

## RESEARCH COMMUNICATION

**Kinetics of elementary  $\text{Ca}^{2+}$  puffs evoked in *Xenopus* oocytes by different  $\text{Ins}(1,4,5)\text{P}_3$  receptor agonists**Jonathan S. MARCHANT\* and Ian PARKER†<sup>1</sup>

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Elementary  $\text{Ca}^{2+}$  puffs form the basic building blocks of global  $\text{Ins}(1,4,5)\text{P}_3$ -evoked  $\text{Ca}^{2+}$  signals. In *Xenopus* oocytes,  $\text{Ca}^{2+}$  puffs evoked by the high-affinity agonist adenophostin were shorter and smaller than puffs evoked by  $\text{Ins}(1,4,5)\text{P}_3$  and the lower

affinity analogue  $\text{Ins}(2,4,5)\text{P}_3$ . Agonist-specific mechanisms, therefore, play a role in shaping local  $\text{Ca}^{2+}$  release events, but termination of  $\text{Ca}^{2+}$  flux is not delimited simply by agonist dissociation.

**INTRODUCTION**

The coordinated activation of  $\text{Ins}(1,4,5)\text{P}_3$  receptors in many cell types generates repetitive oscillations in cytoplasmic  $\text{Ca}^{2+}$  concentration that serve to control the activity of numerous proteins [1,2]. The basic building blocks of these global  $\text{Ca}^{2+}$  signals are 'Ca<sup>2+</sup> puffs', spatially restricted, subcellular  $\text{Ca}^{2+}$  release events, persisting for a few hundred milliseconds, that become progressively recruited by  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release at increasing  $\text{Ins}(1,4,5)\text{P}_3$  concentrations to generate propagating  $\text{Ca}^{2+}$  waves [3–7]. The time-course of individual  $\text{Ca}^{2+}$  puffs recorded at a given release site varies considerably, such that varying amounts of  $\text{Ca}^{2+}$  are liberated [3,8,9]. This variable capacity for local  $\text{Ca}^{2+}$  release is an important factor that controls the initiation of  $\text{Ca}^{2+}$  waves [3]. Consequently, it is important to understand the regulatory mechanisms that act upon  $\text{Ins}(1,4,5)\text{P}_3$  receptors to determine the time-course and magnitude of  $\text{Ca}^{2+}$  puffs and thereby the capacity for initiation of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release [3,10]. One possibility is that  $\text{Ins}(1,4,5)\text{P}_3$  dissociation from its receptor terminates  $\text{Ca}^{2+}$  liberation during a puff, and hence delimits the total  $\text{Ca}^{2+}$  efflux. In that case, the affinity of the challenging agonist (which is partly determined by its dissociation rate) is expected to influence the duration of these events, with higher affinity agonists producing longer durations of  $\text{Ca}^{2+}$  release [11]. To test this prediction, we employed high-resolution laser scanning confocal microscopy [12] to compare the kinetics and magnitudes of  $\text{Ca}^{2+}$  puffs evoked in *Xenopus* oocytes by  $\text{Ins}(1,4,5)\text{P}_3$  with those evoked by other agonists of differing affinities.

**MATERIALS AND METHODS****Materials**

[<sup>3</sup>H] $\text{Ins}(1,4,5)\text{P}_3$  (58 Ci/mmol) was obtained from Amersham (Little Chalfont, U.K.). Caged  $\text{Ins}(1,4,5)\text{P}_3$  [ $\text{Ins}(1,4,5)\text{P}_3$ ,  $P^{4(6)}$ -1-(2-nitrophenyl) ethyl ester] and Oregon Green 488 BAPTA-1 were from Molecular Probes Inc. (Eugene, OR, U.S.A.).  $\text{Ins}(1,4,5)\text{P}_3$  was from American Radiolabelled Chemicals (St. Louis, MO, U.S.A.), D- $\text{Ins}(2,4,5)\text{P}_3$  was purified from bovine

