The mechanism mediating regenerative intercellular Ca^{2+} waves in the blowfly salivary gland

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Intercellular Ca^{2+} signaling in intact salivary glands of the blowfly *Calliphora erythrocephala* was studied by fluorimetric digital imaging combined with microinjection of putative messenger molecules. Iontophoretic injection of D-myo-inositol 1,4,5-trisphosphate (InsP_3) into salivary gland cells evoked regenerative intercellular Ca^{2+} waves that spread through the impaled cell and several rows of surrounding cells. Ca^{2+} increases induced by microinjection of Ca^{2+} ions were confined to the injected cells and their nearest neighbors. Depletion of intracellular Ca^{2+} stores by thapsigargin pretreatment did not alter the time course of the Ca^{2+} increase caused by Ca^{2+} injection. However, activation of Ca^{2+} release became clearly evident when Ca^{2+} was injected in the presence of serotonin (5-HT). Under these conditions, injection of Ca^{2+} triggered intercellular Ca^{2+} waves that consecutively passed through >10 cells. The phospholipase C inhibitor U73122 blocked 5-HT-induced Ca^{2+} increases but did not affect InsP_3 -dependent Ca^{2+} spiking and intercellular Ca^{2+} wave propagation. The results demonstrate that propagation of agonist-evoked Ca^{2+} waves in the blowfly salivary gland requires supra-basal $[\text{InsP}_3]$ but does not depend on feedback activation of phospholipase C. We conclude that the intra- and intercellular transmission of these Ca^{2+} waves is mediated by diffusion of Ca^{2+} and Ca^{2+} -induced Ca^{2+} release via the InsP_3 receptor channel.
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Introduction

One of the most fundamental mechanisms for relaying extracellular signals to the interior of cells is through changes in the free cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (Berridge, 1993). The major pathway of Ca^{2+} elevation in electrically non-excitable cells involves the activation of the inositol 1,4,5-trisphosphate (InsP_3) second messenger system, which releases Ca^{2+} from intracellular Ca^{2+} stores. The resulting increases in $[\text{Ca}^{2+}]_i$ are often temporally 'organized' to form Ca^{2+} oscillations or repetitive Ca^{2+} spikes, and/or spatially coordinated generating intracellular Ca^{2+} waves. In groups of cells, intracellular waves may give rise to intercellular Ca^{2+} waves that propagate through gap junctions. Although such intercellular Ca^{2+} waves have been observed in a large number of systems, including small groups of acutely isolated cells (Sáez *et al.*, 1989;

Yule *et al.*, 1996; Tordjmann *et al.*, 1997), cultured cells (Cornell-Bell *et al.*, 1990; Charles *et al.*, 1991; Boitano *et al.*, 1992; Finkbeiner, 1992; Xia and Ferrier, 1992; Demer *et al.*, 1993; Domenighetti *et al.*, 1998) and intact tissues (Nathanson *et al.*, 1995; Robb-Gaspers and Thomas, 1995; Newman and Zahs, 1997; Zimmermann and Walz, 1997), the underlying mechanisms are still poorly understood. The identity of the chemical signal mediating intercellular Ca^{2+} wave transmission has been the subject of numerous experimental and theoretical studies, and both InsP_3 and Ca^{2+} have been proposed as serving as the intercellular messenger (Sanderson *et al.*, 1990, 1994; Sneyd *et al.*, 1994; Yule *et al.*, 1996; Zimmermann and Walz, 1997). It seems likely, at present, that intercellular Ca^{2+} waves fall into one of at least two different categories whose underlying mechanisms may depend on the type of cell and/or stimulation: Ca^{2+} waves elicited by local mechanical stimulation appear to depend on the local formation and diffusion of InsP_3 , since their amplitude tends to diminish rapidly with distance (Demer *et al.*, 1993; Churchill *et al.*, 1996; Churchill and Louis, 1998). Agonist-evoked intercellular Ca^{2+} waves, on the other hand, can spread over great distances without decrement of amplitude and at a constant velocity, indicating that their propagation involves a regenerative mechanism (Nathanson *et al.*, 1995; Robb-Gaspers and Thomas, 1995; Newman and Zahs, 1997; Zimmermann and Walz, 1997).

