Stimulus-induced Phosphorylation of Vacuolar H\textsuperscript{+}-ATPase by Protein Kinase A*

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Eukaryotic vacuolar-type H\textsuperscript{+}-ATPases (V-ATPases) are regulated by the reversible disassembly of the active V\textsubscript{1}V\textsubscript{0} holoenzyme into a cytosolic V\textsubscript{1} complex and a membrane-bound V\textsubscript{0} complex. The signaling cascades that trigger these events in response to changing cellular conditions are largely unknown. We report that the V\textsubscript{1} subunit C of the tobacco hornworm Manduca sexta interacts with protein kinase A and is the only V-ATPase subunit that is phosphorylated by protein kinase A. Subunit C can be phosphorylated as single polypeptide as well as a part of the V\textsubscript{1} complex but not as a part of the V\textsubscript{1}V\textsubscript{0} holoenzyme. Both the phosphorylated and the unphosphorylated form of subunit C are able to reassociate with the V\textsubscript{1} complex from which subunit C had been removed before. Using salivary glands of the blowfly Calliphora vicina in which V-ATPase reassembly and activity is regulated by the neurotransmitter serotonin, we show that the membrane-permeable cAMP analog 8-(4-chlorophenylthio)adenosine-3',5'-cyclic monophosphate (8-CPT-cAMP) causes phosphorylation of subunit C in a tissue homogenate and that phosphorylation is reduced by incubation with antibodies against subunit C. Similarly, incubation of intact salivary glands with 8-CPT-cAMP or serotonin leads to the phosphorylation of subunit C, but this is abolished by H-89, an inhibitor of protein kinase A. These data suggest that subunit C binds to and serves as a substrate for protein kinase A and that this phosphorylation may be a regulatory switch for the formation of the active V\textsubscript{1}V\textsubscript{0} holoenzyme.

Vacuolar type H\textsuperscript{+}-ATPases (V-ATPases)\textsuperscript{3} are the most versatile proton pumps, being common to all eukaryotic organisms, and are found in endomembrane systems and in the plasma membrane (1–3). V-ATPases are multi-subunit transporters composed of a catalytic ATP-hydrolyzing V\textsubscript{1} complex (~550 kDa), which resides on the cytoplasmic side of the membrane, and a membrane-bound proton-translocating V\textsubscript{0} complex (~250 kDa). V-ATPase-dependent proton pumping is essential for cellular pH homeostasis and creates an electrochemical proton gradient that energizes secondary transport mechanisms in a wide variety of organelles and membrane systems. Acidification of organelles by V-ATPase activity is crucial to several cellular processes such as neurotransmitter uptake into synaptic vesicles, intracellular protein trafficking, and the secretion and activation of lysosomal enzymes for protein processing and degradation (4–7). Located in the plasma membrane of specialized cells, V-ATPases are involved in processes such as cation secretion, bone resorption, renal acidification, and osmoregulation (8–16). With respect to this diversity of functions, mutations in genes encoding V-ATPase subunits obviously lead to several diseases, e.g. osteopetrosis (17) or renal tubular acidosis (18).

Several mechanisms have been proposed for the regulation of V-ATPase activity (3). The most prominent and physiologically relevant mechanism is the reversible disassembly of the V-ATPase holoenzyme into its V\textsubscript{1} and V\textsubscript{0} complexes as discovered in the midgut of the tobacco hornworm Manduca sexta and in the yeast Saccharomyces cerevisiae (19, 20). In both systems, a nutrient drop induced by glucose deprivation in yeast or the cessation of feeding because of molting or starvation in the insect lead to the reversible disassembly of the functional V-ATPase holoenzyme into its inactive V\textsubscript{1} and V\textsubscript{0} complexes (21, 22). Although more recent additional examples support the notion that the reversible disassembly/reassembly is a widely used mechanism for the regulation of V-ATPase activity (23–25), the signaling cascades that trigger the association/dissociation process remain elusive.

