cAMP potentiates InsP3-induced Ca2+ release from the endoplasmic reticulum in blowfly salivary glands
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Abstract

**Background:** Serotonin induces fluid secretion from *Calliphora* salivary glands by the parallel activation of the InsP3/Ca2+ and cAMP signaling pathways. We investigated whether cAMP affects 5-HT-induced Ca2+ signaling and InsP3-induced Ca2+ release from the endoplasmic reticulum (ER).

**Results:** Increasing intracellular cAMP level by bath application of forskolin, IBMX or cAMP in the continuous presence of threshold 5-HT concentrations converted oscillatory [Ca2+]i changes into a sustained increase. Intraluminal Ca2+ measurements in the ER of β-escin-permeabilized glands with mag-fura-2 revealed that cAMP augmented InsP3-induced Ca2+ release in a concentration-dependent manner. This indicated that cAMP sensitized the InsP3 receptor Ca2+ channel for InsP3. By using cAMP analogs that activated either protein kinase A (PKA) or Epac and the application of PKA-inhibitors, we found that cAMP-induced augmentation of InsP3-induced Ca2+ release was mediated by PKA not by Epac. Recordings of the transepithelial potential of the glands suggested that cAMP sensitized the InsP3/Ca2+ signaling pathway for 5-HT, because IBMX potentiated Ca2+-dependent Cl- transport activated by a threshold 5-HT concentration.

**Conclusion:** This report shows, for the first time for an insect system, that cAMP can potentiate InsP3-induced Ca2+ release from the ER in a PKA-dependent manner, and that this crosstalk between cAMP and InsP3/Ca2+ signaling pathways enhances transepithelial electrolyte transport.

**Background**
Calcium ions and cyclic AMP are ubiquitous intracellular messengers that regulate a plethora of cellular processes. Indeed, the stimulation of many non-excitable cells by neurotransmitters or hormones causes the parallel activation of the cAMP and the phosphoinositide signaling pathways [1,2]. The latter culminates in inositol 1,4,5-trisphosphate (InsP3)-induced Ca2+ release through InsP3 receptor Ca2+ channels (InsP3R) from the endoplasmic reticulum (ER) and an elevation in intracellular Ca2+ concentration ([Ca2+]i). InsP3-induced Ca2+ release with or without Ca2+ entry from the extracellular space generates temporally and spatially coordinated Ca2+ signals leading, in many cells, to intracellular Ca2+ oscillations and waves [3-5]. Thus, Ca2+ signals can be spatially compartmentalized and coded by amplitude, frequency, and/or shape: these parameters are important for the specificity of stimulus response coupling [5].

One way of controlling Ca2+ signals can be achieved by cAMP, which has been shown to affect Ca2+ signaling at multiple sites, e.g., at the level of InsP3 generation [6-8]...
and InsP₃-induced Ca²⁺ release from the ER. cAMP exerts its physiological effects through downstream effector proteins, either protein kinase A (PKA) or cAMP-specific guanine nucleotide exchange factors (cAMP-GEF) known as exchange proteins directly activated by cAMP (Epac) [9,10]. Upon activation by cAMP, PKA is able to phosphorylate all three subtypes of vertebrate InsP₃R and thus to modulate InsP₃-induced Ca²⁺ release from the ER [1,11-17]. On the other hand, physiological evidence from pancreatic β cells indicates that Epac sensitizes Ca²⁺-induced Ca²⁺ release (CICR) via InsP₃-R in a cAMP-dependent manner [18].

Although we are beginning to understand the functional consequences of InsP₃ receptor phosphorylation and its effects on InsP₃-induced Ca²⁺ release in some mammalian cell types, little knowledge is currently available about whether cAMP affects InsP₃-induced Ca²⁺ release in invertebrates [19]. Only a single InsP₃-R isoform is expressed in Drosophila melanogaster (DmInsP₃R) [20,21] and Caenorhabditis elegans (CeInsP₃R). InsP₃-R in both species share the main functional properties with mammalian InsP₃-R: InsP₃ sensitivity, single channel conductance, gating, and a bell-shaped Ca²⁺ dependence [22-24]. However, InsP₃-R phosphorylation has not been investigated in these species.

