Rapid activation and partial inactivation of inositol trisphosphate receptors by adenophostin A

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Adenophostin A, the most potent known agonist of inositol 1,4,5-trisphosphate ($InsP_3$) receptors, stimulated $^{45}Ca^{2+}$ release from the intracellular stores of permeabilized hepatocytes. The concentration of adenophostin A causing the half-maximal effect (EC_{50}) was 7.1 ± 0.5 nM, whereas the EC_{50} for $InsP_3$ was 177 ± 26 nM; both responses were positively co-operative. In rapid superfusion analyses of $^{45}Ca^{2+}$ release from the intracellular stores of immobilized hepatocytes, maximal concentrations of adenophostin A or $InsP_3$ evoked indistinguishable patterns of Ca^{2+} release. The Ca^{2+} release evoked by both agonists peaked at the same maximal rate after about 375 ms and the activity of the receptors then decayed to a stable, partially (60 %) inactivated state with a half-time ($t_{1/2}$) of 318 ± 29 ms for adenophostin A and 321 ± 22 ms for $InsP_3$. Dissociation rates were measured by recording rates of $InsP_3$-receptor channel closure after rapid removal of agonist. The rate of adenophostin A dissociation ($t_{1/2}$, 840 ± 195 ms) was only 2-fold slower than that of $InsP_3$ ($t_{1/2}$, 436 ± 48 ms). We conclude that slow dissociation of adenophostin A from $InsP_3$ receptors does not underlie either its high-affinity binding or the reported differences in the Ca^{2+} signals evoked by $InsP_3$ and adenophostin A in intact cells.

Key words: analogue, Ca^{2+} channel, Ca^{2+} mobilization, hepatocyte, kinetics.

INTRODUCTION

Inositol 1,4,5-trisphosphate ($InsP_3$) receptors are intracellular Ca^{2+} channels that are regulated by both $InsP_3$ and Ca^{2+} [1]. Most analyses of $InsP_3$ receptors have used either the endogenous ligand, $InsP_3$, or related inositol phosphates [2], but the demonstration that adenophostins, products of the fungus *Penicillium brevicompactum*, are the most potent known agonists of $InsP_3$ receptors [3] has provided additional opportunities to examine $InsP_3$-receptor behaviour. Adenophostins A and B are not metabolized by the enzymes that degrade $InsP_3$ [4], they do not bind to $InsP_4$ receptors [4], and in both functional and radioligand-binding assays of all three $InsP_3$-receptor subtypes they bind with 10–100-fold greater affinity than $InsP_3$ [3,5–11].

The structure of adenophostin suggests that its glucose 3″,4″-bisphosphate structure and adjacent 2″-hydroxy may mimic the critical 4,5-bisphosphate and 6-hydroxy of $InsP_3$ (Figure 1A), and that the adenine group may increase the strength of the binding either by improving the positioning of the 2′-phosphate (analogous to the 1-phosphate of $InsP_3$) or through a more direct interaction (presumably hydrophobic) with a residue close to the $InsP_3$-binding site of the receptor [4,7,12]. Because syntheses of adenophostins and their analogues involve fewer steps than syntheses of chiral inositol phosphates, adenophostins provide an alternative approach to developing high-affinity selective ligands of $InsP_3$ receptors [8,13].

Many observations are consistent with the notion that adenophostins are simply high-affinity analogues of $InsP_3$. Adenophostin A and $InsP_3$ have similar EC_{50}/K_d ratios [6,14], consistent with the two agonists having similar efficacy. Adenophostin A can, like $InsP_3$, trigger Ca^{2+} oscillations ([15,16], but see [17]), and chronic exposure to either $InsP_3$ or adenophostin A down-regulates type-1 $InsP_3$ receptors [18]. The quantal patterns of Ca^{2+} release mediated by reconstituted type-1 $InsP_3$ receptors are also similar for $InsP_3$ and adenophostin A [5].