The salivary glands of the blowfly Calliphora vicina have served as a model system for the analysis of the regulation of V-ATPase reassembly and activation. V-ATPase activity in these tubiform glands is under the control of the hormone serotonin (5-hydroxytryptamine, 5-HT). In the presence of 5-HT, the V\textsubscript{1} complex reallocates within minutes to the apical membrane and reassociates with the V\textsubscript{0} complex, thus leading to the active holoenzyme (23). Recent results demonstrate that a 5-HT-induced increase in intracellular cAMP induces V-ATPase reassembly (26) and that protein kinase A (PKA) is the downstream target of cAMP in this scenario.\textsuperscript{4} These results lead to the assumption that one or more subunits

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\textsuperscript{3} The abbreviations used are: V-ATPase, vacuolar H\textsuperscript{+}-ATPase; V\textsubscript{1}, V\textsubscript{0}, complex saturated with subunit C; 5-HT, 5-hydroxytryptamine; PKA, protein kinase A; 8-CPT-cAMP, 8-(4-chlorophenylthio)adenosine-3',5'-cyclic monophosphate; GABA, \gamma-amino butyric acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

\textsuperscript{4} J. Rein, M. Voss, B. Walz, and O. Baumann, unpublished results.
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of the V-ATPase become phosphorylated via PKA and that this phosphorylation may act as a trigger for holoenzyme formation in the blowfly salivary gland and possibly other systems.

Regarding the reversible assembly/disassembly, the V₁ subunit C is unique among V-ATPase subunits because it is released to the cytosol upon dissociation of the holoenzyme into its V₁ and V₀ complexes (21, 27). Subunit C is an elongated molecule (28) that appears to bridge the V₁ with the V₀ complex (29). Moreover, subunit C binds to actin filaments, and this interaction may be involved in stabilizing the proton pump in its assembled state (30, 31). These properties make subunit C suited to the control of V-ATPase reassembly state and to the mediation of the relevant cellular signals. Here we test the above-mentioned hypothesis and demonstrate that V-ATPase subunit C becomes phosphorylated by PKA.

EXPERIMENTAL PROCEDURES

Animals and Preparation—M. sexta (Lepidoptera, Sphingidae) was reared at 27 °C under long day conditions (16 h light) at the University of Osnabrück. Blowflies (C. vicina) were reared at 25 °C under a light-dark cycle of 12 h:12 h at the University of Potsdam. At 1–3 weeks after the eclosion of the flies, the abdominal portions of their salivary glands were dissected in physiological saline containing 128 mM NaCl, 2 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 2.7 mM sodium glutamate, 2.7 mM malic acid, 10 mM D-glucose, and 10 mM Tris-HCl, pH 7.2.

Reagents—ProQ Diamond phosphoprotein stain was obtained from Invitrogen, 8-(4-chorophenylthio)adenosine-3',5'-cyclic monophosphate (8-CPT-cAMP) was from Biolog LSI (Bremen, Germany), and H-89 was from Axxora (Grünberg, Germany). 5-HT, protease inhibitor cocktail (catalog no. P8340) and the catalytic subunit of bovine PKA were purchased from Sigma. [γ⁻³²P]ATP was from GE Healthcare (Munich, Germany).

Antibodies—Monospecific polyclonal antibodies directed against the recombinant subunit C from M. sexta were produced in guinea pigs (serum 488–1; Ref. 27). On Western blots of blowfly salivary gland homogenates, these antibodies identified a single intense band at 42 kDa (Figs. 7 and 8) corresponding approximately to the molecular mass of Manduca subunit C (27). Guinea pig polyclonal antibodies against γ-amino butyric acid (GABA) were from Biotrend (Cologne, Germany), rabbit antiserum against the Drosophila PKA catalytic subunit was from Daniel Kalderon (Columbia University, New York, NY), mouse antibody E7 against β-tubulin (32) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA), alkaline phosphatase-conjugated anti-guinea pig antibodies at a dilution of 1:30,000 in 1% gelatin/TNNT buffer. Another membrane without incubation with recombinant subunit C was used as a negative control.

Phosphorylation Assays—For experiments with [³²P]ATP, 6 μM subunit C was incubated with 1.4 μM PKA catalytic subunit in the presence of 5 μCi of [γ⁻³²P]ATP in 50 μl of PKA buffer (20 mM Na-Hepes, pH 7.5, 0.1 mM NaCl, 4 mM MgCl₂, 10 mM dithiothreitol, and 2 mM ATP) for 3 h at 30 °C. The reaction was stopped by adding 0.25 volumes 5 × SDS sample buffer (625 mM Tris-HCl, pH 6.8, 25% sucrose, 10% SDS, 0.025% bromphenol blue, and 10% β-mercaptoethanol). After SDS-PAGE (17.4% total acrylamide concentration [T], 0.4% cross-linker concentration [C]) and Coomassie Blue staining, the gel was exposed to a phosphoscreen and finally analyzed by a phosphorimaging device (Molecular Dynamics, Sunnyvale, CA).