Current models of agonist-induced regenerative intra- and intercellular Ca^{2+} waves have been developed by extending models describing local InsP_3 -mediated Ca^{2+} oscillations into the spatial domain (Cuthbertson and Chay, 1991; Jaffe, 1991, 1993; Meyer and Stryer, 1991; De Young and Keizer, 1992; Atri *et al.*, 1993). These models postulate spatio-temporal fluctuations either of InsP_3 (Meyer and Stryer, 1991) or of Ca^{2+} at constant $[\text{InsP}_3]$ (Goldbeter *et al.*, 1990; Jaffe, 1991; DeLisle and Welsh, 1992; Lechleiter and Clapham, 1992; Atri *et al.*, 1993). Thus, it has been suggested that propagating waves of elevated $[\text{InsP}_3]$ might arise from InsP_3 diffusion, InsP_3 -dependent Ca^{2+} release and feedback activation of phospholipase C (PLC) by released Ca^{2+} (Meyer and Stryer, 1991), InsP_3 , diacylglycerol or other metabolic products of PLC activity (Cuthbertson and Chay, 1991; Sneyd *et al.*, 1994). Alternative models are based on the finding that the opening of the InsP_3 receptor channel is regulated both positively and negatively by Ca^{2+} (Baumann and Walz, 1989; Iino, 1990; Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991). According to these latter models, Ca^{2+} waves propagate in the presence of constant supra-basal $[\text{InsP}_3]$ by Ca^{2+} diffusion and Ca^{2+} -induced Ca^{2+} release via the InsP_3 receptor.

The purpose of the present study has been to dissect the molecular mechanisms underlying agonist-evoked intercellular Ca^{2+} wave propagation by using the intact

blowfly salivary gland as a model system. Previous studies have shown that the temporal characteristics of agonist-induced Ca^{2+} signaling in the secretory portion of the blowfly salivary gland is markedly influenced by the agonist concentration (Rapp and Berridge, 1981; Zimmermann and Walz, 1997; Zimmermann, 1998): stimulation with low doses of serotonin (5-HT, 1–10 nM) evokes repetitive Ca^{2+} spikes and intercellular Ca^{2+} waves mediated by Ca^{2+} fluxes across the membranes of intracellular Ca^{2+} -storing organelles. Application of higher agonist concentrations results in responses consisting of initial transient Ca^{2+} release followed by Ca^{2+} entry across the plasma membrane. Extensive previous work has also demonstrated indirectly that these $[\text{Ca}^{2+}]_i$ changes are mediated by increases in the intracellular concentration of InsP_3 (Fain and Berridge, 1979; Berridge, 1983; Berridge *et al.*, 1983).

We report that intracellular increases of InsP_3 , brought about by local iontophoretic injection of InsP_3 , are sufficient to generate repetitively regenerative intercellular Ca^{2+} waves in the absence of receptor-mediated PLC activation. These InsP_3 -induced intercellular Ca^{2+} waves are not blocked by the inhibition of endogenous PLC activity. The ability of increases of $[\text{Ca}^{2+}]_i$, evoked by microinjection, to trigger Ca^{2+} release and Ca^{2+} waves critically depends on the presence of a low dose of hormone. Taken together, this study has failed to provide evidence for an involvement of feedback activation of PLC in triggering and supporting agonist-evoked intercellular Ca^{2+} waves. Rather, the present findings are consistent with Ca^{2+} acting as the diffusing intra- and intercellular messenger and Ca^{2+} -induced Ca^{2+} release through the InsP_3 receptor acting as the local feedback mechanism.

Results

Morphological characteristics of the blowfly salivary gland

The paired secretory segments of the tubular salivary gland of the blowfly *Calliphora erythrocephala* consist of a single layer of uniformly differentiated cells grouped around a central lumen (Figure 1). Lateral diameters of the secretory cells range from 15 to 20 μm . Morphological data (Oschman and Berridge, 1970; our unpublished data) and the intercellular diffusion of iontophoretically injected Lucifer yellow CH (data not shown) demonstrate that neighboring cells within the epithelium are coupled by gap junctions.

Spatio-temporal characteristics of InsP_3 -induced Ca^{2+} signaling

In order to test whether increases in $[\text{InsP}_3]$ were sufficient to evoke the Ca^{2+} responses elicited by agonist, InsP_3 was injected iontophoretically into individual cells of the intact salivary gland while monitoring $[\text{Ca}^{2+}]_i$ with fura-2 and Ca^{2+} imaging. The injection of InsP_3 indeed mimicked the 5-HT-evoked 'peak-and-plateau' responses in the impaled cell (Figures 2A and 3A). Regular, repetitive Ca^{2+} spikes as seen in the presence of 5-HT were, however, difficult to produce initially. The reason for this might have been that high local $[\text{InsP}_3]$ around the tip of the injection pipette caused Ca^{2+} entry that interfered with coordinated Ca^{2+} spiking. However, a reduction of the

Fig. 1. Differential interference contrast micrograph of a segment of the secretory portion of the blowfly salivary gland. Secretory cells within the tubular single-layered epithelium have a diameter of 15–20 μm . Arrowheads indicate the nuclei of adjacent cells. Scale bar = 100 μm .

