Functional properties of *Drosophila* inositol trisphosphate receptors

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The functional properties of the only inositol trisphosphate (IP₃) receptor subtype expressed in *Drosophila* were examined in permeabilized S2 cells. The IP₃ receptors of S2 cells bound (1,4,5)IP₃ with high affinity ($K_d = 8.5 \pm 1.1$ nM), mediated positively co-operative Ca²⁺ release from a thapsigargin-sensitive Ca²⁺ store ($EC_{50} = 75 \pm 4$ nM, Hill coefficient = 2.1 ± 0.2), and they were recognized by an antiserum to a peptide conserved in all IP₃ receptor subtypes in the same way as mammalian IP₃ receptors. As with mammalian IP₃ receptors, (2,4,5)IP₃ ($EC_{50} = 2.3 \pm 0.3$ μ M) and (4,5)IP₂ (EC_{50} approx. 10 μ M) were approx. 20- and 100-fold less potent than (1,4,5)IP₃. Adenophostin A, which is typically approx. 10-fold more potent than IP₃ at mammalian IP₃ receptors, was 46-fold more potent than IP₃ in S2 cells ($EC_{50} = 1.67 \pm 0.07$ nM). Responses to submaximal concentrations of IP₃ were quantal and IP₃-evoked Ca²⁺ release

was biphasically regulated by cytosolic Ca²⁺. Using rapid superfusion to examine the kinetics of IP₃-evoked Ca²⁺ release from S2 cells, we established that IP₃ (10 μ M) maximally activated *Drosophila* IP₃ receptors within 400 ms. The activity of the receptors then slowly decayed ($t_{1/2} = 2.03 \pm 0.07$ s) to a stable state which had $47 \pm 1\%$ of the activity of the maximally active state. We conclude that the single subtype of IP₃ receptor expressed in *Drosophila* has similar functional properties to mammalian IP₃ receptors and that analyses of IP₃ receptor function in this genetically tractable organism are therefore likely to contribute to understanding the roles of mammalian IP₃ receptors.

Key words: Ca²⁺ mobilization, insect, inositol trisphosphate receptors, kinetics, receptor subtype.

INTRODUCTION

Inositol trisphosphate (IP₃) receptors are tetrameric intracellular Ca²⁺ channels that often provide the link between receptors in the plasma membrane and release of Ca²⁺ from intracellular stores [1]. Purification of a type 1 IP₃ receptor from rat brain [2] was followed by molecular cloning of several closely related subtypes of IP₃ receptor from different species, including three mammalian IP₃ receptor subtypes (types 1–3), and one subtype from each of *Xenopus* oocytes [3], *Caenorhabditis elegans* [4], and *Drosophila melanogaster* [5,6] (reviewed in [7]). The functional significance of IP₃ receptor diversity, which is increased further by alternative splicing and assembly of the subunits into both homo- and hetero-tetrameric channels, has yet to be fully elucidated [7,8].

As the genome sequencing projects for both *C. elegans* [9] and *Drosophila* [10] near completion, it is clear that only a single IP₃ receptor subtype is expressed in each species, the former sharing approx. 54% [4] and the latter [5] approx. 69% amino acid sequence similarity with rat type 1 IP₃ receptors. Although none of the alternative splice sites found in mammalian IP₃ receptors are present in *Drosophila*, the central portion of the receptor does include two sites that are differentially spliced in embryo and adult insects [11]. The expression of single IP₃ receptor subtypes in these genetically tractable organisms provides opportunities to both identify the essential properties of IP₃ receptors and to precisely define the roles of IP₃ receptors in the complex physiological and behavioural responses in which they are thought to be involved [12–14]. Work with *Drosophila* has already proved useful in shedding light on signalling pathways in mammalian cells. *Drosophila* photoreceptors for example, where the photoresponse is mediated by the phosphoinositide cascade,

provided the first evidence that homologues of the *Drosophila* TRP and TRPL proteins might form the channels responsible for capacitative Ca²⁺ entry in mammalian cells [15,16] and established the role of PDZ-domain proteins as scaffolds for signalling proteins [17]. Furthermore, disruption of the *Drosophila* IP₃ receptor gene established that although the IP₃ receptor is essential for metamorphosis, it is not required for phototransduction [13,14]. Despite the success of these strategies in defining the roles of IP₃ receptors in complex physiological responses in *Drosophila* [13,14] and *C. elegans* [12], nothing is known of the functional properties of the IP₃ receptors from either species, aside from the ability of the N-terminus of each receptor to bind IP₃ [4,5].

In the present study, we use a cell line (S2 cells) derived from late embryonic stages of *D. melanogaster* [18] to provide the first functional characterisation of the *Drosophila* IP₃ receptor. We provide evidence that insect IP₃ receptors, like those of mammals, mediate quantal Ca²⁺ release, are biphasically regulated by cytosolic Ca²⁺, are rapidly activated and then partially inactivated by IP₃ and are potently stimulated by adenophostin A. Our results suggest that S2 cells, with the opportunities they provide for manipulating expression of endogenous proteins [19], may be useful in defining both the essential properties and roles of IP₃ receptors.

