Effect of genistein on amylase release and protein tyrosine phosphorylation in parotid acinar cells

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Abstract **We evaluated the role of protein tyrosine phosphorylation in amylase exocytosis from parotid acinar cells by using genistein, a tyrosine kinase inhibitor. Amylase release stimulated by isoproterenol was dose-dependently inhibited by genistein. Genistein also inhibited the exocytosis evoked by dibutyryl- or 8-chlorophenylthio-cAMP. Daidzein, a negative control agent of genistein, elicited no inhibitory effect. Isoproterenol had dual effects on protein tyrosine phosphorylation; it increased the phosphorylation of 190- and 210-kDa proteins and decreased that of a 90-kDa one. The phosphorylation was dose-dependently inhibited by genistein but not by daidzein. These results suggest that protein tyrosine phosphorylation plays a role in the process of amylase exocytosis from parotid acinar cells.**

Key words: Genistein; Tyrosine kinase inhibitor; Tyrosine phosphorylation; Amylase exocytosis; Parotid acinar cell

1. Introduction

Amylase release from parotid acinar cells is a typical model of cAMP-mediated exocytosis [1]. We recently demonstrated that the catalytic subunit of cAMP-dependent protein kinase induces amylase release from streptolysin O-permeabilized parotid acinar cells [2]. In contrast, Ca^{2+}-mobilizing agonists such as cholecystokinin and acetylcholine mainly stimulate amylase release from pancreatic acini, and Ca and protein kinase C seem to be equally important in the regulatory mechanism [3]. However, it was recently found that cholecystokinin and carbachol increased protein tyrosine phosphorylation in addition to serine/threonine phosphorylation and that tyrosine kinase inhibitors, including genistein, significantly inhibited amylase release stimulated by those agonists [4–7]. These results suggest that tyrosine kinases are involved in the regulation of amylase exocytosis from pancreatic acini. Since little is known concerning the role of tyrosine kinases in parotid acini, we studied the effect of genistein on amylase release and tyrosine phosphorylation. The results obtained showed that isoproterenol-induced amylase release and protein tyrosine phosphorylation were dose-dependently inhibited by genistein but not by daidzein.

2. Experimental

2.1. Materials

Genistein and daidzein were purchased from Extrasynthese (Genay, France). Monoclonal anti-phosphotyrosine antibody and agarose-conjugated monoclonal anti-phosphotyrosine antibody (clone 4G10) were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Pefabloc SC was from Merck (Darmstadt, Germany). Polyvinylidene difluoride (PVDF) membranes (Immobilon) were from Millipore (Bedford, MA). Enhanced chemiluminescence (ECL) kit was from Amersham (Little Chalfont, England). Isoproterenol, carbachol, leupeptin, orthovanadate, and Nonidet P-40 were from Sigma (St. Louis, MO). 8-Chlorophenylthio-cAMP was from Boehringer Mannheim (Tokyo, Japan). All other chemicals utilized were the highest grade commercially available.

2.2. Preparation of parotid acini

Rat parotid acini (small acini) were prepared as described previously [8]. In each experiment, parotid glands from one rat were minced finely and incubated with trypsin (0.5 mg/ml) for 10 min at 37°C in minimum essential medium (MEM) buffered with 20 mM Na-Hepes (pH 7.4: MEM-H) containing 0.1% bovine serum albumin (BSA) under 100% O_2 in a metabolic shaker. The tissue was washed once with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution containing 2 mM EGTA and then incubated for 5 min with trypsin inhibitor (0.5 mg/ml) in the same medium. After the tissue had been washed once with MEM-H, it was further incubated for 20 min in MEM-H containing collagenase (130 U/ml), hyaluronidase (0.25 mg/ml), and 0.1% BSA. After digestion, dispersed acini were filtered through two layers of medical gauze, washed 4 times with MEM-H, and suspended in the same medium.

