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cAMP potentiates $InsP_3$-induced Ca^{2+} 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 $InsP_3/Ca^{2+}$ and cAMP signaling pathways. We investigated whether cAMP affects 5-HT-induced Ca^{2+} signaling and $InsP_3$-induced Ca^{2+} 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 $[Ca^{2+}]_i$ changes into a sustained increase. Intraluminal Ca^{2+} measurements in the ER of β-escin-permeabilized glands with mag-fura-2 revealed that cAMP augmented $InsP_3$-induced Ca^{2+} release in a concentration-dependent manner. This indicated that cAMP sensitized the $InsP_3$ receptor Ca^{2+} channel for $InsP_3$. 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 $InsP_3$-induced Ca^{2+} release was mediated by PKA not by Epac. Recordings of the transepithelial potential of the glands suggested that cAMP sensitized the $InsP_3/Ca^{2+}$ signaling pathway for 5-HT, because IBMX potentiated Ca^{2+}-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 $InsP_3$-induced Ca^{2+} release from the ER in a PKA-dependent manner, and that this crosstalk between cAMP and $InsP_3/Ca^{2+}$ 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 ($InsP_3$)-induced Ca^{2+} release through $InsP_3$ receptor Ca^{2+} channels ($InsP_3R$) from the endoplasmic reticulum (ER) and an elevation in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). $InsP_3$-induced Ca^{2+} release with or without Ca^{2+} entry from the extracellular space generates temporally and spatially coordinated Ca^{2+} signals leading, in many cells, to intracellular Ca^{2+} oscillations and waves [3-5]. Thus, Ca^{2+} 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 Ca^{2+} signals can be achieved by cAMP, which has been shown to affect Ca^{2+} signaling at multiple sites, e.g., at the level of $InsP_3$ generation [6-8]

and $InsP_3$-induced Ca^{2+} 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 $InsP_3R$ and thus to modulate $InsP_3$-induced Ca^{2+} release from the ER [1,11-17]. On the other hand, physiological evidence from pancreatic β cells indicates that Epac sensitizes Ca^{2+}-induced Ca^{2+} release (CICR) via $InsP_3$-R in a cAMP-dependent manner [18].

Although we are beginning to understand the functional consequences of $InsP_3$ receptor phosphorylation and its effects on $InsP_3$-induced Ca^{2+} release in some mammalian cell types, little knowledge is currently available about whether cAMP affects $InsP_3$-induced Ca^{2+} release in invertebrates [19]. Only a single $InsP_3R$ isoform is expressed in *Drosophila melanogaster* ($DmInsP_3R$) [20,21] and *Caenorhabditis elegans* ($CeInsP_3R$). $InsP_3R$ in both species share the main functional properties with mammalian $InsP_3R$: $InsP_3$ sensitivity, single channel conductance, gating, and a bell-shaped Ca^{2+} dependence [22-24]. However, $InsP_3R$ phosphorylation has not been investigated in these species.

Since almost nothing is known regarding whether cAMP affects $InsP_3R$ 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 $InsP_3$-induced Ca^{2+} release from the ER and, at low 5-HT concentrations, to intracellular Ca^{2+} oscillations through cyclical Ca^{2+} release from and reuptake into the ER [26,27]. The Ca^{2+} elevation activates transepithelial Cl^- transport, whereas the increase in cAMP level stimulates transepithelial K^+ transport [28-31]. The aim of the present study has been to investigate whether cAMP affects 5-HT-induced Ca^{2+} signaling and $InsP_3$-induced Ca^{2+} release from the ER. We provide evidence that cAMP sensitizes the $InsP_3$-sensitivity of $InsP_3$-induced Ca^{2+} release in a PKA-dependent manner.

Results

cAMP affects 5-HT-induced Ca^{2+} signaling

Threshold concentrations of 5-HT (1–3 nM) induced intracellular Ca^{2+} oscillations, whereas saturating 5-HT concentrations (> 30 nM) produced biphasic Ca^{2+} responses that consisted of an initial transient followed by a plateau of elevated $[Ca^{2+}]_i$ (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 $[Ca^{2+}]_i$ [33]. As shown in Fig. 1A, 3 nM 5-HT induced intracellular Ca^{2+} oscillations, as described previously. Application of forskolin to the bath in the continuous presence of 3 nM 5-HT converted the oscillatory $[Ca^{2+}]_i$ 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 Ca^{2+} elevation produced by 30 nM 5-HT (Fig. 1B), a concentration that saturates the rate of fluid secretion [34].

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

cAMP augments $InsP_3$-induced Ca^{2+} release from the ER

Theoretically, there are two mechanisms for the release of Ca^{2+} from the ER: the $InsP_3R$ and the ryanodine receptor Ca^{2+} channel (RyR). Blowfly salivary glands, however, seem to lack RyR [26], leaving only the $InsP_3R$ as potential target for the cAMP pathway in order to enhance Ca^{2+} release.

