Cyclic AMP binding proteins in saliva

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Summary. Cyclic adenosine 3′,5′-monophosphate (cyclic AMP)-binding proteins which have the characteristics of cyclic AMP-dependent protein kinase (E.C. 2.7.1.37) regulatory subunits, have been identified in rat and human saliva. Concentration of these proteins was increased in rat saliva after stimulation with isoproterenol, suggesting that they were released during exocytosis. Cyclic AMP-dependent protein kinase catalytic activity was not measurable in saliva of either species.
Key words. Cyclic AMP-binding protein; regulation of exocytosis; salivary protein secretion; rat saliva; human saliva; isoproterenol.

The salivary glands synthesize and secrete into the oral cavity numerous proteins and glycoproteins. Several have been identified[1,2] and can be classified into functional categories: digestive enzymes (amylase, DNase, RNase); antibacterial substances (lactoperoxidase, lysozyme, lactoferrin, secretory IgA); and mineral binding proteins (statherin). Other salivary proteins such as the acidic, basic and glycoconjugated, proline-rich proteins have been extensively characterized[3-6], but their in vivo function remains largely unknown.

We report here a previously unrecognized protein component of (rat and human) saliva, which has the physical properties of the regulatory (R) subunit of cyclic AMP-dependent protein kinase (cA-PK; ATP: protein phosphotransferase, E.C. 2.7.1.37). While various types of protein kinases, with multiple functions, exist in almost all cell types and organisms, cA-PK has physical and physiological features that make it uniquely identifiable. First, the R subunits are the only known cyclic AMP-binding proteins in mammalian tissues[7] and second, catalytic activity typically occurs in response to increased cellular concentrations of cyclic AMP resulting from hormonal stimulation. These reactions are shown schematically as follows:

$$1.\ R_2C_2 \underset{-\text{cAMP}}{\overset{+\text{cAMP}}{\rightleftharpoons}} 2(R\cdot\text{cAMP}) + 2C$$

$$2.\ \text{(dephospho)protein} \xrightarrow[\text{cA-PK}]{\text{ATP ADP}} \text{(phospho)protein}$$

where R·cAMP, a regulatory subunit – cyclic AMP complex, and C, the catalytic subunit which catalyzes the formation of a phosphate ester with hydroxyl groups of specific serine or threonine residues, are the dissociated components of the catalytically inactive holoenzyme, R_2C_2[8]. Reaction (2) can be followed by measuring incorporation into protein of [^{32}P] phosphate from ATP labeled in the γ phosphate and has been extensively described[9].

Materials and methods. Parotid saliva was collected with a Lashley cup from humans and by extraoral cannulation from anesthetized rats. The samples were briefly dialyzed (2 h, with 2 changes in a 1:1000 ratio of saliva versus dialysis buffer (5×10^{-2} M tris, pH 7.6, 10^{-6} M $MgCl_2$, 10^{-5} M EDTA, 2×10^{-3} M benzamidine, 10^{-4} M PMSF), and concentrated by membrane filtration (Amicon) to the original volume. Aliquots (500 µl) were used for photoaffinity labeling, using 8-azido adenosine-3′,5′-cyclic monophosphate ([^{32}P], sp.act. 10–25 Ci/mM, ICN). This was carried out by a modified method of Hoyer et al.[10] in a 10^{-1} M tris buffer, pH 7.6, with 5×10^{-3} M $MgCl_2$, 4×10^{-2} M NaCl, and 3×10^{-2} M KCl by incubating 1 h at 4 °C, then irradiating for 10 min with a UV light (254 nm) at a distance of 10 cm. The samples were treated with charcoal (Norite, 2 mg/ml) and concentrated by membrane filtration. For chromatography, combined samples were applied to a S-200 Sephacryl (Pharmacia) column. Similar samples were prepared for electrophoresis, using 8% polyacrylamide, sodium dodecyl sulfate gels (SDS-PAGE) according to Laemmli[11].

