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Evidence against direct involvement of cyclic AMP-dependent protein phosphorylation in the exocytosis of amylase

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To examine whether or not the activation of cyclic AMP-dependent protein kinase is coupled to the exocytosis of amylase from rat parotid cells, the effect of protein kinase inhibitors on amylase release and protein phosphorylation was studied. A membrane-permeable inhibitor of cyclic AMP-dependent protein kinase, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide (H-8), and peptide fragments of the heat-stable protein kinase inhibitor [PKI-(5–24)-peptide and PKI-(14–24)-amide] strongly inhibited cyclic AMP-dependent protein kinase activity in the cell homogenate. However, H-8 had no inhibitory effect on amylase release from either intact or saponin-permeabilized parotid cells stimulated by isoproterenol or cyclic AMP. Moreover, PKI-(5–24)-peptide and PKI-(14–24)-amide did not inhibit cyclic AMP-evoked amylase release from saponin-permeabilized cells, whereas cyclic AMP-dependent phosphorylations of 21 and 26 kDa proteins in intact or permeabilized cells were markedly inhibited by these inhibitors. These results suggest that cyclic AMP-dependent protein phosphorylation is not directly involved in the exocytosis of amylase regulated by cyclic AMP.

INTRODUCTION

Protein phosphorylation stimulated by various protein kinases has been generally recognized to play crucial roles in the regulatory mechanism of diverse cellular functions. Most, if not all, effects of cyclic AMP are also believed to be mediated by the activation of cyclic AMP-dependent protein kinase and the subsequent phosphorylation of specific proteins (Greengard, 1978; Krebs & Beavo, 1979). Amylase secretion from the parotid gland has been extensively studied as a useful model of cyclic AMP-dependent exocytosis (Butcher & Putney, 1980). Although the activation of cyclic AMP-dependent protein kinase and the phosphorylation of some proteins are observed concurrently with amylase release evoked by β-adrenergic agonists or cyclic AMP analogues (Jahn *et al.*, 1980; Kanamori & Hayakawa, 1980; Baum *et al.*, 1981; Dowd *et al.*, 1981; Freedman & Jamieson, 1982; Spearman *et al.*, 1984; Quissel *et al.*, 1985), it was not clear whether or not the activation of the protein kinase was coupled to the exocytosis. In the present study I examined the effect of H-8, a membrane-permeable inhibitor of cyclic nucleotide-dependent protein kinases (Hidaka *et al.*, 1984), and PKI-(5–24)-peptide and PKI-(14–24)-amide, peptide fragments of the heat-stable protein kinase inhibitor (PKI) (Scott *et al.*, 1985; Cheng *et al.*, 1986), on amylase release and protein phosphorylation, and obtained evidence against the direct involvement of cyclic AMP-dependent protein phosphorylation in amylase secretion from rat parotid cells.

EXPERIMENTAL

Assay of amylase release

Rat parotid cells were prepared by collagenase and hyaluronidase digestion (Takuma & Ichida, 1986), and amylase secretion from intact or saponin-permeabilized parotid cells was determined as described previously (Takuma & Ichida, 1988).

Assay of cyclic AMP-dependent protein kinase

Cyclic AMP-dependent protein kinase activity was determined by the method of Glass *et al.* (1978). Parotid cells were homogenized with 150 mM-KCl/20 mM-Hepes/Tris (pH 7.2)/3 mM-dithiothreitol in a Teflon/glass homogenizer. The homogenate was centrifuged at 750 *g* for 10 min and at 100000 *g* for 60 min at 4 °C, and the resulting pellet dissolved in the above buffer and the supernatant were used as enzyme samples. The protein kinase activity was measured in a reaction mixture (100 μl) containing 40 mM-Hepes/Tris, pH 7.2, 0.2 mM-Kemptide (a synthetic heptapeptide corresponding to the phosphorylation site of pyruvate kinase), 0.2 mM-ATP ($\sim$ 2 μCi of [γ-^{32}P]ATP), 10 mM-$MgSO_4$, 1 μM-cyclic AMP, various concentrations of inhibitors and the enzyme. Reaction was started by the addition of enzyme and continued for 1–5 min at 30 °C. At the end of the incubation, 20 μl of the reaction mixture was spotted on to Whatman P81 phosphocellulose paper (2 cm square). The paper was immediately immersed into 30% (v/v) acetic acid and processed as described by Glass *et al.* (1978).