To examine directly whether cAMP augmented $InsP_3$-induced Ca^{2+} release we studied Ca^{2+} release from the ER by intraluminal Ca^{2+} measurements with the low-affinity Ca^{2+}-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 Ca^{2+} ($[Ca^{2+}]_L$) [32,35,36]. Figures 2A and 2B show two representative original recordings of intraluminal Ca^{2+} 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% Ca^{2+} release representing the intraluminal Mag-fura-2 ratio at time zero of the recording, and 100% Ca^{2+} release representing the fluorescence ratio after the loss of intraluminal Ca^{2+} following ionomycin application.

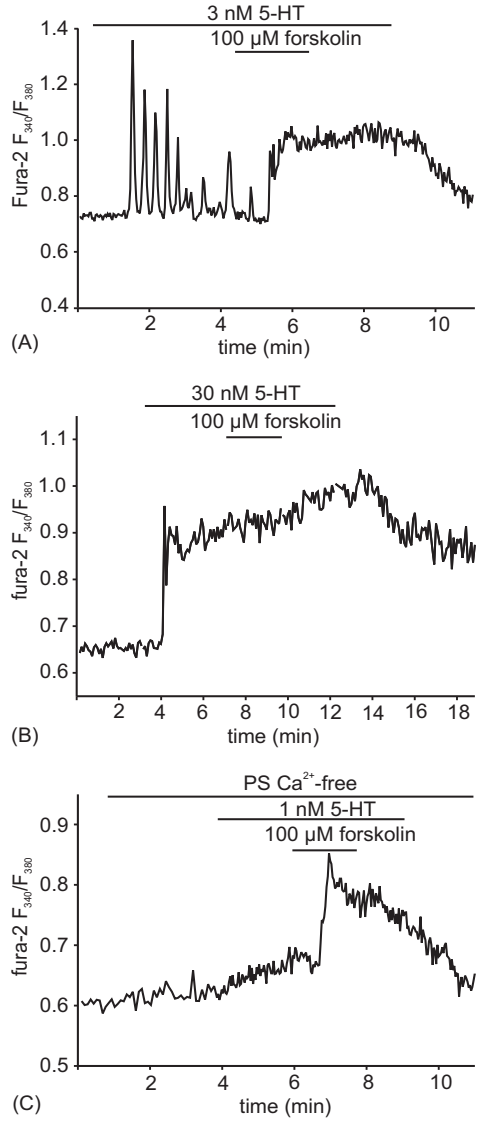

Figure 1
Forskolin augments $[Ca^{2+}]_i$ changes induced by low 5-HT concentrations in *Calliphora* salivary gland cells. (A) Stimulation with 3 nM 5-HT produces intracellular Ca^{2+} oscillations. Application of 100 µM forskolin converts oscillatory $[Ca^{2+}]_i$ changes into a sustained increase (n = 8). (B) Stimulation of the gland with 30 nM 5-HT, a concentration that saturates fluid secretion, produces a biphasic Ca^{2+} response consisting of an initial transient followed by a plateau of elevated $[Ca^{2+}]_i$. The sustained phase of elevated $[Ca^{2+}]_i$ is not effected by forskolin (n = 4). (C) Application of a threshold concentration of 5-HT (1 nM) in Ca^{2+}-free PS (0-Ca, 2 mM EGTA) increases $[Ca^{2+}]_i$ just measurably without triggering Ca^{2+} spikes. Additional application of 100 µM forskolin induces a transient Ca^{2+} elevation, showing that forskolin augments 5-HT-induced Ca^{2+} release, not Ca^{2+} entry (n = 8).

Application of 100 µM cAMP to the permeabilized gland tubules did not induce Ca^{2+} release from the ER, whereas the Ca^{2+}-ionophore ionomycin led to a dramatic loss in intraluminal Ca^{2+} (Fig. 2A). Treatment with 5 µM $InsP_3$, on the other hand, caused a partial Ca^{2+} release, and the subsequent addition of 100 µM cAMP resulted in a further Ca^{2+} release (Fig. 2B), indicating that cAMP had augmented $InsP_3$-induced Ca^{2+} release. In order to obtain the dose-response relationship for the effect of cAMP on $InsP_3$-induced Ca^{2+} release, the cAMP concentration was systematically varied, and Ca^{2+} release (%) (Fig. 2E, squares) was measured after cAMP addition to ICM containing 5 µM $InsP_3$. The sigmoidal dose-response curve fitted to the mean values of the $InsP_3$(+cAMP)-induced Ca^{2+} release gave a mean half maximal cAMP concentration (EC_{50}) of 2.5 µM (Fig. 2E).

