Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 66 (2003) 239–250
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Specific expression of an A-kinase anchoring protein subtype, AKAP-150, and specific regulatory mechanism for Na^+,K^+-ATPase *via* protein kinase A in the parotid gland among the three major salivary glands of the rat

Kinji Kurihara[a,*], Nobuo Nakanishi[b], Osamu Amano[c,1], Miyuki Yamamoto[c], Shoichi Iseki[c]

[a]*Department of Oral Physiology, Meikai University School of Dentistry, 1-1 Keyaki-Dai, Sakado-Shi, Saitama 350-0283, Japan*
[b]*Department of Biochemistry, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan*
[c]*Department of Anatomy, School of Medicine, Kanazawa University, Takara-machi, Kanazawa 920-8640, Japan*

Received 9 July 2002; accepted 21 December 2002

Abstract

We have examined the expression of A-kinase anchoring protein (AKAP) in the three major salivary glands, i.e. the parotid gland (PG), submandibular gland (SMG), and sublingual gland (SLG), of the rat to elucidate the functional relevance between saliva secretion and Na^+,K^+-ATPase regulation by protein kinase A (PKA)-dependent phosphorylation, since an AKAP subtype, AKAP-150, is known to be involved in the regulation of the ATPase in PG. Although AKAP-150 and its mRNA were clearly detected in the PG, they were hardly detectable in either the SMG or SLG. The membrane-bound form of the RII regulatory subunit of PKA, an index for the total amount of AKAP subtypes and therefore of the anchored PKA holoenzyme, was also undetectable in membranes from the SMG and SLG but was found in the PG; though a substantial and comparable amount of Na^+,K^+-ATPase was present in all of these membrane preparations. Incubation with [γ-^{32}P]ATP revealed that Na^+,K^+-ATPase in the PG membranes was quickly phosphorylated upon the addition of cAMP, whereas the ATPases in the membranes from SMG and SLG were not; though they were readily and equally phosphorylated by the exogenously added PKA catalytic subunit. AKAP-150 in the basolateral membranes of PG acinar cells was co-immunoprecipitated with RII by an anti-RII antiserum; and AKAP-150 and Na^+,K^+-ATPase were immunohistochemically co-localized predominantly on the basolateral membranes, suggesting a possibility that the ATPase might directly interact with the AKAP to form an ATPase/AKAP/PKA complex or associate with the AKAP, such association being mediated *via* some scaffolding molecule. Expression of AKAP-150 and quick down-regulation of Na^+,K^+-ATPase by AKAP-anchored PKA in response to cAMP elevation are characteristics specific to PG among the three major salivary glands, suggesting the presence of PG-specific regulatory mechanisms for saliva production/secretion.

Keywords: Na^+,K^+-ATPase; AKAP; PKA; Phosphorylation; Salivary gland

1. Introduction

Na^+,K^+-ATPase is a typical membrane-bound enzyme, and consists of at least two subunits, the catalytic α subunit and the glycosylated β subunit [1–6]. By utilizing the energy of one molecule of ATP, Na^+,K^+-ATPase produces an electrochemical gradient/membrane potential across the plasma membrane by pumping three Na^+ ions out of and two K^+ ions into the cell [7,8]. Na^+,K^+-ATPase is also important for homeostasis of epithelial fluid and electrolyte secretion. In the PG, for example, fluid secretion is

* Corresponding author. Tel.: +81-49-279-2771; fax: +81-49-287-6657.
E-mail address: kkinji@dent.meikai.ac.jp (K. Kurihara).
[1] Present address: Department of Oral Anatomy, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan.

Abbreviations: SMG, submandibular gland; SLG, sublingual gland; PG, parotid gland; PKA, cyclic AMP-dependent protein kinase; AKAP, A-kinase anchoring protein; AGPC, acid guanidinium thiocyanate–phenol–chloroform; RT–PCR, reverse transcriptase–polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAGUK, membrane-associated guanylate kinase; AMPA, α-amino-3-hydroxy-5-methylisoxazolepropionate.

0006-2952/03/$ – see front matter
doi:10.1016/S0006-2952(03)00259-4

regulated by the combined action of four membrane transport systems, i.e. Na^+,K^+-ATPase, Na^+-K^+-$2Cl^-$ cotransporter, and Ca^{2+}-activated K^+ channel in basolateral membranes, and an apical conductive pathway for Cl^-, presumably involving Ca^{2+}-activated Cl^- channel [9]. Na^+,K^+-ATPase activity in intact cells was reported to be inhibited by a protein phosphatase inhibitor, phospho-DARPR-32 (phosphorylated form of a dopamine and cAMP-regulated 32-kDa protein) [10]. Purified Na^+,K^+-ATPase was shown to be phosphorylated *in vitro* by the catalytic subunit of PKA, resulting in a decrease in its activity [11]. Na^+,K^+-ATPase was also phosphorylated *in vivo* when cells were treated with forskolin [12,13] and cAMP analogues [14,15]. Thus, Na^+,K^+-ATPase is thought to be down-regulated by cAMP *via* PKA-dependent phosphorylation [11].

cAMP is known to be one of the major intracellular messengers, and its level is elevated by a wide variety of intercellular signaling molecules, such as hormones, neurotransmitters, and growth factors [16–18]. However, since the PKA catalytic subunit has rather broad substrate specificity, various proteins can be phosphorylated *in vitro* by the kinase regardless of the physiological significance. Non-specific phosphorylation of cellular proteins was also observed *in vivo* by the treatment of cells with membrane-permeable cAMP analogues or forskolin [11–15]. Therefore, for transducing physiological signals, the cAMP/PKA-dependent phosphorylation system must have some mechanism for the preferential phosphorylation of its specific target substrate *in vivo*. Coghlan *et al*. [19], Mochly-Rosen [20], and Scott *et al*. [21–23] demonstrated the role of AKAP, a specific protein that anchors the PKA regulatory subunit RII (and thereby the catalytic subunit bound to RII) on the membrane near its specific target proteins. Since then various AKAP subtypes have so far been identified in various types of cells and shown to associate with cellular and intracellular membrane structures [23,24]. Recently, Dodge and Scott [25] expanded the role of AKAPs to members of multiunit complexes, kinase/phosphatase scaffolding units, containing both upstream activators and downstream targets.

In a previous study [26], we demonstrated that Na^+,K^+-ATPase in basolateral membrane vesicles prepared from acinar cells of the rat PG was much more efficiently phosphorylated by endogenous membrane-bound PKA upon the addition of cAMP than by the addition of an excess of exogenous PKA catalytic subunit and that the endogenous PKA was associated with the membrane by being anchored *via* AKAPs.

In the present study, we examined the mRNA and protein levels of three AKAP subtypes, i.e. AKAP-95, AKAP-150, and AKAP-220, in the SMG, SLG, and PG of rats to investigate the role of AKAPs in the Na^+,K^+-ATPase regulation and saliva production/secretion in those glands. Localization of both AKAP-150 and Na^+,K^+-ATPase in PG acinar cells was also examined by a histochemical method. Expressions of AKAP-95 and AKAP-220 genes were very low in all three salivary glands. Although AKAP-150 was clearly detected in whole PG homogenates and in membranes prepared from the PG, both AKAP-150 mRNA and AKAP-150 protein were unexpectedly quite low in both the SMG and SLG. The results of the present study indicate that AKAP-150 expression and the regulation of Na^+,K^+-ATPase by AKAP-150-anchored PKA are characteristics rather specific to the PG among the three major salivary glands.

2. Materials and methods

2.1. Animals

Male Wistar strain rats were purchased from Tokyo Laboratory Animals Science Co, Ltd. All experiments were carried out in accordance with the guidelines for animal experiments of Kyoto University (1988). All animals were sacrificed at 10 weeks of age in all of the experiments.

