Cooperative activation of IP_3 receptors by sequential binding of IP_3 and Ca^{2+} safeguards against spontaneous activity

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Background: Ca^{2+} waves allow effective delivery of intracellular Ca^{2+} signals to cytosolic targets. Propagation of these regenerative Ca^{2+} signals probably results from the activation of intracellular Ca^{2+} channels by the increase in cytosolic [Ca^{2+}] that follows the opening of these channels. Such positive feedback is potentially explosive. Mechanisms that limit the spontaneous opening of intracellular Ca^{2+} channels are therefore likely to have evolved in parallel with the mechanism of Ca^{2+}-induced Ca^{2+} release.

Results: Maximal rates of $^{45}Ca^{2+}$ efflux from permeabilised hepatocytes superfused with medium in which the [Ca^{2+}] was clamped were cooperatively stimulated by inositol 1,4,5-trisphosphate (IP_3). A minimal interval of ~400 msec between IP_3 addition and the peak rate of Ca^{2+} release as well as an absolute latency of 30 msec before initiation of Ca^{2+} mobilisation indicate that channel opening does not immediately follow binding of IP_3. Although the absolute latency of Ca^{2+} release was unaffected by further increasing the IP_3 concentration, it was reduced by increased [Ca^{2+}].

Conclusions: We propose that the closed conformation of the IP_3 receptor is very stable and therefore minimally susceptible to spontaneous activation; at least three (probably four) IP_3 molecules may be required to provide enough binding energy to drive the receptor into a stable open conformation. We suggest that a further defence from noise is provided by an extreme form of coincidence detection. Binding of IP_3 to each of its four receptor subunits unmasks a site to which Ca^{2+} must bind before the channel can open. As IP_3 binding may also initiate receptor inactivation, there may be only a narrow temporal window during which each receptor subunit must bind both of its agonists if the channel is to open rather than inactivate.

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Background

Cytosolic Ca^{2+} signals evoked by receptors linked to inositol 1,4,5-trisphosphate (IP_3) formation are complex: the signals often occur after a substantial latency [1] and then comprise cycles of Ca^{2+} release from intracellular stores that give rise to Ca^{2+} waves and Ca^{2+} spikes [2]. The IP_3 receptors that mediate these Ca^{2+} responses are homotetrameric or heterotetrameric assemblies of large subunits [3], which form high conductance IP_3-gated Ca^{2+} channels [4]. The stimulatory and inhibitory effects of cytosolic Ca^{2+} on IP_3 receptors [5–7], together with the possibility that responses to IP_3 are positively cooperative [8], are often invoked to explain the complex patterns of Ca^{2+} mobilisation [2].

In intact cells, the long latency and subsequent sigmoidal increase in cytosolic [Ca^{2+}] after addition of agonists [1,2], flash photolysis of caged IP_3 [9–12], or intracellular perfusion with IP_3 [13] are consistent with either positively cooperative activation of IP_3 receptors by IP_3 alone or by positive feedback from cytosolic [Ca^{2+}]. Distinguishing between these mechanisms, however, is difficult in intact cells, in which IP_3 receptor activation inevitably increases cytosolic [Ca^{2+}]. In broken cells, the use of fluorescent indicators to measure rapid rates of Ca^{2+} efflux is prone to similar difficulties. Stopped-flow analyses of permeabilised basophilic leukaemia cells, for example, suggest that gating of IP_3 receptors by IP_3 is positively cooperative [8]. However, results from a variety of tissues using superfusion [5], bilayer recording [14,15], fluorescent indicators [16] or flash photolysis of caged IP_3 in intact cells [12] suggest that responses to IP_3 are either not positively cooperative [5,12,14] or only slightly so [15,16]. Each approach has its limitations and together the results provide no clear indication of whether responses to IP_3 are positively cooperative in the absence of positive feedback from cytosolic Ca^{2+}.

The high degree of temporal and spatial resolution provided by confocal imaging has refined our understanding of the initiation of IP_3-mediated Ca^{2+} signals by suggesting that increasing concentrations of IP_3 progressively recruit a hierarchy of Ca^{2+}-release events. Elementary events or 'Ca^{2+} blips', which may reflect the opening of single IP_3

receptors, are rare in unstimulated cells, but their frequency increases with IP_3 concentration [2,17]. Higher concentrations of IP_3 trigger larger, yet still localised, Ca^{2+} signals ('Ca^{2+} puffs') which result from the opening of clusters of IP_3 receptors. The frequency of these Ca^{2+} puffs also increases with IP_3 concentration, eventually giving rise to the Ca^{2+} waves that spread throughout the cytosol. Ca^{2+} is proposed to provide the link between this hierarchical sequence of Ca^{2+}-release events [2,17]. By virtue of its ability to sensitise IP_3 receptors, Ca^{2+} released from a single receptor within a cluster may recruit neighbouring receptors and thereby transform a blip to a puff, and diffusion of Ca^{2+} from one cluster of receptors to another probably allows the regenerative propagation of a Ca^{2+} wave [2,17].

