Regulation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase by cAMP-dependent protein kinase anchored on membrane via its anchoring protein

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Kurihara, Kinji, Nobuo Nakanishi, and Takao Ueha. Regulation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase by cAMP-dependent protein kinase anchored on membrane via its anchoring protein. Am J Physiol Cell Physiol 279: C1516–C1527, 2000.—Na\textsuperscript{+}-K\textsuperscript{+}-ATPase \(\alpha\)-subunits in basolateral membrane vesicles (BLMVs) purified from rat parotid glands were \(^{32}\text{P}\)-labeled within 5 s by incubation with \(\gamma\text{-}^{32}\text{P}\)ATP at 37°C in the presence of cAMP, but no labeling occurred without cAMP. Phosphorylation of assay and was coimmunoprecipitated by anti-RII antibody. BLMVs was shown by immunoblotting and an RII overlay of A-kinase anchoring protein (AKAP). AKAP-150 protein in membrane-bound endogenous PKA, indicating an involvement kinase anchored on membrane via its anchoring protein.

Regulation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase by cAMP-dependent protein kinase (PKA) (3). Na\textsuperscript{+}-K\textsuperscript{+}-ATPase was identified to be Ser-943 of its \(\alpha\)-subunits (for rat Na\textsuperscript{+}-K\textsuperscript{+}-ATPase) (3, 15). The activity of purified Na\textsuperscript{+}-K\textsuperscript{+}-ATPase was decreased by in vitro phosphorylation of the ATPase by exogenous PKA (3). Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in the cells was also shown to be phosphorylated by the stimulation of the cells with forskolin (2, 17) and cAMP analogs (9, 18). Thus Na\textsuperscript{+}-K\textsuperscript{+}-ATPase is thought to be downregulated by cAMP via PKA-dependent phosphorylation (3).

The action of many hormones is mediated by the generation of the intracellular cAMP. The predominant effect of cAMP is to activate a cAMP-dependent protein kinase (PKA) (34, 45–47). Four molecules of cAMP bind each dormant PKA holoenzyme, activating the kinase by releasing the catalytic subunits from the regulatory subunit-cAMP complex. Two classes of regulatory subunits exist: RI and RII, which form the type I and type II PKA holoenzymes, respectively. Since the PKA catalytic subunit has rather broad substrate specificity, various proteins can be phosphorylated by the kinase in vitro regardless of the physiological significance. Phosphorylation of various cellular proteins was also observed in vivo when cells were treated with membrane-permeable cAMP analogs or forskolin and incubated for a rather long period of time, for example, 30 min or more (2, 3, 9, 17, 18). Therefore, for transducing physiological signals, PKA has to somehow perform preferential phosphorylation of its specific target substrate in vivo. However, it is not yet definitely clear how each target protein is specifically phosphorylated in vivo in response to the increase in cAMP via the activation of PKA, which has broad substrate specificity. Scott et al. (37–39), Coghlan et al. (11), and Mochly-Rosen (33) demonstrated the role of A-kinase anchoring protein (AKAP), a specific protein that anchors the PKA regulatory subunit RI but not RI (and thereby the catalytic subunit bound to RII) on the membrane near its specific target proteins. Thus the cellular location of PKA is dictated by the regulatory subunit: the RI isofrom is thought to be primarily cytoplasmic, whereas a significant proportion of the RII isofrom associates with the plasma membrane, cytoskeletal components, endoplasmic reticulum, se-

A TYPICAL MEMBRANE-BOUND ENZYME, Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, consists of at least two subunits, the catalytic \(\alpha\)-subunit and the glycosylated \(\beta\)-subunit (22, 23, 36, 41). By converting the energy of ATP, Na\textsuperscript{+}-K\textsuperscript{+}-ATPase produces an electrochemical gradient of Na\textsuperscript{+} and K\textsuperscript{+} across the plasma membrane (42). Thus Na\textsuperscript{+}-K\textsuperscript{+}-ATPase maintains the membrane potential of excitable nerve (32, 43, 49) and muscle tissues (32, 49) and is also involved in the reabsorption of Na\textsuperscript{+} in the kidneys (22, 23, 36) and in the salivary glands (24, 26, 27). It was reported that Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in intact cells was inhibited by a protein phosphatase inhibitor, phospho-DAKPR-32 (phosphorylated form of a dopamine- and cAMP-regulated 32-kDa protein) (1). Purified Na\textsuperscript{+}-K\textsuperscript{+}-ATPase was shown to be phosphorylated in vitro by the catalytic subunit of protein kinase A (PKA) exogenously added (3). The phosphorylation site of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase by PKA was identified to be Ser-943 of its \(\alpha\)-subunits (for rat Na\textsuperscript{+}-K\textsuperscript{+}-ATPase) (3, 15). The activity of purified Na\textsuperscript{+}-K\textsuperscript{+}-ATPase was decreased by in vitro phosphorylation of the ATPase by exogenous PKA (3). Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in the cells was also shown to be phosphorylated by the stimulation of the cells with forskolin (2, 17) and cAMP analogs (9, 18). Thus Na\textsuperscript{+}-K\textsuperscript{+}-ATPase is thought to be downregulated by cAMP via PKA-dependent phosphorylation (3).

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cretory granules, and nuclei (12, 13, 29). The anchoring of PKA near its substrate may thus permit rapid phosphorylation of a specific substrate protein on the membrane in response to an increase in intracellular cAMP.

