The Effects of Inositol 1,4,5-Trisphosphate (InsP₃) Analogues on the Transient Kinetics of Ca²⁺ Release from Cerebellar Microsomes

InsP₃ ANALOGUES ACT AS PARTIAL AGONISTS*

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An investigation of the effects of a number of inositol trisphosphate analogues on the transient kinetics of Ca²⁺ release from cerebellar microsomes was undertaken. All the analogues investigated could release the total Ca²⁺ content of the inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) mobilizable Ca²⁺ store; however, their potencies were substantially reduced compared to Ins(1,4,5)P₃. The concentration required to induce half-maximal Ca²⁺ mobilization was 0.14 μM for Ins(1,4,5)P₃, 1.8 μM for 3-deoxyinositol 1,4,5-trisphosphate (3-deoxy-Ins(1,4,5)P₃), 1.0 μM for 2,3-dideoxyinositol 1,4,5-trisphosphate (2,3-dideoxyIns(1,4,5)P₃), 24 μM for 2,3,6-trideoxyinositol 1,4,5-trisphosphate (2,3,6-trIDEOxyIns(1,4,5)P₃), and 2.9 μM for inositol 2,4,5-trisphosphate (Ins(2,4,5)P₃). In all cases and for all concentrations tested, the inositol trisphosphate analogues induced biphasic transient release of Ca²⁺, which could fit to a biexponential equation assuming two independent processes. The rate constants calculated for the release processes were much larger for Ins(1,4,5)P₃ than for the other inositol trisphosphates (the fast phase rate constant varying from 0.3 to 1.6 s⁻¹ and the slow phase from 0.01–0.5 s⁻¹, at concentrations between 0.03 and 20 μM Ins(1,4,5)P₃). The rate constants for all other inositol trisphosphates did not appear to exceed 0.4 s⁻¹ for the fast phase and 0.1 s⁻¹ for the slow phase at their highest concentrations tested. The maximum amplitudes for Ca²⁺ release by the two phases appeared to be similar for all inositol trisphosphates (approximately 45% for the fast phase and approximately 55% for the slow phase). On comparing the rate constants for Ca²⁺ release at inositol trisphosphate concentrations for the analogues which all induced the same extent of Ca²⁺ release, it was apparent that the rates of release were independent of the extent of Ca²⁺ release. As the extent of Ca²⁺ release can be related to degree of occupancy of the binding sites, it is evident that different analogues which occupy the binding site of the receptor to the same extent can induce Ca²⁺ to be released at different rates. We explain this conclusion in terms of partial agonism where inositol phosphates can induce two (or more) occupied states of the channel.

Certain hormones and neurotransmitters induce cells to produce the second messenger inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃),¹ which opens an InsP₃-sensitive calcium channel (the InsP₃ receptor) causing the elevation of cytosolic Ca²⁺ concentrations. This then activates a diverse multitude of cellular processes which depend on the cell type (1). InsP₃-induced Ca²⁺ release is a complex process which, despite considerable study, remains poorly understood. Several studies have demonstrated that the InsP₃-induced Ca²⁺ release is “quantal” in nature where submaximal concentrations of Ins(1,4,5)P₃ are unable to fully discharge the InsP₃-sensitive Ca²⁺ pool unless maximal Ins(1,4,5)P₃ concentrations are added (2, 3). The mechanism for quantal Ca²⁺ release induced by Ins(1,4,5)P₃ remains unknown, even though this process appears to be adopted by other intracellular Ca²⁺ channels (4). Attempts to explain this mechanism have lead to the proposal of several models based on either the existence of heterogeneous Ca²⁺ stores which contain Ca²⁺ channels with different sensitivities to InsP₃, discharging their Ca²⁺ in an all-or-nothing manner (5), or homogeneous Ca²⁺ stores which discharge their Ca²⁺ in a regulated fashion, possibly controlled by luminal Ca²⁺, Ca²⁺ gradients, or limited desensitization (6–8).