Since almost nothing is known regarding whether cAMP affects InsP₃-R function in invertebrates or its possible mode of action, we have studied this interaction in isolated salivary glands of the blowfly Calliphora vicina, a dipteran species closely related to Drosophila. Calliphora salivary glands secrete a KCl-rich saliva when stimulated with the neurohormone serotonin (5-hydroxytryptamine, 5-HT). 5-HT activates, in parallel, the cAMP and the phosphoinositide signaling cascade [25]. The latter leads to InsP₃-induced Ca²⁺ release from the ER and, at low 5-HT concentrations, to intracellular Ca²⁺ oscillations through cyclical Ca²⁺ release from and reuptake into the ER [26,27]. The Ca²⁺ elevation activates transeptoidal CI-transport, whereas the increase in cAMP level stimulates transeptoidal K⁺ transport [28-31]. The aim of the present study has been to investigate whether cAMP affects 5-HT-induced Ca²⁺ signaling and InsP₃-induced Ca²⁺ release from the ER. We provide evidence that cAMP sensitizes the InsP₃-R sensitivity of InsP₃-induced Ca²⁺ release in a PKA-dependent manner.

Results
cAMP affects 5-HT-induced Ca²⁺ signaling
Threshold concentrations of 5-HT (1–3 nM) induced intracellular Ca²⁺ oscillations, whereas saturating 5-HT concentrations (>30 nM) produced biphasic Ca²⁺ responses that consisted of an initial transient followed by a plateau of elevated [Ca²⁺]i (Figs. 1A, B, and [26,27]). To test whether these two types of response patterns were affected by cAMP, we increased the intracellular cAMP by bath application of 10 mM cAMP, 100 μM IBMX, or 100 μM forskolin. These substances/concentrations had no effect on resting [Ca²⁺]i [33]. As shown in Fig. 1A, 3 nM 5-HT induced intracellular Ca²⁺ oscillations, as described previously. Application of forskolin to the bath in the continuous presence of 3 nM 5-HT converted the oscillatory [Ca²⁺] changes into a sustained increase (n = 8). Treatment with cAMP or IBMX had the same effect as forskolin at all tested preparations (cAMP, n = 7; IBMX, n = 5). Forskolin did not affect the sustained Ca²⁺ elevation produced by 30 nM 5-HT (Fig. 1B), a concentration that saturates the rate of fluid secretion [34].

To determine whether the extra Ca²⁺ increase produced by forskolin at low 5-HT concentrations was attributable to Ca²⁺ influx from the extracellular space, we stimulated glands with a sub-threshold concentration of 5-HT (in order to prevent fast Ca²⁺ store depletion [26]) and applied forskolin in Ca²⁺-free PS (no added Ca²⁺, 2 mM EGTA). As seen in Fig. 1C, 1 nM 5-HT was below the concentration that induced marked Ca²⁺ oscillations (in Ca²⁺-containing PS), but application of 100 μM forskolin stimulated a transient Ca²⁺ elevation even in the absence of extracellular Ca²⁺. Taken together, these results suggested that cAMP did not induce Ca²⁺ influx but rather augmented Ca²⁺ release from the ER produced by low 5-HT concentrations.

cAMP augments InsP₃-induced Ca²⁺ release from the ER
Theoretically, there are two mechanisms for the release of Ca²⁺ from the ER: the InsP₃R and the ryanodine receptor Ca²⁺ channel (RyR). Blowfly salivary glands, however, seem to lack RyR [26], leaving only the InsP₃-R as potential target for the cAMP pathway in order to enhance Ca²⁺ release.