Protein phosphorylation in intact cells

The study of protein phosphorylation in intact cells was performed by the method of Jahn *et al.* (1980). Parotid cells were incubated with 0.2–0.4 mCi of [^{32}P]-orthophosphate/ml in phosphate-free medium composed of 140 mM-NaCl, 5 mM-KCl, 20 mM-Hepes, pH 7.4, 1.4 mM-$CaCl_2$, 1 mM-$MgSO_4$, 10 mM-glucose, 0.1% bovine serum albumin and Phenol Red (10 μg/ml) at 37 °C for 60 min. The ^{32}P-labelled cells were washed once

Abbreviations used: PKI, protein kinase inhibitor; H-8, *N*-[2-(methlyamino)ethyl]-5-isoquinolinesulphonamide; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; W-7, *N*-(6-aminohexyl)-5-chloronaphthalene-1-sulphonate.

with normal Hanks medium, preincubated with or without H-8 in Hanks medium for 5 min, and further incubated for 10 min after addition of isoproterenol. After incubation, the medium was removed, and cells were homogenized in 0.3 M-sucrose/2 mM-EDTA/0.2 mM-EGTA/10 mM-Hepes/Tris, pH 7.2, in a Teflon/glass homogenizer. The homogenates were centrifuged at 750 *g* for 10 min, at 15000 *g* for 15 min, and at 100000 *g* for 60 min at 4 °C, and the resulting pellets or supernatants were boiled in 3% (w/v) SDS/2% (v/v) 2-mercaptoethanol/5% (v/v) glycerol/70 mM-Tris/HCl, pH 6.8, for 5 min. SDS/polyacrylamide-gel electrophoresis was carried out (Laemmli, 1970) and autoradiograms were prepared using Fuji X-ray films.

Protein phosphorylation in saponin-permeabilized cells

Parotid cells were washed twice with Ca^{2+}-free medium composed of 140 mM-KCl, 1 mM-$MgSO_4$, 1 mM-EGTA, 40 mM-Hepes/Tris, pH 7.2, 0.1% bovine serum albumin and Phenol Red (10 μg/ml), and incubated at 37 °C for 5 min in the same medium containing 20 μg of saponin/ml, ~ 0.4 mCi of [γ-^{32}P]ATP/ml and with or without PKIs. The incubation was continued for another 10 min after addition of 1 mM-cyclic AMP. After incubation, cells were homogenized and processed as for intact cells.

Materials

Kemptide, cyclic AMP, dibutyryl cyclic AMP and isoproterenol were purchased from Sigma (St. Louis, MO, U.S.A.). H-8, H-7, and W-7 were from Seikagaku Kogyo (Tokyo, Japan). PKI-(5–24)-peptide and PKI-(14–24)-amide were from Peninsula Laboratories (Belmont, CA, U.S.A.). Saponin, collagenase (CLS II) and Hanks medium were from Merck (Darmstadt, Germany), Cooper Biomedical (Malvern, PA, U.S.A.) and Gibco (Chagrin Fall, OH, U.S.A.) respectively. [^{32}P]Orthophosphate and [γ-^{32}P]ATP were from Japan Atomic Energy Research Institute (Tokyo, Japan) and New England Nuclear (Boston, MA, U.S.A.) respectively. All other reagents utilized were the highest grade commercially available.

RESULTS

Effect of H-8

The effect of H-8 on cyclic AMP-dependent protein kinase in soluble and particulate fractions of the parotid cells was examined by using Kemptide as a phosphate acceptor. The enzyme activity was proportional with time at least over a 5 min incubation period and with amount of enzyme protein (0.07–1.4 mg/ml) (results not shown). When H-8 was added, the enzyme activities in the two fractions were uniformly inhibited (Fig. 1).

