Inositol 1,4,5-trisphosphate receptor subtypes differentially recognize regioisomers of D-*myo*-inositol 1,4,5-trisphosphate

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The $Ins(1,4,5)P_3$ regioisomers, $Ins(1,4,6)P_3$ and $Ins(1,3,6)P_3$, which can mimic the 1,4,5-arrangement on the inositol ring of $Ins(1,4,5)P_3$, were examined for Ca^{2+} release by using four types of saponin-permeabilized cell possessing various abundances of receptor subtypes, with special reference to the relation of potency to receptor subtype. $Ins(1,4,6)P_3$ and $Ins(1,3,6)P_3$ were weak agonists in rat basophilic leukaemic cells (RBL cells), which possess predominantly subtype II receptors, with respective potencies of 1/200 and less than 1/500 that of $Ins(1,4,5)P_3$ [the EC_{50} values were 0.2, 45 and more than 100 μM for $Ins(1,4,5)P_3$, $Ins(1,4,6)P_3$ and $Ins(1,3,6)P_3$ respectively]. Similar rank order potencies were also evaluated for the displacement of $[^3H]Ins(1,4,5)P_3$ bound to RBL cell membranes by these regioisomers. However, they caused Ca^{2+} release from GH_3 rat pituitary cells possessing predominantly subtype I receptors more potently; $Ins(1,4,6)P_3$ and $Ins(1,3,6)P_3$ evoked release at respective concentrations of only one-third and one-twentieth that of $Ins(1,4,5)P_3$ (the EC_{50} values were 0.4, 1.2 and 8 μM for $Ins(1,4,5)P_3$, $Ins(1,4,6)P_3$ and $Ins(1,3,6)P_3$ respectively). In COS-1 African green-monkey kidney cells, with the relative abundances of 37 % of the subtype II and of 62 % of the subtype III receptor, potencies of 1/40 and approx. 1/200 for $Ins(1,4,6)P_3$ and $Ins(1,3,6)P_3$ respectively were exhibited relative to $Ins(1,4,5)P_3$ (the EC_{50} values were 0.4, 15 and approx. 80 μM for $Ins(1,4,5)P_3$, $Ins(1,4,6)P_3$ and $Ins(1,3,6)P_3$ respectively). In HL-60 human leukaemic cells, in spite of the dominant presence of subtype I receptors (71 %), similar respective potencies to those seen with COS-1 cells were exhibited (the EC_{50} values were 0.3, 15 and approx. 100 μM for $Ins(1,4,5)P_3$, $Ins(1,4,6)P_3$ and $Ins(1,3,6)P_3$ respectively). These results indicate that these regioisomers are the first ligands that distinguish between receptor subtypes; the present observations are of significance for the future design of molecules with enhanced selectivity.

INTRODUCTION

D-*myo*-Inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$ (**1**); see Figure 1], a product of the receptor-activated hydrolysis of $PtdIns(4,5)P_2$, discharges Ca^{2+} from a non-mitochondrial store site by interacting with a specific receptor that distinguishes between the enantiomers of $Ins(1,4,5)P_3$ [1–3]. Molecular cloning studies have revealed the presence of three subtypes of receptor, together with their splice variants, encoded by separate genes [4,5]. The significance of $Ins(1,4,5)P_3$ receptor diversity has not yet been established. Clearly, however, selective expression of $Ins(1,4,5)P_3$ receptor subtypes influences the action of an agonist at the receptor if the receptor subtypes exhibit either regulatory and/or functional differences. One well-investigated difference in the regulation of the receptors is that subtype I can be phosphorylated by cAMP-dependent kinase activity, which is known to modify the sensitivity of the receptor to $Ins(1,4,5)P_3$ [6,7]. The subtype II and III receptors, however, do not possess the consensus sequence required for the phosphorylation and therefore are not under the same regulatory control. Whether there are functional differences between the receptor subtypes is less clear; however, studies concerning the affinity of the ligand or sensitivity to Ca^{2+} have been reported. For example, Südhof's group reported differences in the affinity for $Ins(1,4,5)P_3$, with a rank order of subtype II subtype I subtype III, by using soluble fusion proteins of the N-terminus of each receptor subtype, but not the whole receptor [5,8]. A similar conclusion was drawn from the studies by Parys et al. [9]: rat basophilic leukemic cells (RBL cells) (predominantly containing the subtype II receptor) had a much higher affinity for