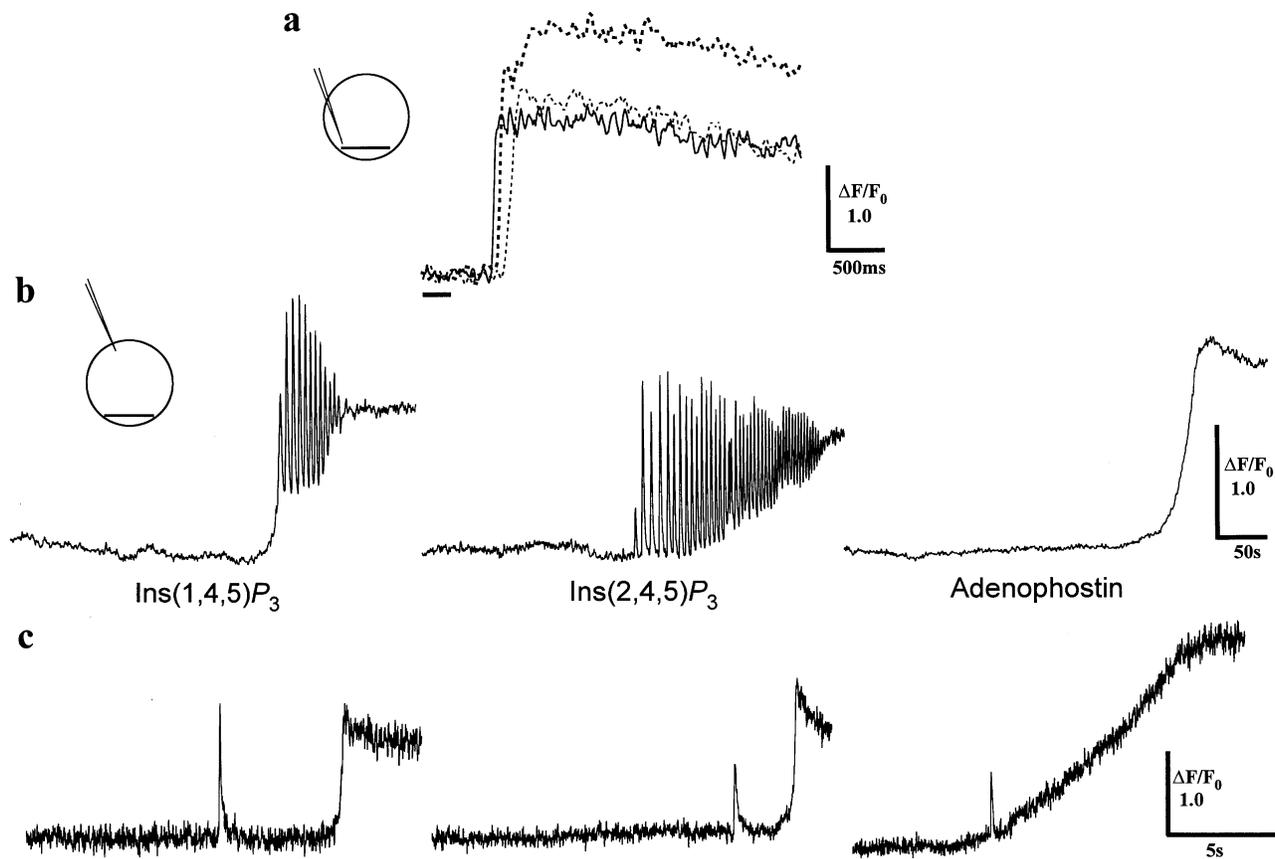
brain [13] and adenophostin A was a generous gift from Dr. M. Takahashi (Sankyo Co., Tokyo, Japan). All other reagents were from suppliers reported previously [8,14].