In the present study, using Na\(^{+}\)-K\(^{+}\)-ATPase-rich basolateral membrane vesicles (BLMVs) purified from rat parotid gland acinar cells, we investigated how Na\(^{+}\)-K\(^{+}\)-ATPase is phosphorylated in response to the increase in cAMP. We found that the Na\(^{+}\)-K\(^{+}\)-ATPase-rich basolateral membrane contained the holoenzyme form of PKA anchored on the membranes via the RII regulatory subunit and that BLMVs indeed contained a functional AKAP subtype, AKAP-150, which could be coimmunoprecipitated by the anti-RII antibody. The PKA anchored to the BLMVs by AKAP/RII quickly resulted in a decrease in Na\(^{+}\)-ATPase-

**MATERIALS AND METHODS**

Preparation of BLMVs. BLMVs were prepared from the parotid glands of 10-wk-old male Wistar strain rats (31) (Tokyo Laboratory Animal Science, Tokyo, Japan). The glands were minced and suspended in homogenization buffer [10 mM HEPES/Tris (pH 7.4), 10% sucrose, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] at 4°C, and minced parotid tissue was then homogenized in a Polytron (Tokyo Laboratory Animal Science, Tokyo, Japan). The parotid glands were minced and suspended in homogenization buffer (Tokyo Laboratory Animal Science, Tokyo, Japan). The glands were minced and suspended in homogenization buffer [10 mM HEPES/Tris (pH 7.4), 10% sucrose, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] at 4°C, and floating tissue fragments were removed by aspiration. The resulting suspension was centrifuged again, and the final pellet was washed with distilled water and boiled with 10 ml/g starting parotid glands. The homogenate was centrifuged at 2,500 \(\times\) g and then incubated for 2 h with the anti-RII antibody. Membrane-bound Na\(^{+}\)-K\(^{+}\)-ATPase was prepared from whole kidneys of male rats by the method of Jorgensen (22). The \(\alpha\)-subunit was then separated from the membrane-bound Na\(^{+}\)-K\(^{+}\)-ATPase by SDS-PAGE (5% acrylamide) using Laemmli’s buffer system (28). Anti-\(\alpha\)-anti-serum was obtained by immunization of a rabbit with the \(\alpha\) protein (24–27).

**Preparation of anti-\(\alpha\), anti-\(\alpha\)-subunit.** An anti-\(\alpha\), antisem was prepared by the method described in our previous report (24–27). In brief, membrane-bound Na\(^{+}\)-K\(^{+}\)-ATPase was prepared from whole kidneys of male rats by the method of Jorgensen (22). The \(\alpha\)-subunit was then separated from the membrane-bound Na\(^{+}\)-K\(^{+}\)-ATPase by SDS-PAGE (5% acrylamide) using Laemmli’s buffer system (28). Anti-\(\alpha\)-anti-serum was obtained by immunization of a rabbit with the \(\alpha\) protein (24–27).

**Western blot analysis of Na\(^{+}\)-K\(^{+}\)-ATPase.** Samples were subjected to SDS-PAGE, and the separated proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane filters in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid and 10% methanol (pH 11) (4). The filter was then stained immunochemically with the anti-\(\alpha\)-anti-serum (24–27). In brief, filters were incubated with 5% skim 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 h with the anti-\(\alpha\)-anti-serum in T-TBS containing 5% skim milk. After being washed with T-TBS, these filters were incubated for 1 h with anti-rabbit IgG goat serum conjugated with horseradish peroxidase (HRP). The filters were then washed, and the signal was detected with enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham RPN 2106; Amersham, UK).

**Phosphopeptide mapping of \([^{32}\text{P}]\text{phospho-Na}^{+}\text{-K}^{+}\text{-ATPase \(\alpha\)-subunit.}\)** \([^{32}\text{P}]\text{phospho-BLMV protein phosphorylated in the presence of cAMP was subjected to SDS-PAGE, and the gels were dried without fixation. The 92-kDa signal, the putative \([^{32}\text{P}]\text{phospho-Na}^{+}\text{-K}^{+}\text{-ATPase \(\alpha\)-subunit, was cut from the gel and swollen with 16 \(\mu\)l (original volume of cut gels) of 125 U/ml V8 protease in 100 mM ammonium carbonate (pH 7.8) (50). The gel pieces were incubated at 37°C for 3 h, and the reaction was then stopped by boiling for 5 min. The peptides extracted from the gel pieces were applied to tricine-SDS-PAGE (16% acrylamide) after being boiled in tricine-SDS-PAGE buffer (35).**
addition of 200 μl of 35% TCA. The inorganic phosphate (P_i) liberated (total ATPase activity) was measured by the procedure of Fiske and Subbarow (16). Ouabain-nonsensitive ATPase activity was measured in the same way as for measurement of total ATPase activity except that 2 mM ouabain was present. The ouabain-sensitive ATPase activity calculated from the two assays was considered as Na\(^+-\)K\(^{-}\)-ATPase.

The amount of enzyme liberating 1 μmol of P_i per minute was defined as 1 unit. Protein amounts were determined by the use of a protein assay kit (Bio-Rad) with bovine serum albumin serving as a standard (5).

**RNA preparation.** Total RNAs were prepared by the method of Chimeczynski and Sacchi (10) using acid guanidinium thiocyanate-phenol-chloroform. Approximately 100-μg amounts of rat brain, kidney, and parotid gland 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 sodium acetate (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.

**RT-PCR and DNA sequencing.** RT-PCR was carried out with a kit that contained a dye terminator for the ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Foster City, CA) according to the following schedule: denaturation, annealing, and elongation at 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s, respectively, for 30 cycles.