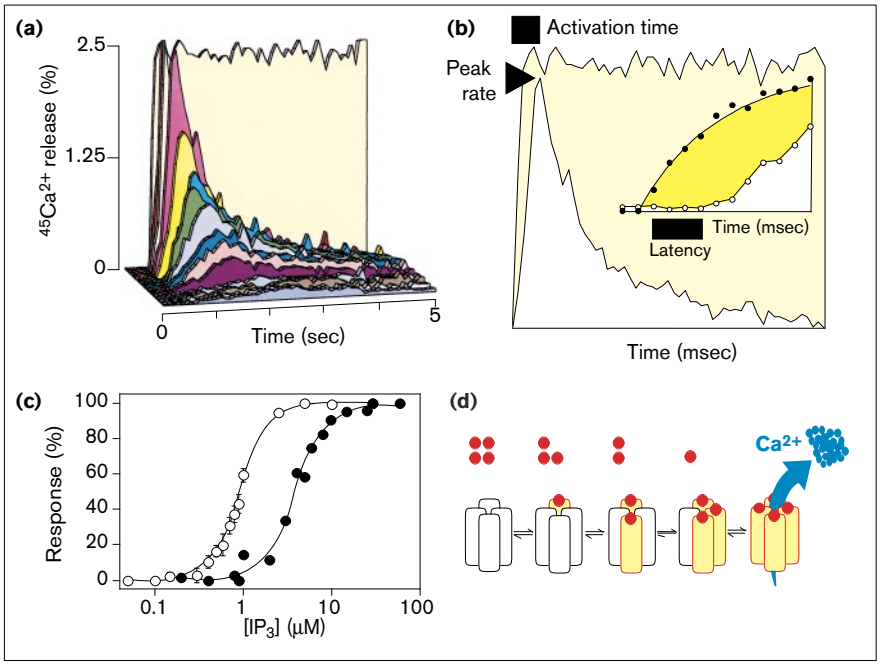
The complex organisation of intracellular Ca^{2+} signals allows effective delivery of Ca^{2+} to targets throughout the cytosol without exposing the cell to potentially damaging increases in cytosolic $[Ca^{2+}]$ [18]; however, the characteristics of IP_3 receptors that provide this complexity may present additional problems. The very large Ca^{2+} conductance of IP_3 receptors [4] together with the positive feedback provided by the ability of the receptors to mediate Ca^{2+}-induced Ca^{2+} release [2,7] suggest that activation of the receptors must be stringently controlled if intracellular Ca^{2+} stores are not to be released inappropriately. These properties of IP_3 receptors suggest that mechanisms that limit spontaneous opening are likely to have evolved in parallel with the Ca^{2+}-induced Ca^{2+} release mechanism. To address these issues, we have used methods that allow the rapid kinetics of unidirectional $^{45}Ca^{2+}$ efflux from permeabilised hepatocytes to be measured during rapid changes of superfusion medium, whilst controlling the composition of this medium precisely.

Results and discussion

Cooperative activation of IP_3 receptors in the absence of Ca^{2+} feedback

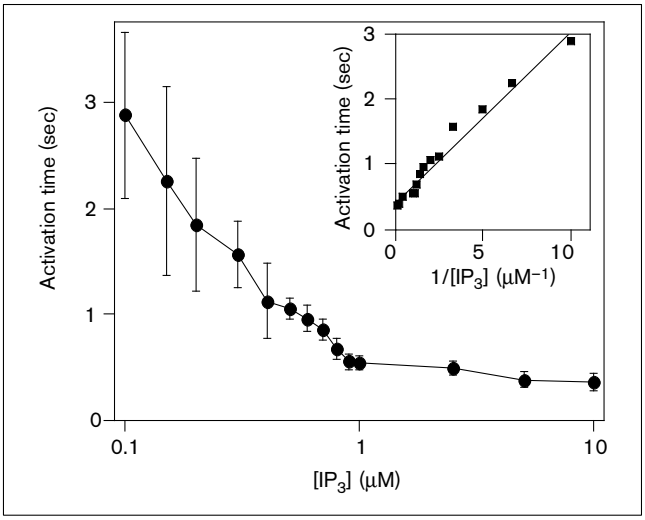
Permeabilised rat hepatocytes immobilised on the filters of our superfusion apparatus responded to the maintained application of various concentrations of IP_3 in cytosol-like medium (CLM) containing 200 nM free $[Ca^{2+}]$ by releasing $^{45}Ca^{2+}$ transiently from their intracellular stores (Figure 1a). The essential characteristics of this response were similar to those observed previously in conventional fluorescence studies [16] and $^{45}Ca^{2+}$-flux assays [19,20]. A saturating concentration of IP_3 (10 μM) released $30 \pm 5\%$ ($n = 10$) of the intracellular Ca^{2+} stores. The response was stereospecific because L-(1,4,5)IP_3 (80 μM) was inactive, and the response could be mimicked by active analogues of IP_3 in the correct order of potency, (1,4,5)IP_3 > 3-deoxy-IP_3 ≥ 2,3-dideoxy-IP_3 > (2,4,5)IP_3. Heparin is a competitive

Figure 1



Cooperative activation of IP_3 receptors by IP_3. **(a)** Permeabilised hepatocytes were superfused (20 psi) with CLM (free $[Ca^{2+}] = 200$ nM) which included one of a range of concentrations of IP_3 (nM: 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2500, 5000, 10000, from front to back) for the period (5 sec) indicated by the ^3H-marker (pale yellow background). The $^{45}Ca^{2+}$ released was collected for 80 msec intervals, and the rate of $^{45}Ca^{2+}$ release during each interval is expressed as a percentage of the total $^{45}Ca^{2+}$ content of the intracellular stores. Each trace is from a single experiment but is representative of at least four individual experiments. **(b)** In this schematic representation, the main panel shows the complete time course of a response to IP_3 illustrating the 'peak rate' of $^{45}Ca^{2+}$ release and the time taken to reach the peak rate, defined as the 'activation time'. The inset shows results collected at higher temporal resolution (see Figure 3) in order to define the 'latency' of the response to IP_3. In both panels, the solid line enclosing the yellow shaded area denotes the arrival of the ^3H-inulin marker and that enclosing the white area denotes $^{45}Ca^{2+}$ release. **(c)** The concentration-dependent effects of IP_3 on the peak rate of $^{45}Ca^{2+}$ release were determined from experiments similar to those shown in (a). Open symbols ($n \geq 4$) show results from CLM containing 1 mM EGTA and 200 nM free $[Ca^{2+}]$, and filled symbols show results from a single experiment in which the CLM contained 20 mM BAPTA and no added Ca^{2+}. In each case, the results are expressed as a percentage of the maximal response. **(d)** The Hill coefficients derived from the curves shown in (c) suggest that three and possibly all four subunits of the tetrameric IP_3 receptor must bind IP_3 before the channel can open and release Ca^{2+} into the cytosol.

Figure 2



Peak rates of IP_3-evoked Ca^{2+} release occur after substantial delays. The interval between the onset of the superfusion with IP_3 (detected by the appearance of ^3H-inulin in the superfusate) and attainment of the peak rate of $^{45}Ca^{2+}$ release (the activation time) was measured from experiments similar to those shown in Figure 1a ($n \geq 4$). The inset shows these activation times (Figure 1b) arbitrarily plotted against $1/[IP_3]$; extrapolation to infinite $[IP_3]$ suggests a minimal activation time of 418 msec.

antagonist of IP_3 receptors and at a concentration of 10 mg/ml blocked the action of 500 nM IP_3 (data not shown). In subsequent experiments, the superfusion apparatus was used to measure the rapid kinetics of IP_3 receptor activation in the absence of feedback effects of cytosolic Ca^{2+}.