**Confocal line-scanning microscopy in *Xenopus* oocytes**

Experiments were performed using defolliculated immature oocytes from *Xenopus laevis*, prepared as detailed previously [8]. Stage V and VI oocytes, bathed in Ringer's solution (120 mM NaCl/2 mM KCl/1.8 mM  $\text{CaCl}_2$ /5 mM Hepes, pH 7.2, at room temperature) were microinjected with the fluorescent calcium indicator Oregon Green 488 BAPTA-1 ( $K_d$  for  $\text{Ca}^{2+}$  ~ 170 nM) and caged  $\text{Ins}(1,4,5)\text{P}_3$  (final intracellular concentrations ~ 40  $\mu\text{M}$  and ~ 5  $\mu\text{M}$  respectively) 1 h before recording images at room temperature. Confocal line-scan imaging and flash photolysis of caged  $\text{Ins}(1,4,5)\text{P}_3$  were achieved using the 'homebrew' confocal microscope and UV illumination system described previously [12]. Recordings were obtained using an Olympus IX70 inverted microscope, with the scan line focused on the underside of the oocyte at the depth of the pigment granules (~ 6  $\mu\text{m}$  inward from the surface membrane), where  $\text{Ins}(1,4,5)\text{P}_3$ -sensitive release sites are localized [15]. Line-scan images were collected at a scan rate of 8 ms per 100  $\mu\text{m}$  line, with a nominal pixel size of 0.13  $\mu\text{m}$  and an integration time of 10  $\mu\text{s}$  per pixel.  $\text{Ins}(1,4,5)\text{P}_3$  receptor agonists were injected into the oocyte by ionophoresis (pipette-negative pulses of about 500 nA and 100–200 ms at 1 Hz, with a steady backing current of about 50 nA to minimize unwanted leakage) from pipettes positioned relative to the scan line using a Narishige hydraulic micro-manipulator. Line-scan  $\text{Ca}^{2+}$ -fluorescence images were constructed and processed using routines written in the IDL programming language (Research Systems Inc., Boulder, CO, U.S.A.). Fluorescence is expressed as a pseudo-ratio ( $F/F_0$  or  $\Delta F/F_0$ ) of fluorescence at a given pixel during a response ( $F$ ) relative to the mean value before stimulation ( $F_0$ ). Fluorescence signal mass [8] provides an estimate of the amount of  $\text{Ca}^{2+}$  released during an elementary event, and was obtained by integration of the fluorescence profile from sharply defined, discrete events through three dimensions. The resulting quantity has units of  $\Delta F/F_0 \times 1 \times 10^{-15}$  litres; where 1 unit corresponds to

Abbreviations used: caged  $\text{Ins}(1,4,5)\text{P}_3$ ,  $\text{Ins}(1,4,5)\text{P}_3$ ,  $P^{4(6)}$ -1-(2-nitrophenyl) ethyl ester;  $[\text{Ca}^{2+}]_{\text{cyt}}$ , cytosolic free  $\text{Ca}^{2+}$  concentration; FKBP, FK506-binding protein molecule.

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**Figure 1** Patterns of  $\text{Ca}^{2+}$  liberation evoked by  $\text{Ins}(1,4,5)\text{P}_3$ ,  $\text{Ins}(2,4,5)\text{P}_3$  and adenophostin in *Xenopus* oocytes

(a) Superimposed records showing the time-course of elevation of  $\text{Ca}^{2+}$  at the centre of a  $100\ \mu\text{m}$  scan line following the ionophoretic release (200 ms pulse, black bar) of  $\text{Ins}(1,4,5)\text{P}_3$  (black line),  $\text{Ins}(2,4,5)\text{P}_3$  (heavy dotted line) and adenophostin (light dotted line) from a pipette positioned near the end of the laser line. The concentration of all analogues in the ionophoretic pipette was  $500\ \mu\text{M}$ . Traces show fluorescence ratios ( $F/F_0$ ) measured from a 3 pixel region. (b) Traces monitor the cytosolic  $\text{Ca}^{2+}$  concentration within a  $2\ \mu\text{m}$  region along the laser scan line following the ionophoretic injection of  $\text{Ins}(1,4,5)\text{P}_3$ ,  $\text{Ins}(2,4,5)\text{P}_3$  and adenophostin from pipettes positioned at the opposite side of the oocyte to the laser line (about 1 mm distant). In each case, ionophoretic pulses (100 ms duration, 1 Hz) began at the start of the trace and continued for the duration of the record. Inset diagrams in (a) and (b) show side views of the oocyte, illustrating the positioning of the ionophoresis pipette relative to the confocal scan line. (c) Fluorescence profiles during continued ionophoresis of low concentrations of (from left to right)  $\text{Ins}(1,4,5)\text{P}_3$ ,  $\text{Ins}(2,4,5)\text{P}_3$  and adenophostin, with the pipettes positioned as in (b), showing discrete elementary events before a  $\text{Ca}^{2+}$  wave. Measurements were made from  $0.4\ \mu\text{m}$  regions, positioned over active puff sites.

