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Evidence for the involvement of protein phosphorylation in cyclic AMP-mediated amylase exocytosis from parotid acinar cells

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Abstract

We evaluated the role of protein phosphorylation in cAMP-mediated amylase exocytosis from parotid acinar cells by using H89, a new protein kinase A (PKA) inhibitor, which is more lipophilic and 25 times more potent than H8. In our previous studies, H8 markedly inhibited protein phosphorylation without decreasing amylase release [Takuma, T. (1988) Biochem. J. 256, 867–871]. These findings were completely reproduced even in the small acini that were prepared by trypsin treatment before collagenase digestion. In the present study, however, H89 strongly inhibited both amylase release and protein phosphorylation in a dose-dependent manner. The inhibibory effect was specific for PKA at least up to 33 μM, since 33 μM H89 did not block amylase release stimulated by PMA. H85, a closely related compound of H89 without inhibitory effect on PKA, did not prevent amylase release or protein phosphorylation at least up to 33 μM. These results suggest that protein phosphorylation by PKA is involved in cAMP-mediated amylase exocytosis. The inhibition of protein phosphorylation by H8 might be insufficient or inadequate for blocking of amylase release.

Key words: Cyclic AMP-dependent protein kinase; Exocytosis of amylase; Protein kinase inhibitor H89; Parotid acinar cell

1. Introduction

Amylase release from parotid acinar cells is a good model of cAMP-mediated exocytosis [1,2]. Except for olfactory cells, where cAMP directly opens cation channels [3], cAMP action is generally believed to be exerted through the activation of PKA, which in turn phosphorylates specific proteins involved in the process of individual cell functions. In the regulation of exocytosis, however, the role of protein phosphorylation has yet to be established, although almost all secretory stimuli affect protein phosphorylation in the direction of increase and/or decrease [4,5]. A crucial role of PKA in the exocytosis from parotid acinar cells has long been postulated, since the activation of PKA and phosphorylation of some proteins were observed concurrently with amylase release evoked by β-adrenergic agonists [6–12]. However, our previous studies suggested that protein phosphorylation is not directly involved in amylase release, since PKA inhibitors H8 and peptide fragments of heat-stable protein kinase inhibitor (PKI-(5–24)-peptide) markedly inhibited protein phosphorylation without decreasing amylase release stimulated by cAMP [13,14]. In addition, protein phosphatase inhibitors (okadaic acid and calyculin A) increased protein phosphorylation but inhibited, rather than enhanced, cAMP-mediated amylase release [15,16].

Nevertheless, studies using various cAMP analogues strongly support the hypothesis that PKA is involved in the process of amylase exocytosis. Namely, (i) suitable pairs of site-selective cAMP analogues synergistically increased amylase release [14,17]; and (ii) cAMP antagonist Rp-cAMPS (Rp-adenosine 3′,5′-phosphorothioate) competitively inhibited amylase release stimulated by cAMP or isoproterenol [18]. Recently O'Sullivan and Jamieson [19] found that H89, a new PKA inhibitor, cleary blocked the stimulatory effect of cAMP on Ca^{2+}-induced amylase release from pancreatic acini. H89 is more lipophilic and 25 times more potent than H8 [20–22]. Thus, we have reexamined the effects of H8 and H89 on amylase release and protein phosphorylation in parotid acini.

2. Experimental

2.1. Materials

H89, H85, and H8 were purchased from Seikagaku Kogyo (Tokyo, Japan). [^{32}P]Orthophosphoric acid was from DuPont-Daiichi (Tokyo). Collagenase (CLS II) was from Worthington (Freehold, NJ, USA). Hyaluronidase (type I-S), trypsin (type III), trypsin inhibitor (type II-S), and phosphate-free Eagle's minimum essential medium (MEM) were from Sigma (St. Louis, MO, USA). All other chemicals utilized were the highest grade commercially available.