$Ins(1,4,5)P_3$ than did cerebellar and A7r5 cells, which contain predominantly subtype I receptor. Conversely, several studies report that subtype I, II and III receptors bind $Ins(1,4,5)P_3$ with very similar characteristics [10,11]. As to the Ca^{2+} sensitivity of the ligand binding, on the other hand, two reports gave similar results: subtype III receptors exhibited a biphasic binding activity, depending on Ca^{2+} concentration ranging from 100 to 700 nM, whereas subtype I did not [12,13]. A greater understanding of the functional and regulatory characteristics that distinguish the $Ins(1,4,5)P_3$ receptor subtypes is obviously required before differences in Ca^{2+} homeostasis between cell types can be categorically attributed to their $Ins(1,4,5)P_3$ receptor subtype diversity.

The finding that $Ins(1,3,4,6)P_4$ provoked Ca^{2+} release, albeit with a lesser potency than $Ins(1,4,5)P_3$, from *Xenopus* oocytes [14] and a permeabilized SH-SY5Y human neuroblastoma cell line [15] was somewhat at odds with established structure–activity precedents, because of the apparent absence of the vicinal 4,5-bisphosphate, which is believed to be an essential requirement for Ca^{2+} release [16]. Later, the activity of this compound was rationalized by envisaging two alternative receptor-binding orientations where the 1,6-vicinal bisphosphate of $Ins(1,3,4,6)P_4$ mimics the normal 4,5-bisphosphate in the $Ins(1,4,5)P_3$-binding orientation. This rationalization predicts that two $Ins(1,4,5)P_3$ regioisomers, namely $Ins(1,4,6)P_3$ (**2**) and $Ins(1,3,6)P_3$ (**3**) (Figure 1) should mobilize Ca^{2+}, with the 1,6-bisphosphate motif in each case mimicking the 4,5-bisphosphate of $Ins(1,4,5)P_3$. Both **2** and **3** have been detected as minor inositol phosphates in avian erythrocytes and as products of $Ins(1,3,4,6)P_4$ dephosphorylation

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by rat brain cytosol [17]; **2** has also tentatively been identified in WRK1 rat mammary tumour cells [18]. The prediction that **2** and **3** should mobilize Ca^{2+} was first corroborated by some of us, with the use of permeabilized RBL cells [19]. In that study, $Ins(1,4,6)P_3$ and $Ins(1,3,6)P_3$ displaced $[^3H]Ins(1,4,5)P_3$ bound to subtype I receptors purified from rat cerebellar membranes with 1/15 and 1/90 the potency of $Ins(1,4,5)P_3$ respectively but each regioisomer could release the Ca^{2+} from permeabilized RBL cells, with a much smaller potency, approx. 1/200 and 1/3000 respectively. The discrepancy in potencies was proposed to be attributed to receptor subtype differences [19]. Recently, some of us further studied the same effect with rabbit platelets and demonstrated that both regioisomers showed almost the same potency between Ca^{2+} mobilization experiments with rabbit platelets and $[^3H]Ins(1,4,5)P_3$ binding with rat cerebellar membranes [20]. According to the reports on relative abundance of $Ins(1,4,5)P_3$ receptor subtypes in many kinds of tissue and cell line [21,22], both cerebellar membranes and platelets contain predominantly the subtype I receptor, whereas RBL cells predominantly contain subtype II. This fact favours the possibility that the subtype difference in the receptor causes the difference in the potencies of Ca^{2+} release by $Ins(1,4,6)P_3$ and $Ins(1,3,6)P_3$.

In the present study we used several cell lines that express different receptor subtypes to gain a further understanding of whether $Ins(1,4,6)P_3$ and $Ins(1,3,6)P_3$ are recognized differently by the receptor, depending on subtype differences.

MATERIALS AND METHODS

Materials

$[^3H]Ins(1,4,5)P_3$ (specific radioactivity 777 GBq/mmol) and $^{45}Ca^{2+}$ (specific radioactivity 1.22 GBq/mg) were purchased from Du Pont–New England Nuclear (Boston, MA, U.S.A.). RBL and HL-60 cells were generously supplied by Dr. M. Abe (Department of Pharmacology, Faculty of Medicine, Fukuoka University, Fukuoka, Japan) and Dr. H. Sumimoto (Department of Biochemistry, Faculty of Medicine, Kyushu University, Fukuoka, Japan) respectively. COS-1 and GH_3 cells were purchased from Riken Cell Bank (Tsukuba, Japan). $Ins(1,4,6)P_3$ [23] and $Ins(1,3,6)P_3$ [24] were synthesized chemically as described and were used as their triethylammonium salts. $Ins(1,4,5)P_3$ was obtained from Dojindo Laboratory (Kumamoto, Japan). Concentrations of aqueous solutions of $InsP_3$ regioisomers were determined by measuring organic phosphate content. All other reagents used were of the highest grade available.