a doubling of fluorescence in a volume of 1 fl, and results from release of roughly  $2 \times 10^{-20}$  moles of  $\text{Ca}^{2+}$  [8].

### Membrane binding assay

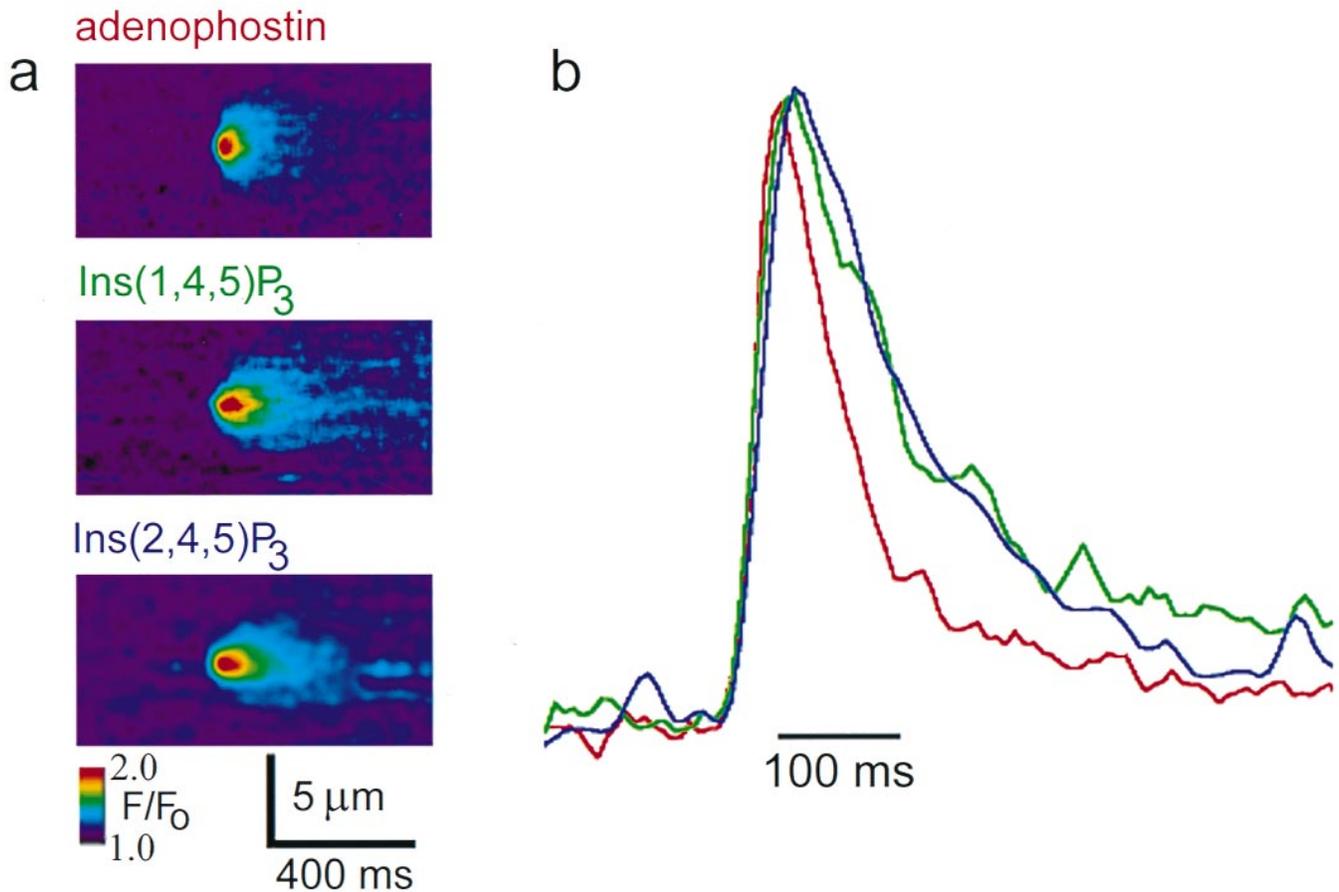
Binding assays were performed on rat cerebellar membranes, as *Xenopus* membrane preparations showed unacceptably low levels of specific binding ( $22.1 \pm 3.0\%$  of total,  $n = 7$  independent preparations). *Xenopus*  $\text{Ins}(1,4,5)\text{P}_3$  receptors share 90% structural identity with cerebellar  $\text{Ins}(1,4,5)\text{P}_3$  receptors and exhibit a similar rank order of analogue potency when binding is assessed in either purified or transfected preparations [16,17]. Membranes prepared from rat cerebellum [14] were stored in liquid nitrogen for up to 2 weeks ( $\sim 0.5$  mg of protein/ml in binding buffer) and equilibrium binding of [ $^3\text{H}$ ] $\text{Ins}(1,4,5)\text{P}_3$  was subsequently measured using a centrifugation assay [14]. Briefly, membranes (0.04 mg of protein/tube) were added to binding buffer (50 mM Tris/HCl/1 mM EDTA, pH 8.3, at  $4^\circ\text{C}$ ), containing [ $^3\text{H}$ ]- $\text{Ins}(1,4,5)\text{P}_3$  (30 nCi, final concentration = 1 nM) and the appropriate concentration of competing ligand. After incubation for 5 min on ice, bound and free [ $^3\text{H}$ ] $\text{Ins}(1,4,5)\text{P}_3$  were separated by centrifugation (200000 g, 5 min,  $0^\circ\text{C}$ ). Competition binding