To examine directly whether cAMP augmented InsP₃-induced Ca²⁺ release we studied Ca²⁺ release from the ER by intraluminal Ca²⁺ measurements with the low-affinity Ca²⁺-indicator dye Mag-fura-2. This dye accumulates within the ER and after β-escin permeabilization of the plasma membrane in an artificial "intracellular medium" (ICM) and loss of cytosolic dye, it monitors intraluminal Ca²⁺ ([Ca²⁺]L) [32,35,36]. Figures 2A and 2B show two representative original recordings of intraluminal Ca²⁺ measurements. In order to facilitate the quantitative evaluation of this type of measurements, we converted Mag-fura-2 fluorescence ratios into a percentage scale, with 0% Ca²⁺ release representing the intraluminal Mag-fura-2 ratio at time zero of the recording, and 100% Ca²⁺ release representing the fluorescence ratio after the loss of intraluminal Ca²⁺ following ionomycin application.
Application of 100 μM cAMP to the permeabilized gland tubules did not induce Ca2+ release from the ER, whereas the Ca2+-ionophore ionomycin led to a dramatic loss in intraluminal Ca2+ (Fig. 2A). Treatment with 5 μM InsP3, on the other hand, caused a partial Ca2+ release, and the subsequent addition of 100 μM cAMP resulted in a further Ca2+ release (Fig. 2B), indicating that cAMP had augmented InsP3-induced Ca2+ release. In order to obtain the dose-response relationship for the effect of cAMP on InsP3-induced Ca2+ release, the cAMP concentration was systematically varied, and Ca2+ release (%) (Fig. 2E, squares) was measured after cAMP addition to ICM containing 5 μM InsP3. The sigmoidal dose-response curve fitted to the mean values of the InsP3(+cAMP)-induced Ca2+ release gave a mean half maximal cAMP concentration (EC50) of 2.5 μM (Fig. 2E).

In order to exclude that the augmentation of InsP3-induced Ca2+ release was not simply the result of the addition of fresh InsP3(+cAMP)-containing ICM, we superfused several preparations with InsP3(no cAMP)-containing ICM twice. A second InsP3 application never increased Ca2+ release induced by a prior InsP3 application (Fig. 2C; n = 5). Moreover, mock stimulation with 10 μM (n = 5) or 100 μM (n = 5) 8-Br-Rp-cAMPS (a competitive antagonist of cAMP binding to PKA) had no significant effect on the InsP3-induced Ca2+ release (Fig. 2D displays a representative original recording with 10 μM 8-Br-Rp-cAMP).

To determine whether cAMP increased the affinity of the InsP3R for InsP3, we examined Ca2+ release induced by increasing InsP3-concentrations in the absence (Fig. 2F, squares) and presence of 100 μM cAMP (Fig. 2F, triangles). The two resulting dose-response curves indicated that cAMP increased the affinity of the InsP3R for InsP3, because cAMP shifted the dose-response curve to lower InsP3 concentrations by about one order of magnitude.

Is the cAMP-dependent augmentation of InsP3-induced Ca2+ release mediated by PKA or EPAC?

The effect of cAMP on InsP3-induced Ca2+ release could be mediated by either PKA or Epac. Both target proteins are expressed in blowfly salivary glands [59]. To distinguish between these possibilities, cAMP-analogs that activate either PKA or Epac or both downstream effectors were used instead of cAMP [39]. These cAMP analogs were applied at concentrations of 10 μM and 100 μM. One problem in the quantitative evaluation of these experiments was, that the Mag-fura-2 fluorescence ratio in the β-escin-permeabilized preparations continuously declined as Ca2+ leaked out of the ER (see, for example, Figs. 2A, B; 3A, C, D), and this decline in fluorescence ratio varied between preparations. Therefore, we did not measure and compare the magnitude of Ca2+ release from the ER (as