2.2. Preparation of membrane vesicles from salivary glands

Membrane vesicles of the PG were prepared by the method of Manganel and Turner [27]. The tissue was homogenized for 10 s at power level 5 in 10 mM HEPES/Tris (pH 7.4), 10% sucrose, 1 mM EDTA, and 0.1 mM phenylmethylsulfonylfluoride (PMSF) with a Polytron (Kinematica Model PT-2000 and aggregate PTA Type 10S). The homogenate was centrifuged at 2500 *g* for 5 min at 4°, after which the supernatant was filtered through a fine 155-μm nylon mesh and centrifuged at 22,000 *g* for 20 min at 4°. The pellet was suspended in the same buffer to 10 mL/g of starting parotid tissue. Percoll was added to this suspension for a final concentration of 16%. This material was centrifuged in 15-mL aliquots at 41,500 *g* for 40 min at 4°. The small band on top of the dense white band was collected and diluted 10-fold with 10 mM HEPES buffer containing 100 mM mannitol, 100 mM KCl, and 1 mM EDTA after which the suspension was adjusted to pH 7.4 with Tris. The membrane vesicle suspension was then washed and stocked after having been resuspended in the same buffer. Membrane vesicles of SMG and SLG were prepared by the method of Jorgensen [1]. Protein content was determined by use of a protein assay kit (Bio-Rad) with BSA serving as a standard [28].

2.3. Phosphorylation of Na^+,K^+-ATPase in membrane vesicles

Membrane vesicles were incubated for 30 s at 37° with 40 μM [γ-^{32}P]ATP (10 Ci/mmol) in a 40-μL incubation mixture containing 20 mM CH_3COONa, 60 mM

CH_3COOK, 60 mM KCl, 10 mM $(CH_3COO)_2Mg$, 1 mM EGTA, 100 mM mannitol, 0.0025% Triton X-100, 10 mM HEPES (adjusted to pH 7.4 with Tris), and 10 μM cAMP (or 16 U PKA catalytic subunit). After the reaction had been stopped by the addition of 200 μL of 18% trichloroacetic acid, the resulting precipitate was washed with distilled water and boiled with SDS–PAGE buffer containing 0.0625 M Tris–HCl (pH 6.8), 1% SDS, and 2.5% 2-mercaptoethanol. The sample was applied to an SDS–PAGE (4–20% gradient gel) according to Laemmli [29]. The phosphorylation pattern of membranes was examined by autoradiography.

2.4. Detection of [E2-P] complex of Na^+,K^+-ATPase

Radioactive phosphate labeling of the [E2-P] complex (form of Na^+,K^+-ATPase transiently phosphorylated at Asp-371 of its α subunit [4]) was carried out as described earlier [30,31]. Eight micrograms of membrane vesicles was incubated at 20° for 5 min with 10 mM Tris (pH 7.25), 3 mM $MgCl_2$ containing 0.005 mg/mL SDS. The membrane vesicles were incubated for 20 s at 0° with 40 μM [γ-^{32}P]ATP (10 Ci/mmol) in a 40-μL incubation mixture also containing 140 mM NaCl, 4 mM ouabain, or 70 mM KCl. The reaction was stopped by adding SDS–PAGE sample buffer. These samples were blotted onto a PVDF filter after having been separated by SDS–PAGE. An autoradiogram was prepared from the blotted filter.

2.5. RNA preparation

Total RNAs were prepared by the method of Chomczynski and Sacchi [32] using AGPC. Approximately 100 mg amounts of rat salivary glands and brain were separately homogenized in 2 mL of guanidinium thiocyanate denaturing solution with the Polytron-aggregate. The homogenate was mixed with 0.2 mL of 2 M CH_3COONa (pH 4.0), 2 mL of water-saturated phenol, and 0.4 mL of 49:1 chloroform/isoamyl alcohol, stood for 15 min on ice, and then centrifuged. RNAs in the aqueous phase were precipitated with isopropanol. These RNAs were dissolved in denaturing solution again and reprecipitated with isopropanol. RNA precipitates were washed with 75% EtOH, air-dried, and dissolved in water.

2.6. Reverse transcriptase–polymerase chain reaction (RT–PCR) and DNA sequencing

RT–PCR was carried out with an RNA PCR Kit (AMV) Ver. 2.1 Code # R019A (Takara Shuzo Co, Ltd. Biomedical Group). Based on the cDNA sequences registered in the Query Gene Bank Database, National Center for Biotechnology, primer pairs were designed and prepared for three AKAP subtypes of rats, i.e. AKAP-95, AKAP-150, and AKAP-220. Primers used to detect isoforms of rat AKAP were the following: AKAP-95, 5′-GCTATGCATGTGGCCTCTTT-3′ (nucleotide positions 220–239) and 5′-TACAGGGCCTGGAGTCGTAG-3′ (399–418); AKAP-150, 5′-CAGGAAGCAAGTGTGCTTGA-3′ (1239–1258) and 5′-GCGCAGTCTCTCCTGATTTC-3′ (1418–1437); AKAP-220, 5′-CGTGACCTTCACAGCAAAGA-3′ (5352–5371) and 5′-TCCTGAGGGACGAAACAAAG-3′ (5532–5551). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer pair used to examine the quality and amounts of prepared mRNA was the following: 5′-TCCCTCAAGATTGTCAGCAA-3′ (506–525) and 5′-AGATCCACAACGGATACATT-3′ (795–814) [33]. Total RNA (1.25 μg each) from salivary glands was used for the RT–PCR, and PCR was performed with a Gene Amp 9600 PCR System (Perkin Elmer) according to the following schedule: denaturation, annealing, and elongation at 94° for 30 s, 60° for 30 s, and 72° for 90 s, respectively, for 30 cycles.

For the sequence analysis, PCR products were reamplified with the above primers in the presence of a dye terminator (BigDye Terminator Cycle Sequencing FS Ready Reaction Kit, #4303573, Perkin Elmer). The DNA sequences were analyzed with an ABI PRISM™ 310 Genetic Analyzer (Perkin Elmer).

2.7. Preparation of anti-α1 antiserum

An anti-α1 antiserum was prepared by the method described previously [26,34,35]. In brief, membrane-bound Na^+,K^+-ATPase was prepared from whole kidneys of male rats by the method of Jorgensen [1]. The α1 subunit was then separated from the membrane-bound Na^+,K^+-ATPase by SDS–PAGE (5% acrylamide) using Laemmli's buffer system [29]. Anti-α1 antiserum was obtained by immunizing a rabbit with the α1 subunit protein.

2.8. Western blot analysis of Na^+,K^+-ATPase, AKAPs, and PKA RII regulatory subunit

Samples were subjected to SDS–PAGE, and the separated proteins were electrophoretically transferred onto PVDF membrane filters in 200 mM glycine, 25 mM Tris, 10% methanol [36]. The filters were then stained immunochemically with the anti-α1 antiserum, anti-AKAP-95, -150, or -220 antiserum, or anti-RII antiserum. In brief, filters were blocked with 5% skimmed milk in T-TBS (10 mM Tris–HCl, pH 7.4, containing 150 mM NaCl and 0.05% Tween 20) at room temperature for 45 min and then incubated for 2 hr with the anti-α1 antiserum, anti-AKAP antisera, or anti-RII antiserum in T-TBS containing 5% skimmed milk. After having been washed with T-TBS, these filters were incubated for 1 hr with anti-rabbit IgG goat serum-horseradish peroxidase (HRP) to stain the Na^+,K^+-ATPase α subunit or incubated with anti-goat IgG donkey serum-HRP to stain AKAPs and the PKA RII regulatory subunit. The filters were then washed, and the signal was detected with ECL+ plus Western blotting detection reagents (Amersham).

2.9. Co-immunoprecipitation of AKAP-150 with PKA regulatory subunit

Membrane vesicles were solubilized in lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 40 mM sodium pyrophosphate, 50mM KH_2PO_4, 10 mM sodium molybdate, 2 mM sodium orthovanadate, 6 μM dithiothreitol, 300 μM PMSF, 100 μM L-tosylamido-2-phenylethyl-chloromethyl ketone, 1.5 μM pepstatin, 1.5 μM leupeptin, and 20 mM Tris–HCl, pH 7.4). Anti-RII antibody was preconjugated to protein G beads as follows: protein G beads were first washed with lysis buffer containing 1% ovalbumin. Then, the beads were incubated for 1 hr with anti-RII antibody or preimmune goat serum in the lysis buffer containing ovalbumin and washed with the same buffer. The protein G beads preconjugated with anti-RII IgG were mixed with the lysate of membranes and incubated for 4 hr at 4°. The beads were collected by centrifugation (10,000 *g* for 1 min at 4°) and washed three times with lysis buffer, with the tube being exchanged for a new one for the last wash. The final pellet of beads was extracted with SDS–PAGE sample buffer and then applied to SDS–PAGE. The separated proteins were transferred to a PVDF filter electrically, and AKAP-150 was detected by Western blot analysis with anti-AKAP-150 antibody.