The sequence analysis, PCR products were reamplified with primers (Table 1) in the presence of dye terminator (BigDye Terminator Cycle Sequencing FS Ready Reaction kit, no. 4303573; Perkin Elmer, Branchburg, NJ). The DNA sequences were analyzed with an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Foster City, CA) according to the following schedule: denaturation, annealing, and elongation at 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s, respectively, for 30 cycles.

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**Results.** Phosphorylation of BLMV proteins by endogenous PKA anchored on the membrane. BLMVs purified from rat parotid glands did not contain any significant amounts of cytosolic proteins, including the free PKA catalytic subunit (31). This was supported by the result that membrane proteins were only faintly phosphorylated by a short-term (30-s) incubation without cAMP in the presence of [γ\(^{32}\)P]ATP (Fig. 1, A and B, lane 1).

Phosphorylation of some of these faintly labeled proteins, such as the 110-kDa band shown later in Fig. 10, seemed to occur independently of cAMP and PKA. However, with the addition of cAMP (10 μM), protein phosphorylation was clearly detected by the 30-s incubation at 37°C: mainly four protein bands with molecular masses of 300 kDa, 180 kDa, 92 kDa, and 50 kDa were phosphorylated (Fig. 1A, lane 2). As described later (Fig. 10), phosphorylation of the 92-kDa protein (Na\(^+-\)K\(^{-}\)-ATPase α-subunit) became detectable 5 s af-

<table>
<thead>
<tr>
<th>Gene</th>
<th>Localization</th>
<th>Product Size, bp</th>
<th>Primer Sequence</th>
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<tr>
<td>AKAP-95</td>
<td>220–239</td>
<td>399–418</td>
<td>5′-CTCATCGCATGTTGCGGCTTTT-3′</td>
</tr>
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<td>732–751</td>
<td>5′-AAGGAGCCGTCGGTGGTAC-3′</td>
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<td>5′-GGGCCTACAGGAAGATGTGA-3′</td>
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<tr>
<td>AKAP-150*</td>
<td>1,043–1,062</td>
<td>1,581–1,600</td>
<td>5′-GCCACTGTAGCTTCCTCTGC-3′</td>
</tr>
<tr>
<td>AKAP-220</td>
<td>5,352–5,371</td>
<td>5,399–418</td>
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<tr>
<td>AKAP-220</td>
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<td>5,532–5,551</td>
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<td>5,581–5,600</td>
<td>5′-GAGGGAGAAGAGGGAAGGA-3′</td>
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*AKAP-150 has been submitted by Y. Takai, M. Irie, A. Toyada, and Y. Hata to the Query GenBank database at the National Center for Biotechnology. AKAP, A-kinase anchoring protein.
enter the addition of cAMP. The phosphorylation of these proteins was inhibited by PKI-(5–24) peptide, a specific inhibitor peptide of PKA, but the phosphorylation of the 110-kDa protein was not inhibited (Fig. 1\textit{A}, lane 3). These results indicate the presence of membrane-anchored PKA in the holoenzyme form in the purified BLMVs.

When the BLMVs were incubated for 30 s with the exogenous PKA catalytic subunit (16 U/40 \(\mu\)l) and \([\gamma-\text{32P}])ATP (Fig. 1\textit{B}, lane 2), BLMV proteins were also phosphorylated, even in the absence of cAMP. The profile of phosphorylation by exogenous PKA was similar to that of the phosphorylation by membrane-anchored endogenous PKA, except that an additional 32P phosphoprotein band of 23 kDa was detected with the exogenous kinase. However, the 23-kDa protein phosphorylation by endogenous PKA was also detectable when prolonged incubation (for example, 30 min) was employed instead of the 30-s one (data not shown). PKI-(5–24) peptide was also inhibitory for the phosphorylation by the exogenous PKA (Fig. 1\textit{B}, lane 3).

Identification of the 92-kDa phosphoprotein as Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\)-subunit. Since Na\(^{+}\)-K\(^{+}\)-ATPase is one of the major proteins in purified BLMV preparations and its \(\alpha\)-subunit has a molecular weight of 92 kDa, the 32P-labeled 92-kDa protein on the SDS-PAGE was assumed to be the Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\)-subunit. To confirm this assumption, we examined the 92-kDa 32P phosphoprotein by using a polyclonal antiserum raised against the \(\alpha\)-isoform of the Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\)-subunit (24–27).

The antiserum reacted only with the 92-kDa protein band on the Western-blotted membrane filter (Fig. 2\textit{B}). The band corresponded to the same exact band detected on the 32P autoradiogram of the same PVDF membrane (Fig. 2\textit{A}).

Although the anti-\(\alpha\)-antiserum was able to react with the \(\alpha\)-isoform protein (24–27) and was able to inhibit Na\(^{+}\)-K\(^{+}\)-ATPase activity (24), immunoprecipitation of the Na\(^{+}\)-K\(^{+}\)-ATPase was not successful with this antiserum. Therefore, we employed peptide mapping of the 92-kDa phosphoprotein with V8 protease for further analysis (Fig. 3).

Proteolytic products from the 92-kDa protein were subjected to tricine-SDS-PAGE/Western blotting. Four
main signals with molecular masses of 25, 20, 18, and 10 kDa and four broad, weak signals (70, 40, 31, and 15 kDa) were detected on the PVDF membrane with the antiserum (Fig. 3, lane 2). It should be noted that the intensity of the signal is not definitely proportional to the amount of each peptide, since the reactivity of this polyclonal antiserum raised against the entire molecule of the α1-subunit with each fragment peptide derived from the α1 may differ from peptide to peptide. The $^{32}$P autoradiogram of the same membrane showed a major strong signal at the position of 20 kDa (Fig. 3, lane 1). Thus the results indicate that almost all of the $^{32}$P phosphopeptides were derived from the α1-subunit of Na$^+$$-$$K^+$-ATPase.