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**Figure 1**

**Forskolin augments [Ca2+]i changes induced by low 5-HT concentrations in Calliphora salivary gland cells.**

(A) Stimulation with 3 nM 5-HT produces intracellular Ca2+ oscillations. Application of 100 μM forskolin converts oscillatory [Ca2+]i changes into a sustained increase (n = 8). (B) Stimulation of the gland with 30 nM 5-HT, a concentration that saturates fluid secretion, produces a biphasic Ca2+ response consisting of an initial transient followed by a plateau of elevated [Ca2+]i. The sustained phase of elevated [Ca2+]i is not affected by forskolin (n = 4). (C) Application of a threshold concentration of 5-HT (1 nM) in Ca2+-free PS (0-Ca, 2 mM EGTA) increases [Ca2+]i just measurably without triggering Ca2+ spikes. Additional application of 100 μM forskolin induces a transient Ca2+ elevation, showing that forskolin augments 5-HT-induced Ca2+ release, not Ca2+ entry (n = 8).
cAMP augments InsP₃-induced Ca²⁺ release from β-escin permeabilized cells, as shown by intraluminal Ca²⁺ measurements with Mag-Fura-2. (A) cAMP does not induce Ca²⁺ release from the ER (n = 4). (B) Application of 5 μM InsP₃ induces Ca²⁺ release from the ER and is augmented by 100 μM cAMP. (C, D) Ca²⁺ release induced by 5 μM InsP₃ is neither enhanced by application of fresh InsP₃ solution (C) nor by mock stimulation with Rp-cAMPS (D). (E) Quantification of the cAMP-dependent augmentation of Ca²⁺ release induced by 5 μM InsP₃ from experiments as shown in B. 0% Ca²⁺ release is the intraluminal Mag-fura-2 ratio at time zero of the recording; 100% Ca²⁺ release is the fluorescence ratio after complete loss of intraluminal Ca²⁺ following ionomycin application. A sigmoidal dose-response curve fitted to mean values (R² = 0.4) of the InsP₃(+cAMP)-induced Ca²⁺ release gives an EC₅₀, cAMP of 2.6 μM. (F) Dose-response relationship for InsP₃-induced Ca²⁺ release in the presence (triangles) and absence (squares) of 100 μM cAMP. The leftward shift of the dose-response relationship indicates sensitization of InsP₃-induced Ca²⁺ release for InsP₃ by cAMP. (E, F) The number of measurements for every data point is given in brackets. Means ± S.D.
InsP₃-induced Ca²⁺ release is augmented by PKA activators and not by Epac activators. (A, C, D) Representative original recordings showing the effects of three cAMP analogs on InsP₃-induced Ca²⁺ release as recorded by intraluminal Ca²⁺ measurements with Mag-Fura-2 in β-escin-permeabilized glands. (B, D, F) Summary of results obtained from experiments as illustrated in A, C and D. Ca²⁺ release is displayed as the change in the rate of the Mag-Fura-2 fluorescence ratio (ΔF₃⁴⁰/F₃⁸⁰·min⁻¹) before and after addition of a cAMP analog as shown in (A), dotted lines. (A, B) The PKA and Epac activator 8-CPT-cAMP augments InsP₃-induced Ca²⁺ release significantly in a concentration-dependent manner. (C, E) Neither 8-pMeOPT-2’-O-Me-cAMP nor the two other Epac activators (8-pHPT-2’-O-Me-cAMP and 8-pCPT-2’-O-Me-cAMP) has an effect on InsP₃-induced Ca²⁺ release. 8-pCPT-2’-O-Me-cAMP was also ineffective in GTP-containing ICM (lowest two bars). (D, F) All three tested PKA activators (6-Phe-cAMP, 6-BNZ-cAMP, 6-MBC-cAMP) augment InsP₃-induced Ca²⁺ release in a concentration-dependent manner. (B, E, F) Means ± S.D., paired t-test, *P < 0.05, **P < 0.01, ***P < 0.001.
above), but rather its rate as measured by the decline in the Mag-fura-2 fluorescence ratio per minute. The rates were obtained from regression lines fitted to the fluorescence traces over a one minute period before and after application of the cAMP analog (see Fig. 3A, dotted lines). As shown in Figs. 3A and 3B, 8-CPT-cAMP, activating both PKA and Epac, augmented InsP3-induced Ca2+ release significantly and in a dose-dependent manner.

Figures 3C–F summarize the effect of three Epac-specific cAMP-analogs and of three PKA-specific analogs on InsP3-induced Ca2+ release. At a concentration of 10 μM none of the Epac activators augmented InsP3-induced Ca2+ release (Figs. 3C, E). The Epac-activator 8-pHPT-2'-O-Me-cAMP produced a slight but significant increase in the rate of Ca2+ release when applied at a concentration of 100 μM, whereas the other two Epac activators were ineffective at 100 μM. Since Epac links cAMP to the activation of the small G protein Rap1 [9,37] and since our ICM did not contain GTP, we tested whether the above Epac activators were ineffective because of the lack of GTP. However, 8-CPT-O-2'-Me-cAMP had also no significant effect on InsP3-induced Ca2+ release when applied in ICM supplemented with 3 mM GTP (Fig. 3E).

In contrast to the Epac activators all tested PKA-specific cAMP analogs augmented InsP3-induced Ca2+ release significantly in a dose-dependent manner (Figs. 3E, F). These findings indicated that the cAMP-dependent augmentation of InsP3-induced Ca2+ release was mediated by PKA rather than Epac.