Cell culture

Cells were grown in α-minimal essential medium supplemented with 10 % (v/v) fetal bovine serum and 20 mM Hepes buffer (pH 7.2) in a humidified incubator aerated with air/CO_2 (19:1), except for HL-60 cells, which were grown in RPMI 1640 medium.

Preparation of membrane fraction of RBL cells

RBL cells (packed volume of approx. 1 ml; approx. 10^9 cells) were sonicated in 5 ml of a solution containing 0.15 M NaCl/20 mM Tris/HCl buffer (pH 7.5) and 1 mM EDTA with a Branson sonifier at half-maximal power three times for 30 s with 1 min intervals. Sonicated preparations were centrifuged at 1200 g at 4 °C for 5 min, followed by a sequential centrifugation at 5000 g for 20 min and then at 100 000 g for 30 min. The resultant pellet was washed once with a large volume of the same solution to obtain the final membrane preparation.

Saponin treatment of cells and Ca^{2+} release experiments

Cells were permeabilized with saponin as described previously [25,26]. Briefly, 10^8 cells were treated with a solution containing 130 mM KCl, 20 mM Tris/maleate buffer, pH 6.8, 5 mM $MgCl_2$, 5 mM ATP, 1 mM EGTA, 0.1 % BSA and 0.1 mg/ml saponin at 37 °C for 5 min, followed by washing twice with 130 mM KCl and 20 mM Tris/maleate buffer and 0.1 % BSA. Permeabilization was confirmed by a Trypan Blue exclusion test; more than 95 % of cells excluded Trypan Blue before a saponin treatment, but cells failed to exclude the dye after the treatment. Permeabilized cells were preincubated in a solution containing 130 mM KCl, 20 mM Tris/maleate buffer, pH 6.8, 5 mM $MgCl_2$, 5 mM NaN_3, 0.12 mM $CaCl_2$ (containing 20 kBq/ml $^{45}Ca^{2+}$), 1 mM EGTA (free Ca^{2+} concentration was calculated as 140 nM) at 37 °C for 2 min. Ca^{2+} accumulation was initiated by the addition of 5 mM ATP and continued for up to 10 min, the time required for maximal accumulation. An aliquot of the mixture was passed through a Whatman GF/C filter (1.2 μm) to determine the Ca^{2+} accumulation. At 11 min after addition of ATP, various concentrations of inositol trisphosphate were added, followed by filtration of an aliquot of the mixture through the filter to determine the remaining Ca^{2+} in permeabilized cells at 1 and 3 min after addition of the ligand. In all cases, two values of the remaining Ca^{2+} at 1 and 3 min did not differ significantly. The same experiments were done in the absence of ATP; the values obtained were subtracted from those in its presence to determine the energized Ca^{2+} accumulation.

RESULTS AND DISCUSSION

Structures

Figure 1 shows the structures of $Ins(1,4,6)P_3$ and $Ins(1,3,6)P_3$, the orientations of which are adjusted for the vicinal 1,6-bisphosphate to mimic the vicinal 4,5-bisphosphate of $Ins(1,4,5)P_3$ in their most likely binding orientations, where the pseudo-2-OH groups are equatorial and the orientations of the pseudo-3-OH and 6-OH groups of $Ins(1,4,6)P_3$ and $Ins(1,3,6)P_3$ respectively are axial, but they are equatorial in $Ins(1,4,5)P_3$.

Ca^{2+} release in RBL cells

Because the previous preparations of synthetic $Ins(1,4,6)P_3$ and $Ins(1,3,6)P_3$ differed [19] from those used in the present study, the potencies of the ligands to evoke Ca^{2+} release were examined