In nonradioactive experiments, phosphoproteins were detected by the Pro-Q Diamond phosphoprotein stain. First, 8 μM V₁ complex from M. sexta was incubated with or without 0.7 μM PKA catalytic subunit in PKA buffer for 3 h at 30 °C. To separate the PKA catalytic subunit from the V₁ complex, the samples were loaded onto a discontinuous sucrose gradient (34) in 16 mM Tris-HCl, pH 8.1, 0.32 mM EDTA, 0.2 mM NaCl, 9.6 mM β-mercaptoethanol and centrifuged at 4 °C for 1.5 h at 310,000 × g. The fractions containing the V₁ complex without the PKA catalytic subunit were collected and concentrated by precipitation with 20% trichloroacetic acid. The pellets were resuspended, heated in SDS sample buffer for 45 s at 95 °C, and loaded onto a gel. To control the phosphorylation reaction, 6 μM recombinant subunit C from M. sexta was incubated with or without 0.7 μM PKA catalytic subunit for 1 h at 30 °C. After SDS-PAGE as above, the gels were analyzed by Pro-Q Diamond phosphoprotein stain and afterward by Coomassie Blue staining.

To check whether subunit C can be phosphorylated after its reassociation with the V₁ complex, the V₁ complex without...
subunit C was incubated with a 10-fold excess of recombinant subunit C for 12 h at 4 °C. V1 complex saturated with subunit C (V1:C) was collected by gel chromatography on a Superdex 200 column. Then 5 μM V1:C complex was incubated with 0.5 μM catalytic subunit of PKA for 2 h at 30 °C and afterward subjected to SDS-PAGE. The gels were stained with Pro-Q Diamond to detect phosphoproteins and afterward with Coomassie Blue. Isolated blowfly salivary glands were homogenized on ice in sample buffer A (20 mM Tris-HCl, pH 7.8, 0.1 mM KCl, 10 mM MgCl2, 10 mM Na2ATP, 5 mM EGTA, 1% Triton X-100), supplemented with protease inhibitor cocktail according to the manufacturer’s instructions and centrifuged for 15 min at 20,000 × g at 4 °C to remove cell debris. The resulting supernatant was divided into pools of equal volumes, supplemented with the respective test reagent(s) (50 μM 8-CPT-cAMP, antibodies), and incubated for 15 or 30 min at 37 °C. The preparations were mixed with sample buffer B (250 mM Tris-HCl, pH 6.8, 5% SDS, 10% β-mercaptoethanol, 10% glycerol) to give a final SDS concentration of 1% and were heated for 5 min at 70 °C. After SDS-PAGE (14.4% T, 0.4% C), phosphoproteins were stained with ProQ Diamond and imaged. Subsequently, the proteins were stained with Coomassie Blue.

**Two-dimensional Electrophoresis**—Two-dimensional electrophoresis was carried out with Mini-PROTEAN 2D and Mini-PROTEAN II cell from Bio-Rad. Isoelectric focusing was performed at 20 °C in denaturing 5% polyacrylamide tube gels (8 M urea, 2% Triton X-100, 2% Servalyt 3/10) with a pH 3–10 gradient. Before the loading of samples, the pH gradient was established by pre-electrophoresis: for 10 min at 200 V, 15 min at 300 V, and 15 min at 400 V. Isolated blowfly salivary glands were incubated at room temperature for 5 min with the respective test reagent(s) (30 mM 5-HT, 100 μM 8-CPT-cAMP, and 50 μM H-89) diluted in physiological saline; a control group was bathed in physiological saline only. Subsequently, the glands were homogenized on ice in sample buffer A and centrifuged for 30 min at 120,000 × g at 4 °C. The supernatants were mixed with sample buffer B to give a final SDS concentration of 1.4% and heated for 5 min at 70 °C. For the alkylation of cysteine residues, iodoacetamide was added to give a final concentration of 6%, and the solution was incubated for 45 min at 30 °C. The samples were diluted in sample buffer C (8 M urea, 2% CHAPS, 2% Servalyt 3/10, 0.0025% bromphenol blue) to give a final SDS concentration of 0.24% to substitute SDS by CHAPS. Proteins (from an equivalent of 5 glands/assay) were separated by the following voltage profile: 10 min at 500 V, 3.5 h at 750 V, and 1 h at 1000 V. Tube gels were rinsed in equilibration buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 0.0025% bromphenol blue) just before SDS-PAGE (10.3% T, 0.3% C). The proteins were electrotransferred onto polyvinylidene difluoride membranes and probed by Western blot analysis as described previously (23).