A more elaborate approach to investigating InsP₃-induced Ca²⁺ release is to study the transient kinetics of this process. Such studies using permeabilized hepatocytes, basophilic leukaemia cells, cerebellar microsomes, and purified-reconstituted InsP₃ receptors have shown InsP₃-induced Ca²⁺ release to be a relatively fast and biphasic process (9–12). These studies have therefore proved useful in aiding our understanding into the mechanism of channel opening.

The use of InsP₃ analogues in studies of InsP₃-induced Ca²⁺ release and binding to the channel have also helped shed light on our understanding of the pharmacological and functional properties of this transport protein (3, 13). Many of these analogues are able to release Ca²⁺ to the same extent and in a similar manner to Ins(1,4,5)P₃, albeit with much lower affinities/potencies and have therefore been classified as “full agonists” (3). In addition, some inositol phosphate analogues are able to bind to the channel and completely displace bound [³H]Ins(1,4,5)P₃, yet unable to induce Ca²⁺ release, and these have been classified as “full antagonists” (3). In this study we have investigated the effects of a variety of inositol trisphosphate analogues (all of which are full agonists) on the transient kinetics of Ca²⁺ release with the view to understanding how these ligands influence the mechanism of channel opening.

MATERIALS AND METHODS

The abbreviations used are: Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; 2,3-deoxyIns(1,4,5)P₃, 2,3-deoxyinositol 1,4,5-trisphosphate; 2,3-dIDEOxyIns(1,4,5)P₃, 2,3-dideoxyinositol 1,4,5-trisphosphate; 3-deoxyIns(1,4,5)P₃, 3-deoxyinositol 1,4,5-trisphosphate; 2,3,6-trIDEOxyIns(1,4,5)P₃, 2,3,6-trideoxyinositol 1,4,5-trisphosphate; IC₅₀, concentration causing half-maximal response.

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¹The abbreviations used are: Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; 3-deoxyIns(1,4,5)P₃, 3-deoxyinositol 1,4,5-trisphosphate; 2,3-dIDEOxyIns(1,4,5)P₃, 2,3-dideoxyinositol 1,4,5-trisphosphate; 2,3,6-trIDEOxyIns(1,4,5)P₃, 2,3,6-inositol 1,4,5-trisphosphate; 

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Bio-Rad, and phosphocreatine, creatine kinase, and ATP were purchased from Boehringer Mannheim. All other reagents were of analytical grade.

In order to avoid any calcium contamination when using the inositol trisphosphate analogues, all InsP₃ analogues were dissolved in double deionized water and treated with Chelex 100 resin prior to use.

Rat cerebellar microsomes were prepared as described previously (14). Briefly, approximately 20 cerebella were homogenized in 10 volumes of buffer containing 0.32 M sucrose, 5 mM Heps, pH 7.4, in the presence of 0.1 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 10 mM pepstatin A, and 50 mM benzamidine and then centrifuged for 10 min at 500 × g. The pellet was resuspended in 5 volumes of the same buffer and again centrifuged as above. The resulting supernatants were pooled and centrifuged for 20 min at 100,000 × g, and the resulting pellet was resuspended in approximately 2 ml of the buffer and snap-frozen in liquid nitrogen and stored at −70°C.

Calcium Uptake and Release Experiments—Ca²⁺ uptake and release were measured as described in Ref. 14. Typically, 300 μg of rat cerebellar microsomes were suspended in 2 ml of 40 mM Tris/phosphate buffer, pH 7.2, at 37°C in the presence of 10 mM phosphocreatine, 10 μg/ml creatine kinase, and 1.25 mM Mg³⁺. The mixture was incubated at 37°C, and Ca²⁺ uptake was initiated by the addition of 1.5 mM MgATP. Ca²⁺ transport across the microsomal membrane was followed by monitoring the fluorescence change of fluo-3 in a Perkin-Elmer LS-50B fluorimeter by exciting at 505 nm and measuring the emission above 515 nm using a cut-off filter. The data were collected, and an average was determined from between 8 and 12 traces. Fluorescence intensities were then correlated to [Ca²⁺] by comparing the traces to identical experiments carried out in a conventional fluorimeter. The traces were analyzed using nonlinear regression analyses programs supplied by Applied Photophysics and Biosoft. InsP₃-induced Ca²⁺ release in the microsomal preparation under study was shown to be biphasic and fit well to a biphasic equation (Equation 2) which assumes two independent processes.