The effect of IP_3 on the peak rate (Figure 1b) of $^{45}Ca^{2+}$ mobilisation, which provides the best indicator of the number of open IP_3 receptors/channels [11], was positively cooperative (Hill coefficient, $n_H = 3.0 \pm 0.3$) and half-maximal (EC_{50}) when the IP_3 concentration was 941 ± 21 nM (Figure 1c). The rapid flow of superfusion medium containing 1 mM EGTA through the small chamber containing the cells minimised any possible contribution of positive feedback from cytosolic Ca^{2+} to the responses to IP_3. Further evidence that the positively cooperative response to IP_3 was an intrinsic property of the receptor was provided by the observation that the response remained positively cooperative ($n_H = 2.4$) when the cells were superfused with CLM containing a much higher concentration (20 mM) of the faster Ca^{2+} buffer, BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N'N'-tetra-acetic acid; Figure 1c) [21]. The two-fold decrease in sensitivity to IP_3 in the presence of BAPTA is a consequence of the ability of BAPTA to act as a weak competitive antagonist of the IP_3 receptor [22]. A recent analysis of the rate of IP_3-evoked $^{45}Ca^{2+}$ release from hepatic microsomes has also suggested a positively cooperative response ($n_H = 3.4$) to IP_3 [23]. We conclude that at least three and possibly all four subunits of the IP_3 receptor must bind IP_3 to initiate opening of the channel (Figure 1d).

Delayed opening of IP_3 receptors

The time taken for IP_3-evoked Ca^{2+} release to rise to its peak (the activation time, Figure 1b) decreased from several seconds at the lowest concentrations of IP_3 to a lower limit of 370 ± 85 msec ($n = 10$) with 10 μM IP_3 (Figure 2). This interval is much longer than the time taken (~15 msec) to reach an IP_3 concentration (2 μM) capable of evoking a maximal rate of $^{45}Ca^{2+}$ release (Figure 1c). The long activation times observed during submaximal stimulation are probably a further reflection of the need for at least three IP_3 molecules to bind to the receptor and cause activation (Figure 1d) [8,23]. Slow binding events alone, however, cannot account for the lack of any further decrease in the activation time upon increasing the superfusing IP_3 concentration from 1 μM to 10 μM (Figure 2). The extrapolated estimate of the minimal activation time at infinite IP_3 concentration was 418 msec (Figure 2, inset). These results suggest that IP_3 binding might not be the sole determinant of receptor activation.

The rapid superfusion and sample collection system allowed us to examine this issue of receptor activation by investigating the effects of IP_3 at higher temporal resolution (9 msec), while simultaneously recording the arrival of IP_3 at the permeabilised cells. Using CLM containing 200 nM free $[Ca^{2+}]$, there was a long delay of > 100 msec between the application of a submaximal concentration of IP_3 (1 μM) and channel opening (Figure 3a). The response to a 250 msec superfusion with a suprasaturating concentration of IP_3 (100 μM) is shown in Figure 3b. Under these conditions, the cells were exposed to ≥ 19 μM IP_3 within 9 msec; this concentration of IP_3 is far higher than that needed to evoke a maximal rate of $^{45}Ca^{2+}$ release (2 μM; Figure 1c). Despite the rapid exposure of the cells to such a high concentration of IP_3, the rate of $^{45}Ca^{2+}$ release did not increase immediately, but increased only after a latency of 34 ± 8 msec ($n = 8$). Further increasing the final IP_3 concentration to 300 μM (i.e. to ≥ 56 μM within 9 msec) failed to reduce this latency, which remained at 31 ± 2 msec ($n = 3$, Figure 3c). Over a 10-fold range of final IP_3 concentrations (30–300 μM), there was no significant difference in the latency between application of IP_3 and the onset of Ca^{2+} release from the intracellular stores: the extrapolated lower limit — the absolute latency — was 29 msec (Figure 3d).

As with any receptor–ligand interaction, the rate of association of IP_3 with its receptor is expected to increase with IP_3 concentration [24]. Our observation that a substantial increase in IP_3 concentration failed to reduce the absolute latency before channel opening therefore indicates that the delay is not due to the kinetics of IP_3 binding to its

receptor. This argument is further supported by the results from rapid analyses of ^{3}H-IP_3 binding to the receptor [24], from which we estimate the half-time for association of 56 μM IP_3 (see above) to be less than 400 μsec.

Slow opening of IP_3 receptors contrasts with rapid gating of plasma membrane ion channels

The long delay between addition of supramaximal concentrations of IP_3 and Ca^{2+} release was unexpected and in marked contrast to the rapid gating of most plasma membrane ligand-gated ion channels, which typically open within 1 msec of maximal stimulation [25,26]. The ion channels most closely related to IP_3 receptors, ryanodine receptors and cyclic GMP-activated channels, also open rapidly (< 1 msec) after maximal stimulation [27,28]. In view of the unusual behaviour of IP_3 receptors, we wanted to eliminate any possible artefactual explanation for our results.

Our first approach involved examining the kinetics of activation of nicotinic acetylcholine receptors, which have previously been well characterised using a variety of methods [25]. The electric organs of the marine ray *Torpedo* are derived from skeletal muscle and provide a uniquely rich source of each of the components of a cholinergic synapse, including the nicotinic acetylcholine receptors that allow the postsynaptic fibre to respond to the acetylcholine released into the synaptic cleft. Passive leakage of $^{22}Na^+$ from vesicles prepared from *Torpedo* electric organs was extremely slow ($t_{1/2}$ > 20 min). During stimulation with acetylcholine (1 mM) in *Torpedo* physiological saline under the same superfusion conditions used to analyse the rapid activation of IP_3 receptors, the rate of $^{22}Na^+$ efflux increased within 9 msec to a peak value that was ~1000-fold greater than the basal leakage rate (Figure 4). In the continued presence of acetylcholine, the rate of $^{22}Na^+$ efflux then decayed with bi-exponential kinetics with half-times of 7 msec and 50 msec, which are comparable to those describing rapid inactivation and desensitisation of nicotinic acetylcholine receptors [29]. By confirming that the nicotinic acetylcholine receptor is maximally opened within 9 msec of superfusion with acetylcholine, these results establish that our apparatus is capable of resolving very rapid changes in ion flux.