Dissociation of the endogenous PKA catalytic subunit. As we demonstrated (Fig. 1), PKA in the holoenzyme form is associated with the BLMV. We then examined whether the catalytic subunit of PKA or the regulatory subunit was anchored on the membrane. BLMVs were incubated with cAMP (10 μM) for 5 min to separate the PKA holoenzyme into the catalytic subunit and regulatory subunits, and the membrane was washed. Subsequently, the BLMVs were incubated with [γ-$^{32}$P]ATP to assess the protein-phosphorylating activity. As shown in Fig. 4, the BLMVs thus pretreated with cAMP and washed lost the activity for phosphorylating membrane proteins including Na$^+$$-$$K^+$-ATPase, even in the presence of cAMP.

On the other hand, BLMVs preincubated without cAMP and washed essentially conserved the protein phosphorylation activity. These results indicate that the PKA catalytic subunit associated with the BLMV by binding to its regulatory subunit, which was anchored to the membrane presumably by AKAP (7, 11–14, 19, 29, 33, 37–39).

Reconstitution of the PKA holoenzyme by anchoring of its catalytic subunit to the BLMV. Since the involvement of AKAP in the phosphorylation of Na$^+$$-$$K^+$-ATPase by endogenous PKA was suggested, we further examined the functional role of AKAP in the regulation of the Na$^+$$-$$K^+$-ATPase phosphorylation (Fig. 5). First, the endogenous PKA holoenzyme bound to BLMVs was separated into its catalytic and regulatory subunits by incubating it with cAMP, and the catalytic subunits were washed away as described in the previous section, resulting in the loss of Na$^+$$-$$K^+$-ATPase phosphorylation. However, the BLMVs thus treated were able to restore the phosphorylation of Na$^+$$-$$K^+$-ATPase by the membrane-bound PKA holoenzyme when the vesicles were incubated with the exogenous PKA catalytic subunit followed by extensive washing for removal of the free catalytic subunits. It should be emphasized that the phosphorylation of Na$^+$$-$$K^+$-ATPase observed in the PKA-reconstituted BLMV was dependent on the presence of cAMP, indicating that the PKA responsible for the Na$^+$$-$$K^+$-ATPase phosphorylation was derived from its holoenzyme form on the membrane. Furthermore, the reconstitution process of BLMV with the PKA catalytic subunit was inhibited by the PKI-(5–24) peptide, indicating that the binding of the PKA catalytic subunit to the BLMV was mediated by the RII regulatory subunit, which was anchored to the membrane by AKAP. The PKI-(5–24) peptide is known to bind to the same site of the PKA catalytic subunit as does the regulatory subunit (8) and thereby inhibits the catalytic activity and the binding of the regulatory subunit to the catalytic subunit. Thus our data indicate that the PKA catalytic subunit reversibly associated with the BLMV, mediated by its regulatory subunit/AKAP, in the absence of cAMP.

mRNAs of AKAP in rat parotid gland. To determine whether AKAP genes are actually expressed in rat parotid glands, we examined mRNAs for AKAP in the
tissue by RT-PCR. On the basis of the cDNA sequences registered in the Query GenBank database, National Center for Biotechnology, two sets of each primer pair were designed and prepared for three AKAP subtypes: AKAP-95, AKAP-150, and AKAP-220 (Table 1). With all of the primer pairs, which were expected to produce large PCR products (553 bp for AKAP-95, 558 bp for AKAP-150, and 251 bp for AKAP-220) and small PCR products (199 bp, 199 bp, and 200 bp for AKAP-95, AKAP-150, and AKAP-220, respectively), DNA products of the expected sizes were obtained when RT-PCR was carried out with RNAs prepared from rat brain, kidney, and parotid gland, though the mRNA levels for AKAP-95 and AKAP-220 in the parotid gland seemed to be less abundant than the level of AKAP-150 mRNA (Fig. 6, A and B). The larger PCR products were tested by the nested PCR method using the respective primer pairs for the smaller PCR products (Fig. 6C). In an additional independent experiment, the DNA sequence of each smaller PCR product in Fig. 6B was analyzed and confirmed to be identical to the corresponding portion of the cDNA sequence for AKAP-95, AKAP-150, or AKAP-220 (data not shown).

Detection of AKAP-150 protein in BLMVs of rat parotid gland. Since the expression of AKAP mRNAs in the parotid gland suggested the presence of AKAP proteins in the BLMVs, we examined BLMV protein by immunoblotting with antibodies to AKAP-95, AKAP-150, and AKAP-220. Anti-AKAP-150 antibody revealed a specific signal at a molecular weight of 150 kDa on a blotting membrane of BLMV, and the antibody also recognized a signal at the same molecular size on the membrane of rat brain homogenate (Fig. 7). On the other hand, signals for AKAP-95 and AKAP-220 proteins were not detectable on the blotting membrane of BLMV (not shown).