For evaluation of the effect of H-8 on amylase release, parotid cells were preincubated with various concentrations of H-8 for 5 min, and amylase release was stimulated by the addition of 10 μM-isoproterenol. Unexpectedly, however, H-8 had no effect on amylase release at any time of incubation or at any dose tested (Figs. 2*a* and 2*b*). The inhibitory effect of H-8 was still not observed when the cells were stimulated by lower concentrations of isoproterenol (Fig. 3).

To make sure that H-8 had access to the cyclic AMP-dependent protein kinase, I tested H-8 with saponin-permeabilized parotid cells. The cells were preincubated with saponin (10 μg/ml) and 0–200 μM-H-8 in Ca^{2+}-free high-K^+ medium for 5 min and further incubated for 15 min after addition of 1 mM-cyclic AMP. Amylase release was not inhibited in these experiments either (Fig. 2*c*). Similarly, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7), a related compound of H-8 and

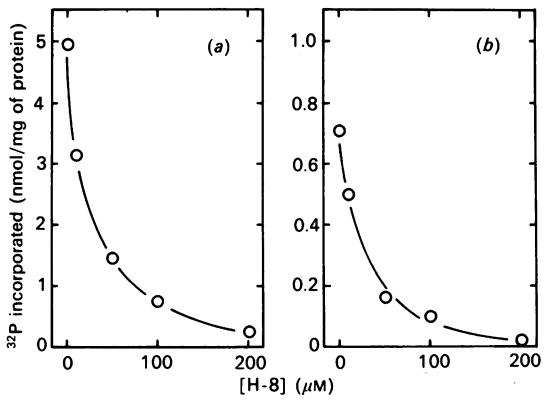

Fig. 1. Effect of H-8 on cyclic AMP-dependent protein kinase activities in soluble (*a*) and particulate (*b*) fractions of rat parotid cells

Parotid cells were homogenized and centrifuged at 750 *g* for 10 min and at 100000 *g* for 60 min. The resulting supernatant (100000 *g*) and pellet (750–100000 *g*) were used as the soluble and particulate fractions respectively. Incubation was carried out with 0–200 μM-H-8 and 1 μM-cyclic AMP at 30 °C for 5 min.

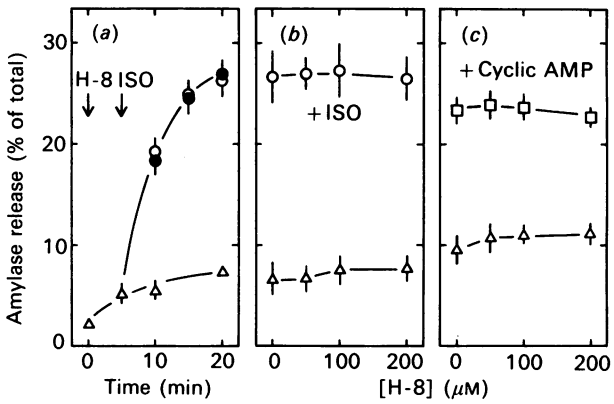

Fig. 2. Effect of H-8 on amylase release from intact (*a* and *b*) or saponin-permeabilized (*c*) parotid cells

(*a*) Parotid cells were preincubated at 37 °C for 5 min in the absence (○, △) or presence (●) of 100 μM H-8 in Hanks balanced salt solution buffered with 20 mM-Hepes, pH 7.4, and further incubated for 15 min after addition of 10 μM-isoproterenol (ISO) (○,●). (*b*) Dose–response curve for H-8. Cells were preincubated with various concentrations of H-8 for 5 min and further incubated for 15 min after addition of 10 μM isoproterenol. △, H-8 alone. (*c*) Cells were preincubated for 5 min with saponin (10 μg/ml) and 0–200 μM-H-8 in Ca^{2+}-free high-K^+ medium and further incubated for 15 min after addition of 1 mM-cyclic AMP. △, H-8 alone. Results are means ± S.D. (n = 6–8).