Fig. 2. Comparison of Ca^{2+} responses evoked by microinjection of InsP_3 and application of 5-HT. (A) Microinjection of InsP_3 (10 mM concentration in the pipette; –10 nA, 100 ms, 0.5 Hz; left panel) and bath application of 100 nM 5-HT in the presence of 2 mM Ca^{2+}_o (right panel). (B and C) Injection of InsP_3 (10 mM concentration in pipette; –10 nA, 100 ms, 0.5 Hz; left panels) and bath application of 5-HT medium (right panels) in nominally Ca^{2+} -free solution.

extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) extended the range of 5-HT concentrations eliciting Ca^{2+} spiking (Zimmermann and Walz, 1997). To exploit this observation, InsP_3 was administered to glands that were bathed in nominally Ca^{2+} -free medium. Under these conditions,

Fig. 3. Spatio-temporal characteristics of InsP_3 -evoked Ca^{2+} responses. (A) Stimulation with InsP_3 delivered at increasing rates and in the presence of 2 mM Ca^{2+}_o (10 mM concentration in pipette; first stimulation –10 nA, 100 ms, 0.5 Hz; second stimulation –1 nA, continuously; third stimulation –5 nA, continuously). The signal was integrated over an area of 5×5 pixels at the injection site. (B–D) Pseudocolor images of $[\text{Ca}^{2+}]_i$ distribution during stimulation with InsP_3 . Images were taken at the peak of the initial Ca^{2+} transient (arrows in A). The size of the activated region increases with the rate of InsP_3 delivery. (E) Space–time plot of the first stimulation shown in (A). The white line in (B) marks the location of pixels selected to construct the plot. (F and G) InsP_3 -evoked Ca^{2+} changes in nominally Ca^{2+} -free solution. (F) Image taken at the peak of the initial Ca^{2+} transient. (G) Space–time plot of the InsP_3 -induced Ca^{2+} elevation. The line selected to construct the plot is shown in (F) (white line). Vertical bars = 100 μm , horizontal bars = 60 s.

the local response to the continuous injection of InsP_3 (10 mM concentration in the pipette; –10 nA, 100 ms, 0.5–1 Hz) resembled responses elicited by 5-HT in low Ca^{2+} media (Figure 2B and C). They consisted of a single initial transient or an initial rapid series of incompletely fused Ca^{2+} spikes followed by repetitive Ca^{2+} spikes (frequency: $6.1 \pm 0.58/\text{min}$; $n = 9$), as long as current was applied to the electrode.

The analysis of the spatio-temporal characteristics of the $[\text{Ca}^{2+}]_i$ dynamics induced by InsP_3 revealed a rapid radial spread of the Ca^{2+} increase after the onset of injection. The velocity of this spread was variable, reaching maximum values that were beyond the temporal resolution of our experiments (i.e. $>30 \mu\text{m/s}$), and was thus considerably faster than the velocity of 5-HT-induced Ca^{2+} waves (12–17 $\mu\text{m/s}$; Zimmermann and Walz, 1997). Regions of elevated $[\text{Ca}^{2+}]_i$ reached radii of 50–70 μm , indicating a Ca^{2+} increase in the first, second and third tier neighbors of the impaled cell. Increasing the rate of InsP_3 delivery further increased the number of cells participating in the Ca^{2+} response (Figure 3B–D). The edges of InsP_3 -induced Ca^{2+} activity usually exhibited oscillatory fluctuations in $[\text{Ca}^{2+}]_i$ (Figure 2E). In nominally Ca^{2+} -free solutions, as in the presence of 2 mM Ca^{2+}_o , InsP_3 -induced Ca^{2+}

elevations initially spread from the impaled cell, thereby reaching radii of up to 70 μm . This corresponded to a spread of the response into the the third and possibly fourth row of neighboring cells. The Ca^{2+} spiking activity following the initial transient Ca^{2+} increase within this region was accompanied by propagating intercellular Ca^{2+} waves (Figures 3G and 4B–D). During individual stimulations, these Ca^{2+} waves were triggered at different locations within the region that displayed the initial Ca^{2+} transient. Their amplitude was unrelated to the distance from the point of initiation (Figure 4) from which they progressed at constant velocity. This indicated that the InsP_3 -induced Ca^{2+} waves were regenerative waves. The mean propagation rate determined in experiments in which the Ca^{2+} waves clearly followed the longitudinal axis of the gland was $13.8 \pm 1.96 \mu\text{m/s}$ ($n = 21$; three glands) and was similar to the velocity of 5-HT-evoked Ca^{2+} waves (12–17 $\mu\text{m/s}$; Zimmermann and Walz, 1997).

Reversal of the current polarity or microinjection of the biologically inactive analog *L*-myo-inositol 1,4,5-trisphosphate (10 mM), Lucifer yellow CH [5% (w/v) in H_2O] or K^+ (3 M KCl in the pipette), when injected under identical conditions, evoked no measurable changes in $[\text{Ca}^{2+}]_i$ ($n = 4$, respectively). This demonstrated the