As we detected in BLMV a protein that reacted with anti-AKAP-150 antibody, we then examined a functional property of the protein by testing to see if it would bind to the RII subunit in an overlay detection assay for AKAP. BLMV proteins separated by SDS-PAGE were blotted onto a PVDF filter, and the filter

![Fig. 6. Expressions of A-kinase anchoring protein (AKAP)-95, -150, and -220 in rat brain, kidney, and parotid gland. PCR was carried out with first-strand DNAs prepared from RNAs of rat brain (B), kidney (K), and parotid gland (PG). A: PCR products amplified with primers for AKAP-95, -150, and -220 cDNAs with product sizes of 553 bp, 558 bp, and 251 bp, respectively. B: PCR products amplified with primers for AKAP-95, -150, and -220 cDNAs with product sizes of 199 bp, 199 bp, and 200 bp, respectively (Table 1). C: nested RT-PCRs of rat AKAP-95, -150, and -220. First PCR products of AKAP-95, -150, and -220 were amplified from first-strand DNAs prepared from kidney, parotid gland, and brain, respectively (with the primers shown in Table 1), the product sizes of which were 553 bp, 558 bp, and 251 bp, respectively. Nested PCR products of AKAP-95, -150, and -220 were amplified from the respective first PCR products (with the primers shown in Table 1), the product sizes of which were 199 bp, 199 bp, and 200 bp, respectively.](image)

![Fig. 7. Western blot analysis of AKAP-150 in BLMV purified from rat parotid gland. BLMV proteins (2 μg) were separated by SDS-PAGE (4–20% gradient gel) on lane 2. Rat brain homogenate (10 μl of the 10% homogenate) was also analyzed on lane 1 as a positive control. Proteins were transferred to a PVDF filter and were immunostained as described in MATERIALS AND METHODS except that the following antibodies, 5,000 times diluted anti-AKAP-150 antibody and 1,000 times diluted anti-goat IgG-HRP (produced by Santa Cruz Biotechnology), were used as first and second antibodies, respectively.](image)
was then incubated with the PKA RII regulatory subunit. Thereafter, the binding of the RII regulatory subunit to AKAPs was analyzed by the anti-RII antibody. The antibody recognized two bands; one was a strong band of the endogenous RII subunit, representing a molecular mass of 60 kDa, and the other was 150-kDa with the same migration as AKAP-150 (Fig. 8). These results indicate that the exogenous RII subunit actually bound to AKAP-150 blotted on the filter, indicating that the BLMV protein detected by the anti-AKAP-150 antibody was a functional AKAP-150 protein molecule that bound to the RII subunit of PKA.

BLMV was further subjected to an analysis for co-immunoprecipitation of AKAP-150 by the anti-RII subunit antibody to examine whether the AKAP-150 formed a complex with the endogenous PKA RII regulatory subunit. Proteins solubilized from BLMVs were incubated with anti-RII antiserum, and the precipitated proteins were then examined by Western blot analysis using the anti-AKAP-150 antibody. AKAP-150 was detected in the immunoprecipitates formed by the anti-RII antibody at the same position as the signal obtained with BLMVs used as a positive control (not shown).

Effect of HT-31 peptide on Na\(^{+}\)-K\(^{+}\)-ATPase phosphorylation by membrane-anchored PKA. We examined the effect of the HT-31 peptide on Na\(^{+}\)-K\(^{+}\)-ATPase phosphorylation on BLMVs. The HT-31 peptide is known to competitively inhibit RII binding to AKAP (6, 20, 48, 52, 53). After the endogenous PKA catalytic subunit in BLMVs was removed by cAMP and washed away, the BLMVs were incubated in the presence or absence of the HT-31 peptide. BLMVs were then incubated with the exogenous PKA catalytic subunit to reconstitute the membrane-anchored PKA holoenzyme. BLMVs thus treated were tested for cAMP-stimulated phosphorylation of Na\(^{+}\)-K\(^{+}\)-ATPase on the membrane. As shown in Fig. 9, the HT-31 peptide decreased the level of Na\(^{+}\)-K\(^{+}\)-ATPase phosphorylation by membrane-anchored PKA, indicating the involvement of AKAP in the regulation of Na\(^{+}\)-K\(^{+}\)-ATPase phosphorylation by cAMP/PKA.

Time course of Na\(^{+}\)-K\(^{+}\)-ATPase phosphorylation by endogenous and exogenous PKA. We examined the time course of phosphorylation of the Na\(^{+}\)-K\(^{+}\)-ATPase α-subunit (92-kDa protein) by membrane-anchored endogenous PKA and by exogenously added PKA. The phosphorylation reaction was terminated by the addition of TCA at various time points after the addition of [γ\(^{32}\)P]ATP and cAMP (Fig. 10, A–C). Furthermore, we measured the intensity of 92-kDa signals on autoradiogram films by computer-assisted image analysis (Fig. 10D). Phosphorylation of Na\(^{+}\)-K\(^{+}\)-ATPase by endogenous PKA was cAMP dependent, became clearly detectable in 5 s, and reached a plateau level within 15 s (Fig. 10, B and D). Phosphorylation by exogenous PKA was also detectable in 5 s, but reached a plateau level much more slowly, i.e., by 30 s (Fig. 10, C and D).

On the other hand, a faint band of 110 kDa was detected even in the absence of cAMP (Fig. 10A). Phosphorylation of this protein was independent of cAMP and not enhanced by the addition of cAMP or exogenous PKA. This result is compatible with the results shown in Fig. 1, which indicated that the 110-kDa protein phosphorylation was insensitive to PKI(5–24) peptide, a PKA inhibitor. Thus the phosphorylation of Na\(^{+}\)-K\(^{+}\)-ATPase in the basolateral membrane is a rapid and highly specific process effected by membrane-anchored endogenous PKA.

After the α-subunit phosphorylation by either endogenous or exogenous PKA had reached its plateau level, these maximal levels were maintained until at least 60 s of incubation (Fig. 10D). We compared the maximum levels of P\(_i\) incorporation into the Na\(^{+}\)-K\(^{+}\)-ATPase α-subunit in the BLMVs after incubation for 1 min at 37°C with [γ\(^{32}\)P]ATP in the presence of cAMP or the exogenous PKA catalytic subunit (Table 2). The
maximal P\(_i\) incorporation into the α-subunit by endogenous PKA was ~70% of that into the α-subunit by exogenous PKA, whereas the total protein phosphorylation (total \(32^P\) incorporation into the membrane proteins) by endogenous PKA was ~2% of that by the exogenous PKA, indicating a molecule-specific action of endogenous (AKAP-associated) PKA.