EXPERIMENTAL

Materials

(1,4,5)IP₃ was from American Radiolabeled Chemicals Inc. (St Louis, MO, U.S.A.) and (2,4,5)IP₃, prepared as previously

Abbreviations used: IP₃, inositol 1,4,5-trisphosphate (unless another isomer is specified); AbC, antiserum that recognizes all IP₃ receptor subtypes; CLM, cytosol-like medium.

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Figure 1 Properties of the Ca^{2+} stores and IP_3 receptors of S2 cells

(A) The $^{45}\text{Ca}^{2+}$ contents of the intracellular stores of S2 cells are shown after the indicated periods of incubation with ATP at 37 °C (●) or 20 °C (○). Results are shown as means $\pm$ S.E.M. of three independent experiments. (B) Lanes were loaded with 100 μg of membrane protein from either rat cerebellum (Cer) or S2 cells as indicated and immunoblotted with AbC. The results are typical of at least 3 similar blots. Molecular-mass markers (kDa) are shown on the left. (C) Specific [^3H]IP $_3$ binding to membranes prepared from S2 cells is shown in the presence of the indicated concentrations of (1,4,5)IP $_3$ (○) or adenophostin A (●). Results are shown as means $\pm$ S.E.M. of three (adenophostin A) or six (IP $_3$) independent experiments.

described [20], was a gift from Robin Irvine (Department of Pharmacology, University of Cambridge, U.K.). $^{45}\text{Ca}^{2+}$ was from ICN Biomedicals (Thame, Oxon., U.K.). Ionomycin and synthetic adenophostin A were from Calbiochem (Nottingham, U.K.). Thapsigargin was from Alomone Labs (Jerusalem, Israel). Other materials were from the suppliers listed previously [21].

Measurement of $^{45}\text{Ca}^{2+}$ fluxes in S2 cells

S2 cells, which were originally derived from unidentified tissues of embryos from *D. melanogaster* [18], were a gift from Dr Raghu Padinjat (Department of Anatomy, University of Cambridge, U.K.). They were maintained at 27 °C in Shields and Sang M3 growth medium (Sigma, Poole, Dorset, U.K.) supplemented with foetal calf serum (12.5%, v/v), penicillin (100 units/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and amphotericin B (0.25 $\mu\text{g}/\text{ml}$) (Sigma). Confluent cultures of S2 cells (passage numbers 11–28) were scraped into growth medium, washed by centrifugation (650 g for 2 min) and resuspended (1.5×10^6 cells/ml) in Ca^{2+} -free cytosol-like medium [CLM: 140 mM KCl, 20 mM NaCl,

2 mM MgCl_2 , 1 mM EGTA and 20 mM Pipes (pH 7.0) at 20 °C]. The plasma membranes of the cells were then permeabilized by incubation with saponin (15 $\mu\text{g}/\text{ml}$) for 8 min in CLM at 20 °C. Cells were washed by centrifugation (650 g for 2 min) and resuspended in CLM containing a free $[\text{Ca}^{2+}]$ of 200 nM (total $[\text{Ca}^{2+}]$, 275 μM), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone ('FCCP', 10 μM) to inhibit mitochondrial function and $^{45}\text{Ca}^{2+}$ (20 $\mu\text{Ci}/\text{ml}$). ATP (1.5 mM), creatine phosphate (5 mM) and creatine phosphokinase (5 units/ml) were then added to allow active uptake of $^{45}\text{Ca}^{2+}$ into the intracellular stores. The $^{45}\text{Ca}^{2+}$ content of the intracellular stores was determined after rapid filtration through Whatman GF/C filters using a Brandel receptor-binding harvester (SEMAT, St Albans, Herts., U.K.), followed by washing (2×5 ml) with cold medium (310 mM sucrose, 1 mM trisodium citrate). Liquid scintillation counting was used to determine the amount of $^{45}\text{Ca}^{2+}$ trapped on each filter. Active $^{45}\text{Ca}^{2+}$ uptake was defined as that which could be released by addition of ionomycin (10 μM).

The effects of IP $_3$ were examined by allowing cells to load to steady-state with $^{45}\text{Ca}^{2+}$ (15 min, see Figure 1A), and then diluting them 2-fold in CLM containing an appropriate free $[\text{Ca}^{2+}]$, thapsigargin (1 μM) to inhibit further Ca^{2+} uptake, and IP $_3$. The incubations, with the exception of those where time courses were examined (see Figure 3), were terminated by rapid filtration after 2 min at 20 °C.

The free Ca^{2+} concentrations of CLM were measured using fura 2 ($K_d^{\text{Ca}} = 288$ nM) as previously described [21].

Rapid kinetics of $^{45}\text{Ca}^{2+}$ release from permeabilized S2 cells

Permeabilized S2 cells were loaded with $^{45}\text{Ca}^{2+}$ (30 $\mu\text{Ci}/\text{ml}$) and then immobilized within a filter sandwich mounted inside a rapid superfusion apparatus, a full description of which was published previously [22]. Briefly, the equipment allows $^{45}\text{Ca}^{2+}$ release from the immobilized cells to be measured with a temporal resolution of up to 9 ms as CLM flows continuously (2 ml/s) over the cells and (with the $^{45}\text{Ca}^{2+}$ released) into a circular fraction collector. Addition of a trace of [^3H]inulin to some of the media allowed changes of media to be precisely related to changes in $^{45}\text{Ca}^{2+}$ efflux. All experiments were carried out at 20 °C, and the $t_{1/2}$ for exchange of media bathing the cells was 36 ± 3 ms ($n = 3$).

