0026-895X/93/040810-08$03.00/0

MOLECULAR PHARMACOLOGY, 44:810-817

Inositol-1,3,4,5-tetrakisphosphate Induces Calcium Mobilization via the Inositol-1,4,5-trisphosphate Receptor in SH-SY5Y Neuroblastoma Cells

ROBERT A. WILCOX, R. A. JOHN CHALLISS, CHANGSHENG LIU, BARRY V. L. POTTER, and STEFAN R. NAHORSKI

Department of Pharmacology and Therapeutics, University of Leicester, Leicester, LE1 9HN, UK (R.A.W., R.A.J.C., S.R.N.), and School of Pharmacy and Pharmacology and Institute for Life Sciences, University of Bath, Bath, BA2 7AY, UK (C.L., B.V.L.P.)

Received May 4, 1993; Accepted July 19, 1993

SUMMARY

myo-Inositol-1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4]-induced Ca^{2+} mobilization was examined in saponin-permeabilized SH-SY5Y cells using *myo*-inositol hexakisphosphate-supplemented buffer to prevent Ins(1,3,4,5)P_4-3-phosphatase-catalyzed back-conversion of exogenous Ins(1,3,4,5)P_4 to *myo*-inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3]. The Ins(1,3,4,5)P_4 concentration-response curve for Ca^{2+} release in SH-SY5Y cells exhibited an EC_{50} of 2.5 μM, compared with 52 nM for Ins(1,4,5)P_3, with the maximally effective concentration of Ins(1,3,4,5)P_4 (100 μM) mobilizing the entire Ins(1,4,5)P_3-sensitive pool. Both Ins(1,3,4,5)P_4- and Ins(1,4,5)P_3-induced Ca^{2+} mobilizations were heparin sensitive. Further, L-*chiro*-inositol-2,3,5-trisphosphorothioate, a recently identified low intrinsic activity Ins(1,4,5)P_3 receptor partial agonist, shifted both the Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 concentration-response curves significantly rightward, with similar potencies. However, binding studies demonstrate that L-*chiro*-inositol-2,3,5-trisphosphorothioate interacts very poorly (IC_{50} > 30 μM) with specific Ins(1,3,4,5)P_4 binding sites that have been previously characterized in pig cerebellum. Carbachol-pretreated SH-SY5Y cells (1 mM, ≥6 hr) exhibit a decrease in Ins(1,4,5)P_3 receptor number, accompanied by both a rightward shift and a reduced maximal Ca^{2+} release in their Ins(1,4,5)P_3 concentration-response curve. Here both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 concentration-response curves were found to exhibit identically reduced maximal Ca^{2+} release responses and about 4-fold rightward shifts in EC_{50} values. Together, these observations provide compelling evidence for our hypothesis that Ins(1,3,4,5)P_4 exhibits weak but full agonist status at Ins(1,4,5)P_3 receptor-operated Ca^{2+} channels in SH-SY5Y cells.

Many cell surface receptors activate (via G proteins) phosphoinositidase C, which catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate to produce the second messengers Ins(1,4,5)P_3 and diacylglycerol (1). Ins(1,4,5)P_3 interacts specifically with a family of Ins(1,4,5)P_3 receptor-operated Ca^{2+} channels to mobilize nonmitochondrial intracellular Ca^{2+} stores (1, 2). Ins(1,4,5)P_3 is rapidly metabolized by 5-phosphatase and 3-kinase activities to form inositol-1,4-bisphosphate and Ins(1,3,4,5)P_4, respectively (3).

In some cell types, Ins(1,3,4,5)P_4 apparently does not affect intracellular Ca^{2+} stores or modulate Ins(1,4,5)P_3-induced Ca^{2+} mobilization (4–7). However, Ins(1,3,4,5)P_4 can directly mobilize Ca^{2+} stores in cerebellar (8) and adrenal (9) microsomes, microinjected *Xenopus* oocytes (10), and permeabilized SH-SY5Y neuroblastoma cells (11, 12). Categorical interpretation of these studies may have been complicated by a number of factors, notably Ins(1,4,5)P_3 contamination of the Ins(1,3,4,5)P_4 (12, 13), endogenous 3-phosphatase activity in the cells (14, 15), or Ins(1,3,4,5)P_4 protection of Ins(1,4,5)P_3 from 5-phosphatase metabolism (16). In all these studies, the maximal concentrations of Ins(1,3,4,5)P_4 (20–30 μM) used mobilized significantly less intracellular Ca^{2+} than could be achieved using Ins(1,4,5)P_3.

In the present study we have utilized conditions under which 3-phosphatase activity is totally suppressed, i.e., using $InsP_6$, and we have shown that pure synthetic Ins(1,3,4,5)P_4 can mobilize the entire Ins(1,4,5)P_3-sensitive Ca^{2+} pool in saponin-permeabilized SH-SY5Y cells. We previously reported that synthetic DL-Ins(1,3,4,5)P_4 mobilized at best only 62% of the intracellular Ins(1,4,5)P_3-sensitive store in permeabilized SH-SY5Y cells (11, 12), with presumed maximally effective concentrations of 10–30 μM. Because we now know that L-Ins(1,3,4,5)P_4 is a very weak Ins(1,4,5)P_3 receptor ligand and

This work was supported by the Science and Engineering Research Council and the Wellcome Trust. B.V.L.P. is a Lister Institute Fellow.

ABBREVIATIONS: Ins(1,4,5)P_3, *myo*-inositol-1,4,5-trisphosphate; Ins(1,3,4,5)P_4, *myo*-inositol-1,3,4,5-tetrakisphosphate; L-ch-Ins(2,3,5)PS_3, L-*chiro*-inositol-2,3,5-trisphosphorothioate; L-ch-Ins(2,3,5)P_3, L-*chiro*-inositol-2,3,5-trisphosphate; $InsP_6$, *myo*-inositol hexakisphosphate; HEPES, 4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HPLC, high performance liquid chromatography; CLB, "cytosol-like" buffer; L-ch-Ins(1,4,6)PS_3, L-*chiro*-inositol-1,4,6-trisphosphorothioate; Ins(1,3,4)P_3, *myo*-inositol-1,3,4-trisphosphate.