The presence of high concentrations of Ca^{2+} buffers within the cytosol substantially slows Ca^{2+} diffusion, such that Ca^{2+} diffuses approximately 20-fold more slowly than IP_3 [30]. We were concerned that, while IP_3 rapidly reached the intracellular stores during our superfusion protocol, much of the $^{45}Ca^{2+}$ released could be trapped by buffers,

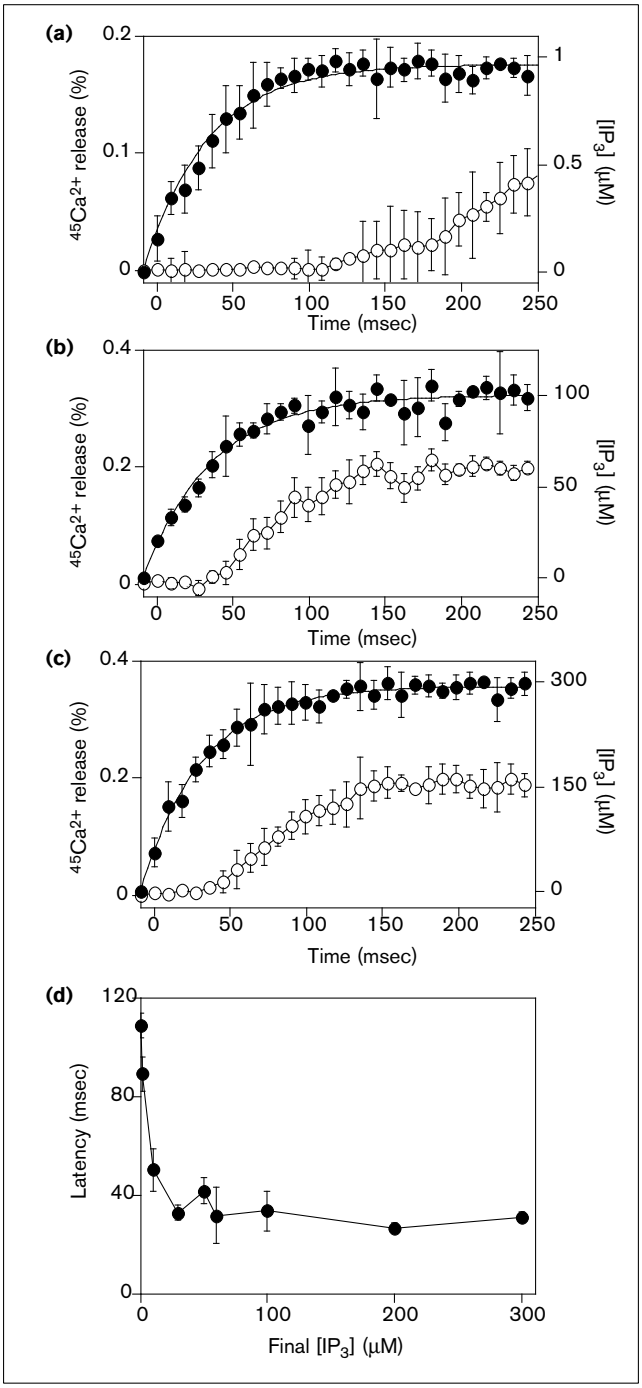
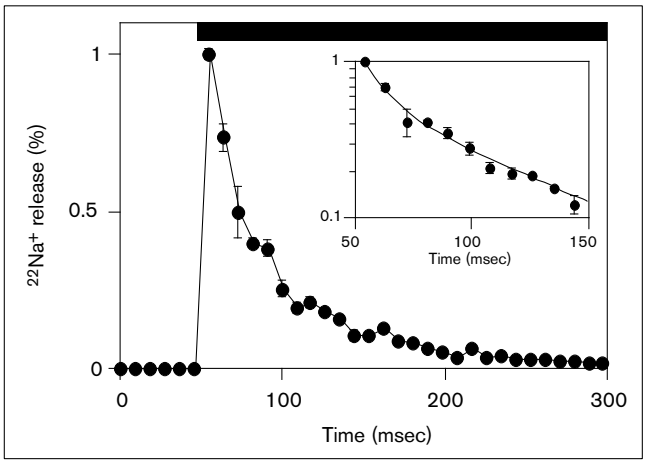


Figure 3

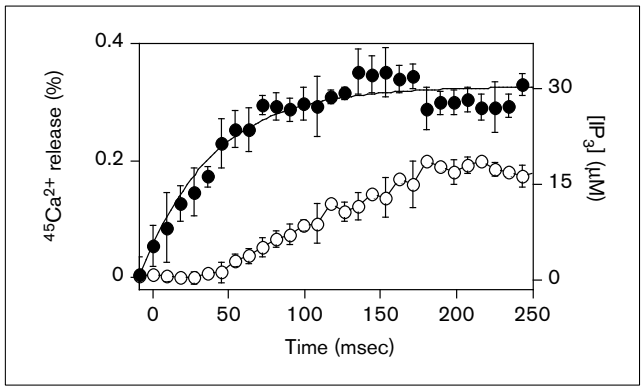
Delayed Ca^{2+} release after maximal stimulation of IP_3 receptors. (a–c) Rates of $^{45}Ca^{2+}$ efflux (open symbols) were recorded at 9 msec intervals in response to rapid superfusion (30 psi) with CLM (free [Ca^{2+}] = 200 nM) containing IP_3 at final concentrations of **(a)** 1 μM, **(b)** 100 μM and **(c)** 300μM. The kinetics of IP_3 delivery to the cells were reported by the rate of appearance of ^{3}H-inulin in the superfusate (filled symbols; $n \geq 3$). **(d)** Experiments similar to those shown in (a–c) were used to establish the effects of IP_3 concentration on the latency. The latency was defined as the interval between the time at which IP_3 was detected in the superfusate and the onset of $^{45}Ca^{2+}$ release (Figure 1b); the latter was described as the first of three consecutive increases in the rolling average of three successive rate measurements ($n \geq 3$). Curve-fitting of these data provided an extrapolated absolute latency of 29 msec at infinite [IP_3].