The size of the $^{45}\text{Ca}^{2+}$ pool in the immobilized cells was calculated by summing all $^{45}\text{Ca}^{2+}$ released during the experiment together with that released at the end when the cells were treated with Triton X-100 (0.5%). The effects of IP $_3$ on rates of $^{45}\text{Ca}^{2+}$ release were expressed after subtraction of the basal rate of $^{45}\text{Ca}^{2+}$ release. The initial basal rate of $^{45}\text{Ca}^{2+}$ release ($0.79 \pm 0.05\%$ per s) was measured at the beginning of each superfusion experiment. Basal rates of $^{45}\text{Ca}^{2+}$ release were slightly faster from IP $_3$ -sensitive stores ($1.66 \pm 0.03\%$ per s; $n = 3$) relative to IP $_3$ -insensitive stores ($0.40 \pm 0.06\%$ per s), the correction for the basal leak was therefore adjusted during a response to accommodate the changing relative contribution from IP $_3$ -sensitive stores. The correction was minor because the IP $_3$ -sensitive stores are a relatively small fraction ($23.1 \pm 0.5\%$; $n = 3$) of the entire stores in superfusion experiments. Rates of $^{45}\text{Ca}^{2+}$ release were expressed either as a percentage of the entire $^{45}\text{Ca}^{2+}$ store or as fractional release rates, i.e. the amount of $^{45}\text{Ca}^{2+}$ released in each interval as a percentage of the amount of Ca^{2+} remaining within the IP $_3$ -sensitive stores at the beginning of that interval. The latter form of analysis provides the clearest indication of any changes in IP $_3$ receptor behaviour (see Results and Discussion section).

[³H]IP₃ binding and antibody methods

S2 cells were scraped into PBS [137 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄ (pH 7.2) at 2 °C], pelleted (1000 *g* for 5 min), washed twice in PBS and resuspended (10⁶ cells/ml) in Ca²⁺-free medium [140 mM KCl, 20 mM NaCl, 20 mM Pipes and 1 mM EDTA (pH 7.0) at 2 °C] containing protease inhibitors (Sigma). The cells were homogenized in an Ultra-Turrax homogenizer (9000 r.p.m. for 9 × 10 s), centrifuged (5000 *g* for 10 min) and the pellet was then resuspended in Ca²⁺-free medium using a homogenizer to give approx. 15 mg of protein in 0.5 ml.

For equilibrium competition binding studies, membranes (300 µg) in a final volume of 200 µl of Tris/EDTA medium [50 mM Tris and 1 mM EDTA (pH 8.3) at 2 °C] were incubated with [³H]IP₃ (1 nM) and appropriate concentrations of a competing ligand for 5 min at 2 °C. Bound and free ligand were separated by centrifugation (20000 *g* for 5 min) and the pellet was then solubilized in 1 ml of EcoScint A (National Diagnostics, Hull, U.K.) for liquid-scintillation counting. Specific [³H]IP₃ binding (approx. 500 d.p.m.) was typically 70 % of total binding.

For immunoblots, membrane proteins were separated by SDS/PAGE (5% gel) transferred to Immobilon membranes (Millipore, Watford, Herts., U.K.), which were blocked by incubation with 5 % (w/v) milk powder in PBS before addition for 1 h of a rabbit anti-peptide serum (AbC) that recognizes a sequence (PMNRYSAQKQFWKA) conserved within the N-terminal domain of all IP₃ receptor subtypes, including that expressed in *Drosophila* (residues 65–78). The properties of AbC were described previously [23,24]. An anti-rabbit antibody coupled to horseradish peroxidase (AbCam, Cambridge, U.K.) was used as the secondary antibody and the blots were developed using Super Signal chemiluminescence reagent (Pierce and Warriner, Chester, U.K.).

RESULTS AND DISCUSSION

Properties of the intracellular Ca²⁺ stores and IP₃ receptors of S2 cells

In the presence of ATP, the intracellular stores of permeabilized S2 cells rapidly accumulated ⁴⁵Ca²⁺ (*t*_{1/2} = 97 ± 13 s at 20 °C; *n* = 3) to reach a steady-state Ca²⁺ content of 96 ± 9 pmol/10⁶ cells within 10 min (Figure 1A). Thapsigargin (1 µM), a selective inhibitor of sarcoplasmic/endoplasmic-reticulum Ca²⁺-ATPases ('SERCA'), inhibited the uptake by 93 ± 1 % (*n* = 3). These results are consistent with those from intact S2 cells where a thapsigargin-sensitive Ca²⁺ store was shown to be 5–10 times larger than a separate acidic, thapsigargin-insensitive Ca²⁺ store [25]. The rate of ⁴⁵Ca²⁺ uptake by permeabilized S2 cells was faster (*t*_{1/2} = 19 ± 3 s; *n* = 3) and the steady-state ⁴⁵Ca²⁺ content greater (212 ± 15 pmol/10⁶ cells) at 37 °C than at 20 °C (Figure 1A), but all subsequent experiments were performed at 20 °C to more realistically mimic the physiological temperature for insect cells.