Ca^{2+}-mobilizing agent (17), the effects we had observed with DL-Ins(1,3,4,5)P_4 were probably mediated exclusively by the D-isomer, suggesting that 5–15 μM was the maximal concentration present in our earlier studies. Furthermore, here we have carefully analyzed the Ins(1,3,4,5)P_4-induced Ca^{2+} mobilization response using the antagonist heparin and the partial agonist L-ch-Ins(2,3,5)PS_3 (18) and have provided evidence that Ins(1,3,4,5)P_4 probably interacts with the Ins(1,4,5)P_3 receptor population to release Ca^{2+} from intracellular stores. This hypothesis was further supported by experiments utilizing carbachol-pretreated SH-SY5Y cells, where the number of Ins(1,4,5)P_3 receptors was significantly reduced (19); these experiments clearly revealed a correlated decrease in both Ins(1,3,4,5)P_4- and Ins(1,4,5)P_3-induced Ca^{2+} release.

Experimental Procedures

Materials. The following reagents were used; $^{45}CaCl_2$ (approximately 1000 Ci/mmol; Amersham International, Buckinghamshire, U.K.) and [^{3}H]Ins(1,3,4,5)P_4 (45 Ci/mmol; Amersham International, Buckinghamshire, U.K.). Disodium ATP, fura-2, carbachol, and EGTA were from Sigma; all other reagents were of the highest purity available. L-ch-Ins(2,3,5)P_3 and L-ch-Ins(2,3,5)PS_3 were synthesized as described previously (20–22), were purified by ion exchange chromatography on Q-Sepharose Fast Flow, and were used as their triethylammonium salts.

Chemically synthesized Ins(1,4,5)P_3 (23) and Ins(1,3,4,5)P_4 (24), as K^+ salts, were obtained from the University of Rhode Island Foundation Chemistry Group and used throughout. Both compounds were extensively characterized by ^{31}P and ^1H NMR and were found to be >99% pure, with no other inositol polyphosphates detectable as contaminants.

Cell culture. SH-SY5Y human neuroblastoma cell monolayers (passages 70–90), initially a gift from Dr J. L. Biedler (Sloane-Kettering Institute, New York, NY) were subcultured and maintained as described (25), with the exception that the culture medium was supplemented with 10% (v/v) newborn calf serum (GIBCO) rather than fetal calf serum. For carbachol pretreatment studies, confluent SH-SY5Y cell monolayers were exposed to carbachol (1 mM) for 6–7 hr. Aliquots of a 200 mM aqueous stock solution were added to the culture medium and gently mixed, to obtain the final carbachol concentration of 1 mM.

$^{45}Ca^{2+}$ mobilization assays. SH-SY5Y cell monolayers were harvested in 25 ml of HEPES-buffered saline, consisting of 10 mM HEPES, 15 mM NaCl, and 0.02% (w/v) EDTA, pH 7.2. The cell suspension was centrifuged at 500 × *g* for 2 min, and the resulting pellet was resuspended in a CLB, consisting of 120 mM KCl, 2 mM Na_2·ATP, 2.4 mM $MgCl_2 \cdot 6H_2O$, 2 mM KH_2PO_4, 5 mM sodium succinate, and 20 mM HEPES, pH 7.2. The free Ca^{2+} concentration of the CLB was buffered between 80 and 150 nM by the addition of 1–2 μM EGTA; this was confirmed fluorometrically in 2-ml samples using fura-2 (250 nM), as described (26). The cells were centrifuged (500 × *g*, 1 min) and washed with CLB twice and were then resuspended in CLB containing 100 μg/ml saponin, at a cell protein concentration of 1.5–2 mg/ml. After exactly 1 min the cells were centrifuged (500 × *g*, 1 min) and the pellet was resuspended to 0.5–0.8 mg/ml in CLB containing 1 μCi/ml $^{45}Ca^{2+}$.

Ins(1,3,4,5)P_4-induced $^{45}Ca^{2+}$ mobilization experiments were performed in CLB supplemented with $InsP_6$ (10 μM final concentration), to preclude the possibility of 3-phosphatase activity generating Ins(1,4,5)P_3 in the permeabilized SH-SY5Y cells. Cells were preincubated for 15 min at 25° to allow ATP-dependent loading of intracellular Ca^{2+} stores, and then 50 μl of cell suspension were added to 50 μl of CLB (with or without 10 μM $InsP_6$) containing the inositol polyphosphates or other agents, in 1.5-ml microcentrifuge tubes. After a 1.5-min incubation at 4°, cells were pelleted by centrifugation (16000 × *g*, 2 min), 250 μl of a silicon oil mixture (Dow-Corning 556/550, 1:1, v/v) were added, and the tubes were recentrifuged (16,000 × *g*, 1 min). Buffer and oil were removed by aspiration, and the tubes were allowed to drain for 20 min. The resulting cell pellets were solubilized in 1 ml of Emulsifier Safe scintillation fluid (Canberra Packard) for 6–8 hr at 4°, and the radioactivity was then counted. All experiments were performed in duplicate. Ionomycin (20 μM, free acid; Calbiochem) was used to define the total releasable $^{45}Ca^{2+}$ pool, and Ins(1,4,5)P_3 (20–30 μM) was used to define the Ins(1,4,5)P_3-sensitive $^{45}Ca^{2+}$ pool; these were included as internal standards in all experiments.

Preparation and analysis of HPLC samples. SH-SY5Y cells were prepared exactly as for the $^{45}Ca^{2+}$ release assay, in CLB or CLB supplemented with $InsP_6$ (10 μM); however, $^{45}Ca^{2+}$ was replaced by an identical concentration of Ca^{2+}. After the 15-min loading period, 100 μl of the permeabilized cell suspension were added to 100 μl of CLB (with or without 10 μM $InsP_6$) containing 3 μM Ins(1,3,4,5)P_4 spiked with [^{3}H]Ins(1,3,4,5)P_4 (6.7 nM or 30 nCi/tube), in 1.5-ml microcentrifuge tubes. The cells were allowed to incubate for 2 min, and incubation was terminated by the addition of 7% (w/v) ice-cold perchloric acid. Boiled cells were included as an appropriate control. Preparation of samples and HPLC analysis of the inositol polyphosphates were performed as described previously (27); 5 ml of Flo-Scint IV (Canberra Packard) were added to each sample and samples were vortexed before scintillation counting.

Preparation of cerebellar 'P_2' membrane fraction. Pig cerebella were obtained from a local abattoir. Portions of cerebellum were either used immediately or frozen in liquid nitrogen and stored at −70°. Cerebellum was chopped with scissors at 4° and homogenized in 20 volumes of 20 mM $NaHCO_3$, 1 mM dithiothreitol, pH 8.0 (Polytron, setting 5, 2 × 15 sec). The homogenate was centrifuged (4000 × *g*, 10 min, 4°). The resulting supernatant was recovered and centrifuged (40,000 ×g 20 min, 4°). The pellet was rehomogenized and the high-speed centrifugation step was repeated twice. The final pellet was resuspended in homogenization buffer at 6–8 mg of protein/ml, snap-frozen in liquid nitrogen, and stored at −20° until required.