Figure 4



Rapid activation of nicotinic acetylcholine receptors. The kinetics of $^{22}Na^+$ efflux from *Torpedo* electric organ vesicles were measured at 9 msec resolution. Acetylcholine at a final concentration of 1 mM in *Torpedo* physiological saline (solid bar) evoked a maximal rate of $^{22}Na^+$ efflux within 9 msec, which then declined towards baseline over the next 250 msec. The inset shows data from the first 100 msec of stimulation plotted semi-logarithmically to illustrate the biphasic decay in the rate of $^{22}Na^+$ efflux ($n = 3$).

Figure 5



Delayed $^{45}Ca^{2+}$ efflux is not a consequence of intracellular Ca^{2+} buffering. Experiments identical to those described in Figure 3 were used to measure the rapid kinetics of $^{45}Ca^{2+}$ efflux from an acellular preparation of intracellular Ca^{2+} stores ('saposomes') prepared from permeabilised rat hepatocytes [20] in response to 30 μM IP_3 ($n = 3$).

and therefore would reach the superfusate only after a considerable delay. Previous rapid kinetic analyses of permeabilised cells have not addressed this concern [8,16]. Several lines of evidence establish that such delays due to diffusion are not the cause of the absolute latency to IP_3. Firstly, the kinetics of washing $^{45}Ca^{2+}$ and ^{3}H-inulin from cells pre-equilibrated with CLM containing the two radiolabels were indistinguishable, indicating that $^{45}Ca^{2+}$ is not selectively bound by the cells. Secondly, during our permeabilisation procedure, hepatocytes lost more than 90% of their lactate dehydrogenase and, as this is a very large cytosolic enzyme (130 kDa), it is unlikely that any mobile Ca^{2+} buffers are retained by the permeabilised cells. Thirdly, the kinetics of $^{45}Ca^{2+}$ efflux decay more rapidly after a brief incubation with IP_3 than after prolonged stimulation, indicating that Ca^{2+} buffering does not limit the temporal resolution of our method (data not shown). Finally, the kinetics of IP_3 receptor activation were identical when we used a cell-free preparation of intracellular Ca^{2+} stores [16] rather than permeabilised cells (Figure 5): using the cell-free preparation, the absolute latency in response to 30 μM IP_3 was 38 ± 6 msec ($n = 3$).

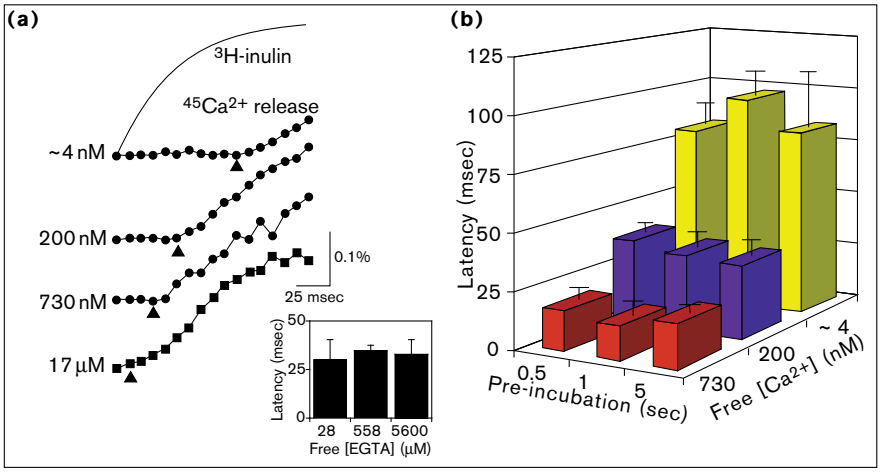
Previous analyses of IP_3-evoked Ca^{2+} release from broken cells [5,23,31] and reconstituted receptors [3] have either lacked the temporal resolution to detect an absolute latency or the latencies observed (20–65 msec) have been impossible to resolve from delays attributable to diffusion barriers or to the properties of fluorescent indicators [8,16,32]. Our methods circumvent both of these limitations and demonstrate that, with Ca^{2+} buffered at a level comparable to that found in the cytosol of unstimulated hepatocytes (200 nM), there is a delay of ~30 msec between IP_3 binding to its receptor and the opening of the intrinsic Ca^{2+} channel of the receptor. This observation is consistent with results from a variety of intact cells, which demonstrate an absolute latency of ~20–30 msec between the release of a high concentration of IP_3 by flash photolysis of its caged precursor and an increase in cytosolic [Ca^{2+}] [10–12]. The kinetics of the activation of IP_3 receptors have yet to be examined using electrophysiological methods [4,14,15].

Ca^{2+} accelerates opening of IP_3 receptors

Low concentrations of ATP potentiate the effects of even maximal concentrations of IP_3 on cerebellar IP_3 receptors [33] by increasing the probability that the channel is open [34]. ATP is also implicated in the gating of ryanodine receptors [21]. However, ATP had no effect on the kinetics of Ca^{2+} mobilisation from permeabilised hepatocytes stimulated with 60 μM IP_3, because the absolute latency, the activation time and the peak rate of $^{45}Ca^{2+}$ release were the same in the absence and presence of 1 mM ATP (data not shown). These results from hepatocytes, in which most IP_3 receptors are type 2, contrast with those from cerebellum, where almost all IP_3 receptors are type 1 [35].