An antiserum (AbC) to a peptide sequence found in all known IP₃ receptor subtypes, including the *Drosophila* IP₃ receptor (see the Experimental section), identified a single band (220 kDa) in immunoblots from membranes prepared from S2 cells (Figure 1B). As expected from the deduced amino acid sequences, the *Drosophila* (2833 residues) and rat cerebellar (type 1, 2750 residues) IP₃ receptors migrated with similar sizes on SDS/PAGE.

Figure 2 Ca²⁺ release from S2 cells evoked by IP₃ and adenophostin A

The effects of the indicated concentrations of adenophostin A (●), (1,4,5)IP₃ (○) or (2,4,5)IP₃ (■) on the Ca²⁺ contents of the intracellular stores are shown following an incubation lasting 2 min. Results are shown as means ± S.E.M. of three independent experiments (most error bars are smaller than the symbols).

In equilibrium competition binding experiments using [³H]IP₃ and membranes prepared from S2 cells, IP₃ bound to a single class of sites [Hill coefficient (*h*) = 0.70 ± 0.05; *B*_{max} = 0.12 ± 0.02 pmol/mg of protein; *n* = 6] with an affinity (*K*_d) of 8.5 ± 1.1 nM (Figure 1C). The affinity for IP₃ is similar to that of the most closely related mammalian IP₃ receptor subtypes measured under the same conditions: type 1 from rat cerebellum (*K*_d = 3.2 ± 0.2; *n* = 6) and type 2 from rat liver (3.09 ± 0.33 nM; *n* = 24) [26]. Furthermore, the ratio of immunostaining with AbC (in arbitrary units) to the *B*_{max} was similar for membranes prepared from S2 cells (AbC/*B*_{max} = 1145 ± 160 units/pmol) and cerebellum (908 ± 6 units/pmol) suggesting that the AbC antiserum, which we have already shown to react equally well with the three mammalian IP₃ receptor subtypes [24], also binds equally to the IP₃ receptor expressed in *Drosophila*.

IP₃ receptors from *Drosophila* and mammals differ in their sensitivities to adenophostin A

A maximally-effective concentration of IP₃ (10 µM) rapidly stimulated the release of 51 ± 1 % (*n* = 3) of the intracellular stores of permeabilized S2 cells. The EC₅₀ of IP₃ after a 2 min incubation was 75 ± 4 nM, and the response was positively cooperative (*h* = 2.1 ± 0.2; *n* = 9) (Figure 2).

Figure 3 Quantal Ca^{2+} release evoked by IP_3 in *Drosophila* S2 cells

Permeabilized S2 cells loaded with $^{45}\text{Ca}^{2+}$ were added to CLM containing thapsigargin ($1 \mu\text{M}$) and the indicated concentrations of IP_3 : 30 nM (●), 70 nM (○), 100 nM (■) or $10 \mu\text{M}$ (□). The $^{45}\text{Ca}^{2+}$ released by IP_3 is shown at each time as a percentage of the $^{45}\text{Ca}^{2+}$ content of cells to which only thapsigargin had been added. Results are means $\pm$ S.E.M. of four to six independent experiments.

Adenophostin A is the most potent known agonist of mammalian IP_3 receptors [27], although its structure, based upon a phosphorylated glucopyranosyl ring with an adenosine attached, is very different from that of IP_3 (Figure 2, lower panel). In most analyses of mammalian cells expressing a variety of IP_3 receptor subtypes, adenophostin A is typically 10-fold more potent than IP_3 in evoking Ca^{2+} mobilization, and that is generally matched by a 10-fold greater affinity than IP_3 for the IP_3 receptor [26,27]. The results shown in Figure 2 demonstrate that, in S2 cells, adenophostin A was 46 ± 2 -fold more potent than IP_3 ; in paired experiments the EC_{50} for IP_3 was 77 ± 1 nM, and for adenophostin A it was 1.67 ± 0.07 nM ($n = 3$). The response to adenophostin A was also significantly more positively co-operative ($h = 3.6 \pm 0.2$) than that to IP_3 ($h = 1.8 \pm 0.1$). In equilibrium competition binding studies, adenophostin A ($K_d = 0.46 \pm 0.11$ nM, $h = 0.80 \pm 0.09$; $n = 3$) bound with 18 ± 5 -fold greater affinity than IP_3 ($K_d = 8.5 \pm 1.1$ nM, $h = 0.70 \pm 0.05$; $n = 6$) to membranes prepared from S2 cells (Figure 1C). In rat hepatocytes (which express largely type 2 IP_3 receptors) examined under identical conditions, adenophostin A was 9.9 ± 1.6 -fold more potent than IP_3 in functional assays, and bound with 6.4 ± 1.1 -fold greater affinity than IP_3 [26]. Many, though not all [27], studies of mammalian cells expressing type 1 [28], type 2 [26] or type 3 [29] IP_3 receptors have likewise concluded that adenophostin A binds to IP_3 receptors with approx. 10-fold greater affinity than IP_3 . These results establish that, in both functional and binding assays, the relative affinity of adenophostin A for *Drosophila* IP_3 receptors is significantly greater than for mammalian IP_3 receptors.