\[
[Ca^{2+}]_{\text{release}} = A_1(1 - \exp(-kt)) + A_2(1 - \exp(-kt)) \quad (\text{Eq.} 2)
\]

where \(A_1, A_2, k_1,\) and \(k_2\) are the amplitudes (relative extent of Ca²⁺ release) and rate constants of Ca²⁺ release for the fast and slow phases, respectively, and \(t\) is the time (s).

Over the Ca²⁺ concentration range for which the ICR was monitored, the fluorescence change when related to \([Ca^{2+}]\) was around the \(K_c\) value for Ca²⁺ binding to fluo-3, and, over this range, the fluorescence was shown to be linearly related to \([Ca^{2+}]\) (linear regression coefficient, \(r > 0.99\)).

results

Fig. 1 shows the dose-dependent response of several InsP₃ analogues on Ca²⁺ release. All the analogues acted as full InsP₃ agonists with respect to the extent of Ca²⁺ release since they all appear to give (or approach) the maximal response to Ins(1,4,5)P₃. In addition, all the analogues exhibited quantal release behavior. In the preparation used in this study, the maximal extent of Ca²⁺ release was 23 ± 3% compared with that released by A23187, which was induced by approximately 3–10 μM Ins(1,4,5)P₃. The concentration of Ins(1,4,5)P₃ causing half-maximal response (IC₅₀) was found to be 0.14 ± 0.03 μM. The deoxy derivatives of InsP₃ were found to be less potent in releasing Ca²⁺. 3-DeoxyInsP₃ had an IC₅₀ of 1.8 ± 0.2 μM, while 2,3-dideoxyInsP₃ had an IC₅₀ of 1.0 ± 0.2 μM. The 2,3,6-trIDEOXYInsP₃ was much less potent, requiring greater than 0.3 mM to reach maximal release (IC₅₀ 24 ± 2 μM). In addition to the deoxyInsP₃ analogues, the isomer Ins(2,4,5)P₃ was also shown to have a lower potency than Ins(1,4,5)P₃, requiring greater than 30 μM for maximum Ca²⁺ release (IC₅₀ for Ca²⁺ release 2.9 ± 0.3 μM). The apparent cooperativity of the extent of Ca²⁺ release was, however, found to be similar for all analogues tested (Hill coefficients were 1.0 ± 0.2 in all cases).

Fig. 2 shows the time-resolved Ca²⁺ release traces induced by Ins(1,4,5)P₃ (Fig. 2A), Ins(2,4,5)P₃ (Fig. 2B), and the three deoxy analogues (3-deoxy-, Fig. 2B; 2,3-dideoxy-, Fig. 2C; 2,3,6-trIDEOXY-, Fig. 2D). As shown, each individual trace could be fitted extremely well to the biphasic equation (Equation 2), and all \(x^2\) values for these fits were less than 0.1. The resulting rates of Ca²⁺ release obtained from these fits show that both fast and slow rate constants increase with