Characterization of Ins(1,3,4,5)P_4 binding sites. Increasing concentrations of the various inositol phosphate analogues investigated were incubated in a total assay volume of 120 μl with 0.6–0.9 nM [^{3}H]Ins(1,3,4,5)P_4 (45 Ci/mmol) in a buffer containing 25 mM sodium acetate, 25 mM KH_2PO_4, 5 mM $NaHCO_3$, 1 mM EDTA, and 0.25 mM dithiothreitol, pH 5.0. Incubations were initiated by addition of 175–200 μg of cerebellar membrane protein and were continued for 30 min at 4°. Bound and free ligand were separated by centrifugation at 12,000 × *g*. After a brief centrifugation (20 sec), 100 μl of silicon oil mixture (Dow-Corning 556/550, 1:1, v/v) were added and the centrifugation was continued for an additional 1.5 min. Samples were immediately returned to an ice-bath and the supernatant and oil layers were removed by aspiration. Pellets were solubilized by addition of 2% (w/v) sodium dodecyl sulfate. Radioactivity was determined after addition of an appropriate scintillation cocktail. Residual bound radioactivity levels in the presence of 100 μM Ins(1,3,4,5)P_4, 1 mM $InsP_6$, or 3 mM 2,3-bisphosphoglycerate were similar (15–25% of total binding) and were defined as nonspecific binding.

Data analysis. EC_{50} and IC_{50} values (concentrations producing half-maximal stimulation and inhibition, respectively) and slope factors were estimated by computer-assisted curve-fitting using GraphPad INPLOT version 3.1 (GraphPad Software). Combined data from the independent experiments (three or more experiments) were expressed as mean ± standard error.

Results

HPLC studies. HPLC of the boiled cell preparations revealed a small quantitiy of contaminating Ins(1,3,4)P_3 (1.4%) and Ins(1,4,5)P_3 (1.2%) present in the [^{3}H]Ins(1,3,4,5)P_4 (three experiments). In control preparations from saponin-permeabilized SH-SY5Y cells allowed to incubate for 2 min with 6.7 nM [^{3}H]Ins(1,3,4,5)P_4 and 3 μM Ins(1,3,4,5)P_4, only 1.7% was metabolized to [^{3}H]Ins(1,4,5)P_3 and 6.8% to [^{3}H]Ins(1,3,4)P_3

(three experiments). The addition of $InsP_6$ (10 μM) completely obliterated Ins(1,3,4,5)P_4-3-phosphatase-catalyzed generation of [^{3}H]Ins(1,4,5)P_3 (1.1%, two experiments) and also inhibited [^{3}H]Ins(1,3,4)P_3 generation (2.2%, two experiments). No inositol bisphosphate peaks were detected above the background counts; the complete details of these findings will be reported elsewhere.

Thus, under the conditions used in the $^{45}Ca^{2+}$ release assay, approximately 15 nM endogenous Ins(1,4,5)P_3 would be produced via 3-phosphatase activity from exogenous Ins(1,3,4,5)P_4 (3 μM) in the permeabilized SH-SY5Y cells. An identical concentration of exogenous Ins(1,4,5)P_3 would mobilize approximately 10–15% of the Ins(1,4,5)P_3-sensitive Ca^{2+} store in these cells (see Fig. 2) and thus produce an artifactual leftward shift in the EC_{50} value for Ins(1,3,4,5)P_4-induced Ca^{2+} release.

Ca^{2+} mobilization by inositol polyphosphates. Ins(1,4,5)P_3 mobilized ≈70% of preloaded $^{45}Ca^{2+}$ from saponin-permeabilized SH-SY5Y cells at 20–22°, with an EC_{50} of 52.1 ± 2.3 nM. The addition of 10 μM $InsP_6$ did not significantly shift the EC_{50} of the Ins(1,4,5)P_3 concentration-response curve, nor did $InsP_6$ cause significant $^{45}Ca^{2+}$ mobilization at concentrations up to 100 μM (Fig. 1; Table 1).

Although Ins(1,3,4,5)P_4 was able to mobilize the entire Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} store of saponin-permeabilized SH-SY5Y cells with an EC_{50} of 879 ± 92 nM, the EC_{50} value was significantly increased in the presence of $InsP_6$ (2.54 ± 0.30 μM) (Fig. 1; Table 1). We hypothesized that the decreased potency of exogenous Ins(1,3,4,5)P_4 in the presence of $InsP_6$ was due to inhibition of 3-phosphatase-catalyzed generation of Ins(1,4,5)P_3. This proposal was supported by our

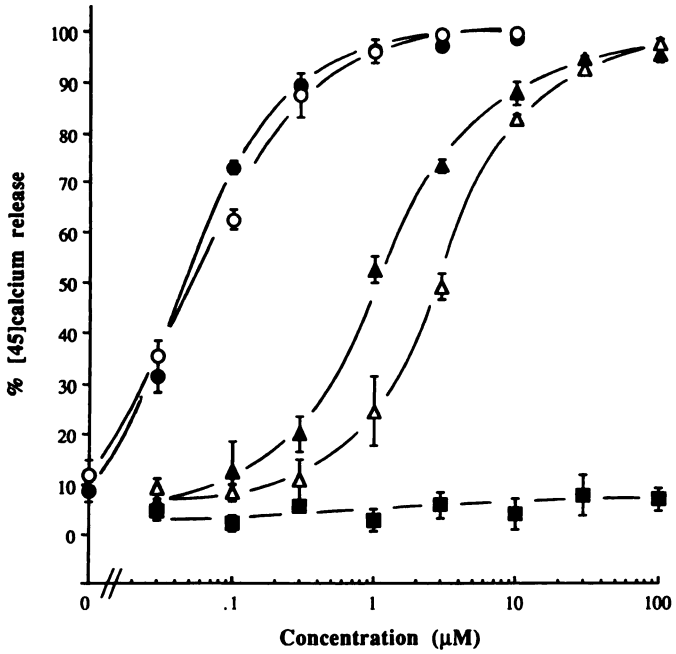

Fig. 1. Effect of $InsP_6$ on Ins(1,4,5)P_3- and Ins(1,3,4,5)P_4-induced Ca^{2+} release. Data indicate the percentage of $^{45}Ca^{2+}$ released at 20–22° from the intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores of saponin-permeabilized SH-SY5Y cells, in the presence of increasing concentrations of Ins(1,4,5)P_3 (●), Ins(1,4,5)P_3 plus $InsP_6$ (10 μM) (○), Ins(1,3,4,5)P_4 (▲), Ins(1,3,4,5)P_4 plus $InsP_6$ (10 μM) (△), or $InsP_6$ (■). Results are shown as mean ± standard error of four independent experiments. Maximal Ca^{2+} release from intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores was defined using an internal standard of 20 μM Ins(1,4,5)P_3 (100% release); see Experimental Procedures for a complete description of the method.