PKA inhibitors block the augmentation of InsP3-induced Ca2+ release by cAMP
To examine by an alternative approach whether the cAMP evoked augmentation of the InsP3-induced Ca2+ release was mediated by PKA, we tested the effect of the competitive antagonist of cAMP-binding to PKA, 8-Br-Rp-cAMPS [39,40], and of the PKA inhibitor H-89 [41] on 8-CPT-cAMP-augmented InsP3-induced Ca2+ release. Both substances reversed the extra-Ca2+ release produced by 8-CPT-cAMP on a background of 5 μM InsP3 (Figs. 4A–D). These results provided further support for our conclusion that the cAMP-evoked augmentation of InsP3-induced Ca2+ release was mediated by PKA.

Discussion
The results of this study provide physiological evidence that cAMP augments InsP3-induced Ca2+ release from the ER in the salivary glands of Calliphora vicina, a dipteran fly closely related to Drosophila melanogaster. Our intraluminal Ca2+ measurements in the ER of permeabilized cells in isolated glands show, in addition, that cAMP increases the affinity of the InsP3R for InsP3 by about a factor of 10. Using cAMP analogs that activate either PKA or Epac and PKA inhibitors we show further that this cAMP effect is mediated by PKA rather than Epac. Finally, intracellular Ca2+ measurements and electrophysiological recordings...
indicate that the cAMP-induced and PKA-mediated sensitization of the InsP₃R for InsP₃ affects Ca²⁺ signaling and transepithelial electrolyte transport.

**cAMP-induced and PKA-mediated augmentation of InsP₃-induced Ca²⁺ release**

All three mammalian InsP₃R subtypes have the potential to undergo phosphorylation by PKA and by some other kinases including PKG, PKC and CaM-kinase [22,45]. The resulting phoregulation of Ca²⁺ release is thought to have profound effects on the spatio-temporal characteristics of Ca²⁺ signals and to provide a potential mechanism of crosstalk between different signaling pathways. Nevertheless, data on the effects of InsP₃R phosphorylation on InsP₃-induced Ca²⁺ release are contradictory (reviewed in [1,46]). Most reports suggest that InsP₃R phosphorylation augments InsP₃-induced Ca²⁺ release (e.g. [12,15,17,47-49]), whereas others indicate that Ca²⁺ release is attenuated [e.g. [14,50]].

Here, we show that cAMP augments InsP₃-induced Ca²⁺ release in permeabilized salivary glands of *Calliphora*, and that the effect of cAMP is mediated by PKA. The cAMP-dependent leftward shift in the dose-response relationship for InsP₃ suggests that the augmentation of Ca²⁺ release is attributable to an increase of about 10-fold in the affinity of the InsP₃R Ca²⁺ channel for InsP₃. We can exclude the possibility that the cAMP-induced augmenta-
tion of Ca\textsuperscript{2+} release results from a stimulation of Ca\textsuperscript{2+} loading of the ER via SERCA, because the intraluminal Ca\textsuperscript{2+} concentration is not affected by cAMP-containing ICM in the permeabilized glands.

The involvement of PKA suggests that the cAMP effect is mediated by phosphorylation of InsP\textsubscript{3}R. However, although six potential PKA phosphorylation sites have been detected in the sequence of *Caenorhabditis elegans* InsP\textsubscript{3}R, no such sites have been identified in *Drosophila melanogaster* InsP\textsubscript{3}R (DmInsP\textsubscript{3}R) \[19,21,22\]. It must be noted, however, that only a single algorithm had been used to search for putative sites for PKA-mediated phosphorylation in the *Drosophila* InsP\textsubscript{3} receptor. We experi-