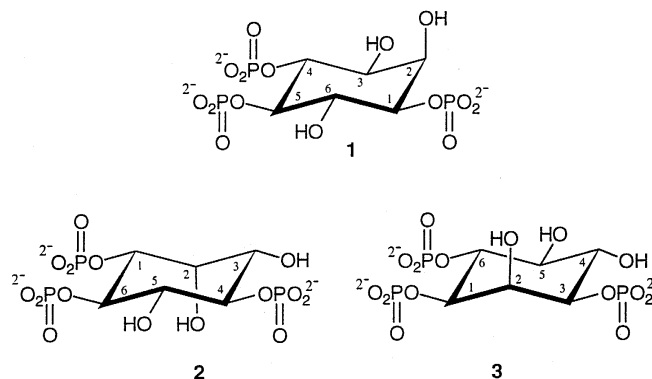**Figure 1   Structures of $Ins(1,4,5)P_3$ (1) $Ins(1,4,6)P_3$ (2) and $Ins(1,3,6)P_3$ (3)**

Regioisomers **2** and **3** are shown, to illustrate the correspondence of their 1,6-bisphosphates to the 4,5-bisphosphates moieties of **1**.
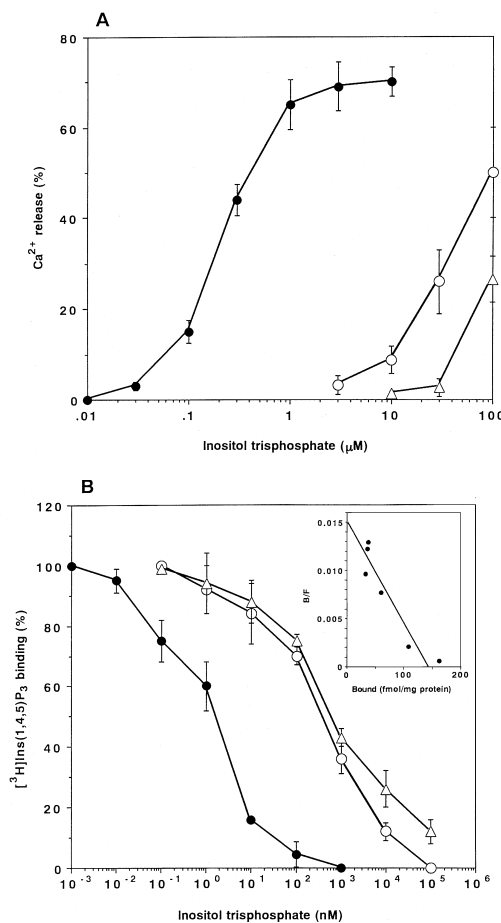
A

B

Figure 2   Ca^{2+} release and [^{3}H]Ins(1,4,5)P_3 binding of RBL cells

Both Ca^{2+} release (**A**) and [^{3}H]Ins(1,4,5)P_3 binding (**B**) were assayed with permeabilized cells and RBL cell membranes respectively, as described in the Materials and methods section. Ca^{2+} accumulation at a free Ca^{2+} concentration of 140 nM was 0.94 ± 0.04 nmol per 10^6 cells (mean $\pm$ S.E.M. for nine determinations), which was determined by subtracting the value in the absence of ATP from that in its presence. The ordinate represents the release of Ca^{2+} as a percentage of the accumulation (mean $\pm$ S.E.M. for three determinations), and the following figures for Ca^{2+} release are similarly depicted. Ins(1,4,5)P_3 (●), Ins(1,4,6)P_3 (○), Ins(1,3,6)P_3 (△). The inset to (**B**) is a Scatchard plot of binding data, indicating that K_d and B_{max} were 1.7 nM and 129 fmol/mg of protein respectively (means of two determinations).

Table 1   Relative abundances of Ins(1,4,5)P_3 receptor subtypes in cells used in the present study

Cell type	Subtype I (%)	Subtype II (%)	Subtype III (%)
RBL*	17.1 ± 6.2	70.0 ± 7.9	12.7 ± 1.6
GH$_3$*	80.0 ± 2.5	5.6 ± 0.4	12.0 ± 0.6
COS-1†	1 ± 1	37 ± 7	62 ± 6
HL-60†	71 ± 7	29 ± 7	0 ± 0

* Values taken from De Smedt et al. [22], in which the estimations were made on the mRNA levels by Southern blotting analyses with a subtype-specific oligonucleotide.

† Values taken from Wojcikiewicz [21], in which the estimations were made on the protein levels by Western blotting analyses with a subtype-specific antibody.

for four determinations) and an increase in free Ca^{2+} to 300 nM slightly increased the binding to $123 \pm 8\%$ (mean $\pm$ S.E.M. for four determinations). In contrast, it has been reported that the subtype III receptor increased the binding activity to a maximum of 350% compared with that in the absence of free Ca^{2+}, whereas the subtype I receptor increased it marginally [12,13]. Therefore it seems that the subtype II receptor behaves like subtype I in its Ca^{2+} sensitivity.