The high-affinity of adenophostin A for IP_3 receptors may result from either its 2'-phosphate moiety more effectively mimicking the 1-phosphate of IP_3 , which is known to improve binding affinity [24], or by an effect mediated by selective recognition of the adenosine moiety of adenophostin A [26]. The

Figure 4 Biphasic regulation of *Drosophila* IP_3 receptors by cytosolic Ca^{2+}

(A) Permeabilized S2 cells loaded with $^{45}\text{Ca}^{2+}$ were added to CLM containing thapsigargin and the indicated concentrations of IP_3 ; the incubations were then terminated after 2 min. The free $[\text{Ca}^{2+}]$ of the CLM was: 85 nM (○), 182 nM (●) or $2 \mu\text{M}$ (■). Results (means $\pm$ S.E.M., $n = 3$) show the Ca^{2+} remaining within the intracellular stores (%). (B) From experiments similar to those shown in (A), the Ca^{2+} released (means $\pm$ S.E.M., $n = 3$) by a submaximal concentration of IP_3 (300 nM) is shown after a 2 min incubation in CLM containing the indicated free $[\text{Ca}^{2+}]$.

former mechanism would imply that changes to the way in which the 1-phosphate group of IP_3 is recognized might be accompanied by changes in the affinity of an IP_3 receptor for adenophostin A. We therefore examined the effects of (2,4,5) IP_3 and (4,5) IP_2 in S2 cells to see if any differences might account for the high-affinity of the *Drosophila* IP_3 receptor for adenophostin A. In parallel $^{45}\text{Ca}^{2+}$ release assays, (2,4,5) IP_3 ($\text{EC}_{50} = 2.31 \pm 0.29 \mu\text{M}$, $h = 1.87 \pm 0.25$; $n = 3$) was 27 ± 4 -fold less potent than (1,4,5) IP_3 ($\text{EC}_{50} = 86 \pm 6$ nM, $h = 1.85 \pm 0.09$; $n = 3$), whereas (4,5) IP_2 ($10 \mu\text{M}$ released $44 \pm 4\%$ of the IP_3 -sensitive stores) was about 100-fold less potent. The relative affinities of both inositol phosphates for *Drosophila* IP_3 receptors are similar to results from mammalian IP_3 receptors, where (2,4,5) IP_3 is typically (10–30)-fold less potent, and (4,5) IP_3 is about 100-fold less potent than (1,4,5) IP_3 [24]. We conclude that, although simple modifications to the 1-phosphate moiety of (1,4,5) IP_3 similarly

Table 1 Effects of cytosolic Ca^{2+} on the sensitivity of the intracellular Ca^{2+} stores to IP_3

From experiments similar to those shown in Figure 4, the EC_{50} and Hill coefficient (h) for IP_3 -evoked Ca^{2+} mobilization and the response to a maximal concentration of IP_3 ($10 \mu\text{M}$) were determined at each of the indicated free $[\text{Ca}^{2+}]$. Results are shown as means $\pm$ S.E.M. of three independent experiments.

Free $[\text{Ca}^{2+}]$ (nM)	EC_{50} for IP_3 (nM)	h	Maximal response to IP_3 (%)
82	96 ± 10	1.6 ± 0.2	47 ± 2
186	62 ± 5	2.6 ± 0.3	49 ± 2
888	286 ± 15	2.4 ± 0.4	44 ± 7
1100	452 ± 32	2.4 ± 0.3	46 ± 2
1250	530 ± 80	2.8 ± 0.9	33 ± 7
1930	697 ± 67	2.4 ± 0.4	44 ± 7

affect binding to mammalian and *Drosophila* IP_3 receptors, adenophostin A binds with unexpectedly high affinity to the *Drosophila* IP_3 receptor.

IP_3 evokes quantal Ca^{2+} release from the intracellular stores of S2 cells

In permeabilized mammalian cells, submaximal concentrations of IP_3 rapidly, but incompletely, empty the IP_3 -sensitive Ca^{2+} stores. This 'quantal' pattern of Ca^{2+} release may require fragmentation of the intracellular Ca^{2+} stores for it to be detected [30], although it has also been observed in intact cells stimulated with Ca^{2+} -mobilizing hormones [31,32]. It appears to be a fundamental property of IP_3 -evoked Ca^{2+} release that may either involve all-or-nothing emptying of stores with different sensitivities to IP_3 [33] or a form of inactivation that leads to closure of the IP_3 receptor before a store has lost its entire Ca^{2+} content [34]. The results shown in Figure 3 establish that, after inhibition of Ca^{2+} uptake, submaximal concentrations of IP_3 stimulate quantal $^{45}\text{Ca}^{2+}$ release from S2 cells. The response to each concentration of IP_3 was essentially complete within 60 s but, whereas a maximally-effective concentration of IP_3 ($10 \mu\text{M}$) released $48 \pm 1\%$ of the stores, sub-maximal concentrations released a lesser fraction of the stores: $4 \pm 2\%$ for 30 nM IP_3 , $24 \pm 3\%$ for 70 nM IP_3 and $31 \pm 3\%$ for 100 nM IP_3 (Figure 3). Our results establish that, even in cells expressing only a single IP_3 receptor subtype, IP_3 -evoked Ca^{2+} release is a quantal process.