2.10. Immunohistochemical study

Male Wistar strain rats of 200–250 g body weight were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and sacrificed by transcardial perfusion with cold physiological saline. They were then further fixed by transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). After perfusion, PG was dissected out and immersed in the same fixative for 4 hr at 4°. The tissue blocks, after having been rinsed overnight in phosphate buffer containing 30% sucrose, were cut on a cryostat into 15-μm sections and then mounted onto gelatin-coated slides.

The sections were treated first with 0.03% Triton X-100 in PBS. After having been washed in PBS, they were incubated with a mixture of anti-AKAP-150 goat polyclonal and anti-Na^+,K^+-ATPase α1 rabbit polyclonal antibodies [26,34,35] diluted at 1:200 overnight at room temperature, following pretreatment with 5% normal porcine serum (Dakopatts). For immunodetection, the sections were incubated with the mixture of Cy3-conjugated anti-goat IgG and FITC-conjugated anti-rabbit IgG (Jackson Immuno Research Laboratories, Inc.) at 1:100 for 1 hr. After having been washed in PBS, the sections were mounted in glycerol for observation under a fluorescence microscope (BX-50/BX-FLA, Olympus). By use of the appropriate excitation filters, the immunostained portions of the cells were visualized in red for Cy3, green for FITC, and yellow for both. For a control, normal rabbit serum or PBS was used instead of the primary antibody.

2.11. Reagents

ATP-Tris salt and PKA catalytic subunit (P-8289) were purchased from Sigma Chemical Co. Precast Tris-glycine and tricine polyacrylamide gels were obtained from Novex. Anti-AKAP-95, -150, -220 antibodies and anti-goat IgG-HRP were purchased from Santa Cruz Biotechnology, Inc. Anti-PKA RII subunit antibody was obtained from Upstate Biotechnology. HT-31 peptide (Asp-Leu-Ile-Glu-Glu-Ala-Ala-Ser-Arg-Ile-Val-Asp-Ala-Val-Ile-Glu-Gln-Val-Lys-Ala-Ala-Gly-Ala-Tyr) and HT-31P peptide (Asp-Leu-Ile-Glu-Glu-Ala-Ala-Ser-Arg-Pro-Val-Asp-Ala-Val-Pro-Glu-Gln-Val-Lys-Ala-Ala-Gly-Ala-Tyr; Carr *et al.* [37]) were synthesized by Sawady Technology Co, Ltd.

3. Results

3.1. mRNAs and proteins for AKAPs in PG, SMG, and SLG

By the RT–PCR method we examined the mRNA levels for AKAP subtypes AKAP-95, AKAP-150, and AKAP-220, in the three major salivary glands of rats. With all of the primer pairs for those AKAP subtypes, DNA products of the expected sizes (199, 199, and 200 bp for AKAP-95, AKAP-150, and AKAP-220, respectively) were obtained when RT–PCR was carried out with RNAs prepared from rat brain used as a positive control. In the PG, mRNAs for all three subtypes of AKAP were detected, though the mRNAs of the other AKAPs, especially AKAP-220, were less abundant than the AKAP-150 mRNA (Fig. 1A). In the SMG and SLG, mRNA levels for all of the AKAP subtypes were quite low compared with those in the PG, when 1.25 μg of total RNA from each gland was used for the RT–PCR analysis; only AKAP-95 mRNA was faintly detected in the SLG sample. The amounts of the GAPDH PCR product appeared to be same among the salivary glands (Fig. 1B). The DNA sequence of each PCR product seen in Fig. 1 was analyzed and confirmed to be identical with the corresponding portion of the cDNA sequence for AKAP-95, AKAP-150, or AKAP-220 (data not shown).

We also examined the expression of AKAP proteins in the salivary glands by immunoblotting with antibodies against AKAP-95, AKAP-150, and AKAP-220 (Fig. 2). Anti-AKAP-150 antibody revealed a specific signal at molecular mass of 150 kDa on blotted membranes bearing both whole PG homogenate and membrane vesicles prepared from the PG. On the other hand, a signal for the AKAP-95 protein was faintly detected on the blotted membrane containing whole PG homogenate, but not on that containing membrane vesicles, indicating that though

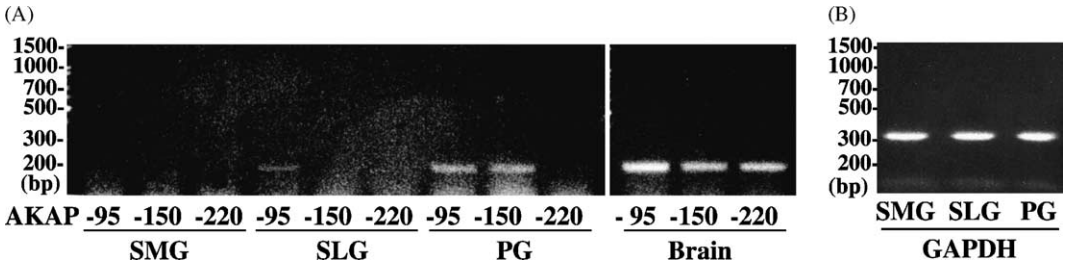

Fig. 1. Expressions of AKAP-95, -150, and -220 (A) and expression of GAPDH (B) in SMG, SLG, and PG of rats. Total RNA (1.25 μg each) from salivary glands was used for the RT–PCR, and PCR was performed as follows: denaturation, annealing, and elongation at 94° for 30 s, 60° for 30 s, and 72° for 90 s, respectively, for 30 cycles. PCR was carried out with first-strand DNAs prepared from RNAs of SMG, SLG, and PG. RNA prepared from rat brain was used as a positive control. Predicted sizes of the PCR products for AKAP-95 [38], -150*, -220 [39], and GAPDH [33] cDNAs were 199, 199, 200, and 309 bp, respectively. *cDNA sequence of AKAP-150 has been submitted by Takai Y, Irie M, Toyoda A, and Hata Y to the Query Gene Bank Database at the National Center for Biotechnology.

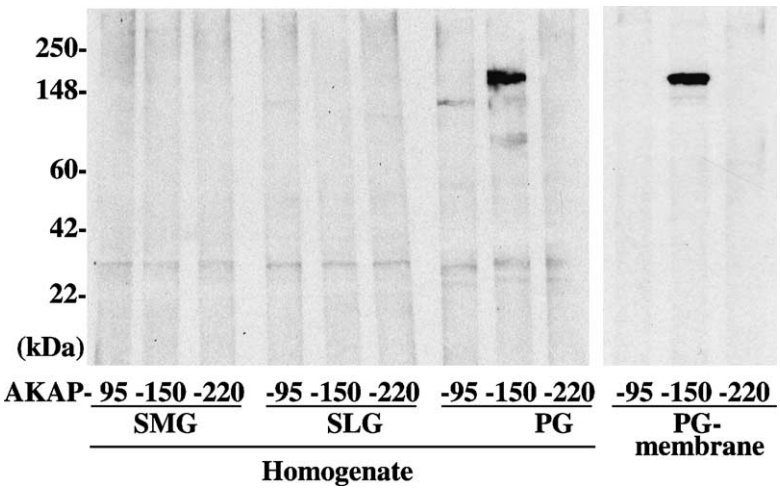

Fig. 2. Western blot analysis of AKAP-95, -150, and -220 proteins in homogenates of rat SMG, SLG, and PG, and in membrane vesicles purified from rat PG. Ten microliters of 10% (w/v) homogenate (SMG, 101 μg protein; SLG, 81.3 μg protein; and PG, 104 μg protein) or 2 μg of protein of membrane vesicles prepared from PGs was separated by SDS–PAGE (4–20% gradient gel). Proteins were transferred to a PVDF filter and then immunostained as described in Section 2, except that the following antibodies, 5000-fold diluted anti-AKAP-95, -150, or -220 antibodies and 10,000-fold diluted anti-goat IgG-HRP produced by Santa Cruz Biotechnology, Inc., were used as first and second antibodies, respectively.

PG cells produced a small amount of AKAP-95 (compared with that of AKAP-150), it was not sorted to the basolateral membranes. AKAP-95 was also faintly detected in the SLG, but not detected in the SMG. AKAP-220 was not detectable in the homogenates (corresponding to 1 mg wet tissue weight) of any of the salivary glands.