However, there are also differences in the responses evoked by adenophostin A and $InsP_3$. In both functional [5,13] and binding assays [5,7] of $InsP_3$ receptors, the interactions with adenophostin A are often more positively co-operative than those with $InsP_3$. It has been suggested that adenophostin A, though not $InsP_3$, can activate store-regulated Ca^{2+} entry [19] without detectably emptying intracellular Ca^{2+} stores [20], although subsequent studies [11,21,22] suggest that the disparity probably results from slow diffusion of adenophostin A within the cytosol allowing localized emptying of stores. Several authors have suggested that high-affinity binding of adenophostin A to $InsP_3$ receptors may impede its diffusion [11,16,22], but the density of $InsP_3$-receptor binding sites ($\leqslant 2$ nM) [23] suggests that they may only minimally influence diffusion of even the lowest concentrations of adenophostin A used (10 nM). Adenophostin A may, of course, bind to other sites and, because it is used at much lower concentrations than $InsP_3$, these sites may significantly deplete the free ligand concentration. The effects of cytosolic Ca^{2+} on $InsP_3$ receptors may also differ according to whether they have $InsP_3$ or adenophostin A bound [24,25]. Finally, the elementary Ca^{2+}-release events, Ca^{2+} puffs, evoked by adenophostin A, decay more rapidly than those evoked by $InsP_3$ [17]. The mechanisms underlying these different effects of $InsP_3$ and adenophostin A are unclear, but they are commonly assumed to be a consequence of adenophostin A dissociating from $InsP_3$ receptors more slowly than does $InsP_3$.

In the present study, we used rapid superfusion methods to compare the kinetics of adenophostin A- and $InsP_3$-evoked Ca^{2+} mobilization from permeabilized hepatocytes and to directly measure the rates of dissociation of each agonist from active $InsP_3$ receptors.

Abbreviations used: CLM, cytosol-like medium; $InsP_3$, inositol 1,4,5-trisphosphate; $t_{1/2}$, half-time.

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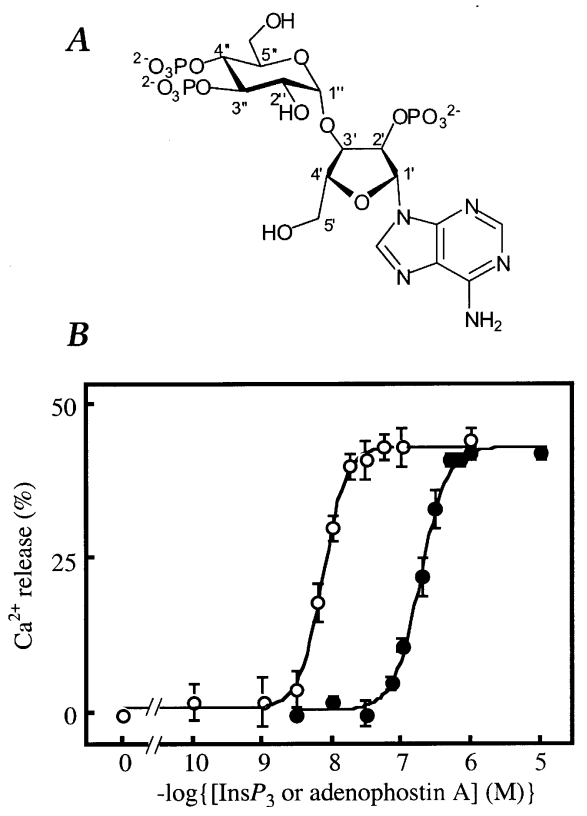

Figure 1 Adenophostin A-evoked Ca^{2+} release from permeabilized cells

(**A**) Structure of adenophostin A. (**B**) Permeabilized hepatocytes loaded to steady state with $^{45}Ca^{2+}$ were stimulated with the indicated concentrations of InsP_3 (●) or adenophostin A (○) for 1 min. Results (means ± S.E.M. of three independent experiments) show the Ca^{2+} released as a percentage of that released by ionomycin (10 μM).