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\text{Rapid}\text{Ca}^{2+}\text{uptake and release across the microsomalembranewasmmeasured}\text{by}\text{comparing}\text{the}traces\text{to}\text{identical}\text{experiments}\text{carried}\text{out}\text{in}\text{a}\text{conventional}\text{fluorimeter.}\text{Thetraces}\text{were}\text{analyzed}\text{using}\text{nonlinear}\text{regression}\text{analyses}\text{programs}\text{supplied}\text{by}\text{Applied}\text{Photophysics}\text{and}\text{Biosoft.}\text{InsP}_3\text{-induced}\text{Ca}^{2+}\text{release}\text{in}\text{the}\text{microsomal}\text{preparation}\text{under}\text{study}\text{was}\text{shown}\text{to}\text{be}\text{biphasic}\text{and}\text{fit}\text{well}\text{to}\text{a}\text{biphasic}\text{equation}\text{(Equation}\text{2})\text{which}\text{assumes}\text{two}\text{independent}\text{processes.}\text{[Ca}^{2+}]_{\text{release}} = A_1(1 - \exp(-kt)) + A_2(1 - \exp(-kt)) \quad (\text{Eq.} 2)
\]

where \(A_1, A_2, k_1,\) and \(k_2\) are the amplitudes (relative extent of Ca²⁺ release) and rate constants of Ca²⁺ release for the fast and slow phases, respectively, and \(t\) is the time (s).

Over the Ca²⁺ concentration range for which the ICR was monitored, the fluorescence change when related to \([Ca^{2+}]\) was around the \(K_c\) value for Ca²⁺ binding to fluo-3, and, over this range, the fluorescence was shown to be linearly related to \([Ca^{2+}]\) (linear regression coefficient, \(r > 0.99\)).
increasing agonist concentration and in some cases appear to saturate (Fig. 3, A and B). The fast phase rate constants for Ca\(^{2+}\) release with Ins(1,4,5)P\(_3\) increases from 0.3 to 1.5 s\(^{-1}\) at concentrations between 30 nM and 20 \(\mu\)M (Fig. 3A) and from 0.02 s\(^{-1}\) to 0.5 s\(^{-1}\) for the slow phase over the same concentration range (Fig. 3B). A Hill coefficient of \(\approx 1.0\) was calculated from the rate constants of the fast phase with Ins(1,4,5)P\(_3\), which is in agreement with the findings of Finch et al. (18), but at variance with the findings presented in Refs. 9 and 10.

Although the deoxyInsP\(_3\) analogues released Ca\(^{2+}\) in a similar fashion to Ins(1,4,5)P\(_3\), with the release process in each case also being biphasic, the rate constants for Ca\(^{2+}\) release were, however, much lower than those of Ins(1,4,5)P\(_3\). Higher concentrations of the InsP\(_3\) analogues were required in order to

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**Fig. 2.** Time-resolved Ca\(^{2+}\) release induced by different analogues. The averaged release traces are plotted as the fractional InsP\(_3\)-induced Ca\(^{2+}\) release (where 1.0 is the amplitude induced by 20 \(\mu\)M Ins(1,4,5)P\(_3\)). A, Ca\(^{2+}\) release induced by Ins(1,4,5)P\(_3\) at concentrations (from top to bottom) of 20 \(\mu\)M, 10 \(\mu\)M, 3.0 \(\mu\)M, 1.0 \(\mu\)M, 0.3 \(\mu\)M, 0.1 \(\mu\)M, and 0.03 \(\mu\)M. B, Ca\(^{2+}\) release induced by 3-deoxyInsP\(_3\) at concentrations (from top to bottom) of 20 \(\mu\)M, 10 \(\mu\)M, 5.0 \(\mu\)M, 1.0 \(\mu\)M, and 0.5 \(\mu\)M. C, Ca\(^{2+}\) release induced by 2,3-dideoxyInsP\(_3\) at concentrations (from top to bottom) of 100 \(\mu\)M, 20 \(\mu\)M, 10 \(\mu\)M, 3.0 \(\mu\)M, and 1.0 \(\mu\)M. D, Ca\(^{2+}\) release induced by 2,3,6-trideoxyInsP\(_3\) at concentrations (from top to bottom) of 250 \(\mu\)M, 150 \(\mu\)M, 100 \(\mu\)M, 50 \(\mu\)M, and 10 \(\mu\)M. E, Ca\(^{2+}\) release induced by Ins(2,4,5)P\(_3\) at concentrations (from top to bottom) of 25 \(\mu\)M, 150 \(\mu\)M, 100 \(\mu\)M, 50 \(\mu\)M, and 10 \(\mu\)M. The solid lines through the traces represent the best fit using Equation 2. In all cases, the \(\chi^2\) values for these fits were always 0.1 or better. (Note that the changes in Ca\(^{2+}\) release with Ins(1,4,5)P\(_3\) are considerably faster than the other analogues and for this reason the recorded time scale is much shorter.)
attain the rate constants for Ca\textsuperscript{2+} release observed with even the lowest concentrations of Ins(1,4,5)P\textsubscript{3} used. When comparing the rate constants for both the fast and slow phases at the maximum concentrations of the analogues used, the values were never greater than 25–30% of those observed with 10 \mu M Ins(1,4,5)P\textsubscript{3}.