data were fitted using non-linear least-squares regression analysis to determine  $\text{IC}_{50}$ , the concentration of competing ligand causing displacement of half the specifically bound radiolabel. All results are presented as means  $\pm$  S.E.M.

### RESULTS

To compare  $\text{Ca}^{2+}$  release events elicited by  $\text{Ins}(1,4,5)\text{P}_3$  with those evoked by agonists of differing affinities, we examined responses to adenophostin A, a metabolically stable glyconucleotide isolated from the fungus *Penicillium brevicompactum*, which is the most potent agonist of  $\text{Ins}(1,4,5)\text{P}_3$  receptors yet identified [18,19], and the lower affinity agonist  $\text{Ins}(2,4,5)\text{P}_3$  [20,21]. The binding affinities for  $\text{Ins}(1,4,5)\text{P}_3$  receptors of these analogues were determined in an equilibrium binding assay using cerebellar membranes, as attempts to measure binding to oocyte membranes were confounded by contamination with endogenous lipids, leading to low levels of specific binding [16,17]. Equilibrium dissociation constants ( $K_d$ ) were:  $1.85 \pm 0.23$  nM, adenophostin A;  $6.55 \pm 0.67$  nM,  $\text{Ins}(1,4,5)\text{P}_3$ ; and  $164.4 \pm 17.5$  nM,  $\text{Ins}(2,4,5)\text{P}_3$  ( $n \geq 3$ ).

The effects of  $\text{Ins}(1,4,5)\text{P}_3$ ,  $\text{Ins}(2,4,5)\text{P}_3$  and adenophostin on