Biphasic regulation of IP_3 -evoked Ca^{2+} release by cytosolic Ca^{2+}

Most IP_3 receptors appear to be both stimulated and inhibited by cytosolic Ca^{2+} [35]. It had been suggested that the type 3 mammalian IP_3 receptor may not be inhibited by Ca^{2+} [36], but subsequent studies [21,37] have suggested that it, like the other mammalian IP_3 receptor subtypes, is biphasically regulated by cytosolic Ca^{2+} . Hence, although the detailed mechanisms may differ between the subtypes [23,35], biphasic modulation of IP_3 receptor activity by cytosolic Ca^{2+} appears to be a ubiquitous feature of mammalian IP_3 receptors. The results shown in Figure 4 demonstrate that this characteristic is also shared with the *Drosophila* IP_3 receptor. As the free $[\text{Ca}^{2+}]$ is increased from 85 nM to $1 \mu\text{M}$ and above, the EC_{50} for IP_3 -evoked Ca^{2+} release first decreases and then increases, whereas the fraction of the intracellular Ca^{2+} stores released by a maximally effective concentration of IP_3 ($10 \mu\text{M}$) remains constant at approx. 45% (Figure 4A and Table 1). Figure 4(B) illustrates the biphasic effect

Figure 5 Rapid kinetics of IP_3 -evoked $^{45}\text{Ca}^{2+}$ release in S2 cells

Permeabilized S2 cells were loaded with $^{45}\text{Ca}^{2+}$ and superfused for 60 s with CLM containing 200 nM free Ca^{2+} and $10 \mu\text{M}$ IP_3 (the latter for the period shown by the dashed line which indicates the arrival of the $[\text{^3H}]\text{inulin}$ included with the IP_3). (A) The $^{45}\text{Ca}^{2+}$ released in each 80-ms interval is shown as a percentage of the total intracellular $^{45}\text{Ca}^{2+}$ store content (rates are expressed as %/s to allow direct comparison with the traces shown in B and C). (B) and (C) The $^{45}\text{Ca}^{2+}$ released in each 1-s interval is shown expressed as either a percentage of the total intracellular $^{45}\text{Ca}^{2+}$ store content (B), or as a fractional release rate (C). The traces are from 3 independent experiments with each point expressed as a mean $\pm$ S.E.M.

of cytosolic Ca^{2+} on the response of S2 cells to a submaximal concentration of IP_3 (300 nM). Furthermore, in keeping with results from mammalian cells [38], pretreatment of permeabilized S2 cells with CLM containing $100 \mu\text{M}$ free Ca^{2+} for 30 s abolished subsequent responses to IP_3 ; the fraction of the Ca^{2+} stores

released by a maximal concentration of IP_3 (10 μM) was reduced from $41 \pm 1\%$ to $6 \pm 2\%$, and the response to a submaximal IP_3 concentration (70 nM) was reduced from $21 \pm 2\%$ to $3 \pm 3\%$ ($n = 3$).

Rapid activation and partial inactivation of *Drosophila* IP_3 receptors by IP_3

Analysis of the rapid kinetics of the Ca^{2+} release evoked by a maximal concentration of IP_3 in S2 cells demonstrated that the rate of $^{45}\text{Ca}^{2+}$ release reached a peak ($3.79 \pm 0.35\%$ per s) within 400 ms, before slowly decaying over several seconds (Figure 5A). The slow decay of the response is shown more completely in Figure 5(B), where the $^{45}\text{Ca}^{2+}$ release was recorded for longer but with lesser temporal resolution (1 s). Because the intracellular stores contain only a limited amount of $^{45}\text{Ca}^{2+}$, there is inevitably an exponential component to the kinetics of IP_3 -evoked Ca^{2+} release, as less Ca^{2+} is available for release at later times. By expressing the results as fractional release rates (see Experimental section), the behaviour of the IP_3 receptor can be isolated from the changing Ca^{2+} content of the stores; fractional rates of $^{45}\text{Ca}^{2+}$ release are expected to remain constant unless the permeability of the stores changes. The results (Figure 5C) demonstrate that the activity of the IP_3 receptors decays mono-exponentially with a $t_{1/2}$ of 2.03 ± 0.07 ($n = 3$) from their initial maximally active state (mediating release of $11.5 \pm 0.1\%$ per s of the IP_3 -sensitive stores) to a less active state ($5.4 \pm 0.1\%$ per s). The rate of $^{45}\text{Ca}^{2+}$ release was then maintained at this reduced rate for as long as the stores retained sufficient Ca^{2+} to allow meaningful analysis. This partial inactivation of the receptor after IP_3 binding is also a feature of the IP_3 receptors expressed in rat hepatocytes [39], although the rate of inactivation is about 10-times slower in *Drosophila*. In hepatocytes, this partial inactivation is probably a direct consequence of IP_3 binding because it still occurs when the Ca^{2+} release evoked by IP_3 binding is prevented [39]. We have not been able to perform similar experiments with S2 cells; partial inactivation of *Drosophila* IP_3 receptors might therefore result either directly from IP_3 binding or as a consequence of the fall in luminal Ca^{2+} concentration regulating the behaviour of the receptor.