In order to exclude that the augmentation of $InsP_3$-induced Ca^{2+} release was not simply the result of the addition of fresh $InsP_3$(+cAMP)-containing ICM, we superfused several preparations with $InsP_3$(no cAMP)-containing ICM twice. A second $InsP_3$ application never increased Ca^{2+} release induced by a prior $InsP_3$ 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 $InsP_3$-induced Ca^{2+} release (Fig. 2D displays a representative original recording with 10 µM 8-Br-Rp-cAMP).

To determine whether cAMP increased the affinity of the $InsP_3R$ for $InsP_3$, we examined Ca^{2+} release induced by increasing $InsP_3$-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 $InsP_3R$ for $InsP_3$, because cAMP shifted the dose-response curve to lower $InsP_3$ concentrations by about one order of magnitude.

Is the cAMP-dependent augmentation of $InsP_3$-induced Ca^{2+} release mediated by PKA or EPAC?

The effect of cAMP on $InsP_3$-induced Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} release from the ER (as

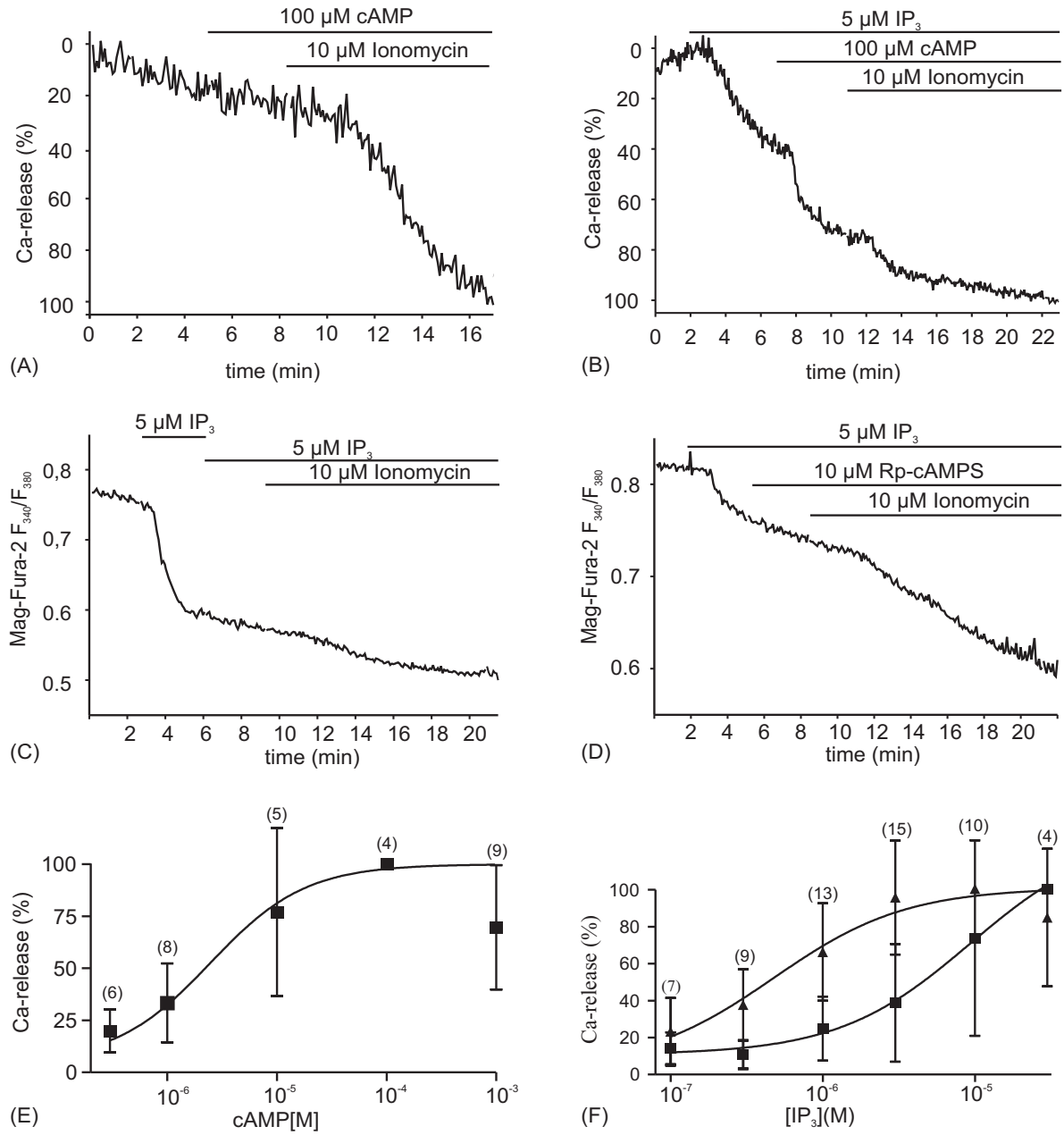

Figure 2

cAMP augments $InsP_3$-induced Ca^{2+} release from β-escin permeabilized cells, as shown by intraluminal Ca^{2+} measurements with Mag-Fura-2. (A) cAMP does not induce Ca^{2+} release from the ER (n = 4). (B) Application of 5 µM $InsP_3$ induces Ca^{2+} release from the ER and is augmented by 100 µM cAMP. (C, D) Ca^{2+} release induced by 5 µM $InsP_3$ is neither enhanced by application of fresh $InsP_3$ solution (C) nor by mock stimulation with Rp-cAMPS (D). (E) Quantification of the cAMP-dependent augmentation of Ca^{2+} release induced by 5 µM $InsP_3$ from experiments as shown in B. 0% Ca^{2+} release is the intraluminal Mag-fura-2 ratio at time zero of the recording; 100% Ca^{2+} release is the fluorescence ratio after complete loss of intraluminal Ca^{2+} following ionomycin application. A sigmoidal dose-response curve fitted to mean values ($R^2 = 0.4$) of the $InsP_3$(+cAMP)-induced Ca^{2+} release gives an $EC_{50,\ cAMP}$ of 2.6 µM. (F) Dose-response relationship for $InsP_3$-induced Ca^{2+} release in the presence (triangles) and absence (squares) of 100 µM cAMP. The leftward shift of the dose-response relationship indicates sensitization of $InsP_3$-induced Ca^{2+} release for $InsP_3$ by cAMP. (E, F) The number of measurements for every data point is given in brackets. Means ± S.D.

