAMP. Moreover, the effects of herbimycin A on DBcAMP-induced phenomena were similar to those on IPR-induced effects, indicating that the target sites of herbimycin A are downstream of cAMP.

Though the receptors and consequent signals of the three agonists used in this study are different, the PTK inhibitors and vanadate had almost the same effects irrespective of the agonists. This finding admits of two interpretations: The inhibitors used affect so many kinases and phosphatases that all three signal transduction pathways may be affected independently. Alternatively, the target kinase(s) and phosphatase(s) are common to the far downstream regions of these signaling pathways and play pivotal roles in ODC induction. Since the targets of inhibitors seem to be relatively far downstream in the signaling pathways, as described above, the latter possibility seems to be the more likely, but further investigations are needed.

We demonstrated that at least five proteins were actually highly phosphorylated in rat parotid glands in response to IPR. Recently, Nakagawa *et al.* (10) claimed that treatment of rat parotid acinar cells with IPR or EGF causes an increase in tyrosine phosphorylation of the p21ras-GTPase activating protein (GAP) of 120 kDa. To clarify the signaling pathways regulating ODC induction and amylase secretion, the phosphoprotein(s) involved in sialagogue-induced processes must be identified.

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