Conclusions

Because most mammalian cells express more than one IP_3 receptor subtype assembled into both homo- and heterotetrameric channels [7], it has proved difficult to establish both the functional significance of IP_3 receptor diversity and the structural determinants of IP_3 receptor function. Relative to mammalian cells, *Drosophila*, and more specifically the S2 cell line derived from *Drosophila* embryonic tissues [18], have the experimental advantages of being both genetically tractable and of expressing only a single IP_3 receptor subtype [5,6]. We have now established that *Drosophila* IP_3 receptors share key functional properties with mammalian IP_3 receptors: they mediate quantal Ca^{2+} release, are biphasically regulated by cytosolic Ca^{2+} , are rapidly and co-operatively activated by IP_3 before partially inactivating and are potentially stimulated by adenylylation. We suggest that S2 cells provide a model system in which to address the roles of IP_3 receptors and the determinants of their behaviour, while avoiding some of the problems associated with mammalian cells.

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REFERENCES

- Berridge, M. J., Lipp, P. and Bootman, M. D. (2000) The versatility and universality of calcium signalling. *Nature Rev. Mol. Cell Biol.* **1**, 11–21.
- Supattapone, S., Worley, P. F., Baraban, J. M. and Snyder, S. H. (1988) Solubilization, purification, and characterization of an inositol trisphosphate receptor. *J. Biol. Chem.* **263**, 1530–1534.
- Kume, S., Muto, A., Aruga, J., Nakagawa, T., Michikawa, T., Furuichi, T., Nakade, S., Okano, H. and Mikoshiba, K. (1993) The *Xenopus* IP_3 receptor: structure, function and localization in oocytes and eggs. *Cell (Cambridge, Mass)* **75**, 555–570.
- Baylis, H. A., Furuichi, T., Yoshikawa, F., Mikoshiba, K. and Sattelle, D. B. (1999) Inositol 1,4,5-trisphosphate receptors are strongly expressed in the nervous system, pharynx, intestine, gonad, and excretory cell of *Caenorhabditis elegans* and are encoded by a single gene (*itr-1*). *J. Mol. Biol.* **294**, 467–476.
- Yoshikawa, S., Tanimura, T., Miyawaki, A., Nakamura, M., Yuzaki, M., Furuichi, T. and Mikoshiba, K. (1992) Molecular cloning and characterization of the inositol 1,4,5-trisphosphate receptor in *Drosophila melanogaster*. *J. Biol. Chem.* **267**, 16613–16619.
- Hasan, G. and Rosbash, M. (1992) *Drosophila* homologs of two mammalian intracellular Ca^{2+} -release channels: identification and expression patterns of the inositol 1,4,5-trisphosphate and the ryanodine receptor genes. *Development* **116**, 967–975.
- Taylor, C. W., Genazzani, A. A. and Morris, S. A. (1999) Expression of inositol trisphosphate receptors. *Cell Calcium* **26**, 237–251.
- Patel, S., Joseph, S. K. and Thomas, A. P. (1999) Molecular properties of inositol 1,4,5-trisphosphate receptors. *Cell Calcium* **25**, 247–264.
- The *C. elegans* Sequencing Consortium. (1998) Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science (Washington, DC)* **282**, 2012–2018.
- Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F. et al. (2000) The genome sequence of *Drosophila melanogaster*. *Science (Washington, DC)* **287**, 2185–2224.
- Sinha, M. and Hasan, G. (1999) Sequencing and exon mapping of the inositol 1,4,5-trisphosphate receptor cDNA from *Drosophila* embryos suggests the presence of differentially regulated forms of RNA and protein. *Gene* **233**, 271–276.
- Dal Santo, P., Logan, M. A., Chisholm, A. D. and Jorgensen, E. M. (1999) The inositol trisphosphate receptor regulates a 50-second behavioral rhythm in *C. elegans*. *Cell (Cambridge, Mass)* **98**, 757–767.
- Acharya, J. K., Jalink, K., Hardy, R. W., Hartenstein, V. and Zuker, C. S. (1997) InsP_3 receptor is essential for growth and differentiation but not for vision in *Drosophila*. *Neuron* **18**, 881–887.
- Venkatesh, K. and Hasan, G. (1997) Disruption of the IP_3 receptor gene of *Drosophila* affects larval metamorphosis and ecdysone release. *Curr. Biol.* **7**, 500–509.
- Hardie, R. C. and Minke, B. (1992) The *trp* gene is essential for light-activated Ca^{2+} channel in *Drosophila* photoreceptors. *Neuron* **8**, 643–651.
- Putney, J. W. (1999) TRP, inositol 1,4,5-trisphosphate receptors, and capacitative calcium entry. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 14669–14671.
- Tsunoda, S. and Zuker, C. S. (1999) The organization of INAD-signaling complexes by a multivalent PDZ domain protein in *Drosophila* photoreceptor cells ensures sensitivity and speed of signaling. *Cell Calcium* **26**, 165–171.
- Schneider, I. (1972) Cell lines derived from late embryonic stages of *Drosophila melanogaster*. *J. Embryol. Exp. Morphol.* **27**, 353–365.
- Clemens, J. C., Worby, C. A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B. A. and Dixon, J. E. (2000) Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6499–6503.
- Irvine, R. F., Brown, K. D. and Berridge, M. J. (1984) Specificity of inositol trisphosphate-induced calcium release from permeabilized Swiss-mouse 3T3 cells. *Biochem. J.* **222**, 269–272.
- Swatton, J. E., Morris, S. A., Cardy, T. J. A. and Taylor, C. W. (1999) Type 3 inositol trisphosphate receptors in RINm5F cells are biphasically regulated by cytosolic Ca^{2+} and mediate quantal Ca^{2+} mobilization. *Biochem. J.* **344**, 55–60.
- Marchant, J. S. and Taylor, C. W. (1997) Cooperative activation of IP_3 receptors by sequential binding of IP_3 and Ca^{2+} safeguards against spontaneous activity. *Curr. Biol.* **7**, 510–518.
- Cardy, T. J. A., Traynor, D. and Taylor, C. W. (1997) Differential regulation of types 1 and 3 inositol trisphosphate receptors by cytosolic Ca^{2+} . *Biochem. J.* **328**, 785–793.
- Nerou, E. P., Riley, A. M., Potter, B. V. L. and Taylor, C. W. (2001) Selective recognition of inositol phosphates by subtypes of inositol trisphosphate receptor. *Biochem. J.* **355**, 59–69.
- Yagodin, S., Pivovarova, N. B., Andrews, S. B. and Sattelle, D. B. (1999) Functional characterization of thapsigargin and agonist-insensitive acidic Ca^{2+} stores in *Drosophila melanogaster* S2 cell lines. *Cell Calcium* **25**, 429–438.
- Correa, V., Riley, A. M., Shuto, S., Horne, G., Nerou, E. P., Marwood, R. D., Potter, B. L. and Taylor, C. W. (2001) Structural determinants of adenylylation activity at inositol trisphosphate receptors. *Mol. Pharmacol.* **59**, 1206–1215.