To examine relative affinities, the displacement of [^{3}H]Ins(1,4,5)P_3 bound to the membrane preparations by Ins(1,4,6)P_3 and Ins(1,3,6)P_3 in addition to unlabelled Ins(1,4,5)P_3 was examined (Figure 2B). The EC$_{50}$ values for displacement were 1.8, 380 and 1100 nM for Ins(1,4,5)P_3, Ins(1,4,6)P_3 and Ins(1,3,6)P_3 respectively, thereby indicating that the potencies of Ins(1,4,6)P_3 and Ins(1,3,6)P_3, relative to Ins(1,4,5)P_3, were approx. 1/200 and 1/350 respectively; the values for both ligands were roughly the same as those for the release of Ca^{2+} described above. The inset to Figure 2(B) shows a Scatchard plot indicating that K_d and B_{max} were 1.7 nM and 129 fmol/mg protein (means of two determinations) respectively.

The results on the relative potency between Ca^{2+} release and [^{3}H]Ins(1,4,5)P_3 binding experiments by Ins(1,4,5)P_3 regioisomers indicate that there is no discrepancy between these two experiments when the same subtype of receptors is used. Taking previous reports that Ins(1,4,6)P_3 and Ins(1,3,6)P_3 in this rank order showed closer ties with Ins(1,4,5)P_3 in binding experiments with rat cerebellar membranes [19,20] and with rabbit platelets in Ca^{2+} release experiments [20] (both tissues predominantly contain subtype I receptors), we then examined the Ca^{2+} release activity with Ins(1,4,5)P_3 regioisomers with other cell types containing different receptor subtypes from those in RBL cells. Table 1 shows the relative abundances of receptor subtypes reported in various cell lines used in the following experiments.

Ca^{2+} release from GH$_3$ cells

GH$_3$ rat pituitary tumour cells are reported to contain more than 80% of the subtype I receptor, the rest being mainly subtype III. As shown in Figure 3, both Ins(1,4,6)P_3 and Ins(1,3,6)P_3 were examined to verify that both are full agonists in releasing Ca^{2+} from permeabilized GH$_3$ cells within the ranges examined and were effective at EC$_{50}$ values of 1.2 ± 0.9 and 8 ± 1.2 μM respectively, whereas the EC$_{50}$ of Ins(1,4,5)P_3 was 0.4 ± 0.1 μM, indicating that the potencies were only one-third and one-twentieth that of Ins(1,4,5)P_3. Experiments on permeabilized rabbit platelets with the same preparations of Ins(1,4,5)P_3 regioisomers as those used here provided similar results: Ins(1,4,5)P_3, Ins(1,4,6)P_3 and Ins(1,3,6)P_3 evoked the release of Ca^{2+} with EC$_{50}$

again with RBL cells, which contain predominantly the subtype II Ins(1,4,5)P_3 receptor. As shown in Figure 2(A), Ins(1,4,6)P_3 and Ins(1,3,6)P_3 were capable of causing Ca^{2+} release from permeabilized RBL cells in a dose-dependent manner. Although neither Ins(1,4,6)P_3 nor Ins(1,3,6)P_3 could be examined up to the maximal effect because of the large amounts of ligands required, the concentrations required for half-maximal effects were 45 ± 10 μM and more than 100 μM respectively, whereas that of Ins(1,4,5)P_3 was 0.2 ± 0.1 μM. Therefore the potencies were approx. 1/200 and approx. 1/500 that of Ins(1,4,5)P_3 respectively, confirming the previous report by some of us with different preparations of the ligands [19], in which relative potencies of 1/175 and approx. 1/3000 respectively were exhibited.

Membrane preparations of RBL cells were next examined for binding of [^{3}H]Ins(1,4,5)P_3. The 100000 **g** precipitates after the 5000 **g** centrifugation of sonicated RBL cells were assayed for binding at 1.3 nM [^{3}H]Ins(1,4,5)P_3 at free Ca^{2+} concentrations of less than 10 nM and 300 nM; we found that binding in the absence of Ca^{2+} was 37 ± 6 fmol/mg of protein (mean $\pm$ S. E. M.
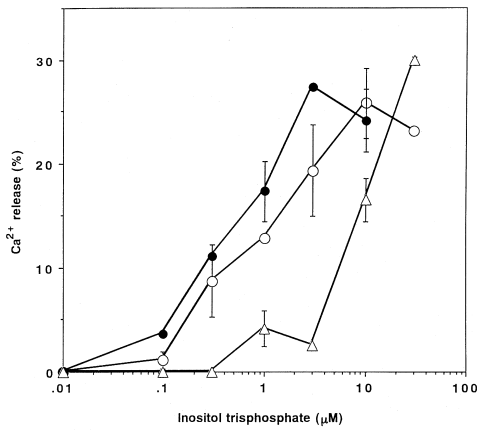
Figure 3 Ca²⁺ release from GH₃ cells