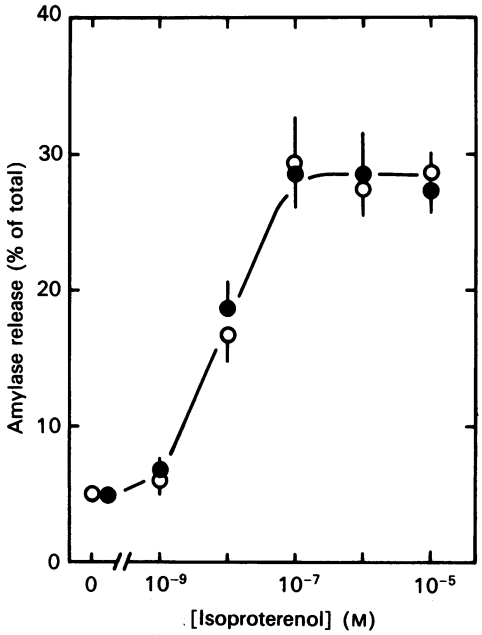

Fig. 3. Effect of H-8 on amylase release from parotid cells stimulated by isoproterenol

Cells were preincubated with (●) or without (○) 100 μM-H-8 for 5 min and further incubated for 15 min after addition of 0–10^{-5} M-isoproterenol. Results are means ± S.D. ($n = 4$).

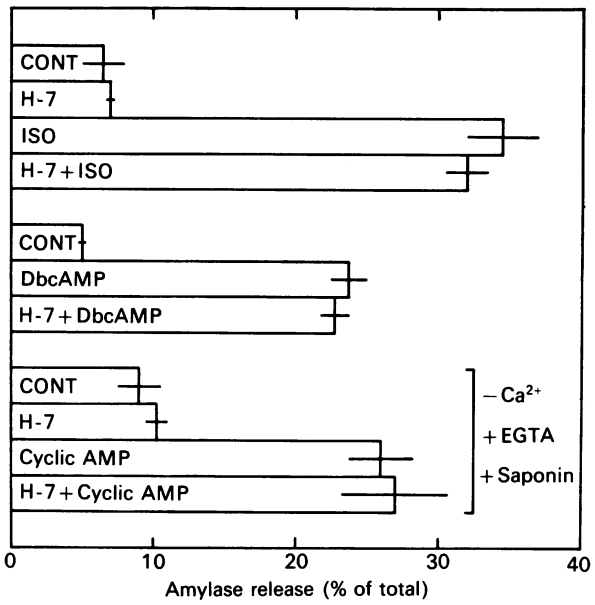

Fig. 4. Effect of H-7 on amylase release from intact or saponin-permeabilized parotid cells

Cells were preincubated with 100 μM-H-7 for 5 min in either normal Hanks medium or Ca^{2+}-free high-K^+ medium containing 1 mM-EGTA and saponin (10 μg/ml). The incubation was continued for another 15 min after the addition of either 1 μM-isoproterenol (ISO) or 1 mM-dibutyryl cyclic AMP (DbcAMP) for intact cells and 1 mM-cyclic AMP for saponin-permeabilized cells. Results are means ± S.D. ($n = 6$). Abbreviation: CONT, control.

widely used as an inhibitor of protein kinase C (Hidaka *et al.*, 1984; Inagaki *et al.*, 1984; Pandol & Schoeffield, 1986), had no inhibitory effect on amylase release stimulated by isoproterenol or dibutyryl cyclic AMP in intact cells or by cyclic AMP in permeabilized cells (Fig. 4).

To examine whether or not H-8 actually exerts its effect as an inhibitor of cyclic AMP-dependent protein kinase inside the cell, I studied the effect of H-8 on protein phosphorylation. ^{32}P-labelled cells were preincubated with 200 μM-H-8 for 5 min and further incubated for 10 min after the addition of 1 μM-isoproterenol. The isoproterenol-evoked protein phosphorylation was clearly found in the particulate fraction but not in the soluble fraction, as previously described (Jahn *et al.*, 1980). In the 15000 *g* pellet fraction there were two phosphorylated proteins whose molecular masses were 21 and 26 kDa (Fig. 5). The phosphorylation of these proteins was markedly inhibited by H-8. The stimulation of protein phosphorylation by isoproterenol or the inhibition of protein phosphorylation by H-8 did not occur after the cells were homogenized (Fig. 5, lanes 5 and 6).