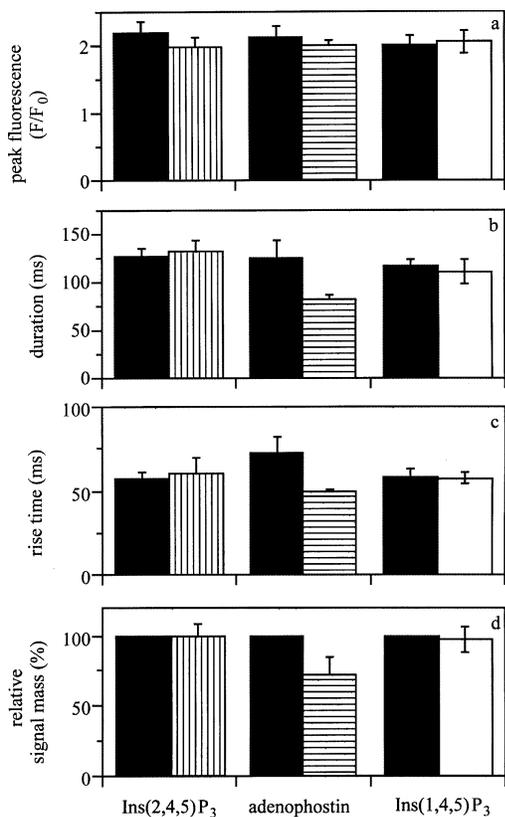
**Figure 2** Ca<sup>2+</sup> puffs evoked by different agonists

(a) Line-scan images show averages of puffs ( $n > 10$  events each, from  $\geq 3$  oocytes) evoked by Ins(1,4,5)P<sub>3</sub>, Ins(2,4,5)P<sub>3</sub> and adenophostin. In each image, distance along the scan line is depicted vertically, and time runs from left to right. Increasingly 'warm' colours correspond to increasing fluorescence ratio ( $F/F_0$ ). Averages were formed by aligning in space and time the peak fluorescence during individual Ca<sup>2+</sup> puffs. (b) Traces show fluorescence ratios ( $F/F_0$ ) measured from a 3 pixel region ( $\sim 0.4 \mu\text{m}$ ) through the point of peak fluorescence on each image: Ins(2,4,5)P<sub>3</sub>, blue line; adenophostin, red line; Ins(1,4,5)P<sub>3</sub>, green line.

cytosolic Ca<sup>2+</sup> concentration ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ) were monitored by ionophoresing each analogue into *Xenopus* oocytes previously micro-injected with the fluorescent Ca<sup>2+</sup> indicator Oregon Green 488 BAPTA-1 and caged Ins(1,4,5)P<sub>3</sub>. Ca<sup>2+</sup>-dependent fluorescence was visualized by line-scan confocal microscopy, imaging along a 100 μm line focused by an inverted microscope in the vegetal hemisphere of the oocytes. With the ionophoretic pipette inserted through the oocyte so that its tip lay adjacent to one end of the laser scan line, ionophoresis of amounts of analogues sufficient to evoke maximal responses triggered rapid Ca<sup>2+</sup> release from the intracellular stores, after a latency that was similarly brief for each analogue (Figure 1a). When the ionophoretic pipette was inserted with its tip positioned near the top of the oocyte ( $\sim 1 \text{ mm}$  from the scan line), the latency to Ca<sup>2+</sup> release for each analogue was much longer and, with Ins(1,4,5)P<sub>3</sub> and Ins(2,4,5)P<sub>3</sub>, repetitive Ca<sup>2+</sup> waves were observed before a sustained elevation in  $[\text{Ca}^{2+}]_{\text{cyt}}$  (Figure 1b). In contrast, repetitive Ca<sup>2+</sup> waves were never observed ( $n = 18$  cells) with a range of adenophostin concentrations (Figure 1b). Furthermore, the rate of rise of  $[\text{Ca}^{2+}]_{\text{cyt}}$  with adenophostin was less abrupt than with the other agonists (Figure 1c) and, even after  $[\text{Ca}^{2+}]_{\text{cyt}}$  had declined almost to the resting level following its peak, responses could no longer be evoked by photolytic release of concentrations of Ins(1,4,5)P<sub>3</sub>

up to 100-fold higher than required previously to mobilize Ca<sup>2+</sup> at the same site.