**RESULTS**

**Interaction of the V-ATPase Subunit C with the Catalytic Subunit of Protein Kinase A**—To test our assumption that subunit C can be phosphorylated by PKA, we initially chose the V-ATPase from midgut plasma membranes of the tobacco hornworm, *M. sexta*, a useful model system for the investigation of this family of proteins. First, we checked whether a direct interaction of subunit C with the catalytic subunit of PKA could be detected by overlay blotting. We used the catalytic subunit of bovine heart PKA (with more than 80% amino acid sequence identity with the respective *Drosophila* enzyme) as an appropriate substitute for the insect PKA. After loading the PKA catalytic subunit onto nitrocellulose and incubation with or without recombinant V-ATPase subunit C from *M. sexta*, strong immuno-signals developed on the membrane incubated with subunit C as compared with the control membrane (Fig. 1). Interaction of the PKA catalytic subunit with the *M. sexta* subunit C was also confirmed by overlay blots after SDS-PAGE (not depicted). These results support the conclusion that the V-ATPase subunit C binds to the catalytic subunit of PKA and thus might also be a physiologically relevant substrate for phosphorylation.

**Phosphorylation of Subunit C by Protein Kinase A**—Phosphorylation was investigated by incubation of subunit C with the catalytic subunit of PKA in the presence of [γ-32P]ATP. Fig. 2 demonstrates that, after SDS-PAGE and phosphorimaging analysis, subunit C is labeled with 32P in a PKA-dependent manner. The upper band at ~42 kDa represents subunit C with a molecular mass of 44 kDa as deduced from the cDNA sequence, whereas the lower band corresponds to the PKA catalytic subunit, which has a molecular mass of about 40 kDa and is known to become autophosphorylated (36).

Phosphorylation of subunit C does not exclude the possibility that other V-ATPase subunits are also phosphorylated, especially in view of a recent report that a WNK (with no K (lysine)) kinase from *Arabidopsis* phosphorylates not only subunit C but also the V1 subunits A, G, and either B or H (37). Therefore, we tested whether other subunits of the V1 complex could also be phosphorylated by PKA. After incubation of the

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**Phosphorylation of V-ATPase Subunit C by PKA**

| PKA-C [μg] | + subunit C | - subunit C |
|------------|------------|------------|
| 1.5        |            |            |
| 6          |            |            |

![Figure 1. Interaction of the V-ATPase subunit C with the catalytic subunit of PKA.](image1)

The 6 μg recombinant subunit C from *M. sexta* was incubated with 1.4 μg PKA catalytic subunit (PKA-C) in the presence of [γ-32P]ATP. After SDS-PAGE and Coomassie staining, the gel was exposed to a phosphoscreen and finally analyzed by phosphorimaging. Different amounts of subunit C were loaded per lane onto the gel (denoted above the image). The arrowheads indicate the PKA catalytic subunit that becomes autophosphorylated.

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**Phosphorylation of subunit C by the catalytic subunit of PKA.** The 6 μg recombinant subunit C from *M. sexta* was incubated with 1.4 μg PKA catalytic subunit (PKA-C) in the presence of [γ-32P]ATP. After SDS-PAGE and Coomassie staining, the gel was exposed to a phosphoscreen and finally analyzed by phosphorimaging. Different amounts of subunit C were loaded per lane onto the gel (denoted above the image). The phosphorimaged gels were exposed to a phosphoscreen and finally analyzed by phosphorimaging. Different amounts of subunit C were loaded per lane onto the gel (denoted above the image). The arrowheads indicate the PKA catalytic subunit that becomes autophosphorylated.
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**FIGURE 3. Subunits of the V₁ complex are not phosphorylated by PKA.** The 8 µM purified V₁ complex from *M. sexta* was incubated with or without 0.7 µM PKA catalytic subunit (PKA-C). After centrifugation in a sucrose gradient, the fractions containing the V₁ complex were collected and subjected to SDS-PAGE. As a control for the phosphorylation reaction, the 6 µM recombinant subunit C from *M. sexta* was incubated with the PKA catalytic subunit. The gels were stained with Coomassie Blue (A) after phosphoprotein staining with ProQ Diamond (B). A molecular mass marker (M) containing two phosphorylated proteins (ovalbumin, 45 kDa; β-casein, 24 kDa) was used. The positions of the V₁ subunits are marked by capital letters (in A, the asterisk denotes a decay product of subunit B).