Effect of heat-stable PKI

To obtain separate lines of evidence using different inhibitors, I examined the effects of PKI-(5–24)-peptide and PKI-(14–24)-amide on cyclic AMP-dependent protein kinase activity in the cell homogenate (750 *g* supernatant). As Fig. 6 shows, the inhibitory effect of PKI-(5–24)-peptide was more than two orders of magnitude stronger than that of PKI-(14–24)-amide; 1 μM-PKI-(5–24)-peptide almost completely inhibited the enzyme activity, but 16 and 8% of the total activity remained at 40 and 100 μM-PKI-(14–24)-amide respectively.

Effects of these peptide inhibitors and H-8 on protein phosphorylation in saponin-permeabilized cells were shown in Fig. 7. In these experiments parotid cells were preincubated for 5 min with 20 μg/ml saponin, 0.4 mCi/ml [γ-^{32}P]ATP and either 10 μM-PKI-(5–24)-peptide, 40 μM-(14–24)-amide or 200 μM-H-8 and further incubated for 10 min after addition of 1 mM-cyclic AMP. The cyclic AMP-dependent phosphorylation of 21 and 26 kDa proteins was similarly detected in permeabilized cells, although some proteins, namely those which were phosphorylated independently of isoproterenol in intact cells, were not detectable. The phosphorylation of 21 and 26 kDa proteins was markedly inhibited by PKI-(5–24)-peptide and H-8, and slightly inhibited by 40 μM-PKI-(14–24)-amide.

Effects of these inhibitors on amylase release from saponin-permeabilized cells are shown in Table 1. Under incubation conditions identical with those used above for protein phosphorylation, except that [γ-^{32}P]ATP was not present, no inhibitory effect of these inhibitors on cyclic AMP-evoked amylase release was detected.

DISCUSSION

The present study has shown that H-8 and PKI-(5–24)-peptide strongly inhibit cyclic AMP-dependent protein phosphorylation in rat parotid cells without affecting cyclic AMP-evoked amylase release. This implies that cyclic AMP-dependent protein phosphorylation is not directly involved in the exocytosis of amylase. In previous studies, the involvement of cyclic

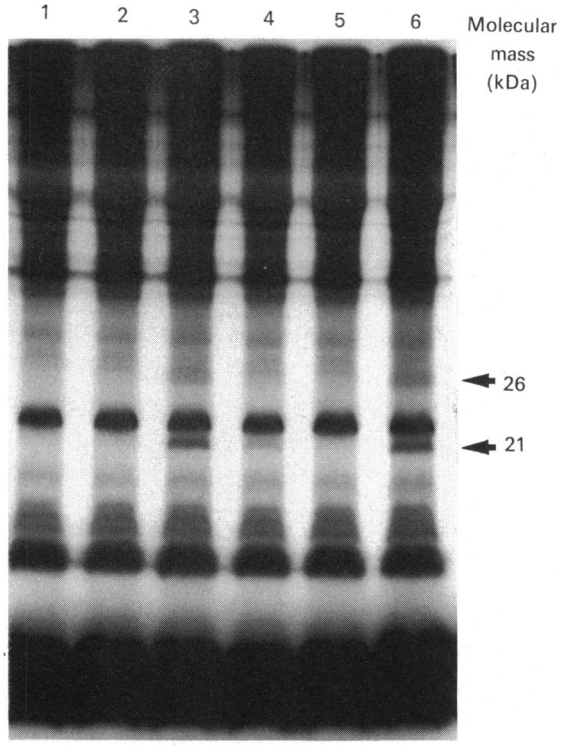

Fig. 5. Effect of H-8 on protein phosphorylation in parotid cells stimulated by isoproterenol

The ^{32}P-labelled cells were preincubated with 200 μM-H-8 in Hanks medium at 37 °C for 5 min, and further incubated for 10 min after addition of 1 μM-isoproterenol. After incubation, cells were homogenized and processed as described in the Experimental section. In some experiments, isoproterenol or H-8 was added after cells were homogenized. The autoradiograms shown are those of the 15000 *g* pellet fraction. Lane 1, control; lane 2, 200 μM-H-8 alone; lane 3, 1 μM-isoproterenol; lane 4, 200 μM-H-8 plus 1 μM-isoproterenol; lane 5, control (1 μM-isoproterenol was added to the homogenate); lane 6, 1 μM-isoproterenol (200 μM-H-8 was added to the homogenate).