3.2. Membrane-bound RII and Na^+,K^+-ATPase in PG, SMG, and SLG

Next we examined the vesicles for the membrane-bound form of the RII regulatory subunit of PKA to assess the total amount of AKAP of any subtypes and to assess the amount of membrane-anchored PKA holoenzyme in membrane vesicles from the salivary glands. Membrane-bound RII was clearly detected in the membrane vesicles from the PG (Fig. 3A). However, it was not detectable in those from the SMG and SLG (Fig. 3A). On the other hand, substantial

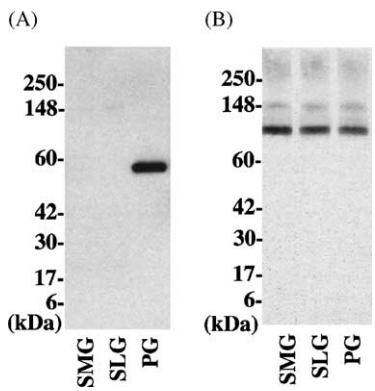

Fig. 3. Analysis of membrane-bound RII regulatory subunit of PKA and Na^+,K^+-ATPase in membrane vesicles from SMG, SLG, and PG. Membrane vesicle proteins (25, 25, and 5 μg from SMG, SLG, and PG, respectively) were separated by SDS–PAGE, transferred to PVDF filters, and then analyzed with antibodies against the RII regulatory subunit of PKA (A) and α subunit of Na^+,K^+-ATPase (B) as described in Section 2.

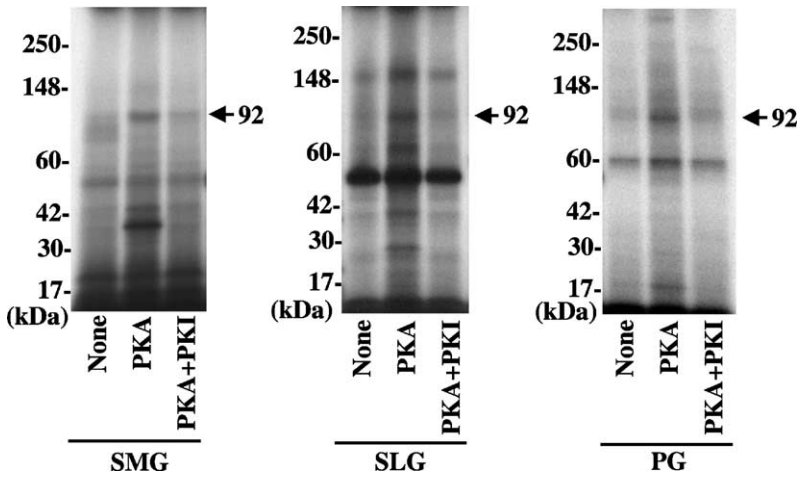

Fig. 4. Phosphorylation of proteins in the membrane vesicles from SMG, SLG, and PG of rats by exogenously added PKA catalytic subunit. Membrane vesicles (40, 40, and 8 μg prepared from SMG, SLG, and PG, respectively) were incubated with [γ-^{32}P]ATP for 30 s as described in the text along with the following additive(s): none (None); 16 U PKA catalytic subunit (PKA); 16 U PKA catalytic subunit plus 10 μM [5–24]PKI peptide (PKA + PKI). The reaction was terminated by the addition of trichloroacetic acid and subsequent heat treatment with SDS–PAGE buffer after a rinse with water. Protein phosphorylation was analyzed by autoradiography after the membrane proteins had been separated by SDS–PAGE. The arrow marked "92" indicates the position of ^{32}P-labeled 92-kDa protein, which was identified as the Na^+,K^+-ATPase α subunit based on the examination shown in Fig. 5.

and comparable amounts of Na^+,K^+-ATPase were contained in all these membrane preparations (Fig. 3B). From these results, the amount of membrane-anchored PKA holoenzyme in both the SMG and SLG was estimated to be quite low, if the proteins were present at all.

3.3. Phosphorylation of Na^+,K^+-ATPase in membranes from PG, SMG, and SLG by exogenous PKA

As shown in Fig. 4, when membranes prepared from SMG, SLG, and PG were incubated with the exogenous PKA catalytic subunit in the presence of [γ-^{32}P]ATP, several membrane proteins were labeled with ^{32}P. The phosphorylation of these proteins was inhibited by PKI peptide, a specific inhibitor of PKA [40], indicating the dependency of the reaction on the PKA added. In all of these membrane preparations a 92-kDa protein was commonly phosphorylated, and was identified as the α1 subunit of Na^+,K^+-ATPase based on its reactivity toward a polyclonal antiserum raised against the α1 isoform of the Na^+,K^+-ATPase α subunit [26].

This identity was further confirmed by assessing the ability of Na^+,K^+-ATPase to form a transient phosphoenzyme [E2-P] complex, which is stabilized by ouabain, by incubating the Na^+,K^+-ATPase in a solution of ATP and Na^+ without K^+ [30]. The [E2-^{32}P] complex of Na^+, K^+-ATPase was formed by incubating membrane vesicles with [γ-^{32}P]ATP, 140 mM Na^+, and 4 mM ouabain without K^+ at 0°, and analyzed by autoradiography of the blotted proteins after SDS–PAGE of the membrane vesicles. A ^{32}P-protein band was observed at the position of 92 kDa (Fig. 5A, +Ouabain), which coincided with the protein phosphorylated by the exogenous PKA catalytic subunit (Fig. 5B, exogenous PKA-dependent phosphorylation).

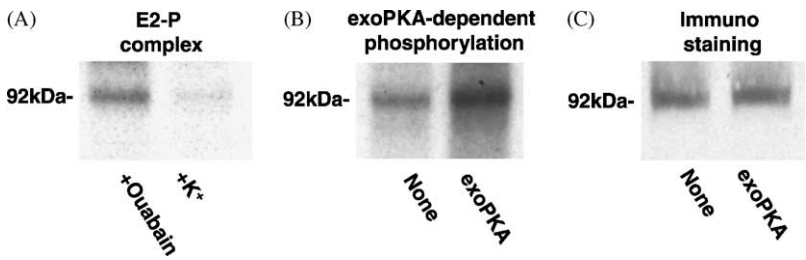

Fig. 5. [E2-P] complex of Na^+,K^+-ATPase and identification of 92-kDa phosphoprotein in membrane preparations by Western blot/immunostaining analysis. (A) [E2-P] complex: SDS-treated membranes from PG were incubated with [γ-^{32}P]ATP at 0° for 20 s in the presence of 140 mM NaCl + 4 mM ouabain (+Ouabain) or in the presence of 70 mM KCl (+K^+). These samples were blotted onto a filter after SDS–PAGE. [E2-P] complexes were detected by autoradiography using the blotted filter. (B) Western blot analysis of 92-kDa phosphoprotein obtained with exogenous PKA-dependent phosphorylation: membrane vesicles were incubated with [γ-^{32}P]ATP for 30 s at 37° in the absence (None) or presence of 16 U/40 μL exogenous PKA (exoPKA). The membrane proteins were blotted onto a PVDF filter after separation by SDS–PAGE, and autoradiography was carried out on the blotted filter. (C) Immunostaining: the filter bearing the blotted protein phosphorylated by exogenous PKA (None and exoPKA) was immunostained with anti-α1 antiserum by the method described in text.

The [E2-^{32}P] complex disappeared when membrane vesicles were incubated in the presence of 70 mM K^+ (Fig. 5A, $+K^+$), indicating one of the very specific characteristics of Na^+,K^+-ATPase that differentiates it from other ATPases or protein kinases. The 92-kDa protein phosphorylated by the exogenous PKA was recognized by anti-α1 antiserum on the same filter used for phosphorylation (Fig. 5C, immunostaining).

