Hormone-induced assembly and activation of V-ATPase in blowfly salivary glands is mediated by protein kinase A

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Rein J, Voss M, Blenau W, Walz B, Baumann O. Hormone-induced assembly and activation of V-ATPase in blowfly salivary glands is mediated by protein kinase A. Am J Physiol Cell Physiol 294: C56–C65, 2008. First published October 31, 2007; doi:10.1152/ajpcell.00041.2007.—The vacuolar H\(^{+}\)-ATPase (V-ATPase) in the apical membrane of blowfly (Calliphora vicina) salivary gland cells energizes the secretion of a KCl-rich saliva in response to the neurohormone serotonin (5-HT). We have shown previously that exposure to 5-HT induces a cAMP-mediated reversible assembly of V\(_{0}\) and V\(_{1}\) subcomplexes to V-ATPase holoenzymes and increases V-ATPase-driven proton transport. Here, we analyze whether the effect of cAMP on V-ATPase is mediated by protein kinase A (PKA) or exchange protein directly activated by cAMP (Epac), the cAMP target proteins that are present within the salivary glands. Immunofluorescence microscopy shows that PKA activators, but not Epac activators, induce the translocation of V\(_{1}\) components from the cytoplasm to the apical membrane, indicative of an assembly of V-ATPase holoenzymes. Measurements of transepithelial voltage changes and microfluorometric pH measurements at the luminal surface of cells in isolated glands demonstrate further that PKA-activating cAMP analogs increase cation transport to the gland lumen and induce a V-ATPase-dependent luminal acidification, whereas activators of Epac do not. Inhibitors of PKA block the 5-HT-induced V\(_{1}\) translocation to the apical membrane and the increase in proton transport. We conclude that cAMP exerts its effects on V-ATPase via PKA.

vacuolar H\(^{+}\)-adenosine 5\(^{\prime}\)-triphosphatase; adenosine 3,5\(^{\prime}\)-cyclic monophosphate; exchange protein directly activated by adenosine 3,5\(^{\prime}\)-cyclic monophosphate; insect

VACUOLAR H\(^{+}\)-ATPASES (V-ATPases) are a family of proton pumps that are present in almost every eukaryotic cell. These large heteromeric protein complexes consist of two multisubunit domains: a cytosolic V\(_{1}\) subcomplex that hydrolyzes ATP, and a membrane-bound V\(_{0}\) subcomplex that transports protons across the membrane (6, 40, 60). Situated on intracellular compartments, V-ATPases acidify early and late endosomes, lysosomes, and Golgi-derived secretory vesicles, providing the driving force and optimal pH for the internalization and degradation (41, 56). V-ATPases have also been identified in the plasma membrane of numerous cell types, contributing to pH homeostasis or extracellular acidification or alkalinization (8, 9, 33). Another important function of V-ATPases, especially in the plasma membrane of epithelial cells, is to energize the membrane for secondary transport mechanisms (68).

A variety of external factors have been reported to modulate and control V-ATPase-mediated proton transport. These include the closely related parameters pH and P\(_{CO}_{2}\), glucose, hormones, and developmental processes (24, 25, 31, 37, 66). One prominent mechanism of V-ATPase regulation is the reversible dissociation of the V-ATPase holoenzyme into its V\(_{0}\) and V\(_{1}\) domains (30, 55, 62, 64, 73). In the dissociated state, V-ATPase is inactive, because the V\(_{0}\) and V\(_{1}\) subcomplexes are unable to transport protons or to hydrolyze ATP (26, 31, 70). However, the intracellular signaling mechanisms that induce V-ATPase assembly/disassembly upon external stimuli remain elusive.

Salivary glands of the blowfly C. vicina provide a model for studying the regulation of V-ATPase by reversible assembly. These paired glands are thin tubules that extend into the abdomen of the animal (47). Their secretory segment consists of uniformly differentiated epithelial cells with an enormously enlarged apical membrane that is densely studded with V-ATPase molecules (73). Within minutes after exposure to the hormone serotonin (5-HT), V\(_{1}\) complexes are recruited from the cytoplasm to the apical membrane, followed by the assembly of V-ATPase holoenzymes, leading to an increase in proton transport across the apical membrane and a luminal acidification (14, 52, 73). The activity of this electrogenic pump establishes an electrochemical proton gradient across the apical membrane; this gradient, in turn, is thought to energize the transport of K\(^{+}\) into the lumen of the gland via a parallel nH\(^{+}\)/K\(^{+}\) antiporter (52, 73). Simultaneous activation of a transepithelial Cl\(^{-}\) flux via Ca\(^{2+}\)-dependent Cl\(^{-}\) channels results in a KCl-rich saliva (1, 4, 5).