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2.2. Preparation of parotid acini

Rat parotid acini were prepared as described previously [13–16]. Briefly, parotid glands were minced finely and incubated for 60 min at 37°C in Hanks' balanced salt solution buffered with 20 mM Na-HEPES (pH 7.4) (HBSS-H) containing collagenase (130 unit/ml), hyaluronidase (0.25 mg/ml), and 0.1% BSA under 100% O_2 in a metabolic shaker. After digestion, the acini were filtered through a nylon mesh, washed 4 times with HBSS-H containing 0.1% BSA, and suspended in the same medium. Acini thus prepared are mentioned sometimes hereafter as large acini.

The other type of acini was prepared according to the method described by Tanimura and Tojyo [23,24]. Briefly, finely minced parotid glands were incubated for 10 min in HBSS-H containing trypsin (0.5 mg/ml) and 0.1% BSA. Tissue was washed once with Ca^{2+}- and Mg^{2+}-free HBSS-H 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 with normal HBSS-H, it was further incubated for 20 min in normal HBSS-H containing collagenase and hyaluronidase as described above. Dispersed acini were filtered through a nylon mesh, washed 4 times with HBSS-H containing 0.1% BSA, and suspended in the same medium. Acini thus prepared are referred to as small acini.

2.3. Amylase release

Parotid acini were preincubated for 15 min at 37°C with various protein kinase inhibitors or their vehicles and further incubated for 15 min after the addition of secretory stimuli. Incubation was terminated by filtration through a glass fiber paper, and the medium was kept for assay of amylase released. For measurement of total amylase activity, acini were incubated with 0.2% Triton X-100 as above. Amylase activity was measured by the method of Bernfeld [25], and the released amylase activity was given as the percentage of the total activity.

2.4. Protein phosphorylation

Small acini were washed twice with phosphate-free MEM buffered with 40 mM Na-HEPES (pH 7.4) containing 0.1% BSA, and incubated for 60 min at 37°C in the same medium containing [^{32}P]orthophosphoric acid (0.2–0.3 mCi/ml). The acini were washed once with the same medium without [^{32}P]orthophosphoric acid, preincubated for 15 min with various protein kinase inhibitors, and further incubated for 15 min after addition of 1 μM isoproterenol. After incubation, acini were homogenized with a solution of 0.3 M sucrose, 10 mM NaF, 10 mM EDTA, 10 mM EGTA, and 10 mM K-phosphate (pH 6.8) in a Teflon–glass homogenizer. The homogenates were centrifuged at 750 × *g* for 10 min and 15,000 × *g* for 15 min at 4°C, and the resulting pellets were boiled with Laemmli buffer [26]. Electrophoresis was carried out on 15% polyacrylamide gel, and autoradiograms were prepared from dried gels by use of Fuji X-ray films.

3. Results

Although the Ki of H89 for PKA has been reported to be 48 nM in a cell-free assay system [20], up to 10 μM H89 had no effect on amylase release from intact parotid acini (large acini) stimulated by 1 μM isoproterenol. Above that concentration, however, H89 dose-dependently inhibited amylase release, and almost completely abolished the release at 100 μM. The IC_{50} was approximately 33 μM (Fig. 1). The inhibitory effect of H89 was more prominent in saponin-permeabilized acini. In these experiments acini were preincubated with H89 for the first 5 min without saponin, and then 5 min with 20 μg/ml saponin, and further incubated for 15 min after the addition of 1 mM cAMP to Ca^{2+}-free KCl medium containing 1 mM EGTA and 10 μM propranolol. Amylase release was almost completely inhibited at 33 μM H89, the IC_{50} value of which decreased to 10 μM (Fig. 1). In the permeabilized acini, however, higher concentrations of H89 markedly increased basal amylase release, suggesting H89 to have some effects on the secretory granule membrane.