**Figure 5**

Effects of IBMX on 5-HT-induced changes in transepithelial potential (TEP) in Ba\textsuperscript{2+}-containing PS. (A, B) Original recordings. The bar graphs (B, D) display and summarize the TEPs recorded at the time points (1-4) as indicated in A and C; means ± S.D. In both groups of experiments (A, C), an initial control stimulation with 30 nM 5-HT produces a biphasic TEP change. The TEP goes negative after superfusion of the preparation with Ba\textsuperscript{2+}-containing PS. Addition of 1 nM and 30 nM 5-HT cause the TEP to go further negative. The TEP recorded in the presence of 1 nM 5-HT (A, B) but not 30 nM 5-HT (C, D) goes further negative by application of 500 μM IBMX in the presence of 5-HT.
enced that, at least for other proteins, results for putative phosphorylation sites vary by using different bioinformatic algorithms [Voss et al., 2007]. Sequence information for Calliphora InsP3R is still lacking but the dipteran fly Calliphora is closely related to Drosophila. Thus, whether fly InsP3 receptor Ca2+ channels can be phosphorylated, or whether the InsP3R in Calliphora differs from that in Drosophila with respect to consensus sites for PKA-mediated phosphorylation remains unknown. Therefore, we cannot yet explain the molecular basis of the cAMP-induced and PKA-mediated sensitization of Ca2+ release in this species. DmInsP3R seems to have consensus sequences for phosphorylation by PKC and CaM-kinase II [21]. The activity of these two kinases can be affected by PKA [17,51-53]. Thus, cAMP might affect DmInsP3R via other kinases or unknown accessory proteins that are phosphorylated by PKA.

**Physiological consequences of cAMP-mediated sensitization of the InsP3R for InsP3**

The cAMP-mediated sensitization of the InsP3R for InsP3 has measurable effects on Ca2+ signaling in Calliphora salivary glands. We have shown that increasing the intracellular cAMP concentration converts baseline Ca2+ spiking induced by threshold concentrations of 5-HT [26] into a sustained Ca2+ elevation. This effect of cAMP on Ca2+ spiking is remarkably similar to that reported for the parotid acinar cell. Here, forskolin potentiates carbachol-induced [Ca2+]i changes, and this potentiation also results from enhanced Ca2+ release attributable to cAMP-dependent and PKA-mediated sensitization of InsP3-induced Ca2+ release from the ER [17]. The enhanced Ca2+ release is probably not the result of a cAMP-dependent stimulation of InsP3 production [17], although cAMP has been shown to potentiate InsP3 production in hepatocytes and parotid acinar cells [54,55]. This possibility can be excluded in Calliphora salivary glands, as IBMX, although it potentiates 5-HT-induced fluid secretion (see below), has no effect on 5-HT-induced [3H]inositol release from isolated glands [56]. Thus, in Calliphora salivary glands, in parotid salivary glands, and in a number of other secretory cell types (such as pancreatic β cells), the InsP3R Ca2+ channel obviously functions as a coincidence detector [18] that monitors a simultaneous increase of InsP3, cAMP, and Ca2+ concentrations, the last-mentioned because InsP3R is also regulated by Ca2+ [reviewed in [22]].

Recordings of the transepithelial potential (TEP) in Calliphora salivary glands indicate that cAMP also augments the Ca2+-dependent transepithelial Cl− transport induced by low 5-HT concentrations, an observation suggesting that the cAMP-dependent enhanced Ca2+ release additionally affects fluid secretion. This notion is supported by experiments dating back more than 30 years. In the early 1970s, Berridge [57,58] found that the phosphodiesterase inhibitor theophylline sensitized 5-HT-induced fluid secretion from Calliphora salivary glands by a factor of about 10.

**Conclusion**

Taking all these data together, we can now ascribe two physiological effects to cAMP in Calliphora salivary glands: (1) the activation of an apical vacuolar-type H+-ATPase [33,59] that energizes the apical membrane for nH+/K+-antiporter-mediated K+ transport, and (2) the augmentation of InsP3-induced Ca2+ release from the ER resulting in enhanced Ca2+ signaling and enhanced transepithelial Cl− transport and fluid secretion. Both actions of cAMP are mediated by PKA, which is present at the sites of these effector proteins, the ER, and the apical membrane [59].

**Methods**

**Animals, preparation and solutions**

The blowfly, Calliphora vicina, was reared at our Institute. Flies were kept at 24–26°C under a 12 h light: 12 h dark cycle. The abdominal region of the tubular salivary glands of adult flies was dissected under physiological solution (PS).