3.4. Phosphorylation of Na^+,K^+-ATPase in membranes by endogenous PKA

We further examined the ability of the membrane preparations to phosphorylate Na^+,K^+-ATPase by endogenous PKA responding to the elevation (addition) of cAMP. As expected from the results shown in Fig. 3A, Na^+,K^+-ATPase (92-kDa protein) in membranes from either the SMG or SLG was not labeled with [γ-^{32}P]ATP upon the addition of cAMP (Fig. 6A). The cAMP-dependently phosphorylated 92-kDa protein in the PG was confirmed to be Na^+,K^+-ATPase by comparison with the protein phosphorylated by the exogenous PKA (Fig. 6B, PKA and cAMP). The cAMP-dependent phosphorylation of the Na^+,K^+-ATPase was inhibited by [5–24]PKI peptide (Fig. 6B, cAMP + PKI), a specific inhibitor of PKA [40], confirming that this phosphorylation was performed by endogenous PKA anchored on the membranes. This result was due to neither the lack of substrate protein nor that of susceptibility of SMG and SLG Na^+,K^+-ATPases to the kinase, since the membrane vesicles contained a substantial amount of Na^+,K^+-ATPase (Fig. 3B) and since the ATPase of all three glands had been readily phosphorylated

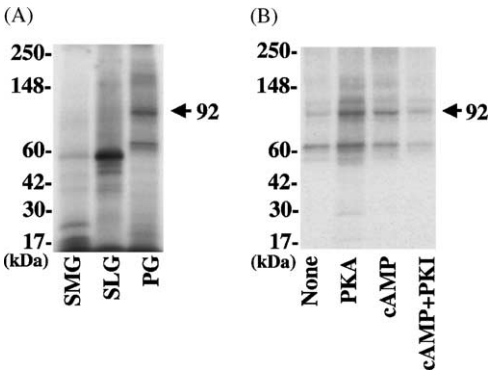

Fig. 6. Phosphorylation of Na^+,K^+-ATPase in membrane vesicles prepared from SMG, SLG, and PG by membrane-anchored endogenous PKA. (A) Na^+,K^+-ATPase phosphorylation by membrane-anchored endogenous PKA triggered by cAMP. Membrane vesicles (40, 40, and 8 μg from SMG, SLG, and PG, respectively) were incubated for 30 s with [γ-^{32}P]ATP and 10 μM cAMP. Na^+,K^+-ATPase phosphorylation was analyzed by SDS–PAGE/autoradiography of the membrane proteins. (B) Effect of [5–24]PKI peptide on cAMP-dependent Na^+,K^+-ATPase phosphorylation in the membrane vesicles from the PG. Eight micrograms of membranes from PG was incubated with [γ-^{32}P]ATP for 30 s as described in the text along with the following additive(s): none (None); 16 U PKA catalytic subunit (PKA); 10 μM cAMP (cAMP); 10 μM cAMP + 10 μM [5–24]PKI peptide (cAMP + PKI).

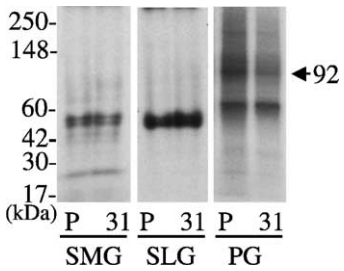

Fig. 7. Effects of HT-31 and HT-31P on cAMP-dependent phosphorylation of Na^+,K^+-ATPase in the membrane vesicles prepared from SMG, SLG, and PG. Membranes (20, 20, and 4 μg from SMG, SLG, and PG, respectively) were incubated for 1 hr at 37° with 20 mM CH_3COONa, 60 mM CH_3COOK, 60 mM KCl, 10 mM $(CH_3COO)_2Mg$, 1 mM EGTA, 100 mM mannitol, 10 mM HEPES (adjusted to pH 7.4 with Tris) in the presence of 100 μM HT-31P peptide (P) or 100 μM HT-31 peptide (31). Then, the samples were washed by centrifugation and subsequently incubated with [γ-^{32}P]ATP for the Na^+,K^+-ATPase phosphorylation reaction in the presence of 10 μM cAMP, as described in the text. The arrow marked "92" indicates the Na^+,K^+-ATPase α subunit.

by the PKA catalytic subunit exogenously added (Fig. 4). Thus, only the PG membranes had a substantial amount of endogenous PKA able to phosphorylate Na^+,K^+-ATPase in the same membranes upon the addition of cAMP (Fig. 6A, PG).

Treatment with HT-31 peptide, an inhibitor of the binding of AKAP to RII, decreased the level of cAMP-dependent phosphorylation of Na^+,K^+-ATPase in the PG (Fig. 7), indicating the involvement of AKAP in retaining the endogenous PKA on the membranes. This was further confirmed by the result of the experiment with HT-31P, a variant of HT-31 with no inhibitory effect [37–41], for Na^+,K^+-ATPase phosphorylation by the endogenous PKA was not affected by this peptide (Fig. 7).

3.5. Immunochemical and immunohistochemical analyses of AKAP-150, RII regulatory subunit, and Na^+,K^+-ATPase in PGs

Since both AKAP-150 and RII were present in PG membranes, as was shown in Figs. 2 and 3, we examined by co-immunoprecipitation analysis whether these two molecules actually formed a complex on the membrane. Proteins solubilized from membrane vesicles were incubated with anti-RII antiserum-coated beads, and the immunocomplexes formed were then examined by Western blot analysis using anti-AKAP-150 antibody (Fig. 8). AKAP-150 was detected in the immunocomplexes formed by anti-RII antibody at the same position (lane 3) as the signal obtained with membrane vesicles used as a positive control (lane 4). AKAP-150 was not detected when normal serum was used to coat the beads (lane 2).

There were large background signals (Fig. 8, lanes 2 and 3) because anti-RII goat antibody was used for immunocomplex formation and anti-goat IgG-HRP was employed as the second antibody for the Western blot analysis. These background signals were also detected with second

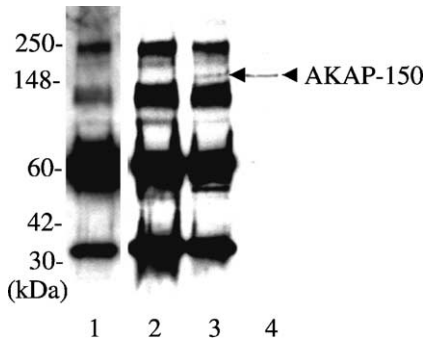

Fig. 8. Co-immunoprecipitation of AKAP-150 in PG acinar cells with anti-RII antibody. Lysates prepared from membrane vesicles of rat PG were incubated with protein G beads preconjugated with either preimmune goat serum (lane 2) or anti-RII goat serum (lanes 1 and 3) to form immunocomplexes. The immunocomplexes were subjected to SDS–PAGE (4–20%). Membrane vesicles of the rat PG were used as a positive control (lane 4) for AKAP-150. After the electrophoresis, immunoprecipitated proteins and membrane vesicles protein were transferred to a PVDF filter and stained with anti-AKAP-150 goat antibody and then with anti-goat IgG-HRP as second antibody, as described in Section 2 (lanes 2–4), or stained with the anti-goat IgG-HRP alone (lane 1).

antibody alone (lane 1), indicating that they were due to the goat IgGs used for the immunocomplex formation. These results indicate that the AKAP-150 formed a complex with the PKA RII regulatory subunit (and therefore with PKA RII catalytic subunit complex = PKA holoenzyme) in basolateral membranes of PG acinar cells *in vivo*.

Finally, we examined the localization of AKAP-150 and Na^+,K^+-ATPase in acinar cells of the rat PG by using an immunohistochemical method (Fig. 9). Sections of rat PG were incubated with a mixture of anti-AKAP-150 goat polyclonal and anti-Na^+,K^+-ATPase rabbit polyclonal antibodies followed by a mixture of Cy3-conjugated anti-goat IgG and FITC-conjugated anti-rabbit IgG to detect AKAP-150 and Na^+,K^+-ATPase, respectively. In acinar cells, Na^+,K^+-ATPase immunoreactivity, visualized in green, was localized predominantly on the basolateral plasma membranes, but not on the apical membrane (Fig. 9A). AKAP-150 immunoreactivity, visualized in red, was likewise localized on the basolateral plasma membranes (Fig. 9B). Furthermore, co-localization of Na^+,K^+-ATPase and AKAP-150 immunoreactivities was visualized as yellow fluorescence (Fig. 9C). These results indicate that Na^+,K^+-ATPase and AKAP-150 were co-localized on the basolateral plasma membrane of acinar cells. Control sections incubated with PBS instead of the primary antibody showed no immunoreaction (not shown).