Fig. 4. Effects of iontophoretic $InsP_3$ injection in nominally Ca^{2+} -free solution. (A) Temporal characteristics of $[Ca^{2+}]_i$ changes induced by $InsP_3$ (–10 nA, 100 ms, 1 Hz) recorded from four different locations (colored boxes in C). The position of the injection pipette is indicated in (C). In this recording, $InsP_3$ leaking from the pipette caused repetitive Ca^{2+} spiking at the injection site before current was applied (black trace). (B) Space–time plot of the Ca^{2+} dynamics; broken lines were fitted to advancing wave fronts and show the linear progression of the intercellular Ca^{2+} waves. The location of the pixels that were selected from the series of two-dimensional images to construct the plot is shown in (C), first image (black line); the time window shown is indicated by the long red arrow in (A). Vertical bar = 100 μm , horizontal bar = 60 s. (C and D) Series of pseudocolor $[Ca^{2+}]$ images depicting propagating intercellular Ca^{2+} waves. Selected time segments are indicated by the shaded areas in (A). Arrowheads mark the sites of wave initiation. Dotted lines indicate the lateral borders of the activated area. Image intervals, 1 s; scale bar in (C) = 100 μm .

specificity of the responses to D-*myo*-inositol 1,4,5-trisphosphate and ensured that the cells were not responding to changes in membrane potential.

In order to test whether the metabolism of $InsP_3$ was necessary to sustain Ca^{2+} spiking and intercellular Ca^{2+} wave propagation, we microinjected the non-hydrolyzable $InsP_3$ analog *myo*-inositol 1,4,5-trisphosphorothioate ($InsP_3S_3$). Iontophoretic injection of $InsP_3S_3$ (concentration in pipette: 1 mM; –10 nA, 100 ms, 1 Hz) resulted in response patterns that qualitatively and quantitatively resembled the responses to $InsP_3$ (Figure 5).

Microinjection of Ca^{2+}

In contrast to $InsP_3$, microinjection of Ca^{2+} ions (10 mM $CaCl_2$ in the pipette, +10 nA, 0.5–1 Hz) resulted in a monotonous and reversible Ca^{2+} increase in the impaled cell, and this spread only to maximum radii of 20–30 μm (i.e. involving the first row of neighboring cells), although the Ca^{2+} concentrations reached in the impaled cells were similar to the Ca^{2+} elevations evoked by the injection of $InsP_3$ (Figure 6A and C). Increasing the rate of Ca^{2+} injection caused greater increases of $[Ca^{2+}]_i$ in the injected cell and a further spread of the Ca^{2+} elevation.

To determine whether intercellular Ca^{2+} waves could be initiated by local Ca^{2+} increases in the presence of supra-basal $[InsP_3]$, Ca^{2+} ions were microinjected while

superfusing the glands with a low concentration of 5-HT (1 or 3 nM). Under these conditions, propagating intercellular Ca^{2+} waves could be elicited repetitively by experimentally increasing $[Ca^{2+}]_i$ (Figure 6B and D). Thus, Ca^{2+} elevations were sufficient to trigger Ca^{2+} waves when $InsP_3$ reached a critical supra-basal concentration.

However, this does not rule out the possibility that the Ca^{2+} elevations, evoked by Ca^{2+} injection, cause the production of $InsP_3$, which in turn triggers the Ca^{2+} waves. It has been shown that PLC can be activated by Ca^{2+} *in vitro* (Rhee *et al.*, 1989) and in specific cells (Harootunian *et al.*, 1991). Thus, in the salivary gland, regeneration of $InsP_3$ induced by increased Ca^{2+} may have played a role in Ca^{2+} wave initiation and propagation.

To test this possibility, the time courses of the Ca^{2+} increases evoked by Ca^{2+} injection were analyzed and compared with the respective Ca^{2+} increases in cells whose Ca^{2+} stores were depleted by a combined treatment with thapsigargin (1 μM) and 5-HT (100 nM) (Figure 7B, left panel, inset). The time courses of the Ca^{2+} increases in both groups of experiments were indistinguishable. They could be well fitted by a single exponential function (lines in Figure 7A and B, right panels) with rate constants of $0.07 \pm 0.027/s$ ($n = 6$) and $0.09 \pm 0.044/s$ ($n = 7$), respectively ($P = 0.32$). Thus, Ca^{2+} release induced by

Fig. 5. Iontophoretic injection of $InsP_3S_3$. (A) Time course of $[Ca^{2+}]_i$ changes evoked by microinjection of $InsP_3S_3$ and integrated over a window of 5×5 pixels (white box in B, first image). (B and C) Series of images showing propagating intercellular Ca^{2+} waves. Image intervals, 1 s; scale bar in (B) = 100 μm .

experimentally increasing $[Ca^{2+}]_i$ was not detectable. In contrast, the time courses of Ca^{2+} increases during injection of Ca^{2+} in the presence of agonist (1 or 3 nM 5-HT, cf. Figure 5B) were strikingly different. Here, the rate of Ca^{2+} increase exhibited a marked acceleration during the first 5 s of injection (Figure 7C), whereas the respective rate continuously decreased in the absence of hormone (Figure 7A and B, right panels). This was clearly indicative of the activation of Ca^{2+} release as a consequence of Ca^{2+} injection in the presence of the hormone.