Table 1. Incorporation of [^{32}P]-8-N_3-cAMP into human and rat parotid salivary proteins and into proteins of rat parotid, liver and heart subcellular fractions

Sample supernatant	cpm incorporated per mg protein		
	600 × g pellet	10,000 × g pellet	10,000 × g supernatant
Human saliva	0	304	16,462
Rat saliva	0	0	3,840
	0	0	3,270
Rat parotid	1,730	4,256	3,476
	1,558	3,270	3,478
Rat liver	5,102	11,524	2,292
	2,830	12,036	1,902
Rat heart	3,858	12,262	4,276
	4,862	12,030	4,952

The values reported are separate aliquots of each sample that were labeled with [^{32}P]-8-N_3-cAMP, treated with charcoal, dialyzed and concentrated in a Minicon concentrator. The human saliva data are averaged values of samples (citric acid-stimulated ductal saliva) from 2 separate individuals. The rat saliva was collected after isoproterenol stimulation (30 mg/kg, i.p.).

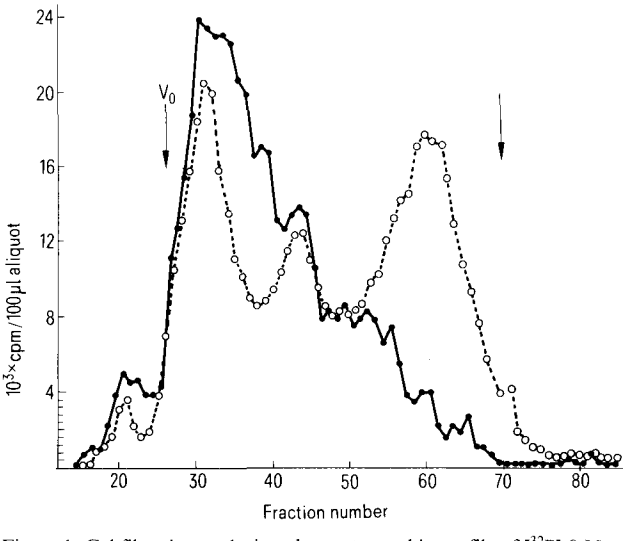

Figure 1. Gel filtration exclusion chromatographic profile of [^{32}P]-8-N_3-cAMP-labeled proteins from human and rat saliva. The samples were prepared as described in 'Materials and methods', then applied to a S-200 Sephacryl column, eluted after Blue Dextran exclusion with the same buffer used for dialysis, and 0.5-ml fractions were collected in the cold and aliquots counted. The buffer contained a protease inhibitor mixture (benzamidine 2×10^{-3} M, PMSF 10^{-4} M). The curve connected with open circles represents the radioactivity peaks obtained by chromatography of rat saliva; the curve connected by closed circles represents comparable peaks from human saliva.

Tissue samples (parotid, liver and heart) were homogenized in 0.25 M sucrose, 0.05 M tris, pH 7.6, 10^{-6} M $MgCl_2$ buffer. Aliquots were removed for measuring total incorporation, the remainder was centrifuged at 600 × g and aliquots of the resuspended pellet were labeled. The 600 × g supernatant fraction was centrifuged at 10,000 × g and the supernatant and resuspended pellet fractions labeled as above. Protein content was measured by the method of Lowry[12].