4. Discussion

Membrane transport systems for Na^+, K^+, and Cl^- are important for electrolyte secretion [9]. Osmotic balance of cells is also important for water secretion, and Na^+,K^+-ATPase is known to be an enzyme that directly regulates cellular osmolarity [7]. Based on the studies from a number of laboratories on the secretion of salt and water by many exocrine epithelia, including salivary glands, the process of saliva production/secretion is thought to conform to the following scheme [9,42,43]: Na^+-K^+-$2Cl^-$ cotransporter located on the basolateral membrane of acinar cells concentrates Cl^- above the electrochemical equilibrium in the cytoplasm. Upon the stimulation with secretagogues, Cl^- exits into the lumen through secretagogue-activated Cl^- channels on the apical membrane. The luminal Cl^- leads to the secretion of salt (NaCl) and osmotically obliged water [43]. Na^+,K^+-ATPase may contribute to this process by providing a driving force (Na^+ gradient) for the Na^+-K^+-$2Cl^-$ cotransporter and by controlling the cellular osmolarity of acinar cells during water transport into the lumen. Na^+,K^+-ATPase is known to be down-regulated by PKA-dependent phosphorylation [10–15]. However, the actual process as to how Na^+,K^+-ATPase is specifically phosphorylated *in vivo* by PKA in response to signaling molecules that elevate the intracellular cAMP has not been definitely clear. We had found earlier that Na^+,K^+-ATPase located on the basolateral membranes of PG acinar cells was regulated by membrane-bound PKA rather than by free cytosolic PKA and that the association of PKA to the membrane was mediated by an AKAP [26].

In the present study, in order to elucidate the functional relevance of the interaction between saliva secretion and

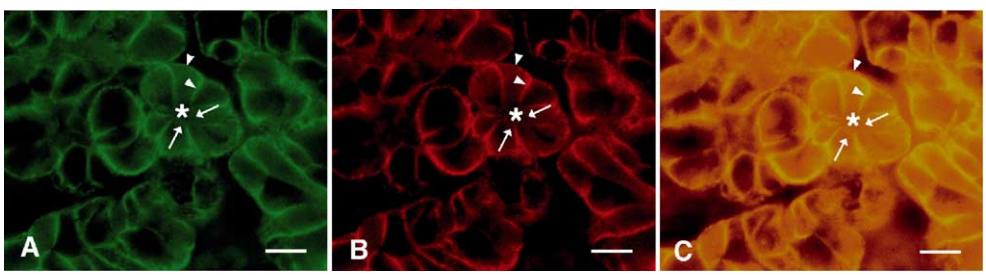

Fig. 9. Light photomicrographs showing the same section of acinar cells in rat PGs stained for Na^+,K^+-ATPase (A), AKAP-150 (B), and both (C). (A) Localization of Na^+,K^+-ATPase was visualized in green with a combination of anti-Na^+,K^+-ATPase rabbit antiserum and FITC-conjugated anti-rabbit IgG. (B) Localization of AKAP-150 was visualized in red with a combination of anti-AKAP-150 antiserum and Cy3-conjugated anti-goat IgG. (C) Co-localization of Na^+,K^+-ATPase and AKAP-150 was visualized in yellow by the double exposure for panels A and B. Note that the immunoreactivity for both Na^+,K^+-ATPase and AKAP-150 is localized predominantly on the basolateral cell membranes (arrowheads), but not on the luminal cell membrane (arrows), lumen (*). Bar = 20 μm.

AKAP/PKA-dependent regulation of Na^+,K^+-ATPase, we examined the expression of AKAP subtypes, AKAP-95, AKAP-150, and AKAP-220, in the three major salivary glands of rats. mRNA and protein of the AKAP-150 subtype were clearly detected in the PG, but the other two subtypes were less abundant there. In both SMG and SLG, AKAP mRNAs and proteins were, unexpectedly, quite low and hardly detectable. The membrane-bound form of the RII regulatory subunit, an index for the amount of total AKAP protein of any subtypes and for the amount of membrane-anchored PKA holoenzyme, was also undetectable in membranes from the SMG and SLG, but was present in the PG, though substantial and comparable amounts of Na^+,K^+-ATPase were contained in all of these membrane preparations. As a consequence, only with the membranes from the PG, in which both the AKAP-150 and membrane-bound RII were clearly detected (Figs. 2 and 3), was Na^+,K^+-ATPase phosphorylated by the endogenous PKA upon the addition of cAMP in the presence of ATP (Fig. 6B). The failure to find the phosphorylation of the SMG or SLG enzyme was due to neither the lack of substrate nor that of susceptibility of either Na^+,K^+-ATPase to the kinase (Fig. 3B). Membrane preparations from any of these glands contained a substantial amount of Na^+,K^+-ATPase that could be readily phosphorylated by the PKA catalytic subunit exogenously added (Fig. 4). Involvement of AKAPs (most probably AKAP-150 subtype) in the regulation of Na^+,K^+-ATPase by the endogenous PKA in PG was also supported by the results of the experiment with HT-31 and HT-31P, an inhibitor peptide of AKAP binding to RII and its variant peptide with no inhibitory action, respectively (Fig. 7).

Furthermore, AKAP-150 on membrane vesicles from PG acinar cells was co-immunoprecipitated with RII regulatory subunit of PKA by an anti-RII antiserum (Fig. 8), indicating that AKAP-150 is indeed functional and forms complexes with RII molecules and therefore with the PKA holoenzyme *in vivo*. AKAP-150 of parotid acinar cells appeared to be predominantly located in basolateral membranes; and Na^+,K^+-ATPase was also present at the same location, as indicated by the histochemical studies (Fig. 9). Our results on the distribution of Na^+,K^+-ATPase agree with those reported by Iwano *et al.* [44], who examined Na^+,K^+-ATPase in acinar and duct cells of the rat PG. Involvement of AKAPs had been reported in a number of PKA-responsive events, such as PKA-mediated inhibition of phospholipase C activity [45], Na^+,K^+-ATPase regulation by PKA-dependent phosphorylation [26], PKA-dependent phosphorylation of β2-adrenergic receptors [46], and PKA phosphorylation of AMPA-responsive glutamate receptor channels [47–49]. Among those systems involving AKAP/PKA, in the one for the β2-adrenergic receptor was demonstrated to be coprecipitated with the AKAP-79 subtype and to directly interact with this AKAP subtype [46]. Another type of association of AKAP with its target protein is one that is mediated *via* scaffolding molecules: AKAP-79/150 binds with members of the MAGUK family of scaffolding molecules, which associate with AMPA-type glutamate receptor channels in the postsynaptic densities [47–49]. The results of our immunochemical and histochemical studies together with the observation made in our previous study that Na^+,K^+-ATPase was quickly and specifically phosphorylated by membrane-anchored PKA [26] suggest a possibility that Na^+,K^+-ATPase somehow physically interacts with the AKAP-150/PKA complex, e.g. by directly binding to form a tri-molecular complex of Na^+,K^+-ATPase/AKAP/PKA or by associating with AKAP-150 *via* scaffolding molecules, such as MAGUKs, as in the case of the glutamate receptor ion channels.

The expression of AKAP (AKAP-150) and the regulation of Na^+,K^+-ATPase by AKAP-anchored PKA are characteristics rather specific to the PG among the three major salivary glands of the rat. Approximately 70% of the Na^+,K^+-ATPase in the basolateral membranes of PG acinar cells was estimated to be associated with AKAP-anchored PKA, based on data from our previous study [26]. Thus, Na^+,K^+-ATPase in PG acinar cells can be considered to be mainly regulated by signaling molecules that elevate the level of intracellular cAMP *via* the action of PKA/AKAP (most probably theAKAP-150 subtype) complexes. This regulatory mechanism for the Na^+,K^+-ATPase might closely correlate with a property of PG distinguishing it from SMG and SLG, and suggests a lack of a rapidly acting regulatory mechanism in the SMG and SLG for regulation of Na^+,K^+-ATPase activity by membrane-anchored PKA in response to a quick and short-term elevation of intracellular cAMP.

Salivary glands consist of acini, striated ducts, and convoluted tubules. Primary saliva (acinus fluid) is produced in acini, and its components are subsequently modified by duct cells during transport in the duct. There are two types of cells in an acinus, serous cells and mucous cells, which produce watery and viscous fluid, respectively. In the PG acinus, only the serous cells are present; and therefore watery saliva is secreted from this gland. Mucous cells are distributed in SLG acini, and thus a slimy viscous saliva is secreted from the SLG. Both types of cells are found in SMG acini, and therefore SMG secretes a moderately viscous saliva from mixed cells [50]. Since the PG contains many more acinar cells compared with the SMG and SLG, it is used as a convenient model for the study of the mechanism and regulation of epithelial fluid and electrolyte secretion. In the PG, Na^+,K^+-ATPase is abundant in basolateral membrane of acinar cells as well as in striated duct cells, whereas in the SMG it is predominantly localized in the striated duct cells. Therefore, it might be possible that the low levels of AKAP-150 mRNA and protein are due to the smaller number of acinar cells per tissue wet weight in the SMG or SLG.