Injection of $InsP_3$ into glands pre-treated with U73122

In a second approach to determine a possible involvement of Ca^{2+} -mediated activation of PLC in Ca^{2+} wave propagation, we attempted to interfere with the production of endogenous $InsP_3$ by directly inhibiting PLC with the aminosteroid U73122 (Bleasdale *et al.*, 1990). As shown in Figure 8, U73122 (10 μM , 10 min pre-incubation) completely abolished 5-HT-dependent Ca^{2+} changes (in six out of six glands), whereas treatment with the inactive structural analog U73343 was without effect. Injection of $InsP_3$ into glands pre-treated with U73122 induced Ca^{2+} responses that resembled the previously observed $InsP_3$ -induced Ca^{2+} changes (Figure 9A and B). The mean frequency of Ca^{2+} spiking was $5.1 \pm 1.68/s$ ($n = 8$; seven preparations). As in the absence of U73122, $InsP_3$ -induced Ca^{2+} spiking in the activated region was temporally and spatially coordinated and was accompanied by the propagation of intercellular Ca^{2+} waves (Figure 9C–E).

Discussion

Intercellular Ca^{2+} waves represent a common mechanism by which cells that are coupled by gap junctions coordinate

responses to various types of external stimuli that activate the phosphoinositide pathway (Berridge, 1993). Although this type of signaling has received considerable attention, the way in which Ca^{2+} waves propagate between cells is so far poorly understood.

The mechanism of intercellular Ca^{2+} wave propagation

The results reported here demonstrate that the microinjection of $InsP_3$ into a single cell of the intact secretory epithelium of the salivary gland in *Calliphora* is able to trigger Ca^{2+} release not only in the impaled cell, but also in a group of neighboring cells. Similar coordinated responses to the local application of $InsP_3$ have been observed in hepatocytes (Sáez *et al.*, 1989), pancreatic acinar cells (Yule *et al.*, 1996), tracheal epithelial cells (Sanderson *et al.*, 1990, 1994) and lens epithelial cells (Churchill and Louis, 1998). These studies were performed either with small groups of cells or by applying single short pulses of $InsP_3$ and thus failed to show whether $InsP_3$ mimicked the effect of agonists in producing repetitive Ca^{2+} spiking, and whether the intercellular spread of the Ca^{2+} signal was the result of: (i) the passive diffusion of $InsP_3$ or a locally produced messenger; or (ii) active intercellular propagation as seen in the presence of agonists. In the salivary gland, responses to the prolonged microinjection of $InsP_3$ consisted of two components. $InsP_3$ injection was followed by initial transient increases in $[Ca^{2+}]_i$ spreading from the impaled cell into neighboring cells. This spread took place at variable rates and probably reflected the intercellular diffusion of $InsP_3$ at the onset of $InsP_3$ injection. Subsequently, trains of Ca^{2+} waves propagated in the presence of continuously elevated $[InsP_3]$ within the region that showed the initial Ca^{2+} transient. These Ca^{2+} waves were propagated at a constant velocity

Fig. 6. Iontophoretic injection of Ca^{2+} . (A and C) Injection of Ca^{2+} (10 mM concentration in pipette; 10 nA, 100 ms, 1 Hz) into glands bathed in nominally Ca^{2+} -free medium causes a monotonous increase in $[\text{Ca}^{2+}]_i$. Regions of increased $[\text{Ca}^{2+}]_i$ involve the impaled cell and its nearest neighbors. (B and D) Injection of Ca^{2+} (10 mM concentration in pipette; 10 nA, 100 ms, 1 Hz) in the presence of a background of 5-HT (1 nM) triggers Ca^{2+} waves that propagate over distances $>150 \mu\text{m}$. Image intervals, 2 s; scale bars in (C) and (D) = 100 μm . The pixel areas used to construct the tracings in (A) and (B) are indicated by the white boxes in (C) and (D). Gray boxes in (A) and (B) mark the time segments shown in (C) and (D).

and with no decrement in amplitude through several cells, demonstrating that elevations of $[\text{InsP}_3]$ were sufficient to support regenerative activity. Such active wave propagation requires a positive feedback step in which the diffusing messenger induces the generation of more messenger. Currently proposed models of intra- and intercellular Ca^{2+} waves differ with respect to this feedback mechanism and incorporate either Ca^{2+} -dependent PLC activation, InsP_3 formation and InsP_3 -induced Ca^{2+} release (Meyer and Streyer, 1991; Sneyd *et al.*, 1994), or Ca^{2+} -induced Ca^{2+} release from InsP_3 -sensitive or insensitive stores (Goldbeter *et al.*, 1990; Atri *et al.*, 1993). Both InsP_3 and Ca^{2+} have been proposed to serve as the intra- or intercellular messenger in this type of Ca^{2+} wave (Meyer and Streyer, 1991; Jaffe, 1993; Sneyd *et al.*, 1994; Yule *et al.*, 1996; Zimmermann and Walz, 1997). An intrinsic feature of all of the above-mentioned models is that local Ca^{2+} spiking and Ca^{2+} wave propagation are closely interrelated events. Furthermore, all models predict that increases in InsP_3 can evoke repetitive Ca^{2+} spiking and regenerative Ca^{2+} waves. In the salivary gland, this is indeed indicated by the finding that local Ca^{2+} spiking induced by either stimulation with hormone or microinjection of InsP_3 is accompanied by intercellular Ca^{2+} waves, unless intercellular communication via gap junctions is blocked (Zimmermann and Walz, 1997; this study). Feed-