To make sure that the inhibitory effect of H89 results from the inhibition of PKA, we examined the effect of H85, which is a compound closely related to H89, but is more than 1000 times less potent for PKA inhibition

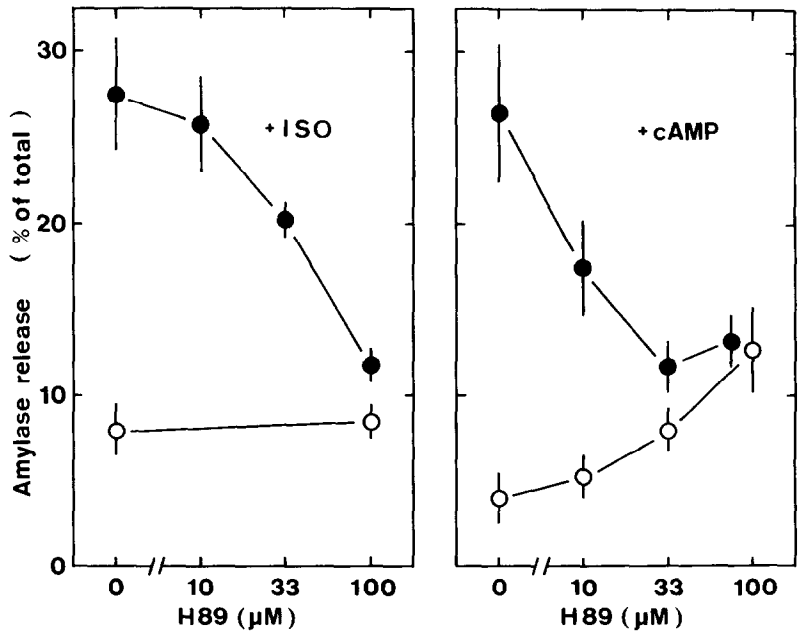

Fig. 1. Effect of H89 on amylase release from intact (left) and saponin-permeabilized (right) parotid acini. Parotid acini were preincubated at 37°C with various concentrations of H89 or its vehicle (1% DMSO) for 15 min in normal HBSS-H or for 10 min in Ca^{2+}-free KCl medium containing 1 mM EGTA, 10 μM propranolol, and 20 μg/ml saponin, and further incubated for 15 min after addition of 1 μM isoproterenol (+ISO) for intact acini or 1 mM cAMP (+cAMP) for permeabilized ones. Open symbols are H89 alone. Results are means ± S.D. (n = 8).

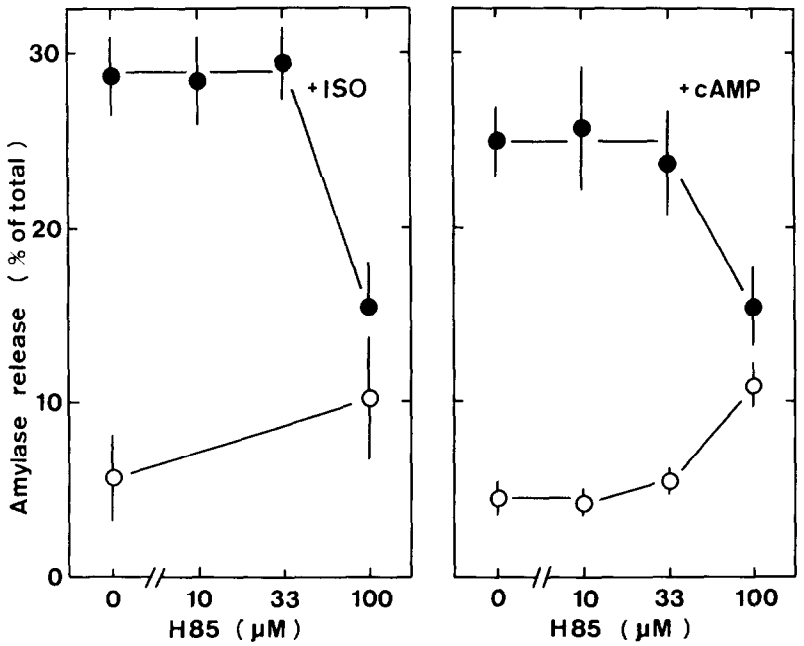

Fig. 2. Effect of H85 on amylase release from parotid acini. Intact (left) and saponin-permeabilized (right) parotid acini were preincubated with various concentrations of H85 or DMSO for 15 min in normal HBSS-H or for 10 min in Ca^{2+}-free KCl medium containing 1 mM EGTA, 10 μM propranolol, and 20 μg/ml saponin, and further incubated for 15 min with 1 μM isoproterenol or 1 mM cAMP. Open symbols are H85 alone. Results are means ± S.D. (n = 8).