Upon binding to two different receptors on the basolateral surface, 5-HT induces an increase in the cytosolic Ca\(^{2+}\) concentration, via the phospholipase C/inositol trisphosphate signaling cascade, and a rise in the intracellular cAMP concentration (2, 3, 21, 72). We have shown previously that cAMP is the factor that induces the assembly and activation of V-ATPase (14). Two downstream signaling pathways should be considered responsible for the mediation of the effect of cAMP on V-ATPase: protein kinase A (PKA) and/or exchange protein directly activated by cAMP (Epac). PKA is a heterotetramer, consisting of two regulatory and two catalytic subunits that form the inactive holoenzyme. Binding of cAMP to two sites on each regulatory subunit induces a conformational change that leads to the dissociation of the two active catalytic subunits (63). The free catalytic subunits can then affect a range of cellular events by phosphorylation of protein substrates. Epac contains a single cAMP-binding domain and a
guanine exchange factor domain. Upon binding of cAMP, Epac activates small Rap proteins, which can affect downstream target proteins (16, 32).

The present study examines the involvement of PKA and Epac in the 5-HT-induced cAMP-dependent assembly and activation of V-ATPase. For this purpose, we have used not only a variety of cAMP analogs that activate either PKA or Epac, but also inhibitors of PKA. We demonstrate that PKA, but not the Epac pathway, regulates reversible V-ATPase assembly and V-ATPase activation.

MATERIALS AND METHODS

Animals and preparation. Blowflies (C. vicina) were reared at our Institute. One to four weeks after eclosion of the flies, the abdominal portions of their salivary glands were dissected in physiological saline (in mM: 128 NaCl, 10 KCl, 2 MgCl₂, 2.7 sodium glutamate, 2.7 malic acid, 10 d-glucose, and 10 Tris·HCl, pH 7.2). In some experiments, the isolated glands were superfused with chloride-free saline (in mM: 128 sodium isothionic acid, 5 K₂SO₄, 2 CaSO₄, 2.7 sodium glutamate, 2.8 malic acid, 3 sodium glutamate, 10 d-glucose, and 10 Tris, pH 7.2).

Reagents. The following primary antibodies were used: rabbit anti-subunit B of Culex quinquefasciatus V-ATPase (22), rabbit anti-subunit A and anti-subunit E of Bos taurus V-ATPase (54), guinea pig anti-subunit d of Manduca sexta V-ATPase, guinea pig anti-subunit C of M. sexta V-ATPase [both antisera provided by H. Wieczorek, University of Osnabrück, Osnabrück, Germany (38)] and mouse monoclonal anti-actin (clone C4; Chemicon, Hofheim, Germany). The cross-reactivity of these antisera with the corresponding V-ATPase subunits in blowfly has been demonstrated (65, 73). AlexaFluor488-, Cy3-, and Cy5-tagged secondary antibodies were obtained from Dianova (Hamburg, Germany) or Invitrogen (Karlsruhe, Germany). 5-N-hexadecanoyl-aminofluorescein (HAF, 20 mM stock solution in DMSO) was purchased from Invitrogen, 5-HT and H-89 were from Sigma (Taufkirchen, Germany), and cAMP analogs were from Biolog Life Science Institute (Bremen, Germany). The cAMP-analogs 8-(4-methoxyphenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (pMeOPT-cAMP) and 8-(4-hydroxyphenylthio)-2',5'-cyclic monophosphate (pHTP-cAMP) were used as Epac activators; N"-mmono-tert-butylcarbamoyladenosine-3',5'-cyclic monophosphate (MBC-cAMP), N"-benzoyladenosine-3',5'-cyclic monophosphate (Bnz-cAMP), and the Sp-isomer of 5-, 6-dichloro-1-β-n-bifuroransobenzimidazole-3',5'-cyclic monophosphorothioate (cBIMPS) were used as PKA activators; 8-(4-chlorophenylthio)adenosine-3',5'-cyclic monophosphate (8-CPT-cAMP) was used as an activator of both PKA and Epac. The Rp-isomer of 8-(4-chlorophenylthio)adenosine-3',5'-cyclic monophosphorothioate (Rp-CPT-cAMP) was used as competitive inhibitor of PKA.