back regeneration of the messenger as an event downstream of receptor-mediated InsP_3 generation in the salivary gland has been demonstrated by the ability of InsP_3 to initiate and maintain repetitive intercellular signaling in the absence of hormone. An important observation in this context is the finding that the number of cells activated by microinjection of InsP_3 depends on the rate of InsP_3 delivery. Since the prolonged injection of InsP_3 creates a region of elevated $[\text{InsP}_3]$ around the tip of the injection electrode, this suggests that the presence of supra-basal $[\text{InsP}_3]$ is a prerequisite for Ca^{2+} waves to propagate (see also Zimmermann and Walz, 1997). However, the establishment of an $[\text{InsP}_3]$ gradient along which the waves might spread does not seem to be a critical requirement, because the pathway of Ca^{2+} waves within the activated region has no strict spatial relationship to the putative concentration gradient emanating from the tip of the injection electrode (cf. Figures 6E and 9C–E). As summarized in Figures 6 and 7, the injection of Ca^{2+} into naive glands fails to initiate Ca^{2+} waves or local Ca^{2+} release, whereas Ca^{2+} injections readily trigger Ca^{2+} release and Ca^{2+} waves in the presence of 5-HT, i.e. at elevated $[\text{InsP}_3]$. This shows that increases in $[\text{Ca}^{2+}]_i$ alone are insufficient to induce Ca^{2+} release. Thus, potentiation of Ca^{2+} release is unlikely to be mediated by Ca^{2+} sensitization of the InsP_3 receptor to basal $[\text{InsP}_3]$ or by

Fig. 7. Temporal analysis of Ca^{2+} increases evoked by Ca^{2+} injections. (A) Left panel: typical time course of $[\text{Ca}^{2+}]_i$ increase (●). Injection started at $t = 0$. Right panel: rates of $[\text{Ca}^{2+}]_i$ increases during the first 20 s of Ca^{2+} injection, taken from six independent experiments (mean $\pm$ SD). (B) $[\text{Ca}^{2+}]_i$ increase induced by iontophoretic injection of Ca^{2+} after Ca^{2+} store depletion with thapsigargin. Inset: glands were incubated from $t = 0$ in 0 Ca^{2+} medium (2 mM EGTA) supplemented with 1 μM thapsigargin. An initial stimulation with 100 nM 5-HT (shaded area) was used to accelerate the emptying of the Ca^{2+} stores, whereas a second stimulus confirmed store depletion. Ca^{2+} was injected for the period indicated by the black bar. Right panel: rates of $[\text{Ca}^{2+}]_i$ increase (mean $\pm$ SD; $n = 7$). Continuous lines in (A) and (B) represent least squares fits of the experimental data to a single exponential (left panels) or linear function (right panels), respectively. (C) Left panel: typical evolution of $[\text{Ca}^{2+}]_i$ during injection of Ca^{2+} in the presence of 1 nM 5-HT. Right panel: the rate of $[\text{Ca}^{2+}]_i$ increase exhibits a marked acceleration during the first 5 s of Ca^{2+} injection. Data points are expressed as mean $\pm$ SD ($n = 6$).

Fig. 8. Effect of the phospholipase C inhibitor U73122 on 5-HT-induced $[\text{Ca}^{2+}]_i$ changes. (A and B) Control stimulations with 10 and 100 nM 5-HT in the presence of vehicle (A, 0.5% dimethylsulfoxide) or the inactive analog U73343 (B, 10 μM). (C) Stimulation in the presence of U73122 (10 μM) fails to elicit $[\text{Ca}^{2+}]_i$ changes. The bathing medium was supplemented with 2 mM CaCl_2 .

the formation of InsP_3 via Ca^{2+} -dependent activation of PLC (Rhee *et al.*, 1989; Harootunian *et al.*, 1991) at basal $[\text{InsP}_3]$.

One possible explanation for the requirement for hormone and/or supra-basal $[\text{InsP}_3]$ in Ca^{2+} spiking and wave propagation is that increases in $[\text{InsP}_3]$ caused by a secondary formation of InsP_3 via Ca^{2+} -dependent activation of PLC add to the elevations in $[\text{InsP}_3]$, generated as a consequence of receptor activation or InsP_3 injection, to

open InsP_3 receptor channels. In this case, Ca^{2+} spikes and waves would be accompanied by synchronous oscillations in $[\text{InsP}_3]$.