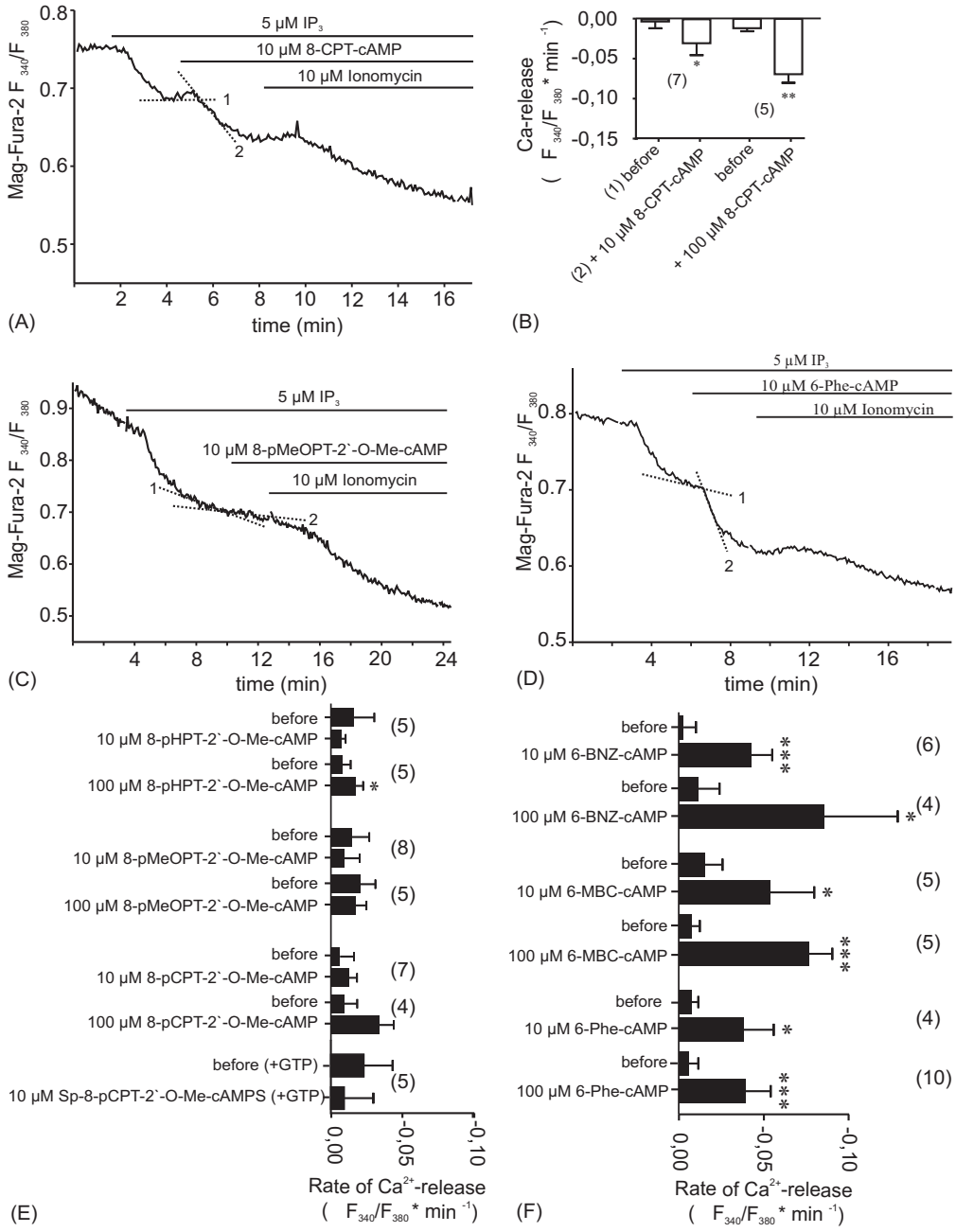

Figure 3

$InsP_3$-induced Ca^{2+} 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 $InsP_3$-induced Ca^{2+} release as recorded by intraluminal Ca^{2+} 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. Ca^{2+} release is displayed as the change in the rate of the Mag-Fura-2 fluorescence ratio ($\Delta F_{340}/F_{380} \cdot min^{-1}$) 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 $InsP_3$-induced Ca^{2+} 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 $InsP_3$-induced Ca^{2+} 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 $InsP_3$-induced Ca^{2+} 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 $InsP_3$-induced Ca^{2+} 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 $InsP_3$-induced Ca^{2+} release. At a concentration of 10 μM none of the Epac activators augmented $InsP_3$-induced Ca^{2+} release (Figs. 3C, E). The Epac-activator 8-pHPT-2'-O-Me-cAMP produced a slight but significant increase in the rate of Ca^{2+} 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 $InsP_3$-induced Ca^{2+} 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 $InsP_3$-induced Ca^{2+}release significantly in a dose-dependent manner (Figs. 3E, F). These findings indicated that the cAMP-dependent augmentation of $InsP_3$-induced Ca^{2+} release was mediated by PKA rather than Epac.

PKA inhibitors block the augmentation of $InsP_3$-induced Ca^{2+} release by cAMP

To examine by an alternative approach whether the cAMP evoked augmentation of the $InsP_3$-induced Ca^{2+} 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 $InsP_3$-induced Ca^{2+} release. Both substances reversed the extra-Ca^{2+} release produced by 8-CPT-cAMP on a background of 5 μM $InsP_3$ (Figs. 4A–D). These results provided further support for our conclusion that the cAMP-evoked augmentation of $InsP_3$-induced Ca^{2+} release was mediated by PKA.

Does cAMP-mediated augmentation of $InsP_3$-induced Ca^{2+} release affect transepithelial electrolyte transport?