than H89 [20]. As shown in Fig. 2, H85 had no inhibitory effect on amylase release up to 33 μM both in intact and saponin-permeabilized acini, but strongly blocked amylase release at 100 μM. 100 μM H85 also increased basal amylase release, as did H89.

Next we examined whether or not the inhibitory effect of H89 is specific for cAMP-mediated amylase release. Fig. 3 shows that 33 μM H89 did not inhibit amylase release stimulated by 1 μM PMA (phorbol 12-myristate 13 acetate), a direct activator of protein kinase C (PKC). The amylase release, however, was markedly inhibited by 100 μM H89, suggesting that the effect of 100 μM H89 is not necessarily specific for PKA.

In our previous studies [13,14], H8 did not inhibit amylase release, although it markedly reduced protein phosphorylation. Parotid acini prepared by collagenase and hyaluronidase digestion are mainly large-complex acini. Thus, the decrease in protein phosphorylation and the normal amylase release might occur independently in different cells (e.g. peripheral cells and interior cells) within the large acini. In order to exclude this possibility, smaller acini were prepared by 10-min treatment with trypsin before collagenase digestion [23,24]. The small acini maintained responsiveness to isoproterenol and 8-chlorophenylthio-cAMP, a membrane-permeable cAMP analogue, but did not respond to cAMP in the presence of 10–20 μg/ml saponin even in the medium supplemented with 1 mM Mg · ATP and 0.2 μM Ca^{2+} (data not shown). Therefore, we could not examine the effects of H8 and PKI-(5–24)-peptide on amylase release from saponin-permeabilized small acini.

Using the small intact acini, we reexamined the effect of H8 at higher concentrations and with longer preincubation than before [13,14]. As shown in Fig. 4, however, up to 400 μM H8 had no inhibitory effect on amylase release. In such acini, 10 μM H89 clearly inhibited amylase release (Fig. 4), although the IC_{50} value of H89 was almost same as with intact large acini (Fig. 1).

Finally we examined the effects of protein kinase inhibitors on protein phosphorylation in intact small acini stimulated by 1 μM isoproterenol. Fig. 5 shows that H89 dose-dependently inhibited phosphorylation of 21- and 26-kDa proteins in the 15,000 × g pellet fraction. Although inhibiting amylase release, 100 μM H85 hardly inhibited protein phosphorylation. Inversely, 200 μM H8 clearly decreased protein phosphorylation, as reported previously [13,14].

4. Discussion

In the present study H89 markedly inhibited both amylase release and protein phosphorylation in parotid acini stimulated by isoproterenol. The inhibitory effect of H89 was dose dependent and specific for PKA at least up to 33 μM, since 33 μM H89 did not inhibit amylase release induced by PMA. In addition, H85, a negative control of H89, which has similar Ki values for various protein kinases except for PKA [20], did not block amylase release up to 33 μM. These results strongly suggest that H89 inhibits cAMP-mediated amylase release through the inhibition of protein phosphorylation by