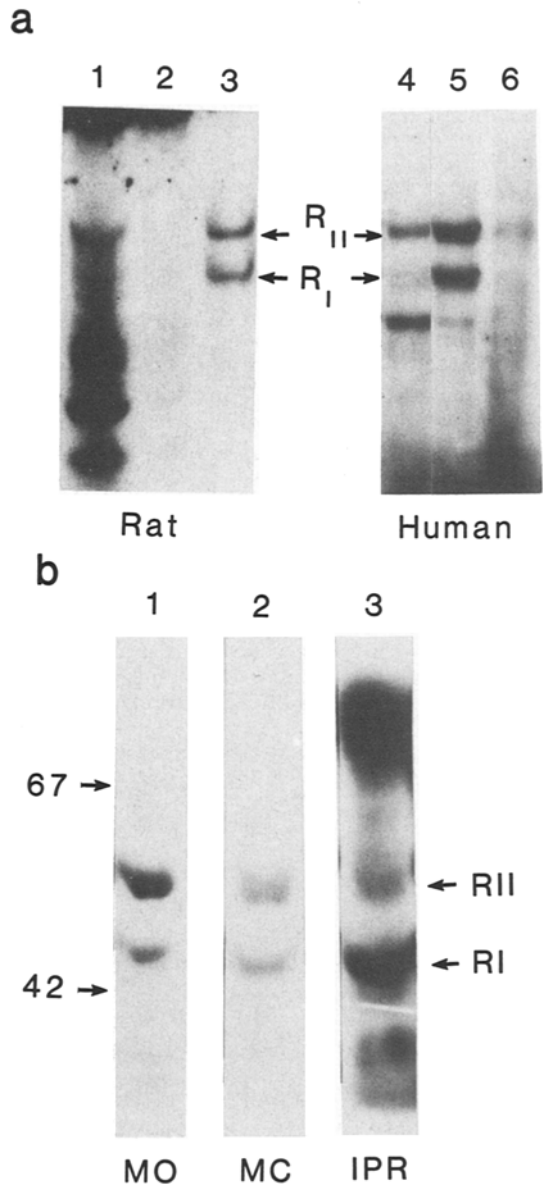

Figure 2. SDS-PAGE and autordiography of [^{32}P]-8-N_3-cAMP-labeled rat and human parotid saliva. *a* Channel 1, unfractionated rat ductal saliva without added protease inhibitors. Channels 2–6, rat and human saliva was fractionated as described in 'Materials and methods'. Fractions 25–50 were combined, concentrated and volumes equivalent to the original saliva applied to each channel (2–6). Samples in channels 2 and 6 were incubated with 10^{-6} M cyclic AMP prior to the addition of the analog, whereas samples in channels 1, 3, 4 and 5 were not. *b* Rat parotid saliva collected in the presence of protease inhibitors from animals injected with 6 mg/kg of methoxamine (MO), 3 × 5 mg/kg methacholine (MC) or 30 mg/kg isoproterenol (IPR), and labeled with 6.2×10^{-7} M [^{32}P]-8-N_3-cAMP. Mobility of R subunits from type I (R_I) and type II (R_{II}) cA-PK are indicated relative to mobilities of proteins of 67 and 42 kdal. R subunits from type I and type II protein kinase (Sigma) were used as controls (not shown).

Results and discussion. Cyclic AMP binding proteins of saliva were identified by using an isotopically labeled analog of cyclic AMP: [^{32}P] 8-azido cyclic AMP (8-N_3-cAMP) which reacts with R subunits in a manner similar to cyclic AMP (illustrated in the dissociative reaction, +cAMP [1]), but which can be covalently bound to the protein by photolysis[10]. There are 2 cA-PK isozyme R subunits which have been extensively characterized. They differ in molecular size; the type I isozyme R subunit (R_I) is approximately 50 kdal and the type II isozyme R subunit (R_{II}) is approximately 55 kdal[13]. These subunits can be isolated by affinity chromatography and identified by electrophoresis[14, 15].

A comparison of chromatographic profiles of [^{32}P]-8-N_3-cAMP-labeled human and rat parotid saliva proteins (collected with a Lashley cup in humans and by extraoral cannulation of the main excretory duct in isoproterenol-stimulated rats) is shown in figure 1. The 2 high molecular weight (> 50 kdal) peaks are similar in both species, and a faster-moving component is present in rat saliva. Fractions 25–50 of both human and rat parotid saliva column eluates were combined, concentrated and subjected to SDS-PAGE, and the labeled proteins visualized by autoradiography of the dried gels (fig. 2a). The autoradiograms show a qualitatively similar banding pattern in both human (channel 4 and 5) and rat (channel 3) parotid saliva. Channel 1 of figure 2a, shows unfractionated rat parotid saliva after electrophoresis and autoradiography and indicates that extensive proteolysis takes place (compared to channel 3 where a sample was fractionated in the presence of protease inhibitors). Channel 4 of figure 2a shows a human parotid saliva sample chromatographed without added protease inhibitors and although total breakdown is less than in the rat the appearance of the pattern indicates that R_I may be more subject to degradation than R_{II}. In other control samples (not shown) a mixture of beef and rat heart protein kinases was labeled and electrophoretically separated to serve as type I and type II standards, respectively. No significant labeling occurred in control experiments where samples were preincubated with unlabeled (10^{-6} M) cyclic AMP prior to incubation with [^{32}P]-8-N_3-cAMP but without photolysis (channels 2 and 6 in fig. 2a).