When the amount of Ins(1,4,5)P<sub>3</sub> injected through the ionophoretic pipette was reduced, discrete elementary events were resolved preceding the initiation of a Ca<sup>2+</sup> wave (Figure 1c). At lower doses still, Ca<sup>2+</sup> puffs were observed as discrete independent events that failed to trigger a global Ca<sup>2+</sup> rise. Because of their resistance to metabolic inactivation [18,22], continued ionophoresis of even low concentrations of Ins(2,4,5)P<sub>3</sub> and adenophostin eventually resulted in the initiation of a widespread Ca<sup>2+</sup> elevation. Nonetheless, discrete Ca<sup>2+</sup> puffs were resolved during the early stages of the response to low doses of all agonists (Figure 1c). Quantitative comparisons of the properties of Ca<sup>2+</sup> puffs evoked by these different agonists were made using four parameters determined from the fluorescence profile of individual events (Figure 2b): peak fluorescence, duration, rise time and 'signal mass' (a measure of the total amount of Ca<sup>2+</sup> liberation). Paired measurements of puffs evoked by ionophoresis of adenophostin or Ins(2,4,5)P<sub>3</sub> were compared with Ca<sup>2+</sup> puffs evoked previously in the same oocytes by photoreleasing low concentrations of Ins(1,4,5)P<sub>3</sub>. Despite the different means of delivery (ionophoresis versus photorelease), this comparison is valid, since ionophoresed or photoreleased Ins(1,4,5)P<sub>3</sub> evoked puffs



**Figure 3** Magnitudes and kinetics of  $Ca^{2+}$  puffs evoked by  $Ins(2,4,5)P_3$ , adenophostin and  $Ins(1,4,5)P_3$

The characteristics of  $Ca^{2+}$  puffs evoked following ionophoresis of  $Ins(2,4,5)P_3$  (vertical striped bars) adenophostin (horizontal striped bars) and  $Ins(1,4,5)P_3$  (clear bars) were compared within the same oocyte with  $Ca^{2+}$  puffs evoked by photorelease of caged  $Ins(1,4,5)P_3$  (black bars). Bars show means  $\pm$  S.E.M. of  $> 50$  individual events in at least three oocytes. (a) Peak fluorescence of  $Ca^{2+}$  puffs expressed as  $F/F_0$ . (b) Durations of puffs, defined as the time for which the fluorescence signal exceeded one-half of the peak amplitude during a puff. (c) Rise times of puffs, measured from baseline to peak fluorescence. (d) Relative signal mass of puffs, expressed as a percentage of that evoked by photoreleased  $Ins(1,4,5)P_3$  in the same cells. Signal mass values (see Materials and methods section) are expressed relative to the amount of  $Ca^{2+}$  released during  $Ins(1,4,5)P_3$ -evoked  $Ca^{2+}$  puffs in the same oocyte.

with identical characteristics (black and clear bars, respectively, in Figure 3).

Similar to the variation in magnitude and kinetics of individual puffs evoked by  $Ins(1,4,5)P_3$  [8], puffs evoked by adenophostin and  $Ins(2,4,5)P_3$  were not stereotyped events. To illustrate the mean characteristics of puffs evoked by each agonist, Figure 2(a) shows average images formed from more than ten individual events, and the corresponding kinetics of the average fluorescence signals are plotted in Figure 2(b). The peak fluorescence changes during puffs were almost identical with all three agonists (Figure 3a), suggesting that the peak rates of  $Ca^{2+}$  liberation were similar [8]. Furthermore, no differences were apparent between  $Ins(1,4,5)P_3$  and  $Ins(2,4,5)P_3$  in terms of half-maximal duration (Figure 3b) or rise time (Figure 3c) of puffs, nor in the amounts of  $Ca^{2+}$  liberated during puffs (Figure 3d). Estimates of  $Ca^{2+}$  liberation based on the fluorescence 'signal mass' [8] yielded values of  $(3.6 \pm 0.2) \times 10^{-19}$  moles of  $Ca^{2+}$  for puffs evoked by  $Ins(1,4,5)P_3$  and  $Ins(2,4,5)P_3$ . On the other hand, puffs evoked by adenophostin showed appreciably faster kinetics (Figure 2; and horizontal striped bars in Figure 3), with shorter

duration and more rapid rise, resulting in a smaller relative signal mass, corresponding to liberation of, on average,  $(2.6 \pm 0.3) \times 10^{-19}$  moles of  $Ca^{2+}$ .