Since there are differences in the potencies between InsP\textsubscript{3} analogues such that different concentrations are required in order to release similar levels of Ca\textsuperscript{2+}, a comparison of the fast and slow rate constants for Ca\textsuperscript{2+} release at analogue concentrations which release 30% and 80% of the total InsP\textsubscript{3}-releasable Ca\textsuperscript{2+} pool was undertaken. Fig. 5, A and B, shows the relationship between the extent of Ca\textsuperscript{2+} release and the rate constants, both at 30% Ca\textsuperscript{2+} release or 80% Ca\textsuperscript{2+} release. This figure clearly demonstrates that there is no direct correlation between the extent of Ca\textsuperscript{2+} release and the rate constants. Different analogues at concentrations which release the same extent of Ca\textsuperscript{2+} do so at different rates.

**DISCUSSION**

The kinetic properties of the InsP\textsubscript{3}-sensitive Ca\textsuperscript{2+} channel remains something of a mystery despite extensive study. The inability of low concentrations of InsP\textsubscript{3} to mobilize all the InsP\textsubscript{3}-sensitive Ca\textsuperscript{2+} stores has led to the development of several theoretical models (6–8), many of which have not held up
to rigorous testing (3). In the hope of gaining further insights into how this channel operates, we employed stopped-flow spectrophotometry to study the transient kinetics of Ca\(^{2+}\) release from cerebellar microsomes using a number of different InsP\(_3\) analogues.

Our data show good agreement with those of a previous study by Kozikowski et al. (19) using these deoxyInsP\(_3\) analogues. They investigated the effects of these analogues on the extent of Ca\(^{2+}\) release and their ability to displace \(^{[3H]}\)Ins(1,4,5)P\(_3\) and showed that Ins(1,4,5)P\(_3\) was the most potent at inducing Ca\(^{2+}\) release, 2,3,6-trideoxyInsP\(_3\) was the least potent, and the others were in between. These results demonstrate the importance of hydroxy groups and their positions on the inositol ring, in activating the channel. Detailed analysis of their data showed a direct correlation between Ca\(^{2+}\) releasing ability (concentration required for half-maximal release) and binding ability (measured as the concentration required to half-maximally displace bound \(^{[3H]}\)Ins(1,4,5)P\(_3\)) (Fig. 6). This correlation, which was initially reported for several other inositol phosphate analogues (20), also holds when comparing our IC\(_{50}\) values for Ca\(^{2+}\) release with the \(K_i\) values determined for binding as given in Ref. 19 (Fig. 6), even though in these studies the binding measurements were done under experimental conditions different from those for Ca\(^{2+}\) release. Such a correlation has led to the proposal that the potency of all inositol phosphates in opening the channel is directly related to occupancy, and, thus, low efficacy inositol phosphates require higher concentrations in order to occupy the binding site before they induce channel opening. It is therefore widely believed that, for full agonists, the extent of occupancy directly relates to the extent of channel opening and therefore presumably the Ca\(^{2+}\) release process would be similar for all analogues which are occupying the receptor to the same extent. However, it is clear from Fig. 5 that the rate constants for Ca\(^{2+}\) release are not the same for any given extent of release, they are dependent upon the structure of the InsP\(_3\) analogue and the concentration used. From this observation it must be concluded that the InsP\(_3\) receptor can distinguish between different InsP\(_3\) analogues occupying the binding site and alter the rate at which Ca\(^{2+}\) flows through the channel accordingly.