Cloning of C. vicina Epac fragment. Degenerate primers (sense: 5'-GTGTTATMCAYGGCAGGG-3'; antisense: 5'--GYTCTCTG-3') corresponding to the highly conserved amino acid sequences VVIHGKG and QEHGKD, respectively, of insect Epac proteins were designed to amplify blowfly Epac fragments. PCR was performed on cDNA made from poly(A)⁺ RNA purified from heads of C. vicina. Amplification was carried out for 2.5 min at 94°C (one cycle), followed by 35 cycles of 40 s at 94°C, 40 s at 45°C, 20 s at 72°C, and a final extension of 10 min at 72°C. A fragment of 211 bp was amplified and cloned into pGEM-T vector (Promega, Mannheim, Germany). Sequencing was performed by AGOWA (Berlin, Germany). The nucleotide sequence of the C. vicina Epac fragment has been submitted to the European Bioinformatics Institute database (accession no. AM902488).

RT-PCR amplification of C. vicina Epac fragments. Total RNA was isolated from heads and salivary glands of C. vicina with TRizol LS Reagent (Invitrogen). The samples were either digested with DNase I (Ambion, Huntington, United Kingdom) to degrade contaminating genomic DNA or with DNase I and RNase cocktail (Ambion) for controls. Epac-specific fragments were amplified from 200 ng total RNA by using the SuperScript One-Step RT-PCR system (Invitrogen). The sense primer was 5'-GTACTGTGGCCACCCCTCAAG-3' and the antisense primer was 5'-GTATTGTCCTAACAATCAGG-3'. Amplification was as follows: cDNA synthesis and denaturation with one cycle of 50°C for 30 min, 94°C for 2 min; PCR amplification with 35 cycles of 94°C for 40 s, 60°C for 40 s, 72°C for 20 s, and a final extension at 72°C for 10 min. Amplified products were analyzed by agarose-gel electrophoresis.

Luminal pH measurements. To monitor pH changes at the luminal surface of the secretory cells, isolated salivary glands were attached to the surface of a glass-bottomed perfusion chamber coated with Cell-Tak (BD Bioscience, San Jose, CA). The chamber was placed on a Zeiss Axioskope 135 inverted microscope, and the luminal surface of the glands was stained with 30 μM HAF in physiological saline as described previously (52). HAF fluorescence was alternately excited through a Zeiss ×20 Fluar, 0.75 numerical aperture objective at wavelengths of 410 and 470 nm provided by a VisiChrome monochromator unit (VisiTech Systems, Puchheim, Germany). The emitted light at wavelengths above 515 nm was detected with a cooled charge-coupled device camera (CoolSnap HQ; Photometrics, Tuscon, AZ). The imaging software MetaFluor 6.1 (Universal Imaging, Downingtown, PA) was used to calculate the ratio of fluorescence excited at 470 nm and 410 nm (F₄₇₀/F₄₁₀ ratio) offline. A drop in the F₄₇₀/F₄₁₀ ratio indicated an acidicification, whereas an increase in the F₄₇₀/F₄₁₀ ratio demonstrated an alkalinalization at the luminal surface (23, 52).

Immunolocalization of the V-ATPase subunits. At least 20 blowflies were dissected for every experiment. One of the pair of salivary glands of each fly was kept as a nonstimulated control. The other glands were incubated for 5 min in 30 mM 5-HT or for 10 min in the other substances. Subsequently, glands were fixed, cryosectioned, and triple labeled with guinea pig anti-subunit d, rabbit anti-subunit B, and mouse anti-actin, or double labeled with guinea pig anti-subunit C and mouse anti-actin as described previously (14). AlexaFluor488 anti-rabbit IgG, Cy3 anti-guinea pig IgG, and Cy5 anti-mouse IgG were used as secondary antibodies for triple labeling, and AlexaFluor488 anti-mouse IgG and Cy3 anti-guinea pig IgG were used for double labeling. Fluorescence images were recorded with a Zeiss LSM 510 confocal microscope equipped with 488-nm, 543-nm, and 633-nm laser lines. Detector gain was adjusted to include all fluorescence intensities within the dynamic range of the imaging system.

The distribution of fluorescence within the cells was analyzed with the imaging software MetaMorph (Universal Imaging) by using the anti-actin image as a reference for the extent of the apical membrane (Fig. 1). Therefore, the anti-V-ATPase image and the corresponding anti-actin image were both thresholded and binarized to provide masks for the entire gland profile (Fig. 1B) and for the apical membrane (Fig. 1E). The anti-V-ATPase image was then multiplied with the masks, and the pixel intensities in the resulting images were integrated and represented total anti-V-ATPase fluorescence (Fig. 1C) and anti-V-ATPase fluorescence at the apical membrane (Fig. 1F), respectively. Cytoplasmic fluorescence was calculated by subtracting the value for apical staining from the total staining intensity.