Alternatively, the requirement for supra-basal $[\text{InsP}_3]$ may be related to the intrinsic sensitivity of the InsP_3 receptor to both InsP_3 and Ca^{2+} (Baumann and Walz, 1989; Iino, 1990; Parker and Ivorra, 1990; Wakui and Petersen, 1990; Bezprozvanny *et al.*, 1991). Thus, the primary effect of elevated $[\text{InsP}_3]$ in mediating Ca^{2+} wave

Fig. 9. Injection of InsP_3 after pre-treatment with the phospholipase C inhibitor U73122. Glands were pre-treated for 10 min with 10 μM U73122 before commencing the injection of InsP_3 . (A) Development of $[\text{Ca}^{2+}]_i$ underneath the black box shown in (C) during three periods of current application (10 mM concentration in pipette; -10 nA, 100 ms, 1 Hz). Lower trace: the recording of $[\text{Ca}^{2+}]_i$ outside the region reactive to the InsP_3 injection (white box in B) shows that the response to 100 nM 5-HT is abolished under these conditions. (B) Evolution of Ca^{2+} during the second period of InsP_3 injection shown in (A) and recorded at four different locations (boxes in C, first image). (C–E) Series of pseudocolor Ca^{2+} images depicting propagating intercellular Ca^{2+} waves. Arrowheads mark the position of advancing wave fronts. Time segments selected for the three series are indicated by the shaded areas in (B). Image intervals, 1 s; scale bar in (C) = 100 μm .

transmission might be to sensitize the InsP_3 receptor, i.e. to transform the Ca^{2+} release mechanism into a state that can be excited by Ca^{2+} . This concept of excitable media was developed originally for intracellular Ca^{2+} waves in *Xenopus* oocytes (Lechleiter *et al.*, 1991; Lechleiter and Clapham, 1992) and predicts Ca^{2+} wave propagation at constant $[\text{InsP}_3]$. The observation that InsP_3 -induced Ca^{2+} spiking and Ca^{2+} wave propagation are not affected by preventing the formation of endogenous InsP_3 via the inhibition of PLC is consistent with this model and strongly suggests that oscillations in InsP_3 do not underlie Ca^{2+} spiking or Ca^{2+} wave propagation in the salivary gland. A similar interpretation has been derived recently for Ca^{2+} spiking in intact and permeabilized hepatocytes (Bird *et al.*, 1997; Hajnóczky and Thomas, 1997).

The present finding that both InsP_3 and Ca^{2+} can diffuse through gap junctions is in agreement with earlier reports (Sáez *et al.*, 1989; Yule *et al.*, 1996) and precludes, by itself, a simple way of distinguishing between the candidate messenger molecules in propagating Ca^{2+} waves. This also applies to the apparently more limited spreading of Ca^{2+} than of InsP_3 (Sáez *et al.*, 1989; Yule *et al.*, 1996; this study), although this repeatedly has been taken as evidence in favor of InsP_3 being the messenger mediating intercellular transmission. Allbritton and co-workers

(1992) have shown that the apparent diffusion coefficient of Ca^{2+} in cytoplasmic extracts of *Xenopus* oocytes is considerably smaller than the diffusion coefficient of InsP_3 . These authors and others have suggested that the 'effective range' of Ca^{2+} diffusion in the cytosol is greatly limited by the presence of endogenous Ca^{2+} buffers and that the experimentally determined diffusion coefficient of Ca^{2+} is incompatible with Ca^{2+} serving as a diffusing messenger in propagating Ca^{2+} waves. However, it has also been pointed out that the diffusion coefficient of Ca^{2+} in the cytosol depends: (i) on Ca^{2+} concentration; and (ii) upon a rapid increase of $[\text{Ca}^{2+}]_i$, changes as a function of time that is determined by the rates of association with endogenous Ca^{2+} buffers (Allbritton *et al.*, 1992; Jaffe, 1993; Wang and Thompson, 1995). Consequently, Ca^{2+} rapidly released from intracellular stores initially will diffuse freely before diffusion is slowed down gradually by binding to mobile or immobile Ca^{2+} buffers, and before the Ca^{2+} is finally removed from the cytosol by active transport mechanisms. Moreover, the distance that the messenger has to spread in a reaction–diffusion system is determined by the distance between sites at which the diffusing species is regenerated. This would correspond to the distances between either neighboring PLC molecules or adjacent Ca^{2+} release sites in an individual cell or at

either side of gap junctions in neighboring cells. Thus, the distances relevant for the spreading of a regenerative intercellular Ca^{2+} wave can be assumed to be substantially shorter than the experimentally observed diffusional spread of microinjected InsP_3 and Ca^{2+} (Wang and Thompson, 1995).