In contrast to the lack of effect of ATP, changing the free [Ca^{2+}] of the superfusing medium significantly altered the absolute latency in response to 60 μM IP_3. When cells were superfused with CLM containing ~4 nM free [Ca^{2+}] for 5 sec before and then during the addition of IP_3 (60 μM), the absolute latency was 84 ± 26 msec, and it decreased to 20 ± 3 msec when the free [Ca^{2+}] was 730 nM (Figure 6a,b). As with the results obtained in CLM containing 200 nM Ca^{2+} (Figure 3d), the longer latency recorded in the presence of ~4 nM free [Ca^{2+}]

Figure 6



Cytosolic Ca^{2+} shortens the absolute latency. **(a)** Using experiments similar to those shown in Figure 3, rates of $^{45}Ca^{2+}$ efflux (filled circles) were recorded from permeabilised cells pre-incubated for 5 sec with CLM containing the indicated free [Ca^{2+}] (~4, 200 and 730 nM) before addition of 60 μM IP_3 to the same medium. The effects of higher free [Ca^{2+}] were examined without pre-incubation by applying 60 μM IP_3 in CLM containing 17 μM Ca^{2+} (bottom trace, solid squares). The solid curve denotes the ^{3}H-inulin content of the superfusate and the arrow heads denote the latency. Each trace is typical of those obtained in at least three independent experiments. **Inset:** absolute latencies during stimulation with 60 μM IP_3 were determined in CLM in which the free [Ca^{2+}] was 200 nM and the total EGTA was varied for both 5 sec before the superfusion with IP_3 and then during the superfusion ($n = 5$). **(b)** The duration of the preincubation with CLM containing different free [Ca^{2+}] (~4–730 nM) was varied (0.5–5 sec) and the latency of the response to 60 μM IP_3 was then measured in the same medium. Prior to the pre-incubation, cells were superfused with CLM containing either 200 nM Ca^{2+} (yellow and red blocks, test Ca^{2+} = ~4 nM and 730 nM, respectively) or ~4 nM Ca^{2+} (purple blocks, test Ca^{2+} = 200 nM). Results are means ± s.e.m. of at least three independent experiments.

could not be reduced by further increasing the IP_3 concentration to 200 μM; under this condition, the latency was 83 ± 8 msec (n = 5).

Pretreatment of the cells with CLM containing much higher free [Ca^{2+}] (17 μM) abolished the response to IP_3, in accordance with the well-documented inhibitory effect of substantial increases in cytosolic [Ca^{2+}] on IP_3 receptor function [5–7,23,36,37]. By simultaneously delivering CLM containing 17 μM free Ca^{2+} and 60 μM IP_3, however, we could observe the effects of very high cytosolic [Ca^{2+}] on channel opening ahead of the more slowly developing Ca^{2+}-mediated inactivation [5]. Under these conditions, the absolute latency was reduced to 6 ± 4 msec (n = 6), and there was no detectable latency in four of these six experiments (Figure 6a), indicating that the channel can open very rapidly when stimulated maximally by both of its agonists.

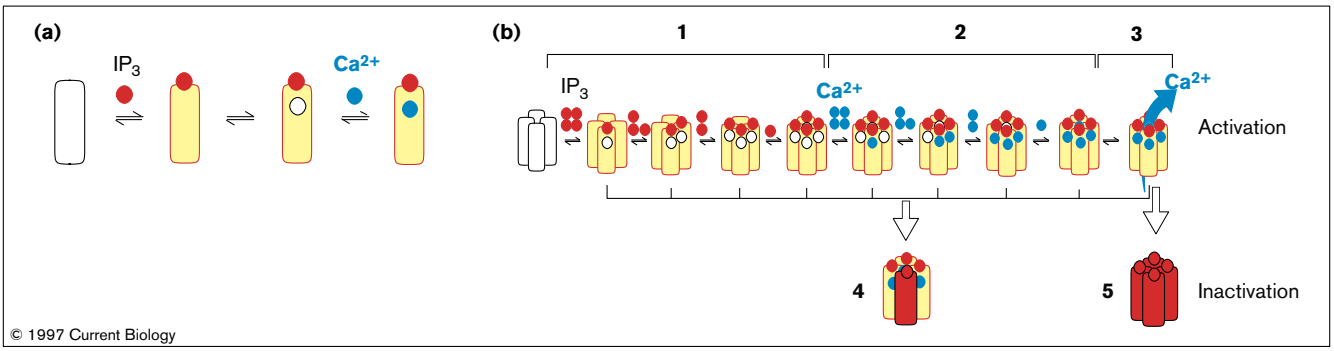
As EGTA is a very weak competitive antagonist of IP_3 receptors (IC_{50} > 20 mM) [22], we considered the unlikely possibility that the effects of changing the free [Ca^{2+}] might be an indirect consequence of the accompanying changes in the free EGTA concentration of the CLM. This possibility was eliminated by the results shown in the inset to Figure 6a which demonstrate that, at a constant free [Ca^{2+}] of 200 nM, there was no difference in the absolute latency of the response to 60 μM IP_3 over a 200-fold range of free EGTA concentration. These results demonstrate that increases in cytosolic [Ca^{2+}] shorten the interval between IP_3 binding to the receptor and channel opening.

A model for receptor activation: opening of IP_3 receptors after sequential binding of IP_3 and Ca^{2+}

For all ligand-gated ion channels, the delay between delivery of the agonist and opening of the channel decreases as the concentration of agonist is increased, until eventually agonist binding is so rapid that the channel-gating processes become rate-limiting [26,28,38]. Nicotinic acetylcholine receptors, for example, open within tens of microseconds of supramaximal stimulation [25], consistent with the ability of proteins to change conformation very rapidly. In medium that mimics the cytosol of an unstimulated cell (free [Ca^{2+}] = 200 nM), the much longer delay of ~30 msec between IP_3 binding and opening of the intrinsic Ca^{2+} channel of the receptor (Figure 3) suggests that a more complex gating process is occurring. In *Xenopus* oocytes, confocal imaging of Ca^{2+} release after flash photolysis of caged IP_3 has also revealed an absolute latency of ~28 msec between maximal stimulation and the first detectable Ca^{2+} mobilisation [12].