Permeabilized GH₃ cells were assayed for Ca²⁺ release at a free Ca²⁺ concentration of 140 nM, at which the ATP-dependent accumulation was 0.21 ± 0.02 nmol per 10^6 cells (mean $\pm$ S.E.M. for five determinations). Data shown are means $\pm$ S.E.M. for three determinations. Symbols: ●, Ins(1,4,5)P_3; ○, Ins(1,4,6)P_3; △, Ins(1,3,6)P_3.
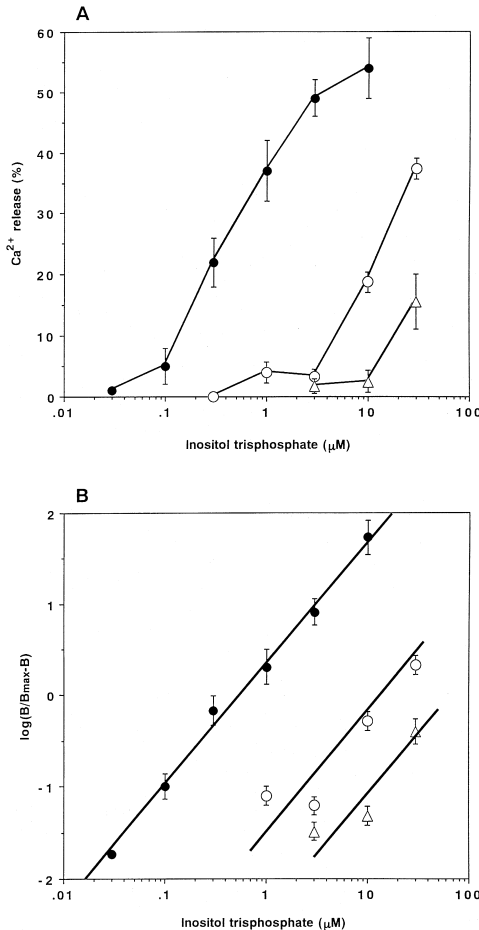

Figure 4 Ca²⁺ release from COS-1 cells

Permeabilized COS-1 cells were assayed for Ca²⁺ release at a free Ca²⁺ concentration of 140 nM, at which the ATP-dependent accumulation was 0.27 ± 0.04 nmol per 10^6 cells (mean $\pm$ S.E.M. for four determinations). Data shown are mean $\pm$ S.E.M. for three determinations (**A**), and were analysed for the Hill plot in (**B**). Symbols: ●, Ins(1,4,5)P_3; ○, Ins(1,4,6)P_3; △, Ins(1,3,6)P_3.
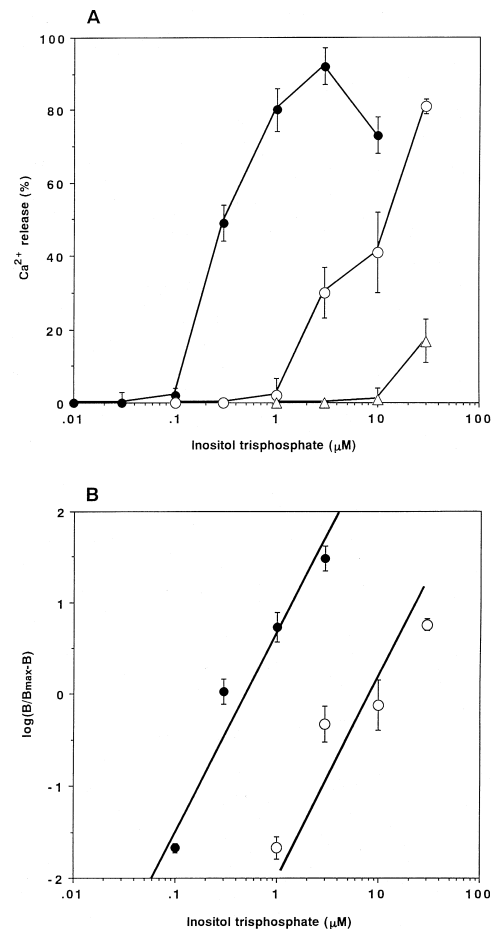