- 27 Takahashi, M., Tanzawa, K. and Takahashi, S. (1994) Adenophostins, newly discovered metabolites of *Penicillium brevicompactum*, act as potent agonists of the inositol 1,4,5-trisphosphate receptor. *J. Biol. Chem.* **269**, 369–372
- 28 Hirota, J., Michikawa, T., Miyawaki, A., Takahashi, M., Tanzawa, K., Okura, I., Furuichi, T. and Mikoshiba, K. (1995) Adenophostin-mediated quantal Ca^{2+} release in the purified and reconstituted inositol 1,4,5-trisphosphate receptor type 1. *FEBS Lett.* **368**, 248–252
- 29 Missiaen, L., Parys, J. B., Sienaeert, I., Maes, K., Kunzelmann, K., Takahashi, M., Tanzawa, K. and De Smet, H. (1998) Functional properties of the type-3 InsP_3 receptor in 16HBE14o- bronchial mucosal cells. *J. Biol. Chem.* **273**, 8983–8986
- 30 Renard-Rooney, D. C., Hajnóczky, G., Seitz, M. B., Schneider, T. G. and Thomas, A. P. (1993) Imaging of inositol 1,4,5-trisphosphate-induced Ca^{2+} fluxes in single permeabilized hepatocytes: demonstration of both quantal and nonquantal patterns of Ca^{2+} release. *J. Biol. Chem.* **268**, 23601–23610
- 31 Muallem, S., Pandol, S. J. and Beeker, T. G. (1989) Hormone-evoked calcium release from intracellular stores is a quantal process. *J. Biol. Chem.* **264**, 205–212
- 32 Bootman, M. D., Berridge, M. J. and Taylor, C. W. (1992) All-or-nothing Ca^{2+} mobilization from the intracellular stores of single histamine-stimulated HeLa cells. *J. Physiol.* **450**, 163–178
- 33 Beecroft, M. D. and Taylor, C. W. (1997) Incremental Ca^{2+} mobilization by inositol trisphosphate receptors is unlikely to be mediated by their desensitization or regulation by luminal or cytosolic Ca^{2+} . *Biochem. J.* **326**, 215–220
- 34 Parys, J. B., Missiaen, L., De Smedt, H., Sienaeert, I. and Casteels, R. (1996) Mechanisms responsible for quantal Ca^{2+} release from inositol trisphosphate-sensitive calcium stores. *Pflügers Arch.* **432**, 359–367
- 35 Taylor, C. W. (1998) Inositol trisphosphate receptors: Ca^{2+} -modulated intracellular Ca^{2+} channels. *Biochim. Biophys. Acta.* **1436**, 19–33
- 36 Hagar, R. E., Burgstahler, A. D., Nathanson, M. H. and Ehrlich, B. E. (1998) Type III InsP_3 receptor channel stays open in the presence of increased calcium. *Nature (London)* **296**, 81–84
- 37 Mak, D.-O. D., McBride, S., Raghiram, V., Yue, Y., Joseph, S. K. and Foskett, J. K. (2000) Single-channel properties in endoplasmic reticulum membrane of recombinant type 3 inositol trisphosphate receptor. *J. Gen. Physiol.* **115**, 241–255
- 38 Adkins, C. E. and Taylor, C. W. (1999) Lateral inhibition of inositol 1,4,5-trisphosphate receptors by cytosolic Ca^{2+} . *Curr. Biol.* **9**, 1115–1118
- 39 Marchant, J. S. and Taylor, C. W. (1998) Rapid activation and partial inactivation of inositol trisphosphate receptors by inositol trisphosphate. *Biochemistry* **37**, 11524–11533

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