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 fluorometric digital imaging combined with microinjection of putative messenger molecules. Iontophoretic injection of D-myoinositol 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 pre-treatment 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 [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. Keywords: Ca$^{2+}$-induced Ca$^{2+}$ release/Ca$^{2+}$ waves/inositol trisphosphate/intercellular

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 ([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 [Ca$^{2+}$]$_i$ are often temporally ‘organized’ to form Ca$^{2+}$ oscillations or repetitive Ca$^{2+}$ spikes, and/or spatially coordinated generating intercellular 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 Streyer, 1991; De Young and Keizer, 1992; Atri et al., 1993). These models postulate spatio-temporal fluctuations either of InsP$_3$ (Meyer and Streyer, 1991) or of Ca$^{2+}$ at constant [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 [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 Streyer, 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 [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
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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 [Ca^{2+}] 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 [Ca^{2+}] to evoke 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 [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 [Ca^{2+}] 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 [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 extracellular Ca^{2+} concentration ([Ca^{2+}]) 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,
the local response to the continuous injection of InsP₃ (10 mM concentration in the pipette; –10 nA, 100 ms, 0.5–1 Hz) resembled responses elicited by 5-HT in low Ca²⁺ media (Figure 2B and C). They consisted of a single initial transient or an initial rapid series of incompletely fused Ca²⁺ spikes followed by repetitive Ca²⁺ spikes (frequency: 6.1 ± 0.58/min; n = 9), as long as current was applied to the electrode.

The analysis of the spatio-temporal characteristics of the [Ca²⁺]i dynamics induced by InsP₃ revealed a rapid radial spread of the Ca²⁺ 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 μm/s), and was thus considerably faster than the velocity of 5-HT-induced Ca²⁺ waves (12–17 μm/s; Zimmermann and Walz, 1997). Regions of elevated [Ca²⁺], reached radii of 50–70 μm, indicating a Ca²⁺ increase in the first, second and third tier neighbors of the impaled cell. Increasing the rate of InsP₃ delivery further increased the number of cells participating in the Ca²⁺ response (Figure 3B–D). The edges of InsP₃-induced Ca²⁺ activity usually exhibited oscillatory fluctuations in [Ca²⁺], (Figure 2E). In nominally Ca²⁺-free solutions, as in the presence of 2 mM CaCl₂, InsP₃-induced Ca²⁺ 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 Ca²⁺ spiking activity following the initial transient Ca²⁺ increase within this region was accompanied by propagating intercellular Ca²⁺ waves (Figures 3G and 4B–D). During individual stimulations, these Ca²⁺ waves were triggered at different locations within the region that displayed the initial Ca²⁺ 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 InsP₃-induced Ca²⁺ waves were regenerative waves. The mean propagation rate determined in experiments in which the Ca²⁺ waves clearly followed the longitudinal axis of the gland was 13.8 ± 1.96 μm/s (n = 21; three glands) and was similar to the velocity of 5-HT-evoked Ca²⁺ waves (12–17 μm/s; Zimmermann and Walz, 1997).

Reversal of the current polarity or microinjection of the biologically inactive analog L-myoinositol 1,4,5-trisphosphate (10 mM), Lucifer yellow CH [5% (w/v) in H₂O] or K⁺ (3 M KCl in the pipette), when injected under identical conditions, evoked no measurable changes in [Ca²⁺], (n = 4, respectively). This demonstrated the
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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$^{2+}$]$_i$ 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$_3$S$_3$). Iontophoretic injection of InsP$_3$S$_3$ (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 ± 0.027/s ($n = 6$) and 0.09 ± 0.044/s ($n = 7$), respectively ($P = 0.32$). Thus, Ca$^{2+}$ release induced by
Fig. 5. Iontophoretic injection of InsP₃S₃. (A) Time course of [Ca²⁺]ᵢ changes evoked by microinjection of InsP₃S₃ and integrated over a window of 5×5 pixels (white box in B, first image). (B and C) Series of images showing propagating intercellular Ca²⁺ waves. Image intervals, 1 s; scale bar in (B) = 100 μm.

eperimentally increasing [Ca²⁺]ᵢ was not detectable. In contrast, the time courses of Ca²⁺ increases during injection of Ca²⁺ in the presence of agonist (1 or 3 nM 5-HT, cf. Figure 5B) were strikingly different. Here, the rate of Ca²⁺ 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 Ca²⁺ release as a consequence of Ca²⁺ injection in the presence of the hormone. 