The effect of cytosolic Ca^{2+} on the latency (Figure 6b) was independent of the duration of the pre-incubation with Ca^{2+} (0.5–5 sec; up to 10 min for 200 nM Ca^{2+}). If the Ca^{2+}-binding site were accessible in the absence of IP_3, it would have equilibrated with Ca^{2+} before addition of IP_3. Under our conditions, superfusion with IP_3 is predicted to achieve maximal occupancy within no more than a few milliseconds (see above); the IP_3 receptor would therefore equilibrate with both of its agonists well within the first 9 msec collection interval. Any delay before channel opening could then only reflect slow conformational changes within the receptor protein and not

Figure 7



Activation of IP_3 receptors by sequential binding of IP_3 and Ca^{2+}. **(a)** The proposed sequence of events leading to IP_3 receptor activation is shown for only a single subunit of the tetrameric IP_3 receptor. Binding of IP_3 rapidly changes the conformation of the receptor exposing a Ca^{2+}-binding site. Ca^{2+} then binds to this newly exposed site, and when all four subunits of the receptor have bound Ca^{2+}, the channel opens allowing Ca^{2+} to pass through. **(b)** When cells are stimulated with supramaximal concentrations of IP_3, as in most of our experiments, binding of IP_3 to its receptor is rapid and very rapidly followed by exposure of the Ca^{2+}-binding site (**1**); shown in more detail in (a). At lower concentrations of IP_3, the initial IP_3-binding steps will be slower. The subsequent binding of Ca^{2+} to the newly exposed Ca^{2+}-binding sites (**2**) is relatively slow if the free cytosolic [Ca^{2+}] is low, but faster when the cytosolic [Ca^{2+}] is increased (Figure 6a). When all four subunits of the receptor have each sequentially bound IP_3 and then Ca^{2+}, the channel rapidly opens allowing Ca^{2+} to be released from the intracellular stores (**3**). In most cells, the resulting rise in cytosolic [Ca^{2+}] causes a delayed inactivation of IP_3 receptors (**5**). Activation steps compete with additional inactivation processes, which remain poorly understood but are probably initiated by IP_3 binding (**4**). We speculate that binding of IP_3 to the first subunit therefore initiates the race between inactivation and channel opening, the latter occurring only if each subunit can bind IP_3 and then Ca^{2+} within the timeframe dictated by the inexorable inactivation.

ligand-binding events. Yet when the free [Ca^{2+}] was very high, the channel opened within 6 ± 4 msec (Figure 6a). We conclude that, under conditions in which further increases of the IP_3 concentration are ineffective because the receptor is already maximally occupied (Figure 3d), and despite the ability of an appropriately liganded channel to open rapidly (Figure 6a), increasing the cytosolic [Ca^{2+}] shortens the delay between addition of IP_3 and Ca^{2+} mobilisation (Figure 6).

These observations indicate that when IP_3 binding is complete, it is the binding of Ca^{2+} that limits the rate of channel opening. The simplest explanation consistent with our observations is that as IP_3 binds to each subunit of the receptor, it evokes a rapid conformational change that exposes a high-affinity Ca^{2+}-binding site to which Ca^{2+} must bind before the channel can open (Figure 7a). The long latencies after addition of supramaximal concentrations of IP_3 (Figures 3,6) reflect the association of Ca^{2+} with this site: the rate of association is slow at submaximal Ca^{2+} concentrations and faster when the cytosolic [Ca^{2+}] is increased (Figure 6).

Multiple IP_3-binding sites allow activation of receptors that are quiet at rest

The commonly espoused advantage of having multiple agonist-binding sites on a receptor is that a response can be evoked within a narrow range of stimulus intensities; however, there are also energetic advantages [39], which are likely to be more important in IP_3 receptor activation. As any small agonist like IP_3 binds first to the inactive state of its receptor and then more tightly to the active conformation, the potential energy released by the improved binding drives receptor activation. For the IP_3 receptor to be largely inactive at rest (i.e. for the closed state to be very stable) and then to be driven into its active state by IP_3, the binding of IP_3 to the active receptor must release substantial binding energy. A small ligand like IP_3 may not form enough contacts with the receptor protein to provide sufficient energy to drive a large change in the conformational equilibrium of the receptor. A similar argument has been extensively developed for the nicotinic acetylcholine receptor [39]. We suggest that a highly stable closed conformation of the IP_3 receptor has evolved to minimise the risk of its spontaneous activation. The energy needed to drive the closed receptor from this stable conformation to its open state cannot be provided by the binding of IP_3 to a single subunit, but requires the binding energy that is released as all four subunits bind IP_3. The four IP_3-binding sites of the receptor therefore provide a defence against noise by allowing the receptor to be stably closed at rest without preventing the receptor from opening in response to IP_3.

Coincidence detection provides a further defence from noise

The delayed inactivation of IP_3 receptors by the increase in cytosolic [Ca^{2+}] that inevitably follows opening of the intrinsic Ca^{2+} channel (Figure 7b) has been observed in many experimental settings [5–7,11,23,36,37]. Such feedback inhibition undoubtedly influences the kinetics of Ca^{2+} release in intact cells [11,37] and has been proposed to contribute both to quantal Ca^{2+} release [31,36] and to

the mechanisms that allow intracellular stores to be replenished between episodes of Ca^{2+} mobilisation [7]. Additional mechanisms may also initiate receptor inactivation before the channel opens, however [23,40].

The mechanisms that we propose are responsible for activation of IP_3 receptors (Figure 7) suggest a very precise form of molecular coincidence detection. In keeping with several previous reports [5–7], we conclude that IP_3 and Ca^{2+} are co-agonists of the receptor, but that they act in strict temporal sequence, because the site to which Ca^{2+} must bind to open the channel is accessible only after IP_3 binding (Figure 7a). Binding of IP_3 has also been proposed to initiate a form of receptor inactivation ([40], but see [41]), which may or may not depend on the presence of cytosolic Ca^{2+} [23,40]. Inactivation of the receptor by IP_3 may allow IP_3 binding to define a temporal window within which the receptor will inactivate unless the newly exposed Ca^{2+}-binding site becomes occupied leading to activation.

We speculate that if inactivation of a single receptor subunit is sufficient to prevent channel opening then the requirements for receptor activation are remarkably stringent. Each of the four subunits of the receptor must first bind IP_3 and then Ca^{2+} within a narrow timeframe if the channel is to open rather than inactivate. In effect, the first IP_3-binding event starts a molecular clock. Such an extreme form of coincidence detection may provide a further defence against spontaneous activation because, unless IP_3 and Ca^{2+} are appropriately presented to the receptor, the channel is more likely to inactivate than open (Figure 7b).