Salivary glands secrete fluid in response to Ca^{2+}-mobilizing rather than to cAMP-generating agonists [51]. However, pertaining to the effect of β-adrenergic stimulation on

PG function, Paulais and Turner [52] demonstrated that the activity of the Na^+-K^+-$2Cl^-$ cotransporter, which is thought to play a central role in the salt and water secretion by many exocrine cells [42,53], was up-regulated by β-adrenergic agonists. Amylase secretion from the PG was stimulated by isoproterenol in a cAMP-mediated manner [54,55]. It was also reported that β-adrenergic receptor stimulation with relatively large doses of isoproterenol induced IP_3 production and Ca^{2+} mobilization in PG acinar cells [56,57]. Thus, it might be possible that β-adrenergic stimulation causes PG saliva secretion, though its effect might be less than that elicited by muscarinic stimulation as reported for the rat SMG [51]. Furthermore, agents that increase intracellular cAMP in the PG were demonstrated to markedly enhance carbachol-induced salivary secretion [58]. Since Na^+,K^+-ATPase is known to directly regulate cellular osmolarity [7], it is of interest to note that β-adrenergic stimulation of PG acini does not result in the cell shrinkage that typically accompanies fluid secretion by exocrine cells [52,59], implying a possible role of down-regulatory mechanism of Na^+,K^+-ATPase, in concert with the up-regulation of Na^+-K^+-$2Cl^-$ cotransporter by cAMP, in controlling the cellular osmolarity during the watery saliva production/secretion in the PG.

Alternatively, AKAP-150/PKA-dependent regulation of Na^+,K^+-ATPase might be related to another characteristic property of the PG that the saliva secretion as affected by autonomic nerve system. Food intake in the mouth and mastication cause both parasympathetic and sympathetic neuronal stimulation, resulting in the profuse watery saliva secretion from the PG. A watery saliva secretion was also observed in the conditioned reflex experiments, as in Pavlov's experiments [50]. There is also a difference in neurons innervating these salivary glands: the PG is under the control of the glossopharyngeal nerve, whereas the SLG and SMG receive fibers from the facial nerve [50]. It might be possible that the expression of AKAP in the PG is associated with the actions of the glossopharyngeal nerve to maintain the ionic balance between the intra- and extracellular fluid during watery saliva production/secretion.

Na^+,K^+-ATPase also functions for generating membrane potential in excitable tissues, and AKAP-150 is known to be abundantly expressed in Purkinje cells and in neurons in the central nervous system, e.g. in the olfactory bulb, basal ganglia, cerebral cortex, and other forebrain regions [37,60–63]. Therefore, elucidating the functional relevance of the interaction between Na^+, K^+-ATPase and AKAP-150 in salivary glands may also contribute to studies on the regulation of neuronal activity involving Na^+,K^+-ATPase in the central nervous system.

Acknowledgments

This work was supported in part by the Japan Salivary Gland Society Award. This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture, Japan (Nos. 10671748, 11671851, 14571774, and 14571775) and by a grant from the Miyata Foundation, Meikai University.

References

[1] Jorgensen PL. Isolation of (Na^+,K^+)-ATPase. In: Fleischer P, editor. Methods in enzymology. Biomembranes, Part B, vol. 32. New York: Academic Press; 1974. p. 277–90.
[2] Jorgensen PL. Mechanism of Na^+, K^+ pump, protein structure and conformations of the pure ($Na^+ + K^+$)-ATPase. Biochim Biophys Acta 1982;694:27–68.
[3] McGrail KM, Sweadner KJ. Immunofluorescent localization of two different Na,K-ATPases in the rat retina and in identified dissociated retinal cells. Neuroscience 1986;6:1272–83.
[4] Shull GE, Greeb J, Lingrel JB. Molecular cloning of three distinct forms of the Na^+,K^+-ATPase α-subunit from rat brain. Biochemistry 1986;25:8125–32.
[5] Specht S, Sweadner KJ. Two different Na,K-ATPases in the optic nerve: cells of origin and axonal transport. Proc Natl Acad Sci USA 1984;81:1234–8.
[6] Sweadner KJ, Gilkeson RC. Two isozymes of the Na,K-ATPase distinct antigenic determinant. J Biol Chem 1985;260:9016–22.
[7] Skou JC. ATP-driven pumps and related transport: the Na^+,K^+-pump. In: Fleischer S, Fleischer B, editors. Methods in enzymology. Biomembranes, Part P, vol. 156. New York: Academic Press, 1988. p. 1–25.
[8] Schwartz A, Lindenmayer GE, Allen JC. The sodium-potassium adenosine triphosphatase: pharmacological, physiological and biochemical aspects. Pharmacol Rev 1975;27:3–134.
[9] Turner RJ. Mechanisms of fluid secretion by salivary glands. Ann NY Acad Sci 1993;694:24–35.
[10] Aperia A, Fryckstedt J, Svensson L, Hemmings Jr HC, Nairn AC, Greengard P. Phosphorylated Mr 32,000 dopamine- and cAMP-regulated phosphoprotein inhibits Na^+,K^+-ATPase activity in renal tubule cells. Proc Natl Acad Sci USA 1991;88:2798–801.
[11] Bertorello AM, Aperia A, Walaas SV, Nairn AC, Greengard P. Phosphorylation of the catalytic subunit of Na^+,K^+-ATPase inhibits the activity of the enzyme. Proc Natl Acad Sci USA 1991;88:11359–62.
[12] Beguin P, Beggah AT, Chibalin AV, Burgener-Kairuz P, Jaisser F, Mathews PM, Rossier BC, Cotecchia S, Geering K. Phosphorylation of the Na,K-ATPase α subunit by protein kinase A and C in vitro and in intact cells. J Biol Chem 1994;269:24437–45.
[13] Fisone G, Cheng SX-J, Nairn AC, Czernik AJ, Hemmings Jr HC, Hoog J-O, Bertorello AM, Kaiser R, Bergmann T, Jornvall H, Aperia A, Greengard P. Identification of the phosphorylation site for cAMP-dependent protein kinase on Na,K-ATPase and effects of site-directed mutagenesis. J Biol Chem 1994;269:9368–73.
[14] Chibalin AV, Vasilets LA, Hennekes H, Pralong D, Geering K. Phosphorylation of Na^+,K^+-ATPase α-subunits in microsomes and homogenates of Xenopus oocytes resulting from the stimulation of protein kinase A and protein kinase C. J Biol Chem 1992;267:22378–84.
[15] Fryckstedt J, Aperia A. Sodium-dependent regulation of sodium, potassium-adenosine-tri-phosphatase (Na^+,K^+-ATPase) activity in medullary thick ascending limb of Henle segments. Effect of cyclic-adenosine-monophosphate guanosine-nucleotide-binding-protein activity and arginine vasopressin. Acta Physiol Scand 1992;144:185–90.
[16] Rall TW, Sutherland EW, Berthet J. The relationships of epinephrine and glucagon to liver phosphorylase. J Biol Chem 1957;224:463–75.
[17] Sutherland EW, Ral TW. Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles. J Biol Chem 1958;232:1077–91.