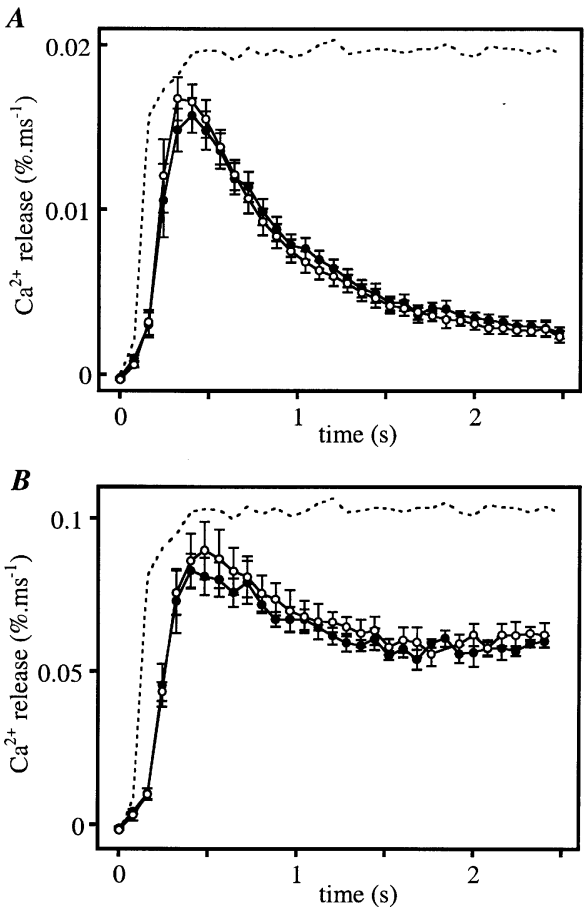

Figure 2 Rapid kinetics of adenophostin A- and InsP_3-evoked Ca^{2+} release

Permeabilized hepatocytes loaded with $^{45}Ca^{2+}$ were superfused with CLM containing either InsP_3 (10 μM, ●) or adenophostin A (10 μM, ○). The broken lines denote the arrival in the superfusate of [^{3}H]inulin (included with the agonists). The rates of $^{45}Ca^{2+}$ release (means ± S.E.M. of three independent experiments) are expressed either as fractions of the entire intracellular Ca^{2+} stores (**A**) or as fractional release rates (**B**, i.e. the $^{45}Ca^{2+}$ released into each fraction as a percentage of that remaining within the agonist-sensitive stores at the beginning of that collection interval).

EXPERIMENTAL PROCEDURES

Materials

Adenophostin A was synthesized and quantified as described previously and was used as the hexakis sodium salt [13]. InsP_3 was from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Thapsigargin was from Alamone Laboratories (Jerusalem, Israel) and ionomycin was from Calbiochem (Nottingham, U.K.).

Measurements of $^{45}Ca^{2+}$ efflux from permeabilized cells

Hepatocytes were isolated from the livers of male Wistar rats (200–300 g) as described previously [26], and permeabilized in Ca^{2+}-free cytosol-like medium (CLM; 100 mM KCl/20 mM NaCl/5 mM $MgCl_2$/1 mM EGTA/20 mM Pipes, pH 7) by incubation with saponin (10 $\mu g \cdot ml^{-1}$) at 37 °C for 8 min. The cells were resuspended (2.2×10^6 cells $\cdot ml^{-1}$) in CLM supplemented with 300 μM $CaCl_2$ (free [Ca^{2+}], 200 nM), ATP (1.5 mM), creatine phosphate (5 mM), creatine phosphokinase (1 unit $\cdot ml^{-1}$), the mitochondrial inhibitor carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 10 μM) and $^{45}CaCl_2$ (5 $\mu Ci \cdot ml^{-1}$; 15 $\mu Ci \cdot ml^{-1}$ for superfusion experiments). After 5 min at 37 °C, during which the intracellular stores were loaded to the steady state with $^{45}Ca^{2+}$, InsP_3 or adenophostin A was added, together with thapisgargin (1 μM) to inhibit further Ca^{2+} uptake. After a further 1 min, the $^{45}Ca^{2+}$ contents of the stores were determined by filtration through Whatman GF/C filters followed by washing with ice-cold sucrose (310 mM) and sodium citrate (10 mM). The actively accumulated $^{45}Ca^{2+}$ content of the stores was defined as that which could be released by addition of ionomycin (10 μM).