V₁ complex with and without the PKA catalytic subunit, sucrose density gradient centrifugation was performed, and fractions containing only the V₁ complex were analyzed by SDS-PAGE. Fig. 3 shows gels after Coomassie staining and after Pro-Q Diamond phosphoprotein staining. The latter stain has, compared with the use of radiolabeled ATP, the advantage that phosphorylated proteins can be detected without prior incubation with a protein kinase and ATP. Thus, Fig. 3 also demonstrates that the recombinant subunit C, which had been expressed in *E. coli*, is not phosphorylated *a priori* and remains nonphosphorylated after incubation with ATP in the absence of the PKA catalytic subunit (Fig. 3B). In the presence of the PKA catalytic subunit, however, a strong phospho-signal could be observed at subunit C (Fig. 3B). In the V₁ complex purified from *M. sexta*, no subunit including subunit C was found to be phosphorylated, either in the absence or in the presence of the PKA catalytic subunit (Fig. 3B). Therefore, we suggest that the V₁ complex purified from the midgut cytosol does not contain phosphorylated subunits and that subunits cannot be phosphorylated by PKA as long as they are part of the complex. This also applies to subunit C, which usually occurs in substoichiometric amounts in the isolated V₁ complex (22).

However, there could be a qualitative difference between the substoichiometric subunit C that remains bound to the V₁ complex purified from *M. sexta* midgut and the subunit C that dissociates upon enzyme disassembly. Hence we reassocated the recombinant subunit C with the V₁ complex from which we had removed before all of subunit C. The resultant V₁C complex was then incubated with the catalytic subunit of PKA. Indeed, subunit C in this complex could be phosphorylated by PKA (Fig. 4), thus confirming our suspicion. Thus, the free cytosolic subunit C as well as subunit C reassocitated with the V₁ complex both appear to be competent for phosphorylation by PKA.

**FIGURE 4. Phosphorylation of subunit C after its reassociation with the V₁ complex.** After incubation of the subunit C free V₁ complex (V₁) with recombinant subunit C, the V₁ complex containing subunit C (V₁C) was collected by gel chromatography. Then 5 µM V₁C complex was incubated with 0.5 µM catalytic subunit of PKA (PKA-C) and afterward subjected to SDS-PAGE. The gel was stained with Coomassie Blue (A) after staining phosphoproteins with Pro-Q Diamond (B). The molecular mass marker (S) contained, in contrast to the marker M in Fig. 3, only one phosphorylated protein (ovalbumin, 45 kDa).

After dissociation of the V₁V₀ holoenzyme, the majority of subunit C occurs freely in the cytosol (30). Therefore, it might be expected that the activation of PKA leads predominantly to the phosphorylation of the free subunit C, which then would have to bind to the V₁ complex in the phosphorylated state. To examine whether phosphorylated subunit C has this ability, we incubated the V₁ complex with either phosphorylated or unphosphorylated subunit C. Already after 30 min of incubation a significant amount of subunit C was found to be part of the V₁ complex, and no difference could be observed between phosphorylated and unphosphorylated subunits C (Fig. 5).

Next we checked whether the V₁V₀ holoenzyme purified from *M. sexta* midgut contained phosphorylated subunits. As Fig. 6 (lane 2) demonstrates, this appeared not to be the case. Incubation of the holoenzyme with the catalytic subunit of PKA led to a phosphorylated band with a molecular mass similar to that of subunit C (Fig. 6B, lane 1). However, after removal of PKA by gradient centrifugation it became clear that the catalytic subunit of PKA, but not subunit C was phosphorylated. Thus, subunit C in the holoenzyme cannot be phosphorylated by PKA.

**FIGURE 5. Reassociation of recombinant subunit C with the V₁ complex is independent of its phosphorylation state.** The 3 µM V₁ complex without subunit C (V₁) was incubated with 25 µM phosphorylated (lane C-P) or unphosphorylated subunit C (lane C), respectively, for 30 min at 4 °C. Unbound subunit C was removed by centrifugation in a sucrose gradient, and after SDS-PAGE, the fractions containing reassoicated V₁C complexes were analyzed by Pro-Q Diamond phosphoprotein staining (B) and finally by Coomassie staining (A). Lane S, molecular mass marker as in Fig. 4. V₁C, V₁ complex reassoicated with phosphorylated subunit C; V₁C, V₁ complex reassoicated with unphosphorylated subunit C.