Other methods. Pelleting assays were performed as described previously (14, 73). Measurements of the transepithelial potential (TEP) were performed by using the oil-gap method (50). Statistical comparisons were made by Student's t-test or the Mann-Whitney test. Only differences with P < 0.05 were considered to be statistically significant.

RESULTS

Presence of PKA and Epac in the blowfly salivary gland. By using antisera against the regulatory subunit and catalytic
regulates the section, and
and anti-vacuolar (V)-ATPase staining between cytoplasm and apical membrane. The pixel intensities within these images were then integrated.

Fig. 1. Analysis of the relative distribution of anti-vacuolar (V)-ATPase staining between cytoplasm and apical membrane. A and D: anti-V-ATPase and anti-actin images recorded by confocal microscopy from a double-labeled section. B and E: the anti-V-ATPase image and the anti-actin image were thresholded and binarized to obtain masks for the entire gland (B) and for the apical membrane (E). Pixels with value 1 are shown in white, and pixels with value 0 are shown in black. C and F: each pixel value in the anti-V-ATPase image was multiplied with the value (0 or 1) of the corresponding pixel in mask A or B, respectively. Image A x mask A (C) represents total V-ATPase staining of the section, and image A x mask B (F) represents V-ATPase staining of the apical membrane. The pixel intensities within these images were then integrated.

subunit of *Drosophila* PKA, we have shown that PKA is present in blowfly salivary glands (65; Voss M, Schmidt R, Walz B, Baumann O, unpublished results). Unfortunately, there are no antibodies against *Drosophila* Epac. To determine whether blowfly salivary glands contain Epac, we amplified from blowfly head cDNA a 208-bp long fragment of Epac, except for the intracellular part of the cAMP binding site (Fig. 2A). With the exception of one conservative substitution, the deduced amino-acid sequence was identical to the corresponding region of *Drosophila* Epac. Moreover, it displayed ~70% identity and ~90% similarity to the corresponding region in rat Epac1 or Epac2, respectively. Notably, the highly conserved Glu residue in the phosphate-binding cassette of PKA is absent not only in vertebrate and *Drosophila* Epac but also in *Calliphora* Epac. It is thought that this replacement of Glu by Gln or Lys contributes to the discriminative power of Epac-selective cAMP analogs (20).

The expression of Epac was determined by RT-PCR. Fragments were amplified on RNA samples isolated from heads and salivary glands (Fig. 2B). Its is unlikely that these fragments are due to genomic DNA because the primers flank an intron that is conserved in *Drosophila melanogaster*, *Drosophila pseudoobscura*, *Anopheles gambiae*, *Aedes aegypti*, and *Tribolium castaneum*. Moreover, RNase treatment of the samples abolished the amplification of any PCR products. Finally, similar results could be obtained by using another combination of primers, resulting in a ~1.7-kb fragment (data not shown). We thus conclude that both PKA and Epac are present in the secretory cells of the blowfly salivary gland.

**Effect of Epac on V-ATPase assembly and activity.** Exposure of isolated salivary glands to 5-HT or to substances that raise the intracellular cAMP level induces a translocation of cytosolic V₁ complexes to the apical membrane and an assembly of V-ATPase holoenzymes (14, 73). To examine whether this effect could be mimicked by activation of Epac within the secretory cells, isolated salivary glands were incubated for 10 min with 100 μM pMeOPT-cAMP or 100 μM pHP-cAMP, i.e., with Epac-specific cAMP analogs (12). Nonstimulated glands and 5-HT-stimulated glands were used as controls. Subsequently, the glands were chemically fixed, and the subcellular distribution of V₀ and V₁ domains was analyzed by immunolabeling of cryosections with antibodies against V₀ subunit d and V₁ subunits B and C.

The V₀ component subunit d was highly concentrated at the apical membrane under all experimental conditions (Fig. 3, A, B, G, and H). Moreover, numerous vesicular structures were stained for subunit d, although the number of these structures varied largely between preparations. V₁ subunits B and C, in