**Injection of InsP₃ into glands pre-treated with U73122**

In a second approach to determine a possible involvement of Ca²⁺-mediated activation of PLC in Ca²⁺ wave propagation, we attempted to interfere with the production of endogenous InsP₃ 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 Ca²⁺ changes (in six out of six glands), whereas treatment with the inactive structural analog U73343 was without effect. Injection of InsP₃ into glands pre-treated with U73122 induced Ca²⁺ responses that resembled the previously observed InsP₃-induced Ca²⁺ changes (Figure 9A and B). The mean frequency of Ca²⁺ spiking was 5.1 ± 1.68/s (n = 8; seven preparations). As in the absence of U73122, InsP₃-induced Ca²⁺ spiking in the activated region was temporally and spatially coordinated and was accompanied by the propagation of intercellular Ca²⁺ waves (Figure 9C–E).

**Discussion**

Intercellular Ca²⁺ 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 Ca²⁺ waves propagate between cells is so far poorly understood.

**The mechanism of intercellular Ca²⁺ wave propagation**

The results reported here demonstrate that the microinjection of InsP₃ into a single cell of the intact secretory epithelium of the salivary gland in Calliphora is able to trigger Ca²⁺ release not only in the impaled cell, but also in a group of neighboring cells. Similar coordinated responses to the local application of InsP₃ 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 InsP₃ and thus failed to show whether InsP₃ mimicked the effect of agonists in producing repetitive Ca²⁺ spiking, and whether the intercellular spread of the Ca²⁺ signal was the result of: (i) the passive diffusion of InsP₃ 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 InsP₃ consisted of two components. InsP₃ injection was followed by initial transient increases in [Ca²⁺]ᵢ spreading from the impaled cell into neighboring cells. This spread took place at variable rates and probably reflected the intercellular diffusion of InsP₃ at the onset of InsP₃ injection. Subsequently, trains of Ca²⁺ waves propagated in the presence of continuously elevated [InsP₃] within the region that showed the initial Ca²⁺ transient. These Ca²⁺ waves were propagated at a constant velocity
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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 [Ca^{2+}]. Regions of increased [Ca^{2+}] 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 μm. 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 [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). Feedback 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 [InsP_3] around the tip of the injection electrode, this suggests that the presence of supra-basal [InsP_3] is a prerequisite for Ca^{2+} waves to propagate (see also Zimmermann and Walz, 1997). However, the establishment of an [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 [InsP_3]. This shows that increases in [Ca^{2+}], 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 [InsP_3] or by

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the formation of InsP₃ via Ca²⁺-dependent activation of PLC (Rhee et al., 1989; Harootunian et al., 1991) at basal [InsP₃].

One possible explanation for the requirement for hormone and/or supra-basal [InsP₃] in Ca²⁺ spiking and wave propagation is that increases in [InsP₃] caused by a secondary formation of InsP₃ via Ca²⁺-dependent activation of PLC add to the elevations in [InsP₃], generated as a consequence of receptor activation or InsP₃ injection, to open InsP₃ receptor channels. In this case, Ca²⁺ spikes and waves would be accompanied by synchronous oscillations in [InsP₃].

Alternatively, the requirement for supra-basal [InsP₃] may be related to the intrinsic sensitivity of the InsP₃ receptor to both InsP₃ and Ca²⁺ (Baumann and Walz, 1989; Iino, 1990; Parker and Ivorra, 1990; Wakui and Petersen, 1990; Bezprozvanny et al., 1991). Thus, the primary effect of elevated [InsP₃] in mediating Ca²⁺ wave
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 [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 [Ca$^{2+}$], 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

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**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 [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 [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.
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 [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 [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 saliva 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 [InsP$_3$]. The present findings suggest that the secretory epithelium of the blowfly saliva 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 [Ca$^{2+}$]$^-$

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 $\mu$M fura-2 acetoxyethyl ester and placed in a superfusion chamber that was loaded with fura-2 during a 20–30 min incubation in 5 $\mu$M fura-2 acetoxyethyl ester and placed in a superfusion chamber that was 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); 1,4-myoinositol 1,4,5-trisphosphate and d-myoinositol 1,4,5-trisphosphate, U73122 and U73343 from Alexis (Grüningen, Germany); and 1,4-myoinositol 1,4,5-phosphoroxythioate from ICN Biomedicals (Eschwege, Germany). All other chemicals were of analytical grade.

Statistical analysis

Where appropriate, data are expressed as means ± 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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