2.3. Amylase release

A suspension of parotid acini (1 ml) was pipetted into a 1.5-ml microcentrifuge tube containing 10 μl genistein or dimethylsulfoxide (DMSO) and preincubated at 37°C for 20 min. The acini were further incubated with various agonists for 20 min. After incubation the tube was mixed and centrifuged at 10,000 rpm for 1 min at room temperature, and the medium was used for assay of amylase released. For measurement of total amylase activity, acini were incubated with 0.2% Nonidet P-40 as above. Amylase activity was measured by the method of Bernfeld [9], and the released amylase activity was given as the percentage of the total activity.

2.4. Tyrosine phosphorylation

Parotid acini were preincubated for 20 min with genistein and further incubated for 5 min with various agonists. After incubation, the acini were pelleted and lysed with lysis buffer containing 40 mM Hepes-Na (pH 7.4), 140 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM sodium orthovanadate, 0.2 mM pefabloc SC, and 10 μg/ml leupeptin. The lysate was centrifuged at 15,000 rpm at 4°C for 5 min in a microcentrifuge, and the resulting supernatant was then used for immunoprecipitation and immunoblotting.

Phosphotyrosine-containing proteins were immunoprecipitated with agarose-conjugated monoclonal anti-phosphotyrosine antibody. The agarose beads were washed 5 times with lysis buffer, suspended in Laemmli buffer [10], and boiled for 5 min. The proteins were resolved by SDS-PAGE on 7.5% gels and then transferred to a PVDF membrane at 100 mA per mini-gel ($90 \times 73 \times 1$ mm) for 100 min in a semi-dry blotter using 0.1 M Tris–0.192 M glycine buffer containing 5% methanol. Phosphoproteins were visualized by immunoblotting with the same monoclonal antibody and the ECL system.

3. Results

3.1. Effects of genistein and daidzein on amylase release

To evaluate the role of tyrosine kinases in amylase release,

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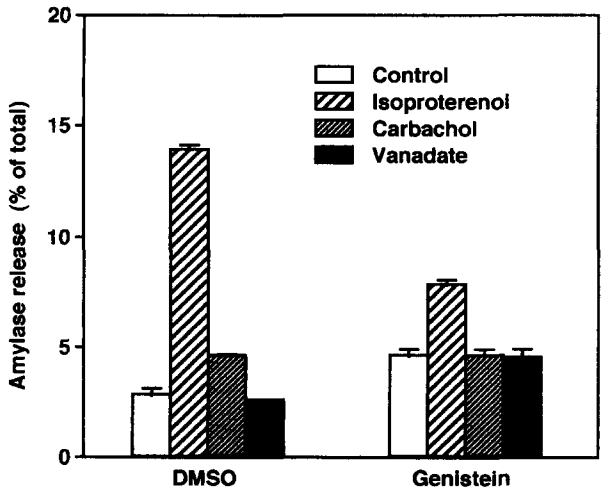

Fig. 1. Effect of genistein on amylase release from parotid acini stimulated by various agonists. The acini were preincubated with 370 μM (100 μg/ml) genistein or 1% dimethylsulfoxide (DMSO) at 37°C for 20 min, and further incubated for 20 min after addition of 1 μM isoproterenol, 10 μM carbachol, or 100 μM sodium orthovanadate. Data shown are means ± S.D. (n = 3) of a representative experiment of two independent experiments.

we examined the effects of genistein on amylase release stimulated by isoproterenol, carbachol, or orthovanadate. As seen in Fig. 1, genistein markedly inhibited amylase release stimulated by isoproterenol. Effects of genistein on the action of other agonists were difficult to detect, since the magnitude of amylase release by those agonists was too small.

The inhibitory effect of genistein on isoproterenol-stimulated exocytosis was dose dependent up to 300 μM; the maximum inhibition was approximately 80% at 300 μM and the IC_{50} value was approximately 120 μM (Fig. 2A). To confirm the effect of genistein is due to inhibition of tyrosin kinase activity, we examined the effect of daidzein, a compound closely related to genistein but without any inhibitory effect on tyrosine kinase activity [11]. As seen in Fig. 2B, daidzein had no inhibitory effect. Genistein also inhibited amylase release stimulated by dibutyryl-cAMP (db-cAMP) and 8-chlorophenylthio-cAMP (cps-cAMP) (Fig. 3). In these experiments, daidzein slightly enhanced amylase release stimulated by cAMP analogs.