[18] Sutherland EW. Study on the mechanism of hormone action. Science 1972;171:401–8.
[19] Coghlan VM, Bergeson SE, Langeberg L, Nilaver G, Scott JD. A-kinase anchoring proteins: a key to selective activation of cAMP-responsive events? Mol Cell Biochem 1993;127/128:309–19.
[20] Mochly-Rosen D. Localization of protein kinases by anchoring proteins: a theme in signal transduction. Science 1995;268:247–51.
[21] Scott JD, Stofko RE, McDonald JR, Comer JD, Vitalis EA, Mangili JA. Type II regulatory subunit dimerization determines the subcellular localization of the cAMP-dependent protein kinase. J Biol Chem 1990;265:21561–6.
[22] Scott JD, Carr DW. Subcellular localization of the type II cAMP-dependent protein kinase. NIPS 1992;7:143–8.
[23] Scott JD, McCartney S. Localization of A-kinase through anchoring proteins. Mol Endocrinol 1994;8:5–11.
[24] Pawson T, Scott JD. Signaling through scaffold, anchoring, and adapter proteins. Science 1997;278:2075–80.
[25] Dodge K, Scott JD. AKAP79 and the evolution of the AKAP model. FEBS Lett 2000;476:58–61.
[26] Kurihara K, Nakanishi N, Ueha T. Regulation of Na^+,K^+-ATPase by cAMP-dependent protein kinase anchored on membrane via its anchoring protein. Am J Physiol 2000;279:C1516–27.
[27] Manganel M, Turner RJ. Coupled Na^+/H^+ exchange in rat parotid basolateral membrane vesicles. J Membr Biol 1988;102:247–54.
[28] Bradford MM. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[29] Laemmli UK. Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[30] Matsuda T, Iwata H, Cooper JR. Specific inactivation of alpha(+) molecular form of ($Na^+ + K^+$)-ATPase by pyrithiamin. J Biol Chem 1984;259:3858–63.
[31] Sen AK, Tobin T, Post RL. A cycle for ouabain inhibition of sodium- and potassium-dependent adenosine triphosphatase. J Biol Chem 1969;244:6596–604.
[32] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
[33] Terada Y, Tomita K, Nonoguchi H, Maruo F. Polymerase chain reaction localization of constitutive nitric oxide synthase and soluble guanylate cyclase messenger RNAs in microdissected rat nephron segments. J Clin Invest 1992;90:659–65.
[34] Kurihara K, Hosoi K, Ueha T, Nakanishi N, Yamada S. Effects of nerve growth factor and dexamethasone on Na^+,K^+-ATPase of cultured PC12h cells. Horm Metab Res 1994;26:14–8.
[35] Kurihara K, Tajima Y, Kawasaki M, Ueha T. Modulation of thyroid hormone-dependent Na^+,K^+-ATPase induction in cultured human submandibular gland cell lines, HSG cells. Oral Oncol 1999;35:368–74.
[36] Bittner M, Kupferer P, Morris F. Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzyloxymethyl cellulose or nitrocellulose sheets. Anal Biochem 1980;102:459–71.
[37] Carr DW, Stofko-Hahn RE, Fraser ID, Cone RD, Scott JD. Localization of the cAMP-dependent protein kinase to the postsynaptic densities by A-kinase anchoring proteins. Characterization of AKAP 79. J Biol Chem 1992;267:16816–23.
[38] Coghlan VM, Langeberg LK, Fernandez A, Lamb NJ, Scott JD. Cloning and characterization of AKAP 95, a nuclear protein that associates with the regulatory subunit of type II cAMP-dependent protein kinase. J Biol Chem 1994;269:7658–65.
[39] Lester LB, Coghlan VM, Nauert B, Scott JD. Cloning and characterization of a novel A-kinase anchoring protein. AKAP 220, association with testicular peroxisomes. J Biol Chem 1996;271:9460–5.
[40] Cheng HC, Kemp BE, Pearson RB, Smith AJ, Misconi L, Van Patten SM, Walsh DA. A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. J Biol Chem 1986;261:989–92.
[41] Lohmann SM, DeCamilli P, Eining I, Walter U. High-affinity binding of the regulatory subunit (RII) of cAMP-dependent protein kinase to microtubule-associated and other cellular proteins. Proc Natl Acad Sci USA 1984;81:6723–7.
[42] Haas M, Forbush III B. The Na-K-Cl cotransporter of secretory epithelia. Ann Rev Physiol 2000;62:515–34.
[43] Kurihara K, Nakanishi N, Moore-Hoon ML, Turner RJ. Phosphorylation of the salivary Na^+-K^+-$2Cl^-$ cotransporter. Am J Physiol Cell Physiol 2002;282:C817–23.
[44] Iwano T, Akayama M, Yamamoto A, Omori K, Kumazawa T, Tashiro Y. Quantitative immunoelectron microscopic localization of (Na^+,K^+)ATPase in rat parotid gland. J Histochem Cytochem 1987;35:871–9.
[45] Dodge KL, Carr DW, Sanborn BM. Protein kinase A anchoring to the myometrial plasma membrane is required for cyclic adenosine 3′,5′-monophosphate regulation of phosphatidylinositide turnover. Endocrinology 1999;140:5165–70.
[46] Fraser ID, Cong M, Kim J, Rollins EN, Daaka Y, Lefkowitz RJ, Scott JD. Assembly of an A kinase-anchoring protein-β2-adrenergic receptor complex facilitates receptor phosphorulation and signaling. Curr Biol 2000;10:409–12.
[47] Colledge M, Dean RA, Scott GK, Langeberg LK, Huganir RL, Scott JD. Targeting of PKA to glutamate receptors through a MAGUK-AKAP complex. Neuron 2000;27:107–19.
[48] Leonard AS, Davare MA, Horne MC, Garner CC, Hell JW. SAP97 is associated with the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor GluR1 subunit. J Biol Chem 1998;273:19518–24.
[49] Gometz LL, Alam S, Smith KE, Horne E, Dell'Acqua ML. Regulation of A-kinase anchoring protein 79/150-cAMP-dependent protein kinase postsynaptic targeting by NMDA receptor activation of calcineurin and remodeling of dendritic actin. J Neurosci 2002;15:7027–44.
[50] Ganong WF. Mouth and esophagus. In: Review of medical physiology, vol. 19. Stamford: Appleton & Lange; 1999. p. 467–70.
[51] Martinez JR, Quissell DO, Wood DL, Giles M. Abnormal secretory response to parasympathomimetic and sympathomimetic stimulation from the submandibular glands of rat treated with reserpine. J Pharmacol Exp Ther 1975;194:384–95.
[52] Paulais M, Turner RJ. β-Adrenergic upregulation of the Na^+-K^+-$2Cl^-$ cotransporter in rat parotid acinar cells. J Clin Invest 1992;89:1142–7.
[53] Kaplan MR, Mount DB, Delpire E. Molecular mechanisms of NaCl cotransport. Ann Rev Physiol 1996;58:649–68.
[54] Butcher FR, Putney JW. Regulation of parotid gland function by cyclic nucleotides and calcium. In: Greengard P, Robison GA, editors. Advances in cyclic nucleotide research, vol. 13. New York: Raven Press; 1980. p. 215–49.
[55] Spearmann TN, Butcher FR. Cellular regulation of amylase secretion by the parotid gland. Handb Physiol 1989;3:63–77.
[56] Takemura H. Changes in cytosolic free calcium concentration in isolated rat parotid cells by cholinergic and beta-adrenergic agonists. Biochem Biophys Res Commun 1985;131:1048–55.
[57] Horn VJ, Baum BJ, Ambudkar IS. Beta-adrenergic receptor stimulation induces inositol trisphosphate production and Ca^{2+} mobilization in rat parotid acinar cells. J Biol Chem 1988;263:12454–60.
[58] Larsson O, Olgart L. The enhancement of carbachol-induced salivary secretion by VIP and CGRP in rat parotids is mimicked by forskolin. Acta Physiol Scand 1989;137:231–6.
[59] Martinez JR, Cassity N, Reed P. Effect of isoproterenol on Cl transport in rat submandibular salivary gland acini. Arch Oral Biol 1988;33:505–9.
[60] Carr DW, Cutler Jr RE, Cottom JE, Salvador LM, Fraser ID, Scott JD, Hunzicker DM. Identification of cAMP-dependent protein kinase holoenzymes in preantral- and preovulatory-follicle-enriched ovaries, and their association with A-kinase-anchoring proteins. Biochem J 1999;344:613–23.

[61] Glantz SB, Amat JA, Rubin CS. cAMP signaling in neurons: patterns of neuronal expression and intracellular localization for a novel protein, AKAP 150, that anchors the regulatory subunit of cAMP-dependent protein kinase II beta. Mol Biol Cell 1992;3:1215–28.

[62] Li Y, Ndubuka C, Rubin CS. A kinase anchor protein 75 targets regulatory (RII) subunits of cAMP-dependent protein kinase II to the cortical actin cytoskeleton in non-neuronal cells. J Biol Chem 1996;271:16862–9.

[63] Zhang Q, Carr DW, Lerea KM, Scott JD, Newman SA. Nuclear localization of type II cAMP-dependent protein kinase during limb cartilage differentiation is associated with a novel developmentally regulated A-kinase anchoring protein. Dev Biol 1996;176:51–61.