The transepithelial potential (TEP) is a sensitive indicator of the transepithelial K^+ and Cl^- transport that results from 5-HT-induced activation of the $InsP_3/Ca^{2+}$ and cAMP signaling pathways, because K^+ transport is activated by cAMP and Cl^- transport is activated by Ca^{2+} [34,38]. We used TEP measurements in order to examine whether cAMP was able to amplify transepithelial Cl^- transport induced (1) by 5-HT concentrations that were just sufficient to stimulate fluid secretion and (2) by saturating 5-HT concentrations. Because cAMP also stimulates transepithelial K^+ transport by activating an apical vacuolar-type H^+-ATPase that energizes K^+ transport [33,42,43], we had to minimize the contribution of transepithelial K^+ transport to 5-HT-induced TEP changes. This was accomplished by using a K^+-free PS containing 7.5 mM of the K^+ channel blocker Ba^{2+} to block basolateral K^+ entry [44], as illustrated in Fig. 5A. A brief control stimulation with 30 nM 5-HT produced a biphasic change of the TEP. The negative-going phase of the TEP change was attributable to transepithelial Cl^- transport, and the positive-going phase was caused by the somewhat delayed transepithelial K^+ transport [34]. Superfusion of the preparation with $BaCl_2$-containig PS caused the TEP to become negative by about 10 mV, because the resting TEP was slightly positive attributable to some transepithelial K^+ transport in the unstimulated gland. Upon application of 1 nM 5-HT to the $BaCl_2$-containing PS, the TEP became more negative (Fig. 5A), as a result of 5-HT-induced Ca^{2+} release [26] and a Ca^{2+}-induced activation of transepithelial Cl^- transport. Most significantly, 500 μM IBMX caused the TEP to become even more negative in the presence of 1 nM 5-HT. The effects of IBMX, 5-HT, and Ba^{2+} were reversible. Fig. 5B summarizes the results of several experiments of this kind and displays the TEP recorded at four selected time points indicated in Fig. 5A. The experiment illustrated in Fig. 5C is identical, except that the preparation was stimulated with 30 nM 5-HT, a concentration that saturates the rate of fluid transport. At this high 5-HT concentration, IBMX caused no further change of the TEP (Fig. 5D).