**PKA-dependent Phosphorylations in Calliphora Salivary Glands—** To examine whether V-ATPase subunit C becomes phosphorylated by PKA within intact cells, we used salivary glands from adult Calliphora erythrocephala. As shown in Fig. 7A, the subunit C from the V-ATPase holoenzyme isolated from salivary glands was phosphorylated in vitro by PKA, as indicated by the Pro-Q Diamond phosphoprotein staining (B)

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glands of the blowfly C. vicina. In this system, V-ATPase assemblies and becomes activated within minutes after exposure to 5-HT (10, 23), and the cAMP/PKA signaling cascade mediates the effect of 5-HT on the V-ATPase (26).4 Experiments with entire glands and subsequent SDS-PAGE and Pro-Q Diamond staining of phosphoproteins indicated that stimulation with 5-HT or the membrane-permeable cAMP analog 8-CPT-cAMP led to a slight increase in phospho-signal at 42 kDa, a position corresponding to V-ATPase subunit C (data not depicted). Based on the results above that free subunit C but not subunit C in the holoenzyme became phosphorylated by PKA, we hypothesized that this 42-kDa phospho-signal should become more intense by using a cellular fraction with an increased amount of free subunit C. Therefore, glands were homogenized on ice and centrifuged, and the resulting supernatant was divided into two aliquots. One sample was supplemented with 50 μM 8-CPT-cAMP, whereas the other served as a control. Fig. 7 shows that incubation with 8-CPT-cAMP for 30 min at 37 °C caused a dramatic increase in phosphoprotein signal in the molecular mass region of 42 kDa. However, although this result is in line with our assumption that V-ATPase subunit C can be phosphorylated by PKA in vivo, we cannot exclude, at this point, the possibility that the prominent phospho-signal at 42 kDa results from other proteins.

**PKA-dependent Phosphorylation of Subunit C in Calliphora Salivary Glands**—To determine whether the phospho-signal at 42 kDa represents phosphorylated V-ATPase subunit C, we tested the effect of antibodies against subunit C on the emergence of the phospho-signal. As shown in Fig. 8, 8-CPT-cAMP led to a strong phospho-signal at 42 kDa. Preincubation of the samples with the antibodies largely abolished the 8-CPT-cAMP-induced increase in the 42-kDa phospho-signal. Other phospho-signals that increased in an 8-CPT-cAMP-dependent manner, however, were unaffected by the antibodies, demonstrating the specificity of the assay. As a further control, the assay was performed in the presence of polyclonal antibodies against an unrelated antigen, viz, GABA. Under these conditions, the 8-CPT-cAMP-induced increase in the 42-kDa phospho-signal was as intense as in the absence of antibodies. We thus conclude that the 42-kDa band that becomes phosphorylated in a PKA-dependent manner represents the V-ATPase subunit C.

Two-dimensional electrophoresis was undertaken as an alternative method to probe the phosphorylation of subunit C by PKA in intact salivary glands of C. vicina. Under control conditions as shown in Fig. 9A, an anti-C immuno-signal in the 42-kDa range was detected after two-dimensional electrophoresis. The signal ran from the edge of the gel near the application point of pH 10 down to a slightly lower pH. We assume that this signal resulted from subunit C, which, on the one
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![Diagram](image)