3.2. *Effects of isoproterenol and genistein on tyrosine phosphorylation*

To evaluate the role of protein tyrosine phosphorylation in amylase exocytosis, we studied the effects of various secretagogs and genistein on tyrosine phosphorylation levels in parotid acini. Tyrosine-phosphorylated proteins in the lysate of parotid acini were immunoprecipitated, resolved on 7.5% polyacrylamide gels, and visualized by immunoblotting. As seen in Fig. 4 (left), isoproterenol had dual effects on tyrosine phosphorylation; it increased phosphorylation of two protein bands, whose molecular masses were apparently 190 and 210 (p190 and p120, respectively, indicated by small arrows), and it decreased the phosphorylation of a 90-kDa protein (p90, indicated by the arrowhead). Carbachol also decreased the phosphorylation of p90 but markedly increased that of a 80-kDa protein (p80) instead of p190 or p210. The p80 was very likely to be protein kinase Cδ as reported recently [12].

To identify the intracellular second messengers that produced those changes in protein tyrosine phosphorylation, we incubated parotid acini with 1 mM 8-chlorophenylthio-cAMP (cps-cAMP), 1 μM phorbol 12-myristate 13-acetate (PMA), or 1 μM A23187 for 5 min, and tyrosine-phosphorylated proteins were analyzed as above. As seen in Fig. 4 (right), cps-cAMP completely mimicked the dual effects of isoproterenol, indicating that those changes are cAMP-dependent events. On the other hand, PMA and A23187 mimicked either one of the two effects of carbachol; PMA enhanced the phosphorylation of p80 and A23187 decreased that of p90.

Genistein dose-dependently inhibited tryosine phosphorylation in the parotid acini (Fig. 5). As expected, daidzein (300 μM) scarcely inhibited tyrosine phosphorylation.

4. Discussion

The present study clearly shows that genistein dose-dependently inhibited amylase release and protein tyrosine phosphorylation in parotid acini stimulated by isoproterenol. The mag-