**Downregulation of Na\(^{+}\)-K\(^{+}\)-ATPase activity via phosphorylation of the enzyme by PKA anchored to BLMVs.** We further examined the effect of phosphorylation of the ATPase by membrane-anchored PKA on the activity of the enzyme (Fig. 11). When Na\(^{+}\)-K\(^{+}\)-ATPase was phosphorylated by incubating the BLMVs with ATP and cAMP, the ATPase activity was decreased to ~80% of that of the control. The addition of PKI-(5–24) peptide along with cAMP blocked this cAMP-dependent inhibition of Na\(^{+}\)-K\(^{+}\)-ATPase, whereas PKI-(5–24) peptide itself did not have any stimulatory or inhibitory effect in this experimental system. Thus the activity of Na\(^{+}\)-K\(^{+}\)-ATPase was quickly downregulated by the action of membrane-anchored PKA in response to the addition of cAMP, in other words, in response to an increase in the cAMP level.

** DISCUSSION **

Salivary glands consist of acini, striated ducts, and convoluted tubules. Since the parotid gland contains many acinar cells, it is a convenient model for the study of the mechanism and regulation of epithelial fluid and electrolyte secretion. Generally, fluid secretion is regulated by the combined action of four mem-

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**Table 2. Phosphorylation of α-subunit of Na\(^{+}\)-K\(^{+}\)-ATPase by PKA and by exogenous PKA**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(P)(_i) Incorporation into Na(^{+})-K(^{+})-ATPase, fmol/mg of BLMV protein</th>
<th>(32^P) Incorporation into Na(^{+})-K(^{+})-ATPase, dpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exogenous PKA</td>
<td>32.1 ± 2.53</td>
<td>620 ± 48.9</td>
</tr>
<tr>
<td>cAMP</td>
<td>23.1 ± 0.489*</td>
<td>447 ± 9.45</td>
</tr>
</tbody>
</table>

Basolateral membrane vesicles (BLMVs; 8 μg) were incubated for 1 min at 37°C with 1 mM \([γ-32^P]ATP\) (1 Ci/mmol) in 40 μl of incubation mixture that contained 20 mM sodium acetate, 60 mM potassium acetate, 60 mM KCl, 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 units of protein kinase A catalytic subunit (exogenous PKA). After incubation, samples were subjected to SDS-PAGE and autoradiograms were then prepared. Radioactive 92-kDa positions were cut from the gel, and the radioactivity was measured in a liquid scintillation counter. The activity of endogenous cAMP-dependent protein kinase in 8 μg of BLMV was estimated to be 0.2–0.3 units. Total \(P\)\(_i\) incorporation into the Na\(^{+}\)-K\(^{+}\)-ATPase α-subunit was calculated based on the \(32^P\) radioactivity of the 92-kDa protein and the specific activity of the [γ-32\(^P\)]ATP substrate. Data indicate means ± SD from 3 independent experiments. *Value is significantly different from that of the exogenous PKA group, as evaluated by Student’s t-test (P = 0.00189).
brane transport systems, i.e., Na\(^+\)-K\(^+\)-ATPase, Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, Ca\(^2+\)-activated K\(^+\) channel in basolateral membranes, and an apical conductive pathway for Cl\(^-\), presumably involving Ca\(^2+\)-activated Cl\(^-\) channels (51). Thus Na\(^+\)-K\(^+\)-ATPase plays a vital role in maintaining homeostasis of epithelial fluid and electrolyte secretion as well as in generating membrane potential in excitable tissues.

In the present study, we investigated the regulatory effect of cAMP on Na\(^+\)-K\(^+\)-ATPase of rat parotid gland acini via the endogenous PKA anchored on the cell membranes. Na\(^+\)-K\(^+\)-ATPase-rich BLMVs used in this study were purified from rat parotid glands and did not contain any significant amounts of free catalytic subunit of PKA. Therefore, without the addition of cAMP, only faint bands of \(^{32}\)P phosphate proteins were detected on the autoradiogram (Fig. 1). However, with the addition of cAMP, protein phosphorylation occurred mainly on four proteins (300 kDa, 180 kDa, 92 kDa, and 50 kDa), including the Na\(^+\)-K\(^+\)-ATPase α-subunit (92 kDa) in the BLMVs, and was inhibited by PKI (5–24) peptide, indicating that the BLMV preparation contained membrane-anchored endogenous PKA in its holoenzyme form.

The phosphorylation profile of BLMV proteins obtained with the membrane-anchored PKA in the presence of cAMP (Fig. 1A) was similar to that of the exogenous PKA catalytic subunit, except for the 23-kDa protein phosphorylated by the latter system (Fig. 1B). This difference might be due to the difference in availability and specificity of the catalytic subunit of PKA for this protein substrate in the endogenous system and in the exogenous system; i.e., the 23-kDa protein might be distant from the anchored endogenous PKA compared with the other proteins, 300 kDa, 180 kDa, 50 kDa, and Na\(^+\)-K\(^+\)-ATPase. Therefore, the endogenous catalytic subunit, which was released from the regulatory subunit anchored on the membrane with the addition of cAMP did not adequately phosphorylate the 23-kDa protein during the relatively short period of incubation (30 s). However, the 23-kDa phosphorylation by endogenous PKA indeed became detectable when the incubation time was prolonged (not shown).