TABLE 1

Inositol polyphosphate-induced $^{45}Ca^{2+}$ mobilization in saponin-permeabilized SH-SY5Y cells at 20–22°C

Results are shown as mean ± standard error of four or more experiments for EC_{50} values, with slope values where appropriate. See Experimental Procedures for additional details.

Inositol polyphosphate	EC_{50}	Slope factor (n_H)
	nM	
Ins(1,4,5)P_3	52.1 ± 2.3	1.39 ± 0.11
Ins(1,3,4,5)P_4	879.2 ± 92.3	1.03 ± 0.09
Ins(1,4,5)P_3 + $InsP_6$ (10 μM)	58.0 ± 6.7	1.10 ± 0.06
Ins(1,3,4,5)P_4 + $InsP_6$ (10 μM)	2,536 ± 303[a]	1.15 ± 0.17
$InsP_6$	$\gg 10^5$	
L-ch-Ins(2,3,5)PS_3	1,595 ± 574	1.37 ± 0.29
Ins(1,4,5)P_3 + L-ch-Ins(2,3,5)PS_3 (30 μM)	854.6 ± 52.7[b]	[c]
Ins(1,3,4,5)P_4 + $InsP_6$ (10 μM) + L-ch-Ins(2,3,5)PS_3 (30 μM)	30,772 ± 6,031[a]	[c]
Ins(1,4,5)P_3 + heparin (50 μg/ml)	345 ± 61[b]	1.48 ± 0.06
Ins(1,3,4,5)P_4 + heparin (50 μg/ml)	11,630 ± 1,040[a]	1.266 ± 0.06

[a] Statistically significant difference from the Ins(1,3,4,5)P_4 control ($p < 0.05$).
[b] Statistically significant difference from the Ins(1,4,5)P_3 control ($p < 0.05$).
[c] Incomplete curves; only EC_{50} values are indicated.

recently reported Ca^{2+} mobilization study conducted at 4°, where in the absence of metabolic activity Ins(1,3,4,5)P_4 was a full agonist and had a similar EC_{50} (2.1 μM) in permeabilized SH-SY5Y cells (17). Further, in the present study direct evidence to support this hypothesis comes from our HPLC studies, which detected a small but significant generation of Ins(1,4,5)P_3 from Ins(1,3,4,5)P_4; this 3-phosphatase activity was obliterated by prior addition of 10 μM $InsP_6$ (see above). Consequently, all subsequent Ins(1,3,4,5)P_4-induced Ca^{2+} mobilization experiments were conducted with $InsP_6$ (10 μM) supplementation.

Heparin (50 μg/ml, M_r 4000–6000) addition to saponized SH-SY5Y cells at 20–22° produced similar rightward shifts in both the Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 concentration-response curves, with respective EC_{50} values shifted to 11.6 μM and 0.35 μM (Fig. 2; Table 1).

We have recently identified an Ins(1,4,5)P_3 receptor partial agonist, L-ch-Ins(2,3,5)PS_3, that at a maximally effective concentration mobilizes only about 30% of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool in SH-SY5Y cells (18). Consequently we used L-ch-Ins(2,3,5)PS_3 at a maximal concentration (30 μM) to probe the interaction of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 at the Ca^{2+}-mobilizing Ins(1,4,5)P_3 receptor in SH-SY5Y cells. L-ch-Ins(2,3,5)PS_3 (30 μM) significantly shifted rightward both the Ins(1,4,5)P_3 (EC_{50}, 52.1 to 855 nM) and Ins(1,3,4,5)P_4 (EC_{50}, 2.5 to 30.8 μM) concentration-response curves, in a manner consistent with its partial agonist action at the Ins(1,4,5)P_3 receptor (Fig. 3). Because L-ch-Ins(2,3,5)PS_3 is a moderate 5-phosphatase inhibitor, control experiments were conducted with a similar compound, L-ch-Ins(1,4,6)PS_3, which is a very potent 5-phosphatase inhibitor (28). L-ch-Ins(1,4,6)PS_3 (≤100 μM) had no intrinsic Ca^{2+} mobilization efficacy, and at 30 μM it failed to shift the Ins(1,4,5)P_3 concentration-response curve significantly.[1] Additionally, increasing concentrations of L-ch-Ins(2,3,5)PS_3 (0.1–30 μM) were able to progressively inhibit the Ca^{2+} mobilization response to a fixed submaximal concentration of Ins(1,3,4,5)P_4 (5 μM),[2] producing an inhibition profile

[1] R. A. Wilcox, unpublished observations.
[2] R. A. Wilcox, unpublished observations.

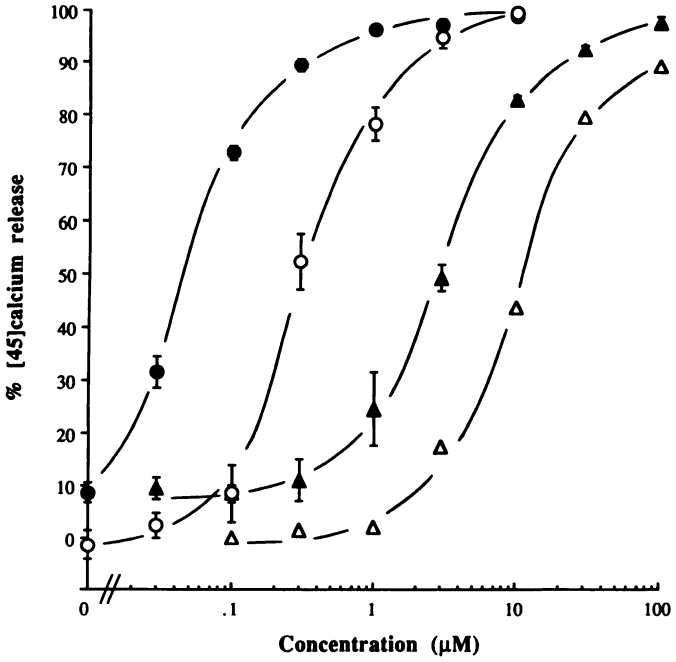

Fig. 2. Effect of heparin on Ins(1,4,5)P_3- and Ins(1,3,4,5)P_4-induced $^{45}Ca^{2+}$ release. Experimental data are as described in Fig. 1, in the presence of increasing concentrations of Ins(1,4,5)P_3 (●), Ins(1,4,5)P_3 plus heparin (50 μg/ml) (○), Ins(1,3,4,5)P_4 plus InsP_6 (10 μM) (▲), or Ins(1,3,4,5)P_4 plus InsP_6 (10 μM) plus heparin (50 μg/ml) (△). Results are shown as mean ± standard error of four independent experiments. Maximal Ca^{2+} release from the intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores was defined using an internal standard of 20 μM Ins(1,4,5)P_3 (100% release); see Experimental Procedures for a complete description of the method.

similar to that we previously reported for L-ch-Ins(2,3,5)PS_3 inhibition of submaximal Ins(1,4,5)P_3 (0.7 μM) (18).