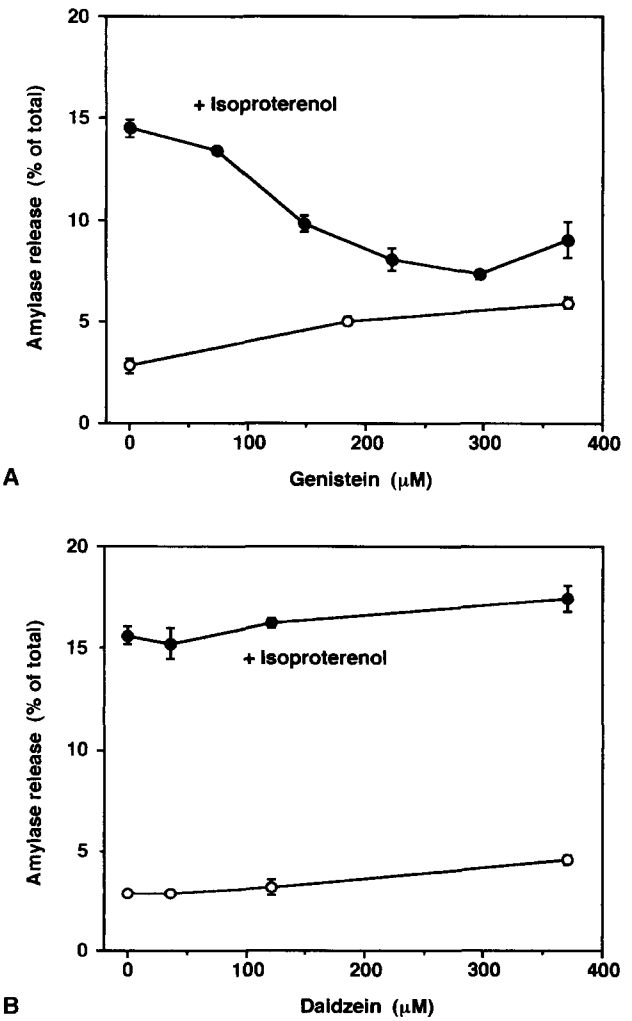

Fig. 2. Effects of genistein and daidzein on amylase release from parotid acini stimulated by isoproterenol. The acini were preincubated with various concentrations of genistein (A) or daidzein (B) at 37°C for 20 min, and further incubated for 20 min after addition of 1 μM isoproterenol. Data shown are means ± S.D. (n = 3) of a representative experiment of 3 independent experiments.

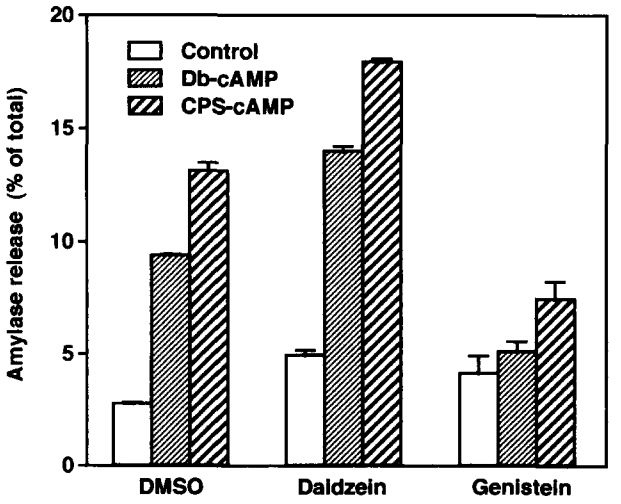

Fig. 3. Effects of genistein and daidzein on amylase release from parotid acini stimulated by membrane-permeable cAMP analogs. The acini were preincubated with 300 μM genistein, 300 μM daidzein, or 1% DMSO at 37°C for 20 min, and further incubated for 20 min after addition of 2 mM dibutyryl-cAMP or 1 mM cps-cAMP. Data shown are means ± S.D. (n = 3) of a representative experiment of 3 independent experiments.

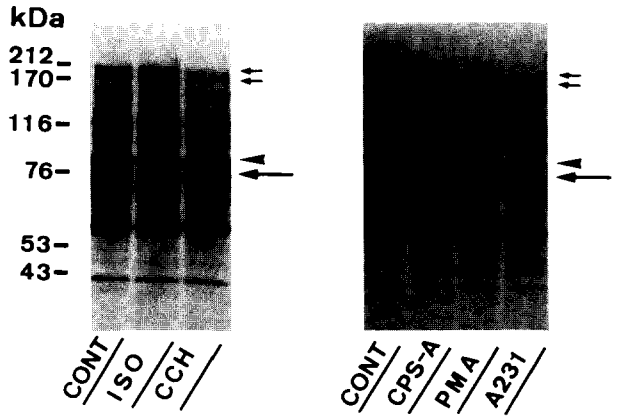

Fig. 4. Effects of various secretagogs on protein tyrosine phosphorylation in parotid acini. Acini were incubated for 5 min with 1 μM isoproterenol (ISO) or 10 μM carbachol (CCH) (the left panel) or 1 mM cps-cAMP (CPS-A), 1 μM PMA, or 1 μM A23187 (A321) (the right panel). After incubation, phosphotyrosine containing proteins were immunoprecipitated by agarose-conjugated monoclonal anti-phosphotyrosine antibody and visualized by immunoblotting as described in section 2. The small arrows indicate 190- and 210-kDa protein. The arrowhead and large arrow indicate 90- and 80-kDa proteins, respectively. CONT, control. The results shown are from two experiments representative of 4 independent experiments.

nitude of the two inhibitory effects seems to be fairly well correlated. The IC_{50} value of 120 μM for amylase release agrees well with the value reported for Ca-induced amylase release from pancreatic acini [5]. Genistein also inhibited amylase release stimulated by membrane-permeable cAMP-analogs, implying that genistein acts at a site distal to cAMP generation. The inhibition of amylase exocytosis is not due to the inhibition of protein kinase A, since genistein scarcely inhibited the enzyme activity in a cell-free assay system even at 100 μg/ml (370 μM) [11]. In addition, those inhibitiory effects of genistein were specific, since daidzein inhibited neither amylase release nor tyrosine phosphorylation. These results suggest that genistein inhibits amylase exocytosis through the inhibition of protein tyrosine phosphorylation.