Among all proteins in the BLMV, the \(^{32}\)P-labeled 92-kDa protein, having a molecular weight identical to that of the α-subunit of Na\(^+\)-K\(^+\)-ATPase, was the only protein reactive with an antisera raised against the α\(_1\)-subunit of Na\(^+\)-K\(^+\)-ATPase of rat (Fig. 2). This polyclonal antibody was specific for the α\(_1\)-isoform of the α-subunit (24–27) and inhibited the Na\(^+\)-K\(^+\)-ATPase activity (24). Therefore, Na\(^+\)-K\(^+\)-ATPase is thought to be phosphorylated by endogenous PKA anchored on the membrane. This was confirmed by the analysis of V8 proteolytic products from the 92-kDa protein. The \(^{32}\)P signal was exclusively detected on the proteolytic peptide fragment of 20 kDa in peptides detected with the anti-α\(_1\) antibody (Fig. 3), indicating that only the α\(_1\)-subunit of Na\(^+\)-K\(^+\)-ATPase was phosphorylated even if other proteins with the same molecular weight were contained in the 92-kDa band.

It is worth noting that phosphorylation of BLMV proteins was carried out in the presence of 0.0025% Triton X-100, since Na\(^+\)-K\(^+\)-ATPase is a typical membrane-bound enzyme with eight transmembrane domains (41). This detergent concentration was slightly higher than the concentration (0.00125%) that gave the maximum ATPase activity, and it did not denature the Na\(^+\)-K\(^+\)-ATPase or change the membrane structure. Although a much higher concentration of Triton X-100 (e.g., 0.05% [15]) is very successful for the purpose of protein phosphorylation, such a high concentration caused a vast increase in the nonspecific protein phosphorylation and entire loss of the Na\(^+\)-K\(^+\)-ATPase activity (unpublished observations). As mentioned formerly, prolonged incubation also caused an increase in phosphorylation of the nonspecific substrates such as the 23-kDa protein. Thus the detergent concentration as well as the incubation time period is a quite important factor in examining the regulation of membrane-bound enzymes by protein phosphorylation.

The catalytic subunit of PKA is assumed to be associated with the BLMV by binding to its regulatory subunit, since Scott et al. (37–39) and Coghlan et al. (11–13) reported the existence of an AKAP that anchored the regulatory subunit to the membrane. We tested this assumption by preincubating the BLMVs with cAMP (Fig. 4). The PKA catalytic subunit was removed from the BLMVs by dissociating it from its regulatory subunit, indicating that the regulatory subunit, but not the catalytic subunit, was directly anchored to the membrane, and supporting the idea that AKAP is involved in the in vivo phosphorylation of Na\(^+\)-K\(^+\)-ATPase by PKA in the rat parotid gland.

Therefore, by performing RT-PCR with primers for AKAP subtypes that are expressed in other rat tissues (11–13, 29, 39), we examined whether or not AKAP mRNA was present in the acinar cells of the rat parotid gland. By this procedure, we detected three subtypes of AKAP mRNAs: those for AKAP-95, AKAP-150, and AKAP-220 (Fig. 6). The mRNAs for AKAP-95 and AKAP-220 were much less abundant than for AKAP-150. We also examined AKAP proteins in BLMVs and detected AKAP-150 protein by immunoblot analysis (Fig. 7). AKAP-95 and AKAP-220 proteins were present in a less than detectable amount, if present at all. The presence of functional AKAP-150 protein in the BLMVs was further evidenced by the result of an RII overlay assay for AKAP: the exogenous RII regulatory subunit bound to the AKAP-150 blotted onto the filter (Fig. 8). Furthermore, AKAP-150 was coimmunoprecipitated with the RII subunit by the anti-RII antibody (data not shown), i.e., the AKAP-150 in BLMV was associated with the endogenous RII subunits of PKA. These results indicate that the PKA RII regulatory subunit was associated with the basolateral membrane via AKAP-150 and that AKAP-150 was functional for anchoring PKA in rat parotid gland acinar cells. The presence of functional AKAP protein, AKAP-150, in BLMVs, together with the fact that incubation of BLMVs with cAMP released free catalytic subunits of PKA, further supports the idea that AKAP is involved.
in the in vivo phosphorylation of the Na\(^+-\)K\(^+\)-ATPase \(\alpha\)-subunit by PKA in the rat parotid gland.

It has already been reported that Na\(^+-\)K\(^+\)-ATPase was phosphorylated in vitro by the PKA catalytic subunit exogenously given (3, 17). Na\(^+-\)K\(^+\)-ATPase was also shown to be phosphorylated in vivo by endogenous PKA when cells were treated with forskolin (2, 17) or membrane-permeable cAMP analogs (9, 18). Nevertheless, the actual process by which Na\(^+-\)K\(^+\)-ATPase is phosphorylated and regulated in cells by hormones or neurotransmitters that elevate the intracellular cAMP level is not yet definitely clear. Since the time period of cAMP elevation in cells by signaling molecules is generally thought to be rather short, if a certain enzyme was regulated by PKA-dependent phosphorylation in response to the transient and short-term increase in cAMP level, it should be quickly and specifically phosphorylated by the kinase. As we demonstrated here, rapid phosphorylation of the \(\alpha\)-subunit of Na\(^+-\)K\(^+\)-ATPase in BLMVs by membrane-anchored endogenous PKA was indeed the case: the phosphorylation was detectable within 5 s on cAMP stimulation in the presence of \(\gamma\)-[\(\beta\)\(\gamma\)]ATP and reached the plateau level in 15 s (Fig. 10). It should be emphasized that the \(\alpha\)-subunit phosphorylation rate by the endogenous PKA in the early phase, for example, during the first 5 s of incubation, was much faster than that of the exogenously added PKA. In these experiments, the activity per one assay of the endogenous PKA was estimated to be 0.2–0.3 U/8 \(\mu\)g of BLMVs (Table 2), and that of the exogenously added PKA was estimated to be 16 U/8 \(\mu\)g of BLMVs.