Rapid kinetics of $^{45}Ca^{2+}$ release from intracellular stores

For measurements of the rapid kinetics of unidirectional $^{45}Ca^{2+}$ efflux, permeabilized hepatocytes loaded with $^{45}Ca^{2+}$ were immobilized rapidly on a sandwich of nitrocellulose and glass-fibre filters (6 mm diameter) and mounted in the chamber of a computer-controlled rapid superfusion apparatus; the details have been described previously [27]. Briefly, the stainless steel and Teflon superfusion chamber (volume < 50 μl) was connected by computer-controlled solenoid valves to four pressurized vessels containing CLM (free [Ca^{2+}], 200 nM) and appropriate additions. Fluid flowed continuously at 2 ml $\cdot s^{-1}$ from one of these vessels, through the immobilized cells and out of the superfusion chamber. The effluent, containing the $^{45}Ca^{2+}$ released from the cells, was collected into vials arranged around the circumference of a circular, variable-speed fraction collector [27]. For most

experiments, the fractions corresponded with intervals of 80 ms, but for the experiments shown in Figure 4 (see below) the intervals were 9 ms. Inclusion of an inert marker ([^{3}H]inulin) allowed the arrival of InsP_3 or adenophostin A to be related precisely to changes in the rate of $^{45}Ca^{2+}$ release. A reed switch reported the position of the turntable to a Viglen III/LS computer, which synchronized rotation of the fraction collector with activation of the solenoid valves. At the end of each experiment, cells were superfused with CLM containing Triton X-100 (0.5 %) to release all $^{45}Ca^{2+}$ remaining within the intracellular stores. All rapid superfusion experiments were carried out at 20 °C.

Analysis of results

Rates of $^{45}Ca^{2+}$ release (into each 80 ms or 9 ms time bin) were expressed initially as percentages of the total $^{45}Ca^{2+}$ content of intracellular stores and then corrected for the small basal leak rate ($\approx 1.2\,\% \cdot s^{-1}$) measured at the beginning of each recording. Because the $^{45}Ca^{2+}$ content of the intracellular stores declines as $^{45}Ca^{2+}$ leaks from them, this form of analysis inevitably includes an exponentially decaying component (Figure 2A). For analysis of inactivation kinetics, therefore, fractional release rates were calculated (i.e. the $^{45}Ca^{2+}$ released into each fraction was expressed as a percentage of that still remaining within the InsP_3- or adenophostin A-sensitive Ca^{2+} stores). With this form of analysis [28], fractional rates of $^{45}Ca^{2+}$ release remain constant unless the InsP_3 receptor changes its behaviour. Least-squares curve-fitting routines (Kaleidagraph, Synergy Software) were used to fit exponential equations to the time courses of the decaying phases of InsP_3- or adenophostin A-evoked $^{45}Ca^{2+}$ release.

RESULTS

Rapid responses to adenophostin A

Our first results (Figure 1B and Table 1) confirm previous observations from both hepatocytes [7] and other cells [4–6,10] by demonstrating that adenophostin A stimulates Ca^{2+} release from intracellular stores at concentrations that are about 25-fold lower than those required for InsP_3-evoked Ca^{2+} mobilization.

Table 1 Adenophostin A- and InsP_3-evoked Ca^{2+} release

EC_{50} values, Hill slopes (h) and maximal effects (percentage of the intracellular Ca^{2+} stores) of the responses to adenophostin A and InsP_3 were determined from the conventional filtration experiments shown in Figure 1(B). Rapid superfusion experiments similar to those shown in Figures 2 and 3 were used to establish the peak rates of InsP_3- and adenophostin A-evoked Ca^{2+} release and the times taken to reach the peak rate, the half-times ($t_{1/2}$) and extents of agonist-induced channel inactivation, and the half-times for channel closure after removal of adenophostin A or InsP_3 after 380 ms. Results are means ± S.E.M. of between three and nine independent experiments.