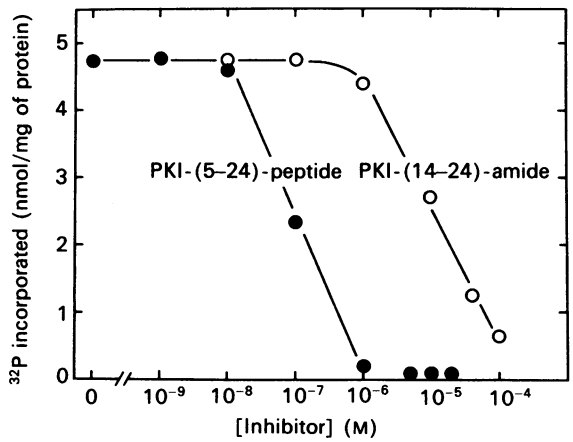

Fig. 6. Effects of PKI-(5–24)-peptide and PKI-(14–24)-amide on the activity of cyclic AMP-dependent protein kinase

Parotid cells were homogenized and centrifuged at 750 *g* for 10 min, and the resulting supernatant was used as an enzyme sample. Incubation was carried out with 1 μM-cyclic AMP and either 0–20 μM-PKI-(5–24)-peptide or 0–100 μM-PKI-(14–24)-amide for 5 min at 30 °C.

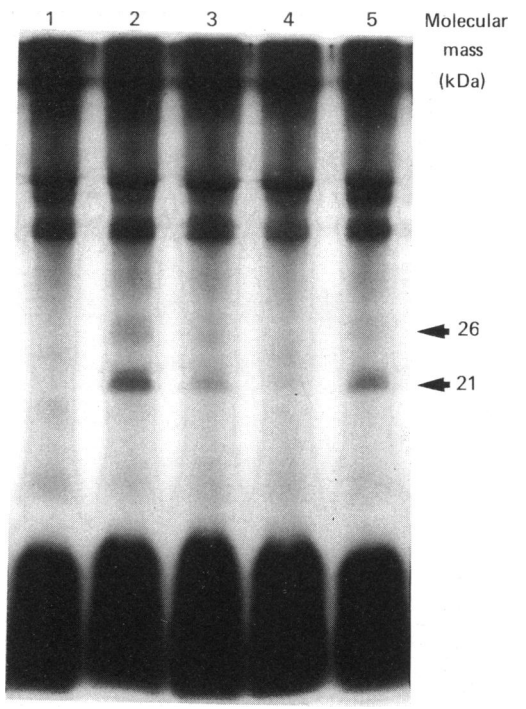

Fig. 7. Effects of PKI-(5–24)-peptide, PKI-(14–24)-amide and H-8 on protein phosphorylation in saponin-permeabilized parotid cells stimulated by cyclic AMP

Cells were preincubated for 5 min with saponin (20 μg/ml), [γ-^{32}P]ATP (0.4 mCi/ml) and either 10 μM-PKI-(5–24)-peptide, 40 μM-PKI-(14–24)-amide or 200 μM-H-8, and further incubated for 10 min after addition of 1 mM-cyclic AMP. After incubation, cells were processed as described in Fig. 5. Lane 1, control; lane 2, cyclic AMP alone; lane 3, PKI-(5–24)-peptide plus cyclic AMP; lane 4, H-8 plus cyclic AMP; lane 5, PKI-(14–24)-amide plus cyclic AMP.

Table 1. Effects of PKI-(5–24)-peptide, PKI-(14–24)-amide and H-8 on cyclic AMP-dependent amylase release from saponin-permeabilized parotid cells

Cells were preincubated with 20 μg of saponin/ml and each inhibitor in Ca^{2+}-free medium at 37 °C for 5 min and further incubated for 15 min after the addition of 1 mM-cyclic AMP. Data shown are means ± S.D. (n = 8).