One possibility to explain such an observation would be to assume the existence of 2 occupied states of the receptor. This assumption is not inconceivable since some inositol phosphates can act as antagonists, e.g., InsP\(_6\), etc. (3). These inositol phosphates were shown to bind to the receptor displacing \(^{[3H]}\)Ins(1,4,5)P\(_3\) without inducing Ca\(^{2+}\) release. Thus, in one state the receptor is occupied by an inositol phosphate antagonist which stabilizes the receptor in an occupied but "unproductive" conformation, while in the other state, stabilized in the presence of Ins(1,4,5)P\(_3\), the receptor adopts an occupied and "productive" conformation which leads to channel opening. Presumably these two states exist due to different contacts being made by the inositol phosphates in the binding domain of the receptor. If these two states exist in dynamic equilibrium, it could be envisaged that some inositol phosphates could in fact induce both conformational states at any given time of occupancy, sometimes the ligand would form contacts which lead to channel opening (productive state), and sometimes it would not (unproductive state). Assuming full occupancy, in such a scheme the extent of Ca\(^{2+}\) release would eventually be the same as that observed for Ins(1,4,5)P\(_3\), but by virtue of the fact that since some of the time the receptor would be in a productive state, eventually all the mobilizable Ca\(^{2+}\) would be released. However, the rates for Ca\(^{2+}\) release would be slower than those observed for Ins(1,4,5)P\(_3\). The rate constants for Ca\(^{2+}\) release would therefore depend upon the forward and backward rate constants for these two states and thus equilibrium constant for the two states induced by a particular inositol phosphate. In such a mechanism, the equilibrium constant for Ins(1,4,5)P\(_3\) would be much greater than that for 2,3,6-trideoxyInsP\(_3\).

An additional mechanism for such a process could depend upon the fact that since multiple conductance states have been reported for the InsP\(_3\) receptor (21), different inositol phos-
phate analogues may preferentially induce the channel to adopt the lower conductance states thus reducing the rate of Ca\(^{2+}\) efflux through the channel. High conductance states would only be induced by Ins(1,4,5)P\(_3\).

As the rate constants for Ca\(^{2+}\) release never reach the values of those observed for Ins(1,4,5)P\(_3\), these analogues must therefore be classified as "partial agonists." Such a model involving two or more occupied states, as outlined here, has also been proposed to explain partial agonism in other types of receptors (22).

Recently, another class of inositol phosphates which includes Ins(1,3,4,6)P\(_4\), l-ch-Ins(2,3,5)PS\(_3\), and d-6-deoxy-myoo-Ins(1,4,5)-PS\(_3\) (3,23,24) have been shown to induce Cu\(^{2+}\) release through the InsP\(_3\) receptor, but are unable to release Ca\(^{2+}\) to the same extent as seen with maximal concentrations of Ins(1,4,5)P\(_3\). These analogues have also been classified as partial agonists. Therefore, in order to avoid confusion between these two classes, we suggest that partial agonists of the type which cannot mobilize all the Ins(1,4,5)P\(_3\) mobilizable Ca\(^{2+}\) pool be classified as class I, whereas partial agonists of the type described in this paper which can mobilize all the Ins(1,4,5)P\(_3\) releasable Ca\(^{2+}\) pool but whose rates are lower than those observed with Ins(1,4,5)P\(_3\), be classified as class 2. The nature of the partial agonism by class 1 is yet to be investigated; however, if we assume that each phase is due to a distinct population of Ca\(^{2+}\) stores which have functionally different InsP\(_3\) receptors, it would be tempting to speculate that these agents may selectively affect only one of these two populations.

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