## DISCUSSION

The mechanisms which regulate the duration of  $Ins(1,4,5)P_3$  receptor openings during a  $Ca^{2+}$  puff, and consequently the amount of  $Ca^{2+}$  released into the cytoplasm, are unresolved [3,8]. In *Xenopus* oocytes, the smallest  $Ins(1,4,5)P_3$ -evoked events liberate  $\sim 2 \times 10^{-20}$  moles of  $Ca^{2+}$  [8], arising from a  $Ca^{2+}$  release flux (current of  $< 0.5$  pA) that is consistent with estimates of calcium current through a single  $Ins(1,4,5)P_3$  receptor under physiological conditions [23,24]. In contrast, larger  $Ca^{2+}$  release events liberate as much as  $5 \times 10^{-18}$  moles of  $Ca^{2+}$ , because several clustered receptors activate synchronously and remain open longer [8]. The time course of  $Ins(1,4,5)P_3$  dissociation from its receptor under physiological conditions ( $t_{1/2} = 125$  ms [25]) is compatible with the durations of  $Ca^{2+}$  liberation during puffs ( $\sim 150$  ms [8]), suggesting that the time course of elementary release events may be determined by the stochastic dissociation of agonist molecules from the  $Ins(1,4,5)P_3$  receptor, in a manner analogous to the agonist-specific open life-time of other ligand-gated channels [11,26].

To assess this possibility, we compared the properties of  $Ca^{2+}$  puffs in *Xenopus* oocytes evoked by three agonists differing by  $\sim 90$ -fold in their affinity for the  $Ins(1,4,5)P_3$  receptor. With similar diffusion-limited association rates [27], the mean open duration of  $Ins(1,4,5)P_3$  receptors/channels is expected to shorten with decreasing agonist affinity, thereby resulting in smaller and briefer puffs. In contrast to this, experimental observations showed the average characteristics of puffs evoked by  $Ins(1,4,5)P_3$  and  $Ins(2,4,5)P_3$  to be almost identical, despite the 25-fold lower affinity of  $Ins(2,4,5)P_3$ , whereas puffs evoked by the high-affinity agonist adenophostin were shorter and involved a smaller liberation of  $Ca^{2+}$  (Figures 2 and 3). We conclude that the duration of a  $Ca^{2+}$  puff bears no simple relationship to the affinity of the agonist that evoked it, and, consequently, propose that regulatory mechanisms other than agonist dissociation must delimit the period of  $Ca^{2+}$  flux through  $Ins(1,4,5)P_3$  receptors at the microscopic level. Possibilities include the processes of  $Ca^{2+}$ -mediated inhibition [28,29] and  $Ins(1,4,5)P_3$ -induced inactivation [30,31], both of which occur sufficiently rapidly to influence  $Ins(1,4,5)P_3$  receptor behaviour on a timescale compatible with the durations of elementary events. Association of  $Ins(1,4,5)P_3$  receptors with FK506-binding protein molecules (FKBP) [32,33] may be involved, as disruption of FKBP association with cardiac ryanodine receptors prolongs the duration of  $Ca^{2+}$  sparks in rat heart [34,35].

Adenophostin is the first compound reported that modulates the time-course of receptor activity during  $Ca^{2+}$  puffs. Furthermore,  $Ca^{2+}$  liberation induced by adenophostin shows characteristics that are qualitatively different from those induced by  $Ins(1,4,5)P_3$  and  $Ins(2,4,5)P_3$ , including its consistent failure to evoke repetitive  $Ca^{2+}$  spikes (Figure 2b) and oscillatory  $Ca^{2+}$ -activated  $Cl^-$  currents [36], and the absence of abrupt, regenerative  $Ca^{2+}$  release in response to gradually increasing concentrations of adenophostin (Figure 2c). The mechanisms underlying the actions of adenophostin remain to be elucidated, but it appears that it may cause  $Ca^{2+}$  release through  $Ins(1,4,5)P_3$  receptors as both discrete  $Ca^{2+}$  puffs, and in a more continuous fashion, without resolvable elementary events.

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