Normal PS contained (mM): 128 NaCl, 10 KCl, 2 CaCl2, 2 MgCl2, 2.8 maleic acid, 3 sodium glutamate, 10 TRIS-HCl, 10 D-Glucose, pH 7.2. Ca2+-free PS was prepared by omitting CaCl2 and adding 2 mM EGTA. "Intracellular-like" medium (ICM) was used for experiments with β-escin-permeabilized preparations and contained (mM): 125 KCl, 20 NaCl, 2 MgCl2, 3 Na2ATP, 0.1 EGTA, 0.06 CaCl2, 10 HEPES at pH 7.3. The free Ca2+ concentration in this medium was determined to be ~250 nM, as noted previously [32]. GTP-ICM contained (mM): 125 KCl, 20 NaCl, 2 MgCl2, 3 Na2ATP, 3 GTP, 0.1 EGTA, 0.06 CaCl2, 10 HEPES at pH 7.3.

**Transepithelial potential recordings**

Because the transepithelial potential (TEP) is a sensitive indicator of the transepithelial K+ and Cl− transport [28,34,38], we used TEP recordings to obtain information about the effects of cAMP on transepithelial Cl− transport that is activated by an increase in intracellular Ca2+ concentration. Isolated salivary gland tubules (ca. 10 mm long) were placed across a narrow paraffin oil gap into a two-well perfusion chamber that was modified according to [28]. One well contained the closed end of the gland tubule and was continuously perfused with PS. The cut end of the salivary gland opened into the other well. Both wells were connected via 3 M KCl agar-bridges and AgAgCl-pellets in microelectrode holders (WPI Int., Berlin, Germany) to a differential amplifier (npi-electronics, Tamm, Germany). Data were sampled and digitized at 2 Hz (A/D-board: DAS-1600; Keithley, Germering, Germany). The software EASYEST (Asyst Software Technolo-
Dye loading and cell permeabilization

For intracellular Ca\(^{2+}\) measurements the dissected glands were loaded with fura-2 by incubation with 5 μM fura-2 acetoxyxymethylene in PS for 40–60 min at room temperature. After dye loading, the gland tubules were mounted on cover slips coated with VectaBond™ (Axoara, Germany) and placed in a superfusion chamber on the stage of a Zeiss Axiovert 135TV epifluorescence microscope. In all experiments, the preparations were continuously superfused with PS (or with Ca\(^{2+}\)-free PS) at a rate of ~1 ml/min.

For intraluminal Ca\(^{2+}\) measurements in the ER the glands were loaded with mag-fura-2 by a 20 min incubation with 1 μM mag-fura-2 AM in PS and subsequently mounted in glass-bottomed perfusion chambers as described above. The glands were then permeabilized for 4–8 min in ICM containing 200 μg ml\(^{-1}\) (w/v) β-escin. After permeabilization, excessive β-escin was washed out with ICM. The progress of permeabilization was monitored by following the decrease in mag-fura-2 fluorescence until the signal had reached a stable level attributable to the loss of cytosolic dye.

Measurements of [Ca\(^{2+}\)], [Ca\(^{2+}\)]\(_{\text{cyt}}\), was measured as described previously [26]. In brief, pairs of fluorescence images, excited at wavelengths of 340 nm and 380 nm (VisiChrome High Speed Poly-chromat System; Visitron Systems, Puchheim, Germany) via a 450 nm dichroic mirror and a Zeiss Fluar 20/0.75 objective, were captured at a rate of 1 Hz with a cooled frame transfer CCD camera (TE/CCD-512FF; Princeton Instruments Corp., Trenton, NJ) via a 515–565 nm bandpass filter. Raw images were processed on a PC by using the software MetaFluor (Universal Imaging Corp., West Chester, PA). Fluorescence ratios (340 nm/380 nm) were calculated after subtraction of background fluorescence and cell autofluorescence both of which were determined at the end of every experiment by quenching fura-2 fluorescence by application of 20 mM MnCl\(_2\).

Statistical analysis

Signal processing and curve fitting were performed by using GraphPad Prism 4 (Version 4.01, GraphPad Software Inc.). Data are expressed as means ± S.D. Statistical comparisons were made by a Student’s paired t-test, and P values < 0.05 were considered significant.

Authors’ contributions

RS carried out all experiments and drafted the manuscript. BW and OB participated in the conception of the project and the design of the experiments, and they helped to write the manuscript. All authors approved the final manuscript.

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