Ins(1,3,4,5)P_4 binding studies. Displacement of specific [^{3}H]Ins(1,3,4,5)P_4 binding by Ins(1,3,4,5)P_4 yielded curves with slope factors significantly less than unity (typically 0.4–0.55), which could be accurately modeled using two-site analysis. Such analysis for the data shown in Fig. 4 yielded K_d values for high and low affinity sites of 2.5 nM and 7.8 μM, respectively; at the concentration of [^{3}H]Ins(1,3,4,5)P_4 used (0.66 nM), the high affinity site accounted for 63% of specific binding.

Comparison of the ability of InsP_6, Ins(1,4,5)P_3, and low molecular weight heparin (M_r 4000–6000) to displace specific [^{3}H]Ins(1,3,4,5)P_4 binding from pig cerebellar membranes was entirely consistent with values reported previously using rat cerebellar membranes and [^{32}P]Ins(1,3,4,5)P_4 as the radioligand (29). As noted previously, the displacement isotherms for these agents yielded slope factors close to unity, suggesting that they did not discriminate between the two populations of binding sites defined by Ins(1,3,4,5)P_4 displacement of specific [^{3}H]Ins(1,3,4,5)P_4 binding.

Both L-ch-Ins(2,3,5)PS_3 and L-ch-Ins(2,3,5)P_3 only weakly displaced [^{3}H]Ins(1,3,4,5)P_4 binding (Fig. 4; Table 2), with respective IC_{50} values of >30 μM and 29.1 ± 3.1 μM. The data presented for L-ch-Ins(2,3,5)PS_3 were obtained using freshly prepared solutions or dilutions made from stock (2 mM) solutions stored at −20° for <14 days. We noted that aqueous solutions of L-ch-Ins(2,3,5)PS_3 stored for >21 days exhibited progressively reduced Ca^{2+} mobilization effects and increased apparent ability to displace [^{3}H]Ins(1,3,4,5)P_4 from specific binding sites. Because these changes were not observed for the

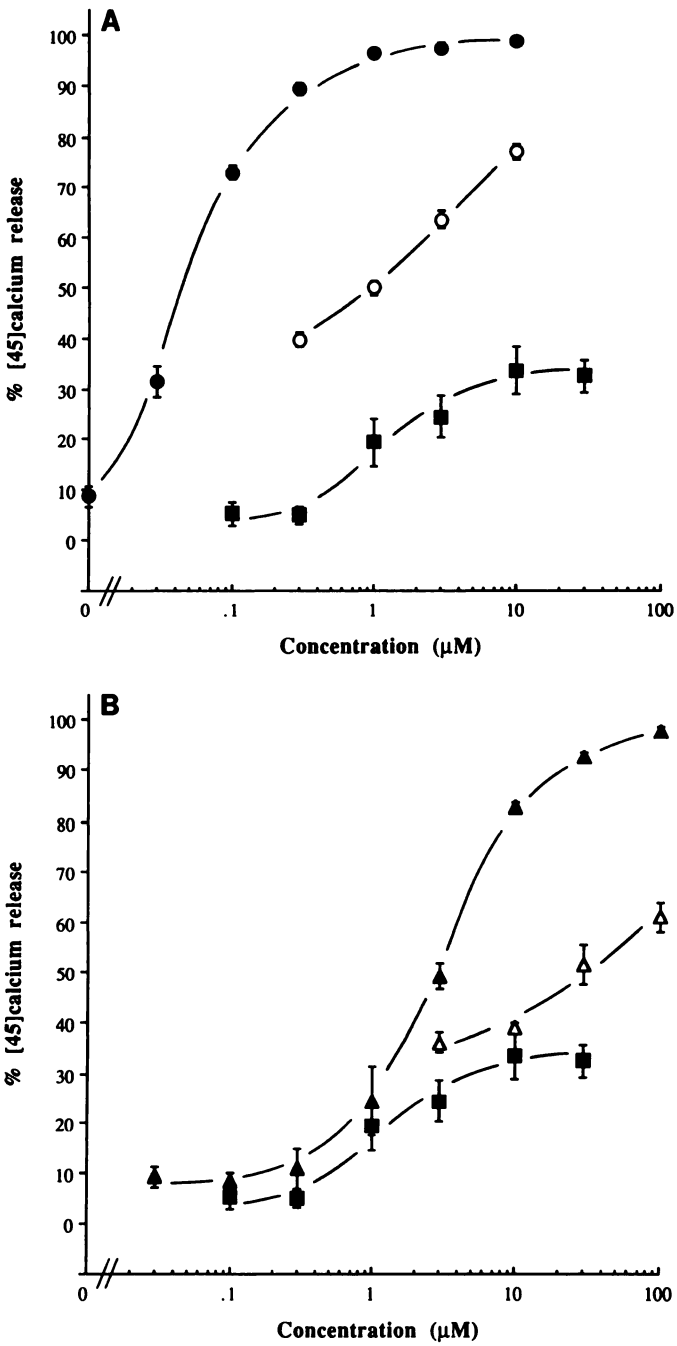

Fig. 3. A, Effect of L-ch-Ins(2,3,5)PS_3 on Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release. Experimental data are as described in Fig. 1, in the presence of increasing concentrations of Ins(1,4,5)P_3 (●), L-ch-Ins(2,3,5)PS_3 (■), or Ins(1,4,5)P_3 plus L-ch-Ins(2,3,5)PS_3 (30 μM) (○). Results are shown as mean ± standard error of four or more independent experiments. Maximal Ca^{2+} release from the intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores was defined using an internal standard of 20 μM Ins(1,4,5)P_3 (100% release); see Experimental Procedures for a complete description of the method. B, Effect of L-ch-Ins(2,3,5)PS_3 on Ins(1,3,4,5)P_4-induced $^{45}Ca^{2+}$ release. Experimental data are as described in Fig. 1, in the presence of increasing concentrations of Ins(1,3,4,5)P_4 plus InsP_6 (10 μM) (▲), L-ch-Ins(2,3,5)PS_3 (■), or Ins(1,3,4,5)P_4 plus InsP_6 (10 μM) plus L-ch-Ins(2,3,5)PS_3 (30 μM) (△). Results are shown as mean ± standard error for three or more independent experiments. Maximal Ca^{2+} release from the intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores was defined using an internal standard of 20 μM Ins(1,4,5)P_3 (100% release); see Experimental Procedures for a complete description of the method.