Fig. 2. Expression of the EPAC gene in blowfly salivary glands. A: amino-acid sequence of part of the cAMP binding site of blowfly Epac [*Calliphora vicina* (C.v.) Epac]. The sequence of the corresponding site is shown for *Drosophila* Epac (D.m. Epac; accession no. NP_724498), rat Epac1 (R.n. Epac1; no. NP_067722), and rat Epac2 (R.n. Epac2; no. XP_215985). For comparison, we show the sequence of the site A and site B of *Drosophila* PKA regulatory subunit type I (D.m. R₁; no. P16905) and type II (D.m. R₂; no. P81900). Residues identical to blowfly Epac are shown as white letters against black; conservatively substituted residues are shaded. The arrow highlights the highly conserved Glu residue in the phosphate-binding cassette (PBC) of PKA that is absent in Epac. B: RT-PCR products amplified from RNA of blowfly head and salivary gland. Amplified fragments have the expected size of 163 bp. Treatment with RNase cocktail before RT-PCR was performed served as a negative control.
contrast, were homogeneously distributed in the cytoplasm of nonstimulated cells, being only slightly enriched at the apical membrane (Fig. 3, C, E, I, and K). After treatment with 5-HT, intense labeling of the apical membrane for subunit B and subunit C was observed, whereas the amount of these subunits in the cytoplasm was severely reduced (Fig. 3, D and F).

Quantitative analysis showed that 33.4 ± 4.2% (subunit B; means ± SD) and 22.8 ± 5.2% (subunit C) of the staining for V1 subunits B and C was associated with the apical membrane of nonstimulated cells and that the relative staining intensity at the apical membrane increased to 66.0 ± 10.0% and 66.2 ± 7.4%, respectively, upon exposure to 5-HT.

The distribution of staining for V0 subunit d, however, was not significantly affected by 5-HT stimulation (Fig. 3, M–O). These data compare well with the results of pelleting assays, demonstrating that the amount of membrane-associated V1 components increases in response to 5-HT by a factor of ~2 (73). Similar results were obtained with 8-CPT-cAMP, a cAMP analog that activates both PKA and Epac (12, 15). We conclude that immunofluorescence localization of V1 components represents an indirect, yet fast method for the analysis of V-ATPase assembly. Unlike 5-HT or 8-CPT-cAMP, the EPAC activators pMeOPT-cAMP (Fig. 3, J, L, Q, and R) or pHPT-cAMP (data not presented) did not induce a significant redistribution of subunits B and C and, thus, a translocation of V1 complexes to the apical membrane for assembly of V-ATPase holoenzymes.

Since a pool of V-ATPase holoenzymes exists at the apical membrane even in nonstimulated cells (14, 52, 73), Epac activation could possibly increase the activity of these pumps without inducing an assembly of additional holoenzymes. To examine this possibility, we analyzed the effect of EPAC activators on the TEP. Activation of transepithelial cation transport, as in the case of 5-HT- and cAMP-induced proton transport via V-ATPase into the lumen of the gland, leads to a positive-going change in TEP. However, neither pMeOPT-cAMP (Fig. 4A) nor pHPT-cAMP (Fig. 5A) changed the TEP, whereas the control substance 8-CPT-cAMP induced a positive phase with an amplitude comparable to that obtained with a saturating 5-HT concentration (Fig. 4B).

Enhanced net proton transport into the gland lumen by increased V-ATPase activity leads to luminal acidification (52). Thus, measurements of pH changes in the gland lumen,
REGULATION OF V-ATPase BY PKA

5,6-dichloro-1-carbamoyladenosine-3'-cyclic monophosphate (8-CPT-cAMP) on the transepithelial potential (TEP) and on luminal surface pH. A and B: treatment with 100 μM pMeOPT-cAMP has no visible effect on TEP, whereas 100 μM 8-CPT-cAMP induces a positive-going change in TEP, indicative of an increase in transepithelial cation transport into the gland lumen. The biphasic TEP change upon 5-HT exposure demonstrates the functionality of the gland and results from Ca2+-induced transepithelial Cl− flux (negative phase) and cAMP-dependent cation transport into the gland lumen (positive phase), the latter persisting for a while after 5-HT washout. C and D: ratiometric measurements of pH changes at the luminal surface in salivary glands with the pH-sensitive dye 5-N-hexadecanoylaminofluorescein (HAF). Treatment with 30 nM 5-HT and 100 μM 8-CPT-cAMP induces a reversible acidification at the luminal surface of the secretory cells, as indicated by the decrease in the F360/F410 fluorescence ratio, whereas 100 μM pMeOPT-cAMP instead induces a small luminal alkalinization.

Epac-activating substances induce neither a significant change in TEP nor luminal acidification. Data are presented as means ± SD. The number of experiments is shown above or below each bar. Bnz-cAMP, N6-benzoyladenosine-3'-5'-cyclic monophosphate; MBC-cAMP, N6-mono-tert-butylcarbamoyladenosine-3'-5'-cyclic monophosphate; cBIMPS, 3, 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole-3'-5'-cyclic monophosphorothioate; pHPT-cAMP, 8-(4-hydroxyphenylthio)-2'-methyadenosine-3'-5'-cyclic monophosphate.