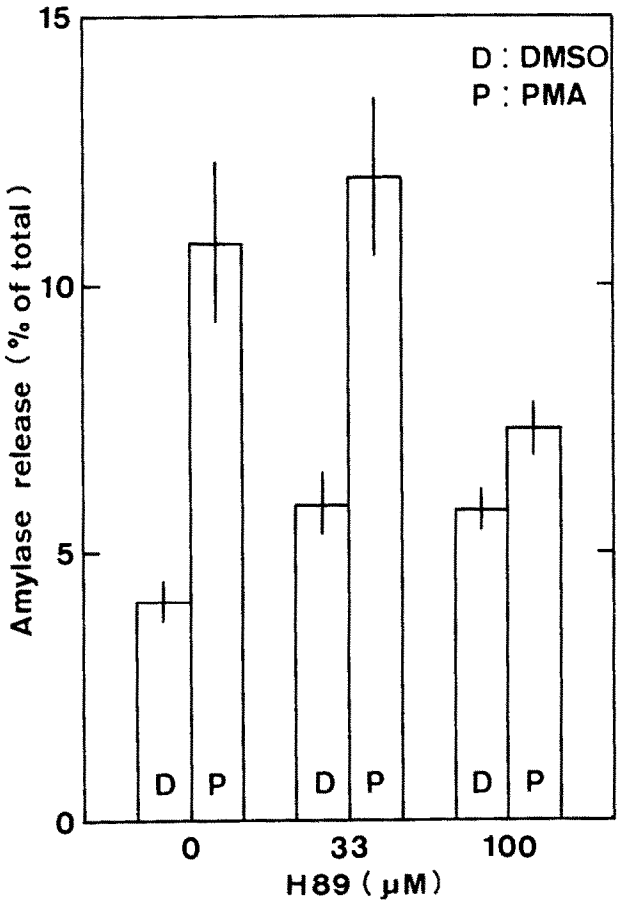

Fig. 3. Effect of H89 on amylase release from parotid acini stimulated by PMA. Intact acini were preincubated with various concentrations of H89 for 15 min, and further incubated for 15 min with 1 μM PMA or its vehicle (DMSO). Resuls are means ± S.D. ($n = 4$).

PKA. This conclusion is quite consistent with previous findings that H89 clearly blocked the stimulatory effect of cAMP on Ca^{2+}-induced amylase release from pancreatic acini [19], but is completely inconsistent with our previous conclusion [13,14].

Our current study, however, also confirmed our previous findings that H8 inhibited protein phosphorylation without decreasing amylase release [13,14]. The inhibition of protein phosphorylation and the normal amylase release are very likely to occur in the same acinar cells, since these phenomena were observed even in the small acini prepared by trypsin treatment prior to collagenase digestion (Figs. 4 and 5). There are several possible reasons why H8 did not block amylase release. Namely, (i) the inhibition of protein phosphorylation by H8 might be insufficient for a detectable decrease in amylase release. This implies that a small increase in protein phosphorylation is enough for the maximal amylase release, although amylase release and phosphorylation of 21- and 26-kDa proteins were well correlated in the absence of protein kinase inhibitors [14]. (ii) Whereas H8 inhibited 21- and 26-kDa proteins, it would not block phosphorylation of the unidentified proteins that is responsible for exocytosis, as suggested by O'Sullivan and Jamieson [19]. This presumes that there are intracellular compartments of PKA and its substrates, which are accessible for H89 but not for H8 because of their differences in lipophilicity or other reasons.

In the present study 100 μM H85 strongly blocked cAMP-mediated amylase release. This inhibitory effect is unlikely to be related to PKA, since the K_i of H85 for PKA is more than 100 μM [20]. Indeed, 100 μM H85