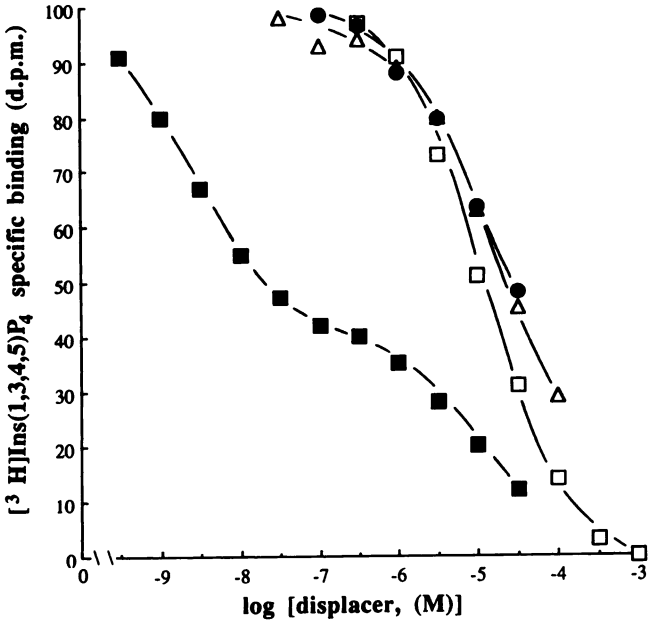

Fig. 4. Displacement of specific [^{3}H]Ins(1,3,4,5)P_4 binding from pig cerebellar membranes. Total [^{3}H]Ins(1,3,4,5)P_4 added was 66 nM (7886 dpm); total bound counts and nonspecific binding were 2345 ± 120 dpm and 582 ± 42 dpm, respectively. Data were normalized to the maximal specific binding value. [^{3}H]Ins(1,3,4,5)P_4 was displaced by Ins(1,3,4,5)P_4 (■) in a manner consistent with a two-site curve fit, resulting in high and low affinity sites of 2.5 nM (K_H) and 7.8 μM (K_L). Inositol trisphosphate analogues shown include Ins(1,4,5)P_3 (△), L-ch-Ins(2,3,5)PS_3 (●), and InsP_6 (□). Results are shown as mean ± standard error of three independent experiments. IC_{50} values for the other inositol polyphosphates and heparin are presented in Table 2; see Experimental Procedures for additional details.

TABLE 2

IC_{50} estimates for binding affinity obtained by displacement of specific [^{3}H]Ins(1,3,4,5)P_4 binding in pig cerebellum

Results are shown as mean ± standard error of three or more experiments (n) for IC_{50} values, with slope values (n_H) where appropriate. See Experimental Procedures for additional details.

Inositol polyphosphate	IC_{50}	Slope factor (n_H)	n
	μM		
Ins(1,4,5)P_3	15.1 ± 2.9	0.87 ± 0.08	3
InsP_6	11.3 ± 1.9	0.92 ± 0.04	3
L-ch-Ins(2,3,5)P_3	>30[a]		3
L-ch-Ins(2,3,5)PS_3	29.1 ± 6.0	0.88 ± 0.06	4
Heparin (M_T 4000–6000)	12.0 ± 3.1[b]	1.23 ± 0.11	3

[a] 45.9 ± 7.0% displacement at 30 μM.
[b] IC_{50} expressed in μg/ml.

structurally similar L-ch-Ins(2,3,5)P_3, these changes are probably due to slow decomposition of L-ch-Ins(2,3,5)PS_3, and we therefore suggest that only fresh solutions of this analogue should be used.

Ins(1,3,4,5)P_4-induced Ca^{2+} release in carbachol-pretreated SH-SY5Y cells. Here we utilized carbachol (1 mM, 6–7 hr)-pretreated SH-SY5Y cells to assess whether Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 exhibited similar EC_{50} shifts and maximal $^{45}Ca^{2+}$ release profiles. The maximal Ins(1,4,5)P_3 (30 μM) release response was reduced from 70.1 ± 1.2% (eight experiments) to 40.6 ± 1.9% (eight experiments) of preloaded $^{45}Ca^{2+}$, whereas the EC_{50} value was shifted about 4-fold rightward (Fig. 5), broadly confirming previous findings (19). Further, Ins(1,3,4,5)P_4-induced Ca^{2+} release curves exhibited almost identical modifications after carbachol pretreatment. The