Although most plasma membrane ligand-gated ion channels open very rapidly [25,26], the receptors for N-methyl-D-aspartate (NMDA) are conspicuously slower [42]. Intriguingly, these receptors, like IP_3 receptors, behave as coincidence detectors [43], allowing the passage of ions only after binding of both glutamate and glycine has activated the receptor and depolarisation has displaced Mg^{2+} from within the channel.

Conclusions

The rather unusual features of IP_3 receptor activation — the need for each of its four subunits to first bind IP_3 and then Ca^{2+} — safeguard against spontaneous activation. These adaptations allow IP_3 receptors to be stable in their closed state and then to open only when both IP_3 and Ca^{2+} are presented within a short interval timed from the binding of IP_3 to the first subunit of the receptor.

Materials and methods

Materials

IP_3 was obtained from American Radiolabeled Chemicals, L-IP_3 from LC Laboratories, and 2,3-dideoxy-IP_3 and 3-deoxy-IP_3 from Calbiochem. Acetylcholine, BAPTA and heparin were from Sigma. All other reagents were from suppliers reported previously [19,44].

Rapid superfusion apparatus

Rapid changes of the medium superfusing permeabilised rat hepatocytes were achieved using apparatus modified from [45]. The permeabilised cells were immobilised within a sandwich of filters (Schleicher and Schuell) selected to maximise flow rate and sample retention while minimising dead space, shear forces and switching artefacts. The filter chamber was linked by four solenoid valves (response times < 2 msec) to pressurised vessels containing superfusion media. Superfusion protocols and the rotation of the fraction collector were coordinated by computer. The stream of effluent from the chamber was collected into 100 samples during a single rotation of a variable-speed turntable (9–3000 msec per fraction), the velocity of which was verified both electronically and with an optical tachometer. The high flow-rate (2ml/sec) and small dead volume of the chamber containing the cells (10 μl) allowed rapid exchange of media with half-times for mixing of 30 ± 4 msec and 46 ± 6 msec when media were delivered at 30 psi and 20 psi, respectively ($n \geq 6$). Inclusion of trace amounts of an inert marker (^{3}H-inulin) in appropriate media allowed the timing of solution exchange to be precisely related to changes in $^{45}Ca^{2+}$ efflux. The entire apparatus was maintained at 20°C in a thermostatically regulated cabinet.

$^{45}Ca^{2+}$ efflux from permeabilised hepatocytes

Isolated hepatocytes were prepared by collagenase digestion of the livers of male Wistar rats and stored at 4°C in Eagle's minimal essential medium buffered with $NaHCO_3$ (26 mM) for up to 24 h [19]. Hepatocytes (2×10^6/ml) were permeabilised by incubation with saponin (10 μg/ml) in cytosol-like medium (CLM: 140 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM PIPES, pH7 at 37°C). After permeabilisation, cells were washed and resuspended (at 10^7 cells/ml) in CLM supplemented with $CaCl_2$ (300 μM; free $[Ca^{2+}] = 200$ nM at 37°C). The intracellular Ca^{2+} stores were loaded to steady-state levels with $^{45}Ca^{2+}$ (1–2 nmol/10^6 cells) by incubating the cells at 37°C with $^{45}CaCl_2$ (100 μCi/ml), ATP (7.5 mM), phosphocreatine (15 mM), creatine kinase (15 U/ml) and FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; 10 μM). After 5 min, cells were immobilised by filtration onto a prerinsed filter triplet, and rapidly secured into the chamber. The superfusion fluid flow rate was adjusted during two prewashes (5 sec) with CLM containing 200 nM free Ca^{2+} at 20°C, which removed $^{45}Ca^{2+}$ that was not trapped within intact intracellular stores. The superfusion protocol was then initiated. These steps were completed within 20 sec, and as the half-time for passive efflux of $^{45}Ca^{2+}$ from the intracellular stores was 162 ± 13 sec at 20°C ($n = 3$), the $^{45}Ca^{2+}$ content of the intracellular stores at the onset of the experiment was ≥ 92% of the steady-state level. At the end of each run, cells were superfused with CLM containing Triton X-100 (0.05%) to release all $^{45}Ca^{2+}$ remaining within the intracellular stores. The radioactivity (^{3}H and $^{45}Ca^{2+}$) of each sample was determined by liquid scintillation counting in EcoScint-A scintillation cocktail. The free Ca^{2+} concentrations of the superfusion media were determined both fluorimetrically using fura-2 ($K_d^{Ca} = 135$ nM at 20°C) or fluo-3 ($K_d^{Ca} = 400$ nM at 22°C) and with a Ca^{2+}-selective macro-electrode (Ingold).

$^{22}Na^+$ efflux from Torpedo *electroplax vesicles*

Vesicles rich in nicotinic acetylcholine receptors, which had been prepared from *Torpedo* electric organ, were a generous gift from Nigel Unwin (Laboratory of Molecular Biology, Cambridge). The vesicles (~3 mg protein/ml) were passively loaded with $^{22}Na^+$ (40 μCi/ml) by incubation for 48 h at 4°C in *Torpedo* physiological saline (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM Na_2HPO_4, pH 7) and extravesicular $^{22}Na^+$ was then removed by passing the vesicles over a 3 ml Sephadex G50 ion-exchange column. After a further 30 min incubation at 4°C to allow damaged vesicles to lose their $^{22}Na^+$, the vesicles were immobilised on a filter array (3 HAWP filters) and transferred to the superfusion apparatus.

Analysis of results

All traces were corrected for the unstimulated rate of $^{45}Ca^{2+}$ efflux and the amount of $^{45}Ca^{2+}$ in each sample was then expressed as a fraction of the total $^{45}Ca^{2+}$ content of the intracellular stores [5,23]. Concentration–response relationships were fitted to a four-parameter logistic equation using a non-linear curve-fitting program (Kaleidagraph, Abeldeck Software) as previously described [26]. Computer-assisted curve-fitting (Kaleidagraph and Microsoft Excel) with > 200 iterations was used to fit exponential equations. The statistical significance of mono-exponential

and multi-exponential fits was assessed according the 'extra sum of squares' principle; $p < 0.05$ was considered significant. All data are expressed as means ± s.e.m.

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