Furthermore, in the absence (or on the removal) of cAMP, the PKA catalytic subunit was able to reversibly bind to the BLMV via its regulatory subunit/AKAP complex, as was demonstrated in Fig. 5. This PKA anchoring process was clearly inhibited by the PKI peptide. The PKI peptide is a part of the regulatory subunit of PKA and binds to the catalytic subunit of PKA at the same site where the regulatory subunit/AKAP complex binds to the catalytic subunit, but PKI does not contain the amino acid sequence needed for binding with AKAP. Thus in the experimental system employed in Fig. 5, PKI blocked the phosphorylation of the \(\alpha\)-subunit of Na\(^+-\)K\(^+\)-ATPase by inhibiting the anchoring of PKA to the BLMV and not by directly preventing the phosphorylation reaction, since PKI was washed out before the phosphorylation reaction, indicating the functional implication of AKAP in the regulation of Na\(^+-\)K\(^+\)-ATPase activity.

We also examined the effect of HT-31 peptide on the cAMP/PKA-dependent Na\(^+-\)K\(^+\)-ATPase phosphorylation. The HT-31 peptide has an amino acid sequence identical to the part of AKAP that binds with the RII subunit, and therefore the peptide can competitively inhibit the binding of RII to AKAP (6, 20, 48, 52, 53). Incubation of BLMVs with the HT-31 peptide resulted in a decrease in cAMP-stimulated Na\(^+-\)K\(^+\)-ATPase phosphorylation by membrane-bound PKA (Fig. 9), also supporting the involvement of AKAP in the regulation of Na\(^+-\)K\(^+\)-ATPase via its cAMP/PKA-dependent phosphorylation.

Although the rate of phosphorylation of the Na\(^+-\)K\(^+\)-ATPase \(\alpha\)-subunit by the endogenous PKA was much faster than that of the exogenously added PKA, the maximal levels of the \(\alpha\)-subunit phosphorylation by AKAP-associated endogenous PKA (0.2–0.3 units) and exogenous PKA (16 units) were calculated to be 23.1 and 32.1 fmol P\(_i\) incorporated per milligram of protein of BLMV, respectively (Table 2). Taking into consideration that a certain part of AKAP-associated PKA might be for phosphorylating membrane proteins other than Na\(^+-\)K\(^+\)-ATPase, the above values indicate that PKA associated with Na\(^+-\)K\(^+\)-ATPase-specific AKAP efficiently and quickly phosphorylated the \(\alpha\)-subunit in the presence of an adequate amount of cAMP. It is likely that the level of PKA-dependent protein phosphorylation is regulated by the level of cAMP rather than by the arbitrary phosphorylation efficiency of PKA once activated. Therefore, the results might suggest that ~70% (23.1/32.1) of the Na\(^+-\)K\(^+\)-ATPase molecules on the BLMV are associated with the PKA holoenzyme via AKAP. The Na\(^+-\)K\(^+\)-ATPase activity was downregulated by phosphorylation of the \(\alpha\)-subunit by endogenous PKA, as its activity was decreased to 80% by the action of endogenous PKA (Fig. 11). Bertorello et al. (3) reported that maximally 40% of the Na\(^+-\)K\(^+\)-ATPase activity was inhibited when it was phosphorylated in vitro in the presence of an excess amount of the PKA catalytic subunit exogenously added. Our result of 20% inhibition of the Na\(^+-\)K\(^+\)-ATPase activity by endogenous PKA, where ~70% of the \(\alpha\)-subunit was estimated to be phosphorylated, might be also compatible with the result of 40% inhibition by Bertorello et al. (3).

The results obtained in the present study indicated that, on the addition of cAMP, the \(\alpha\)-subunit of Na\(^+-\)K\(^+\)-ATPase in BLMV was quickly and selectively phosphorylated by the membrane-anchored endogenous PKA with a high efficiency, resulting in the inhibition of its activity. The presence of a functional AKAP subtype, AKAP-150, in BLMVs was also demonstrated. By the removal of cAMP, the free catalytic subunit of PKA reversibly became anchored to the BLMV via binding with the regulatory subunit/AKAP complex. PKI blocked the Na\(^+-\)K\(^+\)-ATPase phosphorylation by inhibiting this process of PKA anchoring to the membrane, even though it was not contained in the phosphorylation reaction mixture. Inhibition of RII association with AKAP by the HT-31 peptide also resulted in a decrease in membrane-bound PKA-dependent phosphorylation of Na\(^+-\)K\(^+\)-ATPase. Thus Na\(^+-\)K\(^+\)-ATPase might be downregulated in vivo by signaling molecules that employ cAMP as an intracellular messenger via the action of PKA anchored on the membrane by AKAP rather than by the free cytosolic ones with a broad substrate specificity.

The functional relevance of the interaction between Na\(^+-\)K\(^+\)-ATPase and the AKAP-150 subtype is of great interest and should be further elucidated because Na\(^+-\)K\(^+\)-ATPase is an essential component in maintaining...
homeostasis of epithelial fluid and electrolyte secretion, as well as in generating membrane potential in excitable tissues such as neurons, and because AKAP-150 is reported to be abundant in Purkinje cells and in neurons of olfactory bulb, basal ganglia, cerebral cortex, and other forebrain regions (19).

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