The distribution of [^{32}P]-8-N_3-cAMP label in saliva and in soluble and particulate cell fractions is shown in table 1. No significant counts sediment at 600 × g or 10,000 × g from saliva of either rats or humans, eliminating the possibility that salivary cA-PK R subunits are present due to membrane 'shedding' or to cell fragments present in the sample. A comparison of the distribution in cellular compartments of rat parotid, heart and liver shows that in most tissues the cyclic AMP binding proteins are associated with a particulate fraction (when fractionation is carried out so as to preserve organelle integrity [see Mednieks et al.[16, 17]]). Our findings also indicate that R subunits in saliva are present either in the dissociated form or that the C subunits are absent. The absence of cA-PK activity in saliva, despite its presence in a granule enriched subcellular fraction[17] of parotid acinar cells, may indicate that either the C subunit is an integral granule membrane

Table 2. Effect of in vivo stimulation with cholinergic and α- and β-adrenergic agonists on total [^{32}P]-8-N_3-cAMP incorporation into saliva

	cpm [^{32}P]-8-N_3-cAMP/mg protein
Methacholine	28,600
Methoxamine	14,840
Isoproterenol	78,250

The samples were labeled with 6.2×10^{-7} M [^{32}P]-8-N_3-cAMP as described in 'Materials and Methods'. Aliquots (20 µl) were counted from saliva samples from rats injected with the drugs listed in the table. The values are means of quadruplicate samples, representative of 2 consecutive experiments.

protein and is not secreted, or if it is secreted it is inactive in saliva due either to extensive degradation or to the presence of an inhibitor.

Covalent binding of [^{32}P]-8-N_3-cAMP with specific proteins of rat parotid saliva after stimulation with α-adrenergic and cholinergic agonists is $< 40\%$ (19% with methoxamine; 36.5% with methacholine) of that found in isoproterenol-stimulated saliva (table 2 and fig. 2b). The presence of R subunits at much higher levels in saliva after β-adrenergic stimulation suggests that they are released during exocytosis. Their presence at lower levels in saliva after α-adrenergic and cholinergic stimulation, which cause low but measurable levels of exocytosis, supports such a mechanism.

Protein secretion by the rat parotid gland is hormonally regulated via β-adrenergic receptors[18]. Receptor reactions which activate adenylate cyclase result in a rapid increase in levels of cyclic AMP, and activation of cA-PK. Subsequent phosphorylative modification of specific intracellular proteins is one of the steps which leads to exocytotic release of the stored secretory granule content. We have previously shown[17] that cA-PK holoenzyme and R_I subunits are associated with particulate cytoplasmic components (both microsomal and secretory granule cell fractions) in rat parotid cells. Association of cA-PK with the secretory granules would position the enzyme at an optimal site for phosphorylation of cytoplasmic or membrane proteins involved in exocytosis. At present it is not clear whether the R subunits are loosely associated with the granule membrane, or whether they are granule 'content' protein. The apparent increase of R_I in stimulated saliva (fig. 2a, channel 5) and in granules of in vivo stimulated animals[17], may be related to its physical properties of interchain disulfide bonding[19] and involvement in the cyclic AMP binding sites in protein kinase activation[20]. The R_I subunit may also be involved in hormonally stimulated enzyme translocation within the responsive cell and represent an intracellular mechanism of action in exocytosis.

The presence of R subunits in saliva provides an easily obtainable source of this protein for secretion or binding studies, and for purification for immunological and molecular biology studies. The physiologic function of the R subunit in saliva, however, remains enigmatic. Cyclic AMP has repeatedly been shown to be present in saliva[21-23]; to what extent it is in the free form compared to a protein-bound form to the R subunit has not been clearly demonstrated. The release of the R subunits along with secretory proteins may be a mechanism for 'unloading' intracellular cyclic AMP. The presence of R subunits in saliva may have important implications for the growth and metabolism of the oral microbial flora by serving to regulate the levels of cyclic AMP in saliva, or may reflect changes in the regulation of secretory responses in disease processes.

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Antibacterial activity in the egg mass of a sea hare

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Summary. The eggs of a sea hare, *Aplysia kurodai*, contained antibacterial factors which probably play a role in the defense of eggs against bacterial infection. The active factors were composed of several heat-labile proteins, unrelated to lysozyme, and were produced in the albumen gland.

Key words. Sea hare eggs; *Aplysia kurodai;* antibacterial activity.

Sea hares belong to the subclass Opisthobranchia of the gastropod molluscs. In recent years, they have attracted the interest of many workers investigating chemical defense mechanisms[1,2]. The eggs of sea hares are laid in gelatinous strings. There does not appear to be bacterial infection causing the eggs to deteriorate before they hatch as swimming veliger larvae. In addition, the egg mass seems to be rejected as a food source by carnivorous fish. These observation led us to look for physiologically active components in the egg mass of *Aplysia kurodai,* and we found potent agglutinins which could ag-