These findings suggest that activation of Epac induces neither V-ATPase assembly nor an increase in V-ATPase-mediated proton transport.

Effect of PKA activators on V-ATPase assembly and activity. To determine whether PKA was involved in V-ATPase regulation, we examined the effect of PKA activators on V-ATPase assembly and ion transport activity. In TEP measurements, exposure to 100 μM MBC-cAMP, cBIMPS, or Bnz-cAMP consistently led to a positive-going TEP change, although response kinetics and amplitudes differed between these PKA activators (Fig. 5A and Fig. 6, A and C). All PKA activators also led to luminal acidification, as visualized in HAF-stained glands (Fig. 5B and Fig. 6, B and D). With respect to both TEP measurements and luminal pH measurements, cBIMPS appeared to be the most effective cAMP analog, because it induced a faster and larger response than MBC-cAMP or Bnz-cAMP. To test whether these luminal pH changes were attributable to V-ATPase activity, MBC-cAMP was applied to salivary glands that had been preincubated with concanamycin A, a specific inhibitor of V-ATPase (19). Superfusion with concanamycin A induced a slight alkalinization (Fig. 7), indicative of a blockage of basal V-ATPase activity within non-stimulated glands (52, 73). Under these conditions, MBC-cAMP did not cause luminal acidification, demonstrating that the effect of PKA activators on luminal pH changes was mediated by changes in V-ATPase activity. Incubation of salivary glands with MBC-cAMP or cBIMPS also induced a redistribution of V1 subunits B and C from the cytoplasm to the apical membrane (Fig. 6, E–V), indicative of an assembly of V-ATPase holoenzymes.

In its deactivated state, the PKA holoenzyme forms a tetrameric complex of two regulatory and two catalytic subunits. Each regulatory subunit has two binding sites (sites A and B) that bind cAMP in a cooperative manner and that differ in their affinities for different cAMP analogs. Thus, if two cAMP analogs with opposite site selectivity are applied simultaneously, a synergistic effect is to be expected (18, 43, 48). To examine whether such a synergistic effect occurred with respect to V-ATPase assembly and activation, Calliphora salivary glands were simultaneously exposed to MBC-cAMP and...
cBIMPS, having a preference for site A or site B, respectively. As shown in Fig. 8, A and B, these substances produced little or no change in luminal pH if applied alone at a concentration of 60 μM of the PKA-activating cAMP analogs MBC-cAMP or cBIMPS. However, simultaneous exposure to both analogs led to a fast acidification at the luminal side of the epithelium, similar to 5-HT (Fig. 8, C and D). Immunofluorescence labeling demonstrated further that the combination of 60 μM MBC-cAMP with 20 μM cBIMPS was sufficient to induce an enrichment of V₁ subunits B and C at the apical membrane to an extent similar to that observed at a saturating 5-HT concentration (Fig. 8, E–G).

Pelleting assays were performed to examine the assembly status of V-ATPase by an alternative method. We have shown previously that stimulation with 5-HT or experimental conditions that raise the intracellular cAMP level induce a redistribution of V₁ components from the soluble fraction to the membrane fraction (14, 73). Similarly, incubation of salivary glands with 100 μM cBIMPS led to an increase in the relative amount of membrane-associated V₁ components. In control glands, ~96% of V₀ subunit d but only ~35% and ~48% of the V₁ components subunit A and subunit E were detected in the pellet (Fig. 9). These numbers compare well with those of our previous assays (14, 73). Upon exposure to cBIMPS, the relative amount of subunits A and E within the pellet fraction was significantly increased and accounted for ~70% and 71%, respectively, of the total amount of these proteins. These results corroborated...
orate the conclusion that PKA activation induces an assembly of V-ATPase holoenzymes.

**Effect of PKA-specific inhibitors on 5-HT-induced activation and assembly of V-ATPase.** The above results demonstrate that V-ATPase can be activated by PKA. To determine whether 5-HT induced V-ATPase assembly and activation via the PKA pathway, we examined the effect of PKA inhibitors on the 5-HT-induced responses with regard to TEP, luminal pH, and V₁ distribution. Since only the positive TEP component (resulting from V-ATPase-driven cation transport) was of interest, experiments were performed in Cl⁻-free physiological saline to suppress the contribution of Ca²⁺-dependent Cl⁻ transport to the changes in TEP (1, 5). As shown in Fig. 10A, 30 μM H-89, a specific blocker for the catalytic PKA subunit (11), reduced the 5-HT-induced positive TEP phase by 80.3 ± 2.5% (n = 6) (Fig. 10B). Finally, treatment with 5-HT did not lead to a redistribution in V₁-subunits B and C in the presence of H-89 (Fig. 10, C–E). Similar results were observed with 100 μM Rp-CPT-cAMPS (data not presented), a cAMP analog that binds to PKA but that does not induce dissociation of the holoenzyme. We thus conclude that PKA is not only able to activate V-ATPase, but is also required to mediate 5-HT-induced V-ATPase assembly and activation.