	InsP_3	Adenophostin A
Conventional filtration experiments		
EC_{50} (nM)	177 ± 26	7.1 ± 0.5
h	2.5 ± 0.1	2.7 ± 0.4
Maximum response (%)	42 ± 1	44 ± 2
Rapid superfusion experiments		
Peak rate (% · ms^{-1})	0.10 ± 0.01	0.10 ± 0.01
Time to peak (ms)	390 ± 20	360 ± 30
Inactivation (%)	45 ± 6	40 ± 6
$t_{1/2}$ for inactivation (ms)	321 ± 22	318 ± 29
$t_{1/2}$ for decay after agonist washout (ms)	436 ± 48	840 ± 195

Responses to both agonists were positively co-operative (Hill coefficient, h, $\geqslant 2.5$), and maximal concentrations of each caused release of a similar fraction of the intracellular Ca^{2+} stores (Table 1).

In superfusion experiments, maximal concentrations of either adenophostin A or InsP_3 (10 μM) caused a rapid increase in the rate of $^{45}Ca^{2+}$ release, which peaked after about 375 ms, and then abruptly declined with bi-exponential kinetics (Figure 2A). By expressing responses as fractional $^{45}Ca^{2+}$ release rates (see the Experimental procedures section), we removed the element of the decay resulting from loss of $^{45}Ca^{2+}$ from finite stores and unmasked the changing behaviour of the InsP_3 receptors. The results (Figure 2B) demonstrate that responses to InsP_3 and adenophostin A were indistinguishable, with each causing the activity of the InsP_3 receptors to decline with a half-time ($t_{1/2}$) of about 320 ms to a level corresponding to about 60 % of that observed at the peak of the response (Table 1).

Rapid dissociation of adenophostin A from InsP_3 receptors

Both functional (Table 1) and radioligand-binding [7] analyses are consistent with adenophostin A binding to hepatic InsP_3 receptors with at least 10-fold greater affinity than InsP_3. Because differences in rates of dissociation are often major determinants of the affinity of small ligands for their receptors [29], we expected adenophostin A to dissociate from InsP_3 receptors more slowly than InsP_3. Others have made a similar assumption when interpreting the effects of adenophostin A in intact cells [11,17]. The rapid mixing time provided by the superfusion apparatus ($t_{1/2}$, 46 ± 5 ms) allowed the rates of channel closure to be measured after rapid removal of InsP_3 or adenophostin A. After removal of either InsP_3 or adenophostin A at the peak of the response (380 ms after agonists were first added), the rate of $^{45}Ca^{2+}$ release decayed more quickly than in the continued presence of agonist. To ensure that the concentrations of both agonists had fallen below levels capable of evoking a response before we assessed rates of channel closure, we used the amounts of [^{3}H]inulin detected in the perfusate to identify the fractions in which the concentration of ligand first fell below 4 nM. The subsequent rates of $^{45}Ca^{2+}$ release were used to determine the time course of channel closure. The results (Figure 3) indicate that the channels close more slowly after removal of adenophostin A than after removal of InsP_3, consistent with the greater affinity of the former for InsP_3 receptors, but the 1.9-fold slower rate of adenophostin A dissociation is still much faster than expected from its more than 10-fold greater affinity for InsP_3 receptors (Table 1).

Rapid association of InsP_3 and adenophostin A

We attempted to resolve whether adenophostin A associated with InsP_3 receptors more rapidly than InsP_3. Cells were rapidly ($t_{1/2}$, 46 ms) stimulated with a high concentration (50 μM) of either agonist in CLM containing a free [Ca^{2+}] of 100 μM, and the $^{45}Ca^{2+}$ released was collected at intervals of 9 ms. High concentrations of both Ca^{2+} and agonist were necessary because channel opening is slow when the free [Ca^{2+}] is low [30], but the receptors are inhibited by high free [Ca^{2+}] unless they have agonist bound [31]. The results (Figure 4) demonstrate that, under these conditions, both the latency before channel opening (18 ± 3 ms for InsP_3, 21 ± 5 ms for adenophostin A) and the initial change in rate of $^{45}Ca^{2+}$ release (370 ± 53 % · s^{-2} for InsP_3, 320 ± 70 % · s^{-2} for adenophostin A) were similar.