In order for a system to use a specific substance as an intra- and/or intercellular messenger in repetitively propagating regenerative Ca^{2+} waves, it needs to set up periodically a concentration gradient for this substance. So far, oscillations in $[\text{InsP}_3]$ associated with repetitive agonist-evoked Ca^{2+} spiking or Ca^{2+} waves have not been demonstrated (see above). Furthermore, the present experiments show that Ca^{2+} spiking and the spread of intercellular Ca^{2+} waves is insensitive to the inhibition of PLC. Since PLC activity is necessary for the production of such oscillatory changes in $[\text{InsP}_3]$, it is unlikely that InsP_3 diffusion plays a role in the present type of Ca^{2+} wave.

The present data are, therefore, consistent with intercellular Ca^{2+} waves in the blowfly salivary gland being propagated by the mechanism originally proposed for intracellular Ca^{2+} waves by Lechleiter and co-workers (Lechleiter *et al.*, 1991; Lechleiter and Clapham, 1992). This mechanism involves intercellular diffusion of Ca^{2+} and Ca^{2+} -induced Ca^{2+} release from intracellular Ca^{2+} stores by way of InsP_3 receptor channels, which are Ca^{2+} sensitized by the presence of supra-basal $[\text{InsP}_3]$. The present findings suggest that the secretory epithelium of the blowfly salivary gland forms a functional continuum in which Ca^{2+} waves in and between individual cells are propagated by the same mechanism. This mechanism may represent a fundamental method by which groups of cells and tissues coordinate their responses to agonists and hormones.

Materials and methods

Preparation of salivary glands and measurement of $[\text{Ca}^{2+}]_i$

Preparation and Ca^{2+} imaging were performed as previously described (Zimmermann and Walz, 1997; Zimmermann, 1998). Briefly, glands were loaded with fura-2 during a 20–30 min incubation in 5 μM fura-2 acetoxymethyl ester and placed in a superfusion chamber that was mounted on the stage of a Zeiss Axiovert 135TV inverted epifluorescence microscope. Pairs of fluorescence images, excited at wavelengths of 340 and 380 nm, were captured via a 515–565 nm bandpass filter at a rate of 1 Hz and with a cooled frame transfer CCD camera (TE/CCD-512EFT, Princeton Instruments Corp., Trenton, NJ). Raw images were processed offline on a PC using the imaging software Metafluor (Universal Imaging Corp., West Chester, PA). Free cytosolic Ca^{2+} concentrations were determined according to Grynkiewicz *et al.* (1985) by using a mixed *in vitro-in vivo* procedure as previously described (Zimmermann and Walz, 1997).

Space-time plots of the Ca^{2+} dynamics were constructed from series of Ca^{2+} images by sequentially extracting the $[\text{Ca}^{2+}]$ underneath a 1 pixel (3.8 μm) wide line projected onto the glands. Propagation rates were determined from space-time plots by fitting a straight line to the leading edge of the respective wave.

Microinjection

Conventional microelectrodes were used for microinjections. Their tip resistance was 80–120 M Ω when filled with 3 M KCl. The substance to be injected (aqueous solution, 1–2 μl) was passed into the electrode tips from the rear of the electrode and backfilled with 500 mM KCl. A chloridized silver wire was glued into the pipette and connected to the headstage of a BRAMP-01 intracellular amplifier (npi electronic, Tamm, Germany). Cells were impaled with the microelectrodes which were connected to a Narishige micromanipulator mounted onto the stage of

the imaging system. Monitoring of the membrane potential during the experiments with an oscilloscope verified the intracellular location of the micropipette tip. Iontophoretic microinjection of the test agents was performed by applying current pulses to the micropipette.

Solutions and drugs

Standard Ringer solution contained (in mmol/l): 128 NaCl, 10 KCl, 2 CaCl_2 , 2 MgCl_2 , 2.7 sodium glutamate, 2.7 maleic acid, 10 Tris and 10 D-glucose, pH 7.2. Nominally Ca^{2+} -free Ringer was obtained by omitting CaCl_2 from this solution. In some experiments, remaining traces of contaminant Ca^{2+} were eliminated by the addition of 2 mM EGTA to nominally Ca^{2+} -free Ringer solution. Glands were superfused continuously at 1 ml/min. Solution changes were accomplished rapidly by means of a six-port valve. All experiments were performed at ambient temperature. Unless otherwise stated, each trace shown is representative of at least four independent experiments.

Chemicals were purchased from the following suppliers: fura-2 AM and Lucifer yellow CH from Molecular Probes (Leiden, The Netherlands); thapsigargin from Calbiochem (Bad Soden, Germany); L-myo-inositol 1,4,5-trisphosphate and D-myo-inositol 1,4,5-trisphosphate, U73122 and U73343 from Alexis (Grünberg, Germany); and D,L-myo-inositol 1,4,5-phosphorothioate from ICN Biomedicals (Eschwege, Germany). All other chemicals were of analytical grade.

Statistical analysis

Where appropriate, data are expressed as means $\pm$ SD. Statistical comparisons were made by an independent Student's *t*-test, using two-tailed values. *P* values of <0.05 were considered as being significant.

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