**FIGURE 9.** Detection of modified subunit C by two-dimensional electrophoresis. Salivary glands were incubated without (control) or with 30 nM 5-HT, 100 μM 8-CPT-cAMP, and 50 μM of the PKA inhibitor H-89, as indicated; subsequently glands were homogenized, centrifuged and subjected to two-dimensional electrophoresis. The molecular mass region between 40 and 46 kDa was analyzed by immunoblotting with anti-subunit C. A, nonstimulated glands (control) were incubated with or without H-89 for 5 min; separated proteins are denoted by arrowheads. B and C, salivary glands were incubated with 5-HT or 8-CPT-cAMP for 5 min, and subsequently, stimulated glands were incubated for 5 min with or without 8-CPT-cAMP in the presence of 5-HT or 8-CPT-cAMP. D, salivary glands were incubated with 30 nM 5-HT for 5 min and then washed with physiological saline for 10 min. E, Western blot, demonstrating that the total amount of subunit C does not change in response to 8-CPT-cAMP treatment with H-89 prevented the development of these signals in both cases. Fig. 9E demonstrates that the stimulus-induced differences in immuno-signal were not due to or accompanied by changes in the total amount of subunit C within the glands. These results corroborate our hypothesis that a 5-HT stimulus, followed by an increase in cAMP concentration, leads to a PKA-dependent phosphorylation of the V-ATPase subunit C in C. vicina salivary glands and suggest further that subunit C has more than one phosphorylation site. Finally, phosphorylation of subunit C is a reversible process because the additional immuno-signals disappeared after washout of 5-HT (Fig. 9D).

**DISCUSSION**

This is the first demonstration that the V-ATPase subunit C binds the PKA catalytic subunit and is a substrate for this protein kinase. Remarkably, the phosphorylation of subunit C in salivary glands of C. vicina correlates with the hormone-induced reassembly and activation of the V-ATPase in the apical membrane of the secretory epithelial cells. We therefore suggest that phosphorylation of subunit C is not only an initial event in the association process of the V-ATPase complexes but also a key regulatory mechanism of V-ATPase activity.

Vacuolar H⁺-ATPases are evolutionary related to ATP-synthases (F-ATPases) found in bacteria, mitochondria, and chloroplasts (39). The overall structure of V- and F-ATPases is characterized by a peripheral ATP-binding complex and a proton-translocating integral membrane complex (2, 40–42). Both the ATP-binding subunits and the proteolipid subunits display a high sequence homology between V- and F-ATPases. Therefore, a function can be assigned to V-ATPase subunits by homology to those of the F-ATPase. The V-ATPase subunit C, however, is exceptional because it has no equivalent among the F-ATPases (41). Thus, subunit C may lend properties to V-ATPases distinguishing them from F-ATPases. Aside from its actin binding properties (30, 31), subunit C may be a likely candidate for the control of V-ATPase disassembly/reassembly, a mode of regulation unique to V-ATPases (43). The position of subunit C in the V-ATPase holoenzyme, close to subunit a of the V0 complex and to subunits E and G of the V1 complex (29, 44), make it ideally suited to regulate interactions between the V1 and V0 complexes and thus to control the formation of active V-ATPase holoenzymes. This suggestion is strengthened by the finding that disassembly of the V1V0 holoenzyme appears to coincide with the dissociation of subunit C from the V1 complex (21, 27).

Here, we demonstrate, for the first time, that subunit C of the V-ATPase is a target for the cAMP/PKA messenger system. This intracellular signaling cascade has been shown to control V-ATPase activity in several systems (45–49) including C. vicina salivary glands (26).

Using recombinant V-ATPase subunit C from M. sexta and the bovine PKA catalytic subunit, we have shown that these proteins can interact physically and that subunit C is phosphorylated by PKA. Because of the high amino acid sequence identity of mammalian and insect PKA catalytic subunits, our findings imply that the PKA-dependent phosphorylation of subunit C is not an artifact but reflects a real property of the M. sexta
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FIGURE 10. Distribution of subunit C and PKA catalytic subunit (PKA-C) in control and 5-HT-treated (30 nm, 5 min) blowfly salivary glands. Cross-sections through salivary glands were triple-labeled with antibodies against actin (blue), subunit C (green), and PKA-C (red) by procedures as described previously (26). Labeling for actin reveals infoldings of the apical membrane (arrowheads). Asterisks indicate the lumen of the gland. Bar, 25 μm.

subunit C. Binding of the PKA catalytic subunit to subunit C may also occur in C. vicina salivary glands, as indicated by the parallel redistribution of these proteins upon cell stimulation (Fig. 10). In nonstimulated secretory inactive cells, subunit C and PKA are distributed in the cytoplasm, whereas the major amount of V-ATPase protein is dissociated into V$_i$ and V$_o$ complexes (26). Following a 5-HT stimulus, V-ATPase subunit C and the PKA catalytic subunit both become enriched at the highly enfolded apical membrane. Binding of these proteins to each other leads to subunit C phosphorylation, as demonstrated by our assays with M. sexta subunit C and the bovine PKA catalytic subunit. Similarly, incubation of intact salivary glands with the cAMP analog 8-CPT-cAMP or the hormone 5-HT leads to a PKA-dependent increase in subunit C phosphorylation.