**DISCUSSION**

The reversible assembly of V-ATPase holoenzymes is a widespread mechanism for the control of V-ATPase activity. This mode of V-ATPase regulation has been revealed in yeast (30) and in various animal cells, such as midgut epithelial cells of the tobacco hornworm *M. sexta* (62, 69), mammalian dendritic cells (64), and renal epithelial cells (55). However, the intracellular signaling pathways that govern the assembly and disassembly of functional V-ATPase holoenzymes upon external stimuli have remained elusive so far.

*Calliphora* salivary glands represent an example of the regulation of V-ATPase activity via assembly/disassembly. Here, the neurohormone 5-HT induces the assembly and activation of apical V-ATPase (73). 5-HT-induced V-ATPase assembly and activation is, in turn, regulated in a cAMP-dependent manner, because 5-HT leads to a rise in intracellular cAMP (3, 28), and V-ATPase assembly and H⁺ pumping can be elicited by artificially raising cAMP (14). Two target enzymes may pass information of an increase in cytosolic cAMP level to the V-ATPase. First, cAMP may activate PKA (63, 67), leading to a phosphorylation of substrate proteins. Second, cAMP may activate Epac, which catalyzes the exchange of GDP for GTP on Rap-like small G proteins (7, 16, 51). These proteins may then bind to V-ATPase directly or activate interacting proteins or downstream protein kinases (27). Both PKA (65) and Epac (present study) are present within the secretory cells of the blowfly salivary gland.

To examine the possible involvement of these target enzymes in the cAMP-mediated activation of V-ATPase, we have used cAMP analogs that activate either PKA or Epac. Exposure of isolated glands to various Epac activators induces no changes in TEP or luminal acidification, indicating that activation of Epac does not increase proton pumping across the apical membrane into the gland lumen. Moreover, Epac activators do not induce the recruitment of V₁ subunits from the cytoplasm to the apical membrane for holoenzyme assembly. Although pMeOPT-cAMP and pHPT-cAMP could be unable

![Fig. 7. Effect of the V-ATPase inhibitor concanamycin A on luminal pH changes elicited by MBC-cAMP. The dashed line indicates basal fluorescence ratio. Incubation with concanamycin A (1 μM) induces a slight increase in the F₄₁₀/F₄₁₀ fluorescence ratio, indicative of alkalinization because of the inhibition of basal V-ATPase activity. Superfusion with 100 μM MBC-cAMP under these conditions does not lead to acidification, demonstrating that V-ATPase accounts for the MBC-cAMP-induced H⁺ transport across the apical membrane.](http://ajpcell.physiology.org/)

![Fig. 8. Synergistic effect of PKA activators on luminal pH changes and V₁ distribution. A–C: luminal pH changes in response to 60 μM MBC-cAMP (A), 20 μM cBIMPS (B), or both substances applied simultaneously (C). D: quantitative analysis of the luminal pH changes in response to 60 μM MBC-cAMP, 20 μM cBIMPS, or both substances. At these concentrations, MBC-cAMP or cBIMPS alone induces only a small decrease in luminal pH. Combination of both analogs leads to strong luminal acidification that exceeds the acidification induced by 5-HT (=100%). E–G: Analysis of the intensity of V-ATPase labeling (images not shown) on the apical membrane (solid bars) and in the cytoplasm (open bars). Treatment with 60 μM MBC-cAMP plus 20 μM cBIMPS induces V₁ translocation to the apical membrane. Values are means ± SD (n = 6). *P < 0.05, **P < 0.005.](http://ajpcell.physiology.org/)
to activate Calliphora Epac, this possibility seems unlikely because the cAMP binding site is highly conserved between mammalian Epac and fly Epac. In particular, Calliphora Epac, like Drosophila and vertebrate Epac, lacks the Glu residue that is present in the cAMP-binding pocket of PKA; this residue may prevent the binding of cAMP analogs with a 2'-O-methyl substitution to PKA (20). Moreover, agonists of mammalian Epac have also been reported to activate Drosophila and crayfish Epac (10, 71). The inability of Epac agonists to mimic the effects of 5-HT or cAMP on V-ATPase thus suggests that Epac is not directly involved in the activation pathway for V-ATPase.