Figure 5 Ca²⁺ release from HL-60 cells

Permeabilized HL-60 cells were assayed for Ca²⁺ release at a free Ca²⁺ concentration of 140 nM, at which the ATP-dependent accumulation was 0.27 ± 0.06 nmol per 10^6 cells (mean $\pm$ S.E.M. for six determinations). Data shown are mean $\pm$ S.E.M. for three determinations (**A**), and were analysed for the Hill plot in (**B**). Symbols: ●, Ins(1,4,5)P_3; ○, Ins(1,4,6)P_3; △, Ins(1,3,6)P_3.

values of 0.69 μM, 1.56 μM (one-half the potency) and 8.05 μM (one-twelfth the potency) respectively [20]. Platelets are known to possess the subtype I Ins(1,4,5)P_3 receptor; however, the relative abundance of this subtype in platelets is not known [27].

Ca²⁺ release from COS-1 cells and HL-60 cells

COS-1 African green-monkey kidney cells possess subtype III Ins(1,4,5)P_3 receptor as the most abundant subtype; subtype II comprises 37% and subtype I is negligible (Table 1). Ca²⁺-releasing activity by Ins(1,4,5)P_3 regioisomers in permeabilized COS-1 cells is shown in Figure 4(A). The concentration required for half-maximal release of Ca²⁺ was 0.4 ± 0.1 μM for Ins(1,4,5)P_3. Neither Ins(1,4,6)P_3 nor Ins(1,3,6)P_3 was examined up to the doses to evoke the maximal effect. However, if the same EC₅₀ as that seen with Ins(1,4,5)P_3 applies to the results obtained with Ins(1,4,6)P_3 and those expected for Ins(1,3,6)P_3, then 15 ± 5 μM and approx. 80 μM are the EC₅₀ values for Ins(1,4,6)P_3 and Ins(1,3,6)P_3 respectively.

Table 2   Summary of results: EC$_{50}$ values for Ca^{2+} release from different cell types

EC$_{50}$ values for Ca^{2+} release were calculated from each graph. The numbers in parentheses represent potencies relative to Ins(1,4,5)P_3.

Isomer	EC$_{50}$ (μM)			
	RBL	GH$_3$	COS-1	HL-60
Ins(1,4,5)P_3	0.2 $\pm$ 0.1	0.4 $\pm$ 0.1	0.4 $\pm$ 0.1	0.3 $\pm$ 0.1
Ins(1,4,6)P_3	45 $\pm$ 10 (1/230)	1.2 $\pm$ 0.9 (1/3)	15 $\pm$ 5 (1/38)	15 $\pm$ 3 (1/50)
Ins(1,3,6)P_3	> 100 (< 1/500)	8 $\pm$ 1.2 (1/20)	$\sim$ 80 ($\sim$ 1/200)	$\sim$ 100 ($\sim$ 1/300)

Because cell types more abundant in subtype III have not yet been characterized, the behaviour of the pure form of this receptor subtype to the Ins(1,4,5)P_3 regioisomers could not be examined. (Wojcikiewicz [21] reported that RINm5F rat insulinoma cells possess mainly subtype III Ins(1,4,5)P_3 receptor at an abundance of 96 $\pm$ 1 %, whereas De Smedt et al. [22] reported the relative abundances in the same cells of 29.2 %, 6.1 % and 59.7 % for subtypes I, II and III respectively. This discrepancy did not encourage us to examine this cell line to evaluate the character of subtype III.) However, it could be inferred from the present study that the subtype III Ins(1,4,5)P_3 receptor responds to Ins(1,4,6)P_3 and Ins(1,3,6)P_3 with a moderate potency; higher concentrations of Ins(1,4,6)P_3 and Ins(1,3,6)P_3 than those seen with GH$_3$ cells here and with platelets reported previously [20], but lower concentrations than those seen with RBL cells ([19], and the present paper) were needed. The Hill plot depicted in Figure 4(B) shows a Hill coefficient of 1.2 $\pm$ 0.2, indicating that there is no apparent interaction between subtypes II and III.