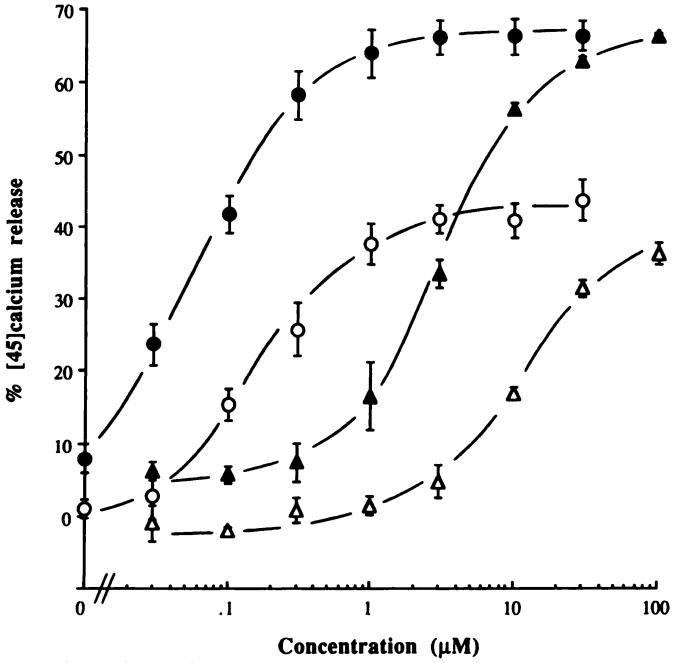

Fig. 5. Effect of carbachol pretreatment on Ins(1,4,5)P_3- and Ins(1,3,4,5)P_4-induced $^{45}Ca^{2+}$ release. The data indicate the percentage of $^{45}Ca^{2+}$ released at 20–22° from the intracellular Ins(1,4,5)P_3-sensitive Ca^{2+} stores of saponin-permeabilized control SH-SY5Y cells (●, ▲) or SH-SY5Y cells pretreated with carbachol (1 mM) for 6–7 hr (○, △), in the presence of increasing concentrations of Ins(1,4,5)P_3 (●, ○) and Ins(1,3,4,5)P_4 plus InsP_6 (10 μM) (▲, △). Results are shown as mean ± standard error of four independent experiments. Carbachol pretreatment reduced the maximal Ins(1,4,5)P_3 (30 μM) release response from 70.1 ± 1.2% (eight experiments) to 40.6 ± 1.9% (eight experiments) of preloaded $^{45}Ca^{2+}$; the mean EC_{50} and slope factor values were 233.9 ± 65.9 nM and 1.19 ± 0.18, respectively. The maximal Ins(1,3,4,5)P_4 (100 μM) release response was reduced to 36.6 ± 1.4% (four experiments) of preloaded $^{45}Ca^{2+}$, which was not significantly different from that released by the maximally effective concentration of Ins(1,4,5)P_3 (30 μM) included as an internal control (37.5 ± 1.1%, four experiments). The mean EC_{50} and slope factor values were 10,972 ± 1,160 nM and 1.62 ± 0.23, respectively. Control EC_{50} values and slope factors are shown in Table 1; see Experimental Procedures for a complete description of the method.

maximal Ins(1,3,4,5)P_4 (100 μM) release response was reduced to 36.6 ± 1.4% (four experiments) of preloaded $^{45}Ca^{2+}$, which was not significantly different from the release induced by the maximally effective concentrations of Ins(1,4,5)P_3 (30 μM) used as internal controls (37.5 ± 1.1%, four experiments); the EC_{50} value for Ins(1,3,4,5)P_4-induced Ca^{2+} release was also increased about 4-fold (Fig. 5).

Discussion

The metabolism of Ins(1,3,4,5)P_4 to Ins(1,3,4)P_3 by Ins(1,4,5)P_3/Ins(1,3,4,5)P_4-5-phosphatase isoenzymes is well characterized, but recently Ins(1,3,4,5)P_4-3-phosphatase-catalyzed metabolism of Ins(1,3,4,5)P_4 to Ins(1,4,5)P_3 has been detected in several cells and tissues, including saponin-permeabilized SH-SY5Y cells (3, 11). Because Ins(1,4,5)P_3 potently mobilizes intracellular Ca^{2+} stores in permeabilized SH-SY5Y cells, 3-phosphatase-catalyzed generation of contaminating Ins(1,4,5)P_3 from exogenous Ins(1,3,4,5)P_4 results in a shift to the left of the Ins(1,3,4,5)P_4 concentration-response curve. To inhibit Ins(1,3,4,5)P_4 metabolism we have previously conducted Ins(1,3,4,5)P_4-induced Ca^{2+} mobilization experiments at 4°

(12). Here we used $InsP_6$ as an inhibitor of Ins(1,3,4,5)P_4-3-phosphatase activity (3), to allow all subsequent Ins(1,3,4,5)P_4-induced Ca^{2+} mobilization experiments to be conducted at room temperature without the artifactual complication of steady state Ins(1,4,5)P_3 generation.

In recent studies we have reported Ins(1,4,5)P_3 receptor binding studies in cerebellum using the D- and L-isomers of Ins(1,3,4,5)P_4 and have detected a marked (>30-fold) stereoselectivity favoring the D-isomer (17). Surprisingly, however, specific Ins(1,3,4,5)P_4 binding sites in cerebellum exhibited a limited stereospecificity, with L-Ins(1,3,4,5)P_4 possessing only about 10-fold lower affinity than the D-isomer (29, 30). Further, we demonstrated that maximally effective concentrations of Ins(1,3,4,5)P_4 (30–100 μM) mobilized the entire Ins(1,4,5)P_3-sensitive Ca^{2+} store in saponin-permeabilized SH-SY5Y cells, with an EC_{50} of 2.1 μM at 4° (17). L-Ins(1,3,4,5)P_4 *per se* was unable to mobilize Ca^{2+} and, in coincubation studies, did not affect Ins(1,3,4,5)P_4-induced Ca^{2+} release, thus paralleling the activities detected in Ins(1,4,5)P_3 but not Ins(1,3,4,5)P_4 binding studies (17). These observations led us to suspect that Ins(1,3,4,5)P_4-induced Ca^{2+} mobilization might be mediated via the Ins(1,4,5)P_3 receptor. The present studies provide strong evidence for this hypothesis. Ins(1,3,4,5)P_4 mobilized the entire Ins(1,4,5)P_3-sensitive Ca^{2+} store in SH-SY5Y cells in a concentration-dependent manner, with an EC_{50} of 2.5 μM, being about 50-fold weaker than Ins(1,4,5)P_3. Furthermore, heparin, a potent and competitive agonist at the Ins(1,4,5)P_3 receptor (31), produced a similar rightward shift of both the Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 concentration-response curves. However, heparin potently inhibits both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 binding to specific binding sites, with respective IC_{50} values in rat cerebellum of 10.2 and 19.3 μg/ml (29). Consequently, we recognize the possibility that heparin could independently inhibit Ca^{2+} mobilization via both the Ins(1,4,5)P_3 receptor and a putative Ins(1,3,4,5)P_4-specific receptor, with similar affinities. To further investigate our hypothesis, we therefore used the Ins(1,4,5)P_3 receptor partial agonist L-ch-Ins(2,3,5)PS_3 (18).

L-ch-Ins(2,3,5)PS_3 produced similar rightward shifts of both the Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 concentration-response curves. Radioligand binding studies have shown that L-ch-Ins(2,3,5)PS_3, L-ch-Ins(2,3,5)P_3, and Ins(1,4,5)P_3 all interact very weakly with Ins(1,3,4,5)P_4 binding sites from pig cerebellum, whereas at bovine adrenal cortex Ins(1,4,5)P_3 binding sites L-ch-Ins(2,3,5)P_3 and L-ch-Ins(2,3,5)PS_3 possess relatively high affinity (18, 22). Consequently, the shift in Ins(1,3,4,5)P_4 [and Ins(1,4,5)P_3]-induced Ca^{2+} mobilization concentration-response curves produced by L-ch-Ins(2,3,5)PS_3 would appear to be derived solely from competition at a population of Ins(1,4,5)P_3 receptors. These experiments appear to provide almost unequivocal evidence that Ins(1,3,4,5)P_4 acts specifically at Ins(1,4,5)P_3 receptors to induce Ca^{2+} release from the intracellular Ca^{2+} stores of saponized SH-SY5Y cells.