Two-dimensional electrophoresis of Calliphora salivary glands has revealed three different spots on the membrane for subunit C, possibly representing nonphosphorylated, mono-phosphorylated and bi- or oligophosphorylated states of this protein. Subunit C may thus have at least two phosphorylation sites for PKA. Likewise, the WNK kinase can phosphorylate Arabidopsis subunit C at multiple sites in vitro (37). The identity of PKA phosphorylation sites in subunit C is as yet unknown. By using bioinformatic algorithms (KinasePhos, NetPhosK, GPS), several putative phosphorylation sites for PKA can be identified in subunit C of M. sexta, D. melanogaster and other organisms, but the results vary dependent on the program used.

What is the physiological consequence of subunit C phosphorylation? In nonstimulated glands, phosphorylated subunit C is present in negligible amounts. Similarly, only a minor fraction of V-ATPase molecules is assembled and active under these conditions (10, 23, 26). Upon exposure to saturating concentrations of 5-HT or 8-CPT-cAMP (10, 50), a fraction of subunit C becomes phosphorylated via PKA. Likewise, saturating 5-HT levels induce a reassembly of the majority of V$_i$ and V$_o$ complexes to the active V$_i$V$_o$ holoenzyme (23, 26). Moreover, subunit C becomes phosphorylated within minutes after exposure to 8-CPT-cAMP or 5-HT and thus within a similar time scale as that for the PKA-dependent reassembly and activation of the V-ATPase on the apical membrane (10, 26). Finally, PKA activity is absolutely required for 5-HT-dependent regulation of V-ATPase holoenzyme reassembly. Because our data demonstrate that subunit C is the only V-ATPase component that becomes phosphorylated by PKA and phosphorylation can only occur on subunit C that is free or bound to V$_i$ complex, it may be concluded that this phosphorylation of subunit C is an initial and essential event in V$_i$V$_o$ holoenzyme reassembly.

In this scenario, the following model seems possible. Upon hormonally induced activation of PKA via cAMP, PKA phosphorylates subunit C, most of which is found occurring freely in the cytosol. Subunit C, V$_i$ complex, and the catalytic subunit of PKA then become enriched on the apical membrane. This process may be mediated by the binding capability of subunit C as well as subunit B in the V$_i$ complex to actin (30, 31, 51), and by a stimulus-induced reorganization of the actin filament system, as shown for Malpighian tubules of the yellow fever mosquito A. aegypti (52). The resulting enhanced concentration of the V$_i$ complex and of subunit C near the apical membrane increases the probability of their physical interaction and, in addition, the interaction with the membrane-bound V$_o$ complex. We suggest that the phosphorylation of subunit C is an important step that facilitates the reassociation of the reaction partners to an active V$_i$V$_o$ holoenzyme. Upon reassembly, subunit C becomes dephosphorylated by an as yet unidentified phosphatase within the secretory cells. Our finding that C subunit in the holoenzyme is dephosphorylated suggests that dephosphorylation does not directly lead to V-ATPase disassembly. The signals for disassembly and inactivation of the V-ATPase remain enigmatic. The redistribution of the PKA catalytic subunit together with its substrate subunit C to the apical membrane (Fig. 10) may fasten C subunit phosphorylation and V-ATPase reassembly and thus support the maintenance of a large number of V-ATPase molecules in the holoenzyme state as long as the 5-HT stimulus persists.

Although the reversible disassembly/reassembly is a widespread mode of V-ATPase regulation, we do not know whether phosphorylation by PKA is a universal property of subunit C, especially if we take into consideration that subunit C is not a highly conserved V-ATPase subunit. Although the amino acid sequence identities within insects are 80% and more, the M. sexta subunit C is clearly less similar to mammalian subunits C and is only 35% identical to the yeast protein. Because we have

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5. M. Voss, R. Schmidt, B. Walz, and O. Baumann, unpublished results.
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no information about the sites of PKA-dependent phosphorylation, we cannot determine whether these residues are conserved among subunit C orthologs. Last but not least, because subunit C has been shown to occur in a tissue-specific mode in various isoforms (53), we are left with the possibility that subunit C heterogeneity between organisms, and even within one organism, contributes to differences in V-ATPase regulation, either by the involvement of different PKA isoforms or even by other protein kinases.

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