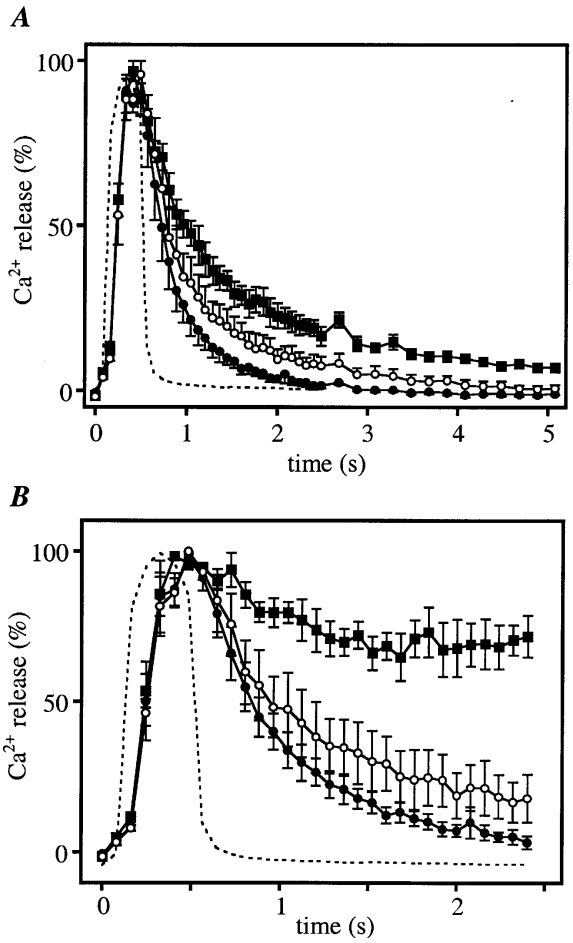

Figure 3 Kinetics of channel closure after rapid removal of adenophostin A or InsP_3

Cells were superfused with adenophostin A (10 μM, ○) or InsP_3 (10 μM, ●) for 380 ms, or with InsP_3 for 5 s (10 μM, ■). $^{45}Ca^{2+}$-release rates (means ± S.E.M. of three experiments) were calculated as either fractions of the total $^{45}Ca^{2+}$ contents of the stores (**A**), or as fractional release rates (**B**), and then, for greater clarity, the rates were expressed as percentages of the peak rate. The broken lines denote the arrival in the superfusate of [^{3}H]inulin (included with the agonists).

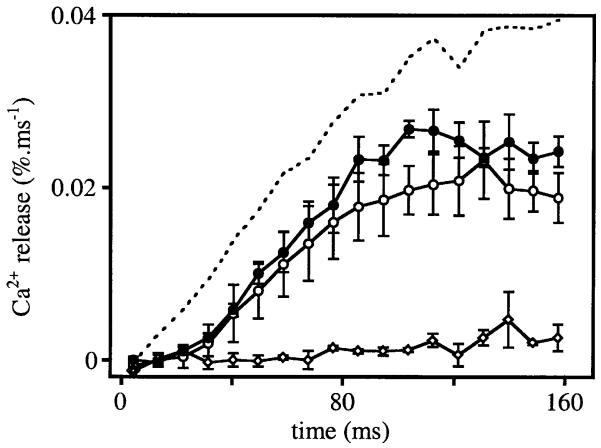

Figure 4 Rapid opening of InsP_3 receptors by high concentrations of InsP_3 or adenophostin A with Ca^{2+}

Cells were stimulated with adenophostin A (50 μM, ○) or InsP_3 (50 μM, ●) in CLM containing 100 μM free Ca^{2+}. The rate of $^{45}Ca^{2+}$ release was measured at intervals of 9 ms. The increase in free [Ca^{2+}] alone had no significant effect on the rate of $^{45}Ca^{2+}$ release (◇). Results are shown as means ± S.E.M. of four independent experiments ($n = 3$ for high Ca^{2+} alone). The broken line denotes the arrival in the superfusate of [^{3}H]inulin (included with the stimuli).