The results of these TEP measurements indicate that an increase in intracellular cAMP concentration (by application of the phosphodiesterase inhibitor IBMX) augments the effect of a threshold concentration of 5-HT on transepithelial Cl^- transport. This result is in agreement with above finding that cAMP sensitizes the $InsP_3R$ Ca^{2+} channel for $InsP_3$. The physiological consequence of $InsP_3R$ sensitization is measurable only when the glands are stimulated by low 5-HT concentrations.

Discussion

The results of this study provide physiological evidence that cAMP augments $InsP_3$-induced Ca^{2+} release from the ER in the salivary glands of *Calliphora vicina*, a dipteran fly closely related to *Drosophila melanogaster*. Our intraluminal Ca^{2+} measurements in the ER of permeabilized cells in isolated glands show, in addition, that cAMP increases the affinity of the $InsP_3R$ for $InsP_3$ 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 Ca^{2+} measurements and electrophysiological recordings

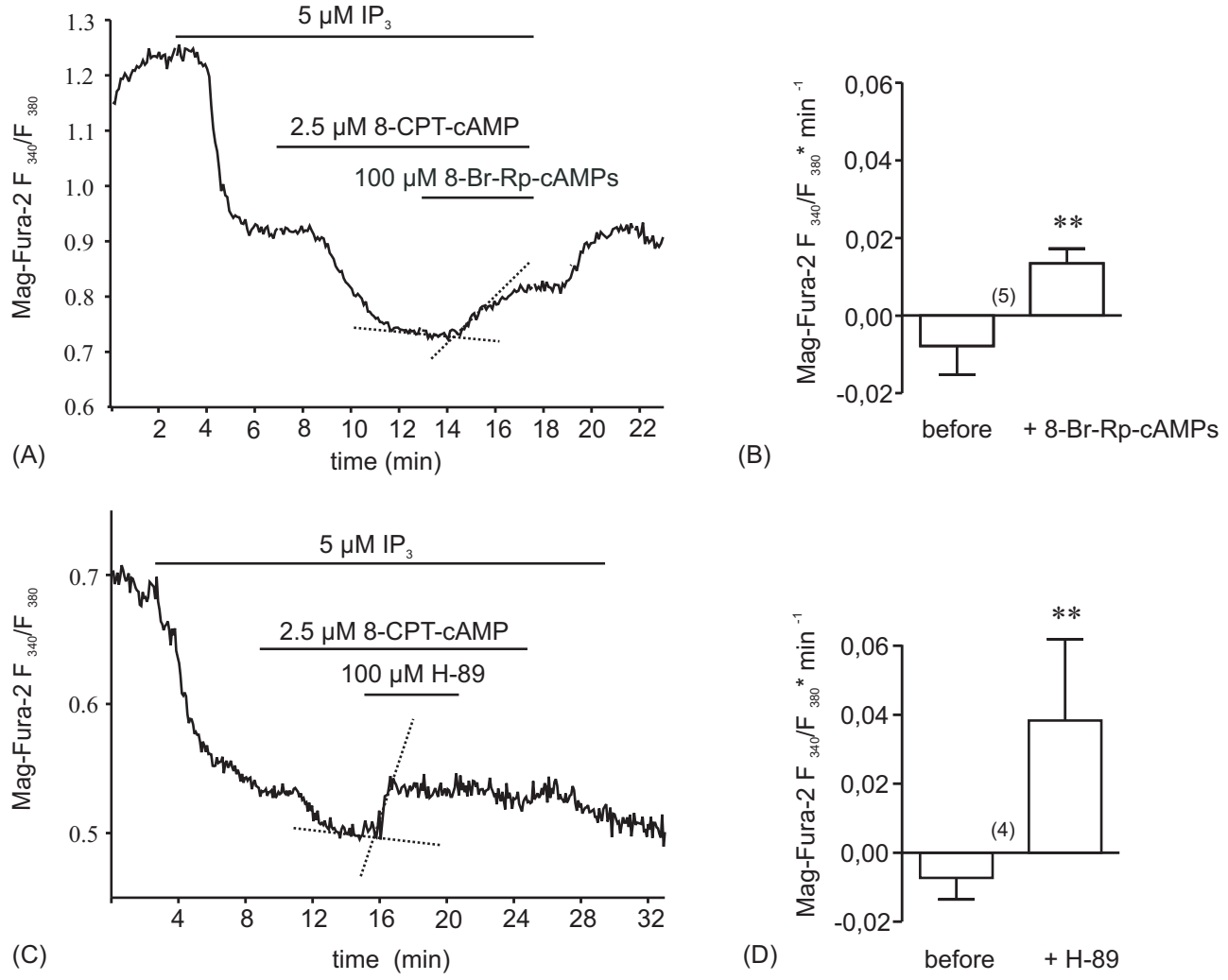

Figure 4

The competitive antagonist of cAMP-binding to PKA, 8-Br-Rp-cAMPS (A, B), and the PKA inhibitor H-89 (C, D) reverse augmentation of $InsP_3$-induced Ca^{2+} release caused by 8-CPT-cAMP. Graphs constructed as described for Fig. 3.