Treatment	Amylase release (% of total/20 min)
Control	13.2 ± 1.8
Cyclic AMP (1 mM)	25.5 ± 1.8
+ PKI-(5–24)-peptide (10 μM)	24.3 ± 1.6
+ PKI-(14–24)-amide (40 μM)	25.6 ± 2.8
+ H-8 (200 μM)	23.8 ± 2.2

AMP-dependent protein kinase and protein phosphorylation in the exocytotic process of amylase was indirectly suggested by an intimate correlation between amylase release and protein phosphorylation during stimulation or inhibition by agonists or antagonists (Jahn *et al.*, 1980; Kanamori & Hayakawa, 1980; Baum *et al.*, 1981; Dowd *et al.*, 1981; Freedman & Jamieson, 1982;

Spearman *et al.*, 1984; Quissell *et al.*, 1985). However, the protein phosphorylation is an almost inevitable phenomenon when the cellular cyclic AMP level is increased. Moreover, cyclic AMP affects diverse cellular functions in the salivary gland besides exocytosis. In the present study the close correlation between amylase release and protein phosphorylation was confirmed in the absence of H-8 or PKI-(5–24)-peptide but not in their presence.

Since the inhibitory effect of H-8 is competitive with respect to ATP (Hidaka *et al.*, 1984), fairly high concentrations of H-8 [as compared with the K_i (1.2 μM) reported for cyclic AMP-dependent protein kinase] were utilized in the present study. Nevertheless, the inhibition of protein phosphorylation was not complete in intact cells, probably because the ATP concentration in intact cells was higher than that used for the assay of the enzyme *in vitro*. When the inhibitor was utilized in saponin-permeabilized cells, however, almost complete inhibition was observed (Fig. 7). On the other hand, the inhibitory mechanism of the heat-stable inhibitor of cyclic AMP-dependent protein kinase is completely different; the effect of PKI-(5–24)-peptide is competitive with respect to the protein substrate (Cheng *et al.*, 1986). In saponin-permeabilized cells PKI-(5–24)-peptide was slighty less effective than H-8, probably because the accessibility of the PKI to the enzyme or the protein substrate was lower than that of H-8 in permeabilized cells. Anyway, the two inhibitors with different inhibitory mechanisms showed similar effects on the protein phosphorylation and amylase release. This strongly supports the above conclusion.

It has been reported that calmodulin antagonists such as trifluoperazine or W-7 inhibit the exocytotic process in various cells, including amylase release from parotid cells stimulated by isoproterenol or dibutyryl cyclic AMP (Kanagasuntheram & Teo, 1983; Tojyo *et al.*, 1987). I have also confirmed the inhibitory effect of W-7 on amylase release from both intact and saponin-permeabilized cells (30–40 % inhibition at 100 μM-W-7). Although calmodulin has been suggested to be involved in the exocytotic process, the inhibitory mechanisms of these agents is still unclear, since calmodulin is involved in diverse cellular structures and functions. Moreover, it is uncertain that these compounds act only on calmodulin (Sakata *et al.*, 1987). The interpretation of the present findings seems to be very simple. Since H-8 and PKI-(5–24)-peptide had no effect on amylase release, cellular changes evoked by these agents were unequivocally excluded from the exocytotic process of amylase.

Although it is not the purpose of this paper to propose an alternative mechanism of cyclic AMP-dependent exocytosis, it has been shown that the type II regulatory subunit (RII) of cyclic AMP-dependent protein kinase, a major acceptor protein of cyclic AMP, binds to various proteins including cytoskeletal proteins (Hathaway *et al.*, 1981; Weber *et al.*, 1982; Lohmann *et al.*, 1984; Gergely & Bot, 1977; Khatra *et al.*, 1985; Jungensen *et al.*, 1985). Complexes of cyclic AMP with RII and other cellular components might have some regulatory roles in exocytosis independent of protein phosphorylation.

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