DISCUSSION

It had been widely and reasonably assumed that the high affinity of adenophostin A for InsP_3 receptors resulted from a slow dissociation rate, and that the slow dissociation accounted for the different responses to InsP_3 and adenophostin A observed in intact cells. It was, for example, proposed to explain the slow diffusion of adenophostin A in the cytosol allowing it to generate sustained but spatially restricted Ca^{2+} mobilization [11] (see the Introduction). Surprisingly, Ca^{2+} puffs in *Xenopus* oocytes are briefer when evoked by adenophostin A than when evoked by InsP_3 [17], leading the authors to conclude that agonist dissociation (assumed to be slower for adenophostin A) was not responsible for limiting the duration of elementary Ca^{2+} release events. We, however, have suggested that agonist dissociation rates are indeed likely to control the duration of these events [31], because InsP_3 receptors only partially desensitize [28] (Figure 2B) and feedback inhibition of InsP_3 receptors by cytosolic Ca^{2+} occurs only after InsP_3 has dissociated [31].

Our present results resolve these inconsistencies by providing the first rapid measurements of InsP_3 receptor activation, inactivation and closure in response to adenophostin A. Conventional measurements of the extents of Ca^{2+} mobilization confirmed that responses to adenophostin A occurred at 25-fold lower concentrations than responses to InsP_3 (Figure 1 and Table 1), but high-resolution measurements of the responses evoked by maximal concentrations of each agonist in normal CLM were indistinguishable. Both agonists caused the same peak rate of Ca^{2+} release, suggesting that each has similar efficacy; the times taken to reach the peaks were the same for InsP_3 and adenophostin A, and each then caused a rapid ($t_{1/2} \approx$ 320 ms) partial inactivation of the receptor (Table 1). The only discernible difference between InsP_3 and adenophostin A is the 1.9-fold slower rate of dissociation of the latter from the InsP_3 receptor. Such a modest difference in dissociation rate cannot explain the 25-fold greater sensitivity of cells to adenophostin A; nor is the disparity likely to result from different patterns of Ca^{2+} release by InsP_3 and adenophostin A: both evoke quantal Ca^{2+} release [5] and InsP_3 receptors behave similarly whether occupied by InsP_3 or adenophostin A (Figure 3). We instead speculate that adenophostin A may have a $\approx$ 10-fold faster rate of association with InsP_3 receptors than does InsP_3.

With a faster association rate, we might have expected that the time taken to reach the peak rate of Ca^{2+} release would be shorter for adenophostin A than for InsP_3 ($k_{obs} = k_1$[agonist]$-k_{-1}$). Yet, the results suggest similar rates of channel opening after addition of InsP_3 or adenophostin A (Figure 2). However, our previous studies established that InsP_3 binding causes a Ca^{2+}-binding site to be unmasked and that Ca^{2+} must bind to that site before the channel can open [30]. At normal cytosolic Ca^{2+} concentrations (free [Ca^{2+}] = 200 nM, as used in the present experiments), the Ca^{2+}-binding step becomes rate-limiting for channel opening after maximal stimulation by InsP_3. The experiments shown in Figure 2 would not, therefore, have been expected to resolve whether InsP_3 and adenophostin A differ in their rates of association with the InsP_3 receptor. However, even when cells were stimulated rapidly with InsP_3 or adenophostin A in the

presence of a much higher [Ca^{2+}] (100 μM), there was no discernible difference in the initial rates of channel opening (Figure 4). At present, we can only speculate on the likely reasons for our inability to detect a difference in the rates of association of InsP_3 and adenophostin in these functional assays. It may be that InsP_3 and adenophostin do associate at similar rates; a conclusion that would be difficult to reconcile with the higher affinity of adenophostin A, but only modestly slower dissociation rate (Table 1). Alternatively, if the rate of Ca^{2+} association is slower than that of InsP_3 and adenophostin, even when the free [Ca^{2+}] is high, then any difference in the association rates of InsP_3 and adenophostin A may be masked.

We conclude that maximal concentrations of adenophostin A and InsP_3 cause very similar patterns of Ca^{2+} release, and whereas the agonists differ substantially in their affinities for hepatic InsP_3 receptors, the difference cannot be explained adequately by the modest differences in the rates of dissociation of adenophostin A and InsP_3 from active InsP_3 receptors.

This work was supported by Programme grants from The Wellcome Trust to C.W.T. (039662) and B.V.L.P. We thank Dr R. Marwood for provision of synthetic adenophostin A.

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Received 23 May 2000/4 October 2000; accepted 17 October 2000

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