HL-60 human leukaemic cells contain both subtypes I and II, with relative abundances of 71 % and 29 % respectively. The higher abundance of the subtype I Ins(1,4,5)P_3 receptor suggested that Ins(1,4,6)P_3 and Ins(1,3,6)P_3 would be effective in releasing Ca^{2+} from permeabilized HL-60 cells at lower concentrations than those seen with RBL and COS-1 cells. As shown in Figure 5(A), however, these regioisomers evoked the release of Ca^{2+} at EC$_{50}$ values of 15 $\pm$ 3 μM and approx. 100 μM (estimated as described for COS-1 cells above), indicating that the values were almost the same as those obtained with COS-1 cells. For clarity, the EC$_{50}$ values for the Ca^{2+} release obtained in the present study are summarized in Table 2.

However, it also seemed that permeabilized HL-60 cells responded to increasing concentrations of Ins(1,4,6)P_3 biphasically (Figure 5A). Therefore subtype I, the most sensitive to Ins(1,4,6)P_3, and subtype II, with the least sensitivity, responded independently to Ins(1,4,6)P_3 for channel opening. This does not contradict recent studies that the Ins(1,4,5)P_3 channel is a heterotetrameric complex of different subtypes [28,29], because it has been reported that the channel incorporated into lipid planar membranes exhibited four conductance levels, indicating that the channel opens in an additive manner [30]. Alternatively, the concentration dependence of Ca^{2+} release in HL-60 cells was somewhat steeper than those obtained with other cell types, suggesting some co-operativity in the process. The Hill coefficient calculated from the plot depicted in Figure 5(B) was 2.3 $\pm$ 0.5, indicating that there might be some co-operativity in the opening of the channel of the heterotetramer made from subtypes I and II. The relative abundances of these subtypes suggest that, if uniformly distributed, the channel is formed from three molecules of subtype I plus one molecule of subtype II. Therefore it could be that the subtype II Ins(1,4,5)P_3 receptor behaves negatively,

overcoming the dominant subtype I receptor's sensitivity to Ins(1,4,6)P_3 and Ins(1,3,6)P_3.

This co-operativity is unique to HL-60 cells, because the Hill coefficients were 1.5 $\pm$ 0.3, 0.97 $\pm$ 0.2 and 1.2 $\pm$ 0.2 for RBL, GH$_3$ and COS-1 cells respectively (results for the former two cell types are not shown). Ca^{2+} concentrations at 300–500 nM around the Ins(1,4,5)P_3 receptor have been reported to positively regulate the release of Ca^{2+} caused by the ligand [31–33], thus producing an apparent co-operativity. In other words, Ca^{2+} from the endoplasmic reticulum released by Ins(1,4,5)P_3 makes the receptor channel more sensitive for the further release of Ca^{2+} despite there being no co-operativity in the channel opening. However, this notion is unlikely because cell types other than HL-60 did not show any co-operativity despite the use of the same experimental protocol. RBL cells were the most prominent for Ca^{2+} efflux/influx among cells used in the present study. Furthermore in the experiments with ^{45}Ca^{2+} as a tracer it could be supposed that no marked changes in free Ca^{2+} concentration occur because of a strong buffering action of EGTA, but those with a fluorescent dye produce a big change. Thus the co-operativity observed in HL-60 is likely to be an intrinsic character, indicating that the co-operativity in the Ca^{2+}-channel opening depends on the combination of receptor subtypes. Obviously further studies, paying special attention to the high rate of Ca^{2+} release, are required before a conclusion on co-operativity can be drawn.

In conclusion, the present study presents evidence that Ins(1,4,5)P_3 receptor subtypes might have different affinities for Ins(1,4,6)P_3 and Ins(1,3,6)P_3, two regioisomers of Ins(1,4,5)P_3; subtype I has the highest affinity for these isomers; subtype III has an intermediate affinity and subtype II has the lowest. As shown in Figure 1, Ins(1,4,6)P_3 and Ins(1,3,6)P_3 have an axial hydroxy group at a pseudo-3-position and a pseudo-6-position respectively, in addition to a pseudo-2-position's equatorial hydroxy group, which has been shown not to be involved in recognition [34]. Therefore it is possible that subtype I is most able to accommodate the increased bulk owing to the axial hydroxy group, although structural arguments alone might not be sufficient to explain these effects. These are the first ligands to have been demonstrated to distinguish between receptor subtypes; the present observations will be of significance for the future design of molecules with enhanced selectivity. The next step will be to examine binding to pure cloned subtypes of the Ins(1,4,5)P_3 receptor.

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