indicate that the cAMP-induced and PKA-mediated sensitization of the $InsP_3R$ for $InsP_3$ affects Ca^{2+} signaling and transepithelial electrolyte transport.

cAMP-induced and PKA-mediated augmentation of $InsP_3$-induced Ca^{2+} release

All three mammalian $InsP_3R$ subtypes have the potential to undergo phosphorylation by PKA and by some other kinases including PKG, PKC and CaM-kinase [22,45]. The resulting phosphoregulation of Ca^{2+} release is thought to have profound effects on the spatio-temporal characteristics of Ca^{2+} signals and to provide a potential mechanism of crosstalk between different signaling pathways. Nevertheless, data on the effects of $InsP_3R$ phosphorylation on $InsP_3$-induced Ca^{2+} release are contradictory (reviewed in [1,46]). Most reports suggest that $InsP_3R$ phosphorylation augments $InsP_3$-induced Ca^{2+} release (e.g. [12,15,17,47-49]], whereas others indicate that Ca^{2+} release is attenuated [e.g. [14,50]].

Here, we show that cAMP augments $InsP_3$-induced Ca^{2+} 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 $InsP_3$ suggests that the augmentation of Ca^{2+} release is attributable to an increase of about 10-fold in the affinity of the $InsP_3R$ Ca^{2+} channel for $InsP_3$. We can exclude the possibility that the cAMP-induced augmenta-