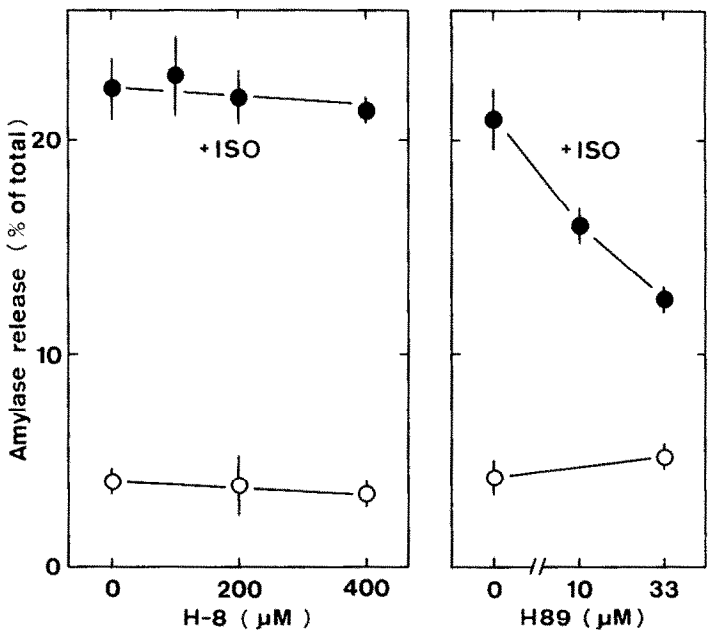

Fig. 4. Effects of H8 and H89 on amylase release from intact small acini. The small acini were preincubated with various concentrations of H8 or H89 for 15 min, and further incubated for 15 min with 1 μM isoproterenol. Results are means ± S.D. ($n = 6$).

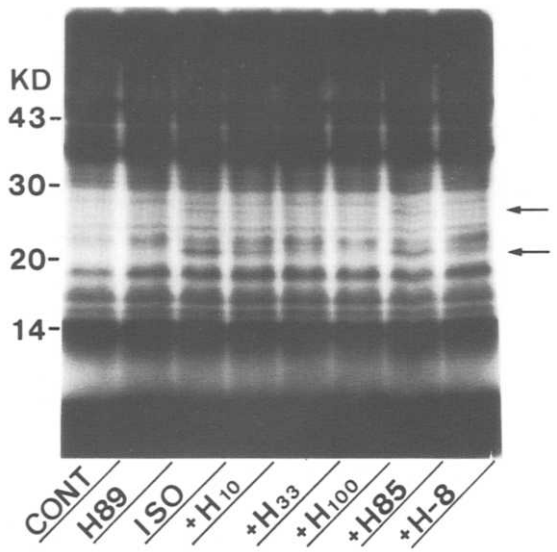

Fig. 5. Effects of H89, H85, and H8 on protein phosphorylation in intact small acini stimulated by isoproterenol. The ^{32}P-labelled small acini were preincubated with 10–100 μM H89, 100 μM H85, or 200 μM H8 for 15 min in phosphate-free MEM, and further incubated for 15 min after addition of 1 μM isoproterenol. Proteins in the 15,000 × g pellet fractions were separated by SDS-PAGE on 15% gel, and phosphorylated proteins were detected by autoradiography. CONT, 1% DMSO alone; H89, 100 μM H89 alone; ISO, 1 μM isoproterenol alone; + H 10, + H 33, and + H 100 are 10–100 μM H89 plus 1 μM isoproterenol, respectively; + H85, 100 μM H85 plus 1 μM isoproterenol; + H-8, 200 μM H8 plus 1 μM isoproterenol. Arrows indicate 21 and 26 kDa proteins.

hardly inhibited protein phosphorylation evoked by isoproterenol. Similarly the effect of H89 is not necessarily specific for PKA, since 100 μM H89 inhibited PMA-induced amylase release. Since H85 and H89 are structurally related, 100 μM H85 and H89 are very likely to share the same inhibitory mechanism that is presently unknown. As discussed above, the inhibition of amylase release by up to 33 μM H89 is highly possible to be mediated by the inhibition of PKA. However, the possibility is not completely ruled out that H89 at lower than 33 μM still maintains the unidentified inhibitory action of 100 μM H85 and H89, although 33 μM H89 did not inhibit PMA-mediated amylase release.

Taken as a whole, the present results and previous findings in pancreatic acini [19] strongly support the conclusion that protein phosphorylation by PKA is involved in the process of cAMP-mediated amylase exocytosis. In the two exocrine glands, however, the role of PKA in the exocytosis is not necessarily the same, since cAMP is the main stimulant for amylase release in parotid glands, whereas cAMP is a poor stimulant by itself but a modulator of Ca^{2+}-induced exocytosis in the pancreas. Anyway, the complete demonstration of the validity of this hypothesis demands data showing that amylase release is induced by direct introduction of PKA catalytic subunits into acinar cells of those glands.

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