On the other hand, we have presented several lines of evidence in favor of the cAMP/PKA pathway as a control of V-ATPase assembly and activation. First, PKA-specific cAMP analogs lead to a recruitment of V1 subunits to the apical membrane, indicative of V-ATPase assembly, and to an increase in V-ATPase-dependent proton transport into the lumen of the gland. The various PKA agonists differ in their effectiveness, with cBIMPS being the only agonist that fully mimics (at a concentration of 100 μM) the effect of 5-HT or cAMP on V-ATPase. This is consistent with the findings of Døskeland and Øgreid (17), who have proposed that site-B-selective analogs (cBIMPS; 18) are more effective in activating PKA than site-A-selective analogs (MBC-cAMP and Bnz-cAMP; 45), because site B is more exposed and the binding of a ligand to this site leads to conformational changes that then enable access to site A. Differences in membrane permeability and in resistance to phosphodiesterases may contribute to the different effectiveness of the cAMP analogs. Second, a combination of site-A-selective MBC-cAMP and site-B-selective cBIMPS leads to a synergistic effect on proton pumping and V-ATPase assembly. This synergistic effect is characteristic of PKA (44, 53). Finally, PKA inhibitors H-89 and Rp-CPT-cAMPS abolish the effect of 5-HT on V-ATPase assembly and proton pumping. We thus conclude that PKA is an essential component in the signaling cascade from the 5-HT receptor to V-ATPase in Calliphora salivary glands. This conclusion is supplemented by our finding that blowfly salivary glands contain PKA at a high concentration, localized throughout the cytoplasm and at the apical membrane, viz. close to unassembled V1 sectors and to V-ATPase holoenzymes (M. Voss, R. Schmidt, B. Walz, O. Baumann, unpublished results). A modulatory role of Epac or other signaling pathways in V-ATPase regulation, however, cannot be excluded.

The cAMP/PKA pathway as a regulator of V-ATPase may be common in animals, although the exact method of regulation may vary. For example, in murine peritoneal macrophages, interleukin 1 causes an increase in V-ATPase activity by way of both the cAMP/PKA pathway and protein kinase C. Here, enhanced V-ATPase activity occurs after a lag period of several hours and is attributable to the increased expression of V-ATPase subunits (8). Moreover, in the gill epithelium of the crab Eriocheir sinensis and in the Malpighian tubules of Drosophila, a rise in the intracellular cAMP level causes an activation of V-ATPase, although the mode of V-ATPase regulation is unknown for these systems (13, 34, 42, 46). Finally, in mammalian epididymis, an intracellular cAMP rise occurs in response to alkaline luminal pH and induces the integration of additional V-ATPase molecules from an internal
vesicular pool into the apical membrane and hence a rise in V-ATPase activity (49). Notably, a cytoplasmic vesicular V₀ pool of variable extent is present in Calliphora salivary glands (14), and eBIMPS treatment leads to a slight but significant shift in the amount of V₀ from the cytoplasmic pool to the apical membrane (Fig. 6). Cycling of V₀ between an internal pool and the apical membrane may thus be an additional method of V-ATPase regulation in Calliphora salivary glands; this is also regulated by cAMP/PKA and may provide long-term effects.

What is the substrate of PKA with respect to V-ATPase regulation? Evidence has been presented that subunit B and C and possibly other subunits of the V₁ complex can be phosphorylated in vitro by WNK kinase (29) or by the clathrin assembly protein AP50 (39). However, whether these reactions occur in vivo and whether they influence V-ATPase assembly and/or activity are unknown. Recently, we have shown that subunit C of the insect M. sexta is the only V-ATPase component that can be phosphorylated by PKA (65). In accordance with these findings, subunit C becomes phosphorylated upon stimulation of blowfly salivary glands with either 5-HT or 8-CPT-cAMP (65). Interestingly, subunit C makes contact with both the V₁ and the V₀ domain (29a) and is thus ideally positioned for the control of interaction between both components. It may thus be concluded that phosphorylation of subunit C is a regulatory key event in the PKA-dependent reassembly of V-ATPase holoenzymes. We cannot exclude, however, that V-ATPase-interacting proteins, such as RAVE, aldolase, and phosphofructokinase (35, 36, 57–59, 61), contribute to the PKA-dependent regulation of V-ATPase assembly and activity.

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