In pancreatic acini, however, genistein markedly inhibited amylase release evoked by Ca, but not by phorbol ester or cAMP [4–6], indicating that the inhibitory effect of genistein is strictly selective concerning secretory stimuli and cells. It is presently unknown why genistein is able to inhibit cAMP-mediated amylase release in parotid acini but not in pancreatic ones. From their study using pancreatic acini, Lutz et al. [4] suggested that the tyrosine phosphorylation pathway functions to amplify the secretory response rather than to provide an obligate signal for the secretion. It this is true, tyrosine phosphorylation might amplify cAMP-mediated exocytosis instead of the Ca-induced one in parotid acini, where indeed cAMP-induced release is much more extensive than the Ca-induced one. Since genistein markedly inhibited major secretory pathways in the two exocrine glands, the two secretory cells might share a common regulation (amplification) mechanism involving tyrosine kinases.

In the present study, for the first time, we show that isoproterenol and cps-cAMP had dual effects on protein tyrosine phosphorylation, causing an increase in the phosphorylation of p190 and p210 and a decrease in that of p90. The dephosphorylation of p90 is not specific to cAMP, since carbachol and A23187 also decreased the phosphorylation. Recently carbachol and phorbol ester were found to induce tyrosine phosphorylation of protein kinase Cδ, although the role of this phosphorylation in exocytosis remains to be elucidated [12]. In this study, we confirmed that carbachol and PMA increased the phosphorylation of p80. So far the phosphorylation of p190 and p210 seems to be a cAMP-specific event; and

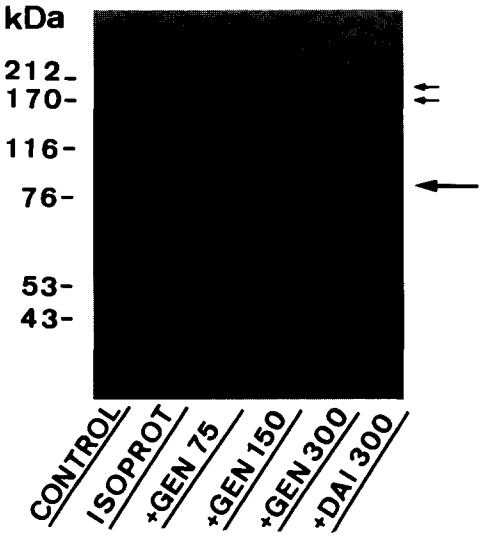

Fig. 5. Effects of genistein and daidzein on protein tyrosine phosphorylation in parotid acini. Acini were preincubated with 0, 75, 150, or 300 μM genistein or 300 μM daidzein at 37°C for 20 min, and further incubated for 5 min after addition of 1 μM isoproterenol. After incubation, phosphotyrosine containing proteins were immunoprecipitated and visualized as described in Fig. 4. The results shown are from an experiment representative of two independent experiments.

hence the two proteins might be involved in the regulatory process of amylase exocytosis from parotid acini. However, it is also possible that these proteins are engaged in other cellular functions. For instance, chronic administration of isoproterenol into rats induces hyperplasia and hypertrophy of parotid glands [13], although in our in vitro incubation system MAP kinase was not activated (unpublished observation). In general, the signaling pathway from classical second messengers, including cAMP, Ca, and diacylglycerol, to tyrosine kinases or tyrosine phosphatases is poorly understood. Thus, further study is necessary to delineate the role of tyrosine phosphorylation in cAMP-mediated secretion and growth in parotid acinar cells.

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