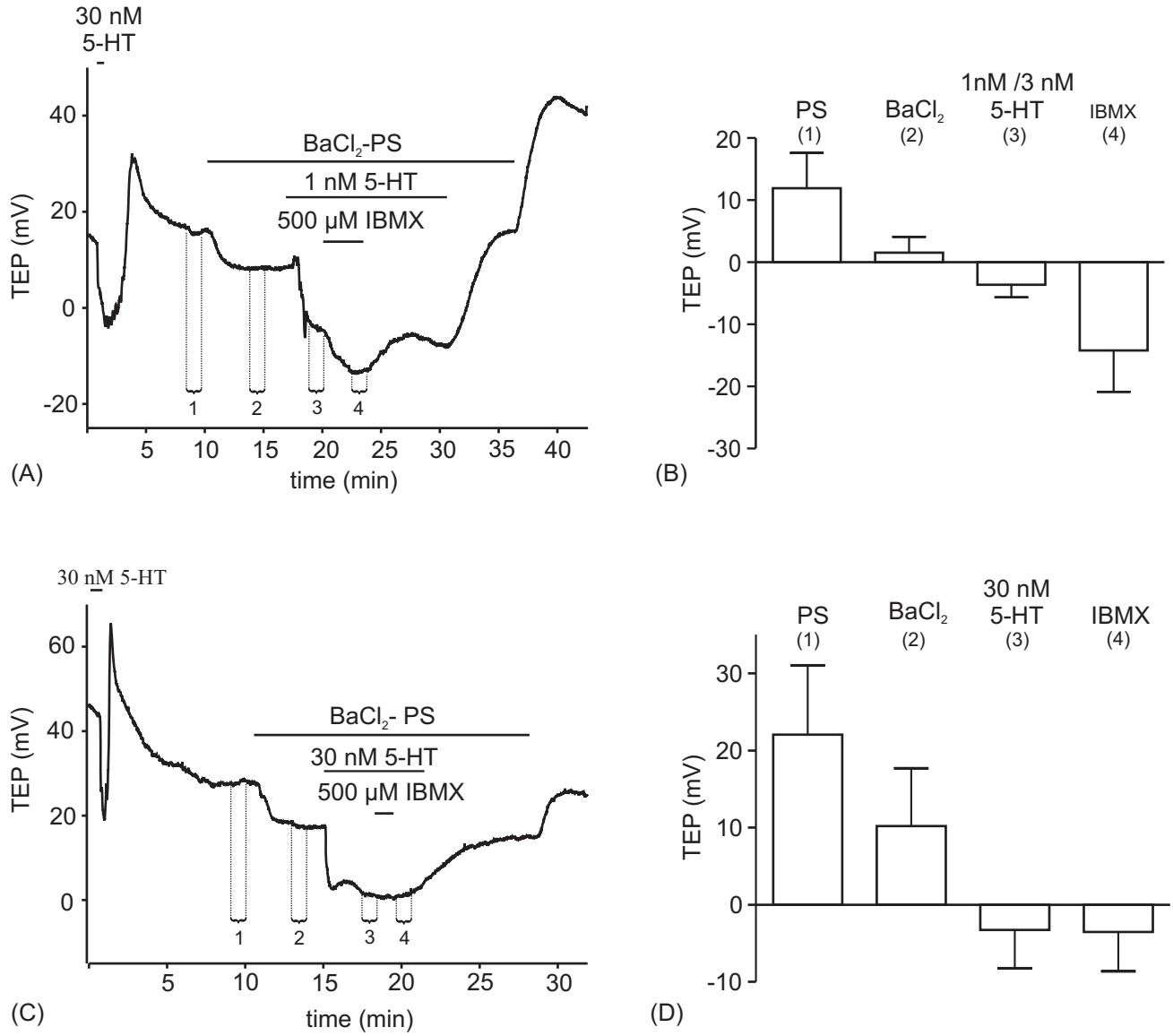

Figure 5

Effects of IBMX on 5-HT-induced changes in transepithelial potential (TEP) in Ba^{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^{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.

tion of Ca^{2+} release results from a stimulation of Ca^{2+} loading of the ER via SERCA, because the intraluminal Ca^{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 $InsP_3R$. However, although six potential PKA phosphorylation sites have been detected in the sequence of *Caenorhabditis elegans* $InsP_3R$, no such sites have been identified in *Drosophila melanogaster* $InsP_3R$ ($DmInsP_3R$) [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* $InsP_3$ receptor. We experi-

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* $InsP_3R$ is still lacking but the dipteran fly *Calliphora* is closely related to *Drosophila*. Thus, whether fly $InsP_3$ receptor Ca^{2+} channels can be phosphorylated, or whether the $InsP_3R$ 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 Ca^{2+} release in this species. $DmInsP_3R$ 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 $DmInsP_3R$ via other kinases or unknown accessory proteins that are phosphorylated by PKA.

Physiological consequences of cAMP-mediated sensitization of the InsP3R for $InsP_3R$ for $InsP_3$

The cAMP-mediated sensitization of the $InsP_3R$ for $InsP_3$ has measurable effects on Ca^{2+} signaling in *Calliphora* salivary glands. We have shown that increasing the intracellular cAMP concentration converts baseline Ca^{2+} spiking induced by threshold concentrations of 5-HT [26] into a sustained Ca^{2+} elevation. This effect of cAMP on Ca^{2+} spiking is remarkably similar to that reported for the parotid acinar cell. Here, forskolin potentiates carbachol-induced $[Ca^{2+}]_i$ changes, and this potentiation also results from enhanced Ca^{2+} release attributable to cAMP-dependent and PKA-mediated potentiation of $InsP_3$-induced Ca^{2+} release from the ER [17]. The enhanced Ca^{2+} release is probably not the result of a cAMP-dependent stimulation of $InsP_3$ production [17], although cAMP has been shown to potentiate $InsP_3$ 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 [^{3}H]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 $InsP_3R$ Ca^{2+} channel obviously functions as a coincidence detector [18] that monitors a simultaneous increase of $InsP_3$, cAMP, and Ca^{2+} concentrations, the last-mentioned because $InsP_3R$ is also regulated by Ca^{2+} [reviewed in [22]].

Recordings of the transepithelial potential (TEP) in *Calliphora* salivary glands indicate that cAMP also augments the Ca^{2+}-dependent transepithelial Cl^- transport induced by low 5-HT concentrations, an observation suggesting that the cAMP-dependent enhanced Ca^{2+} 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 $InsP_3$-induced Ca^{2+} release from the ER resulting in enhanced Ca^{2+} 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 $CaCl_2$, 2 $MgCl_2$, 2.8 maleic acid, 3 sodium glutamate, 10 TRIS-HCl, 10 D-Glucose, pH 7.2. Ca^{2+}-free PS was prepared by omitting $CaCl_2$ 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 $MgCl_2$, 3 Na_2ATP, 0.1 EGTA, 0.06 $CaCl_2$, 10 HEPES at pH 7.3. The free Ca^{2+} concentration in this medium was determined to be ~250 nM, as noted previously [32]. GTP-ICM contained (mM): 125 KCl, 20 NaCl, 2 $MgCl_2$, 3 Na_2ATP, 3 GTP, 0.1 EGTA, 0.06 $CaCl_2$, 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 Ca^{2+} 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-

gies Inc., Rochester, NY) was used for data acquisition and storage, and SigmaPlot 8.0 software for offline data analysis.

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 acetoxymethylester in PS for 40–60 min at room temperature. After dye loading, the gland tubules were mounted on cover slips coated with VectaBond™ (Axxora, Grünberg, 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 $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured as described previously [26]. In brief, pairs of fluorescence images, excited at wavelengths of 340 nm and 380 nm (VisiChrome High Speed Polychromator 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-512EFT; 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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