Additional evidence that Ins(1,3,4,5)P_4-induced Ca^{2+} release was mediated via the Ins(1,4,5)P_3 receptor population comes from the Ca^{2+} mobilization responses of carbachol-pretreated cells. A recent report from this laboratory has demonstrated that pretreatment of SH-SY5Y cells with carbachol for ≥6 hr produces an approximate 50% reduction in the number of Ins(1,4,5)P_3 binding sites, while leaving the receptor affinity relatively unaffected (19). This loss in sites has been confirmed by immunoblot studies using a monoclonal antibody directed against the carboxyl terminus of the Ins(1,4,5)P_3 receptor (32). Further, this down-regulation was accompanied by an approximately 50% reduction of the maximal Ins(1,4,5)P_3-induced Ca^{2+} release and a 3–4-fold rightward shift in the EC_{50} value (19). Here we have shown that pretreatment of SH-SY5Y cells with carbachol (1 mM, 6–7 hr) produced parallel effects on both Ins(1,4,5)P_3- and Ins(1,3,4,5)P_4-induced Ca^{2+} mobilization, again suggesting interaction of the tetrakisphosphate with the Ins(1,4,5)P_3 receptor population. The possibility that carbachol pretreatment could result in parallel down-regulation of both the Ins(1,4,5)P_3-specific population and an independent putative Ins(1,3,4,5)P_4-specific receptor population of course remains. However, in the face of all the other accumulated evidence on stereospecificity and antagonist and partial agonist inhibition characteristics of the responses, we believe this possibility becomes increasingly remote.

Given that Ins(1,3,4,5)P_4 possesses all the critical structural motifs required for effective interaction with the Ins(1,4,5)P_3 receptor (33, 34), perhaps the proposal that the addition of a 3-phosphate group to the Ins(1,4,5)P_3 molecule simply produces a weaker ligand and agonist operating via the same receptor should meet only limited resistance. Indeed, because Ins(1,3,4,5)P_4 is a specific and relatively potent ligand at Ins(1,4,5)P_3 recognition sites (17, 29), if Ins(1,3,4,5)P_4 completely failed to mobilize intracellular Ca^{2+} stores then we should expect it to act as an Ins(1,4,5)P_3 receptor antagonist. This role was tentatively suggested when 20 μM Ins(1,3,4,5)P_4 failed to produce detectable Ca^{2+} fluxes in reconstituted vesicles containing purified type I Ins(1,4,5)P_3 receptors (35). However, this conflicts with the observation that Ins(1,3,4,5)P_4 can effectively mobilize intracellular Ca^{2+} (with an EC_{50} of 10 μM) from membrane fractions prepared from transfected fibroblasts overexpressing type I Ins(1,4,5)P_3 receptors (36).

Two possibilities remain regarding how Ins(1,3,4,5)P_4-induced Ca^{2+} mobilization might occur, i.e., (a) Ins(1,3,4,5)P_4 interacts directly with the Ins(1,4,5)P_3 receptor to mobilize intracellular sequestered Ca^{2+} or (b) Ins(1,3,4,5)P_4 enhances the ability of suboptimal levels of Ins(1,4,5)P_3 to evoke Ca^{2+} mobilization, just as subthreshold concentrations of Ins(1,4,5)P_3 potentiate subsequent Ins(1,4,5)P_3 transients in *Xenopus* oocytes (37, 38). The latter seems unlikely as an exclusive and direct mechanism, because Ins(1,3,4,5)P_4 has been shown to induce Ca^{2+} mobilization from microsomal preparations, which are presumably free of cytosolic Ins(1,4,5)P_3 (8, 9). However, Ins(1,3,4,5)P_4 might indirectly potentiate Ins(1,4,5)P_3-induced Ca^{2+} release by mobilizing intracellular Ca^{2+} stores to produce a modest increase over the basal cytosolic Ca^{2+} concentration. The relationship between Ins(1,4,5)P_3 receptor sensitivity and cytosolic Ca^{2+} concentration is "bell-shaped" (39), with modest increases in Ca^{2+} concentration (up to 200–300 nM) sensitizing the receptor to Ins(1,4,5)P_3 (39–41). Low concentrations of Ins(1,3,4,5)P_4 have been reported to lower the threshold concentration for Ins(1,4,5)P_3-induced Ca^{2+} mobilization, in both microinjected *Xenopus* oocytes (10) and digitonin-permeabilized L1210 lymphoma cells (42). In *Xenopus* oocytes higher concentrations of Ins(1,3,4,5)P_4 evoked independent Ca^{2+} mobilization and inhibition of Ins(1,4,5)P_3 responses, with these responses being independent of extracellular Ca^{2+} (10) and thus unrelated to the phenomenon of Ins(1,3,4,5)P_4-induced Ca^{2+} entry (43). The proposal that

$Ins(1,3,4,5)P_4$ potentiates $Ins(1,4,5)P_3$ responses via a Ca^{2+}-dependent receptor sensitization may be valid if, after transient stimulation of cell surface receptors, $Ins(1,3,4,5)P_4$ levels are sustained longer than are $Ins(1,4,5)P_3$ levels. $Ins(1,4,5)P_3$/$Ins(1,3,4,5)P_4$ mass assays have indicated parallel increases of both inositol polyphosphates after agonist stimulation (44–48), and in some cell types $Ins(1,3,4,5)P_4$ concentrations exhibit a slower decay than do $Ins(1,4,5)P_3$ concentrations (49).

The obvious question arises of whether the high affinity $Ins(1,3,4,5)P_4$-specific binding site in pig cerebellum represents an $Ins(1,3,4,5)P_4$ receptor with a biological function. Theibert *et al.* have utilized an $Ins(1,3,4,5)P_4$ affinity column to isolate several $Ins(1,3,4,5)P_4$-binding proteins solubilized from crude particulate fractions prepared from rat brain membranes, although only two exhibited stringent specificity for $Ins(1,3,4,5)P_4$ (50). Irvine (51) has suggested that a putative $Ins(1,3,4,5)P_4$ receptor-operated Ca^{2+} channel has a role in Ca^{2+} entry across the plasma membrane, and indeed an $Ins(1,3,4,5)P_4$-activated Ca^{2+} channel has been identified in endothelial cells (52). However, $Ins(1,3,4,5)P_4$ has also been observed to specifically inactivate a K^+ channel via an inositol polyphosphate receptor isolated from bovine cerebellar microsomes (53). Thus, $Ins(1,3,4,5)P_4$ could potentially produce a number of biological responses via a variety of putative $Ins(1,3,4,5)P_4$ receptors. Here we have provided evidence that $Ins(1,3,4,5)P_4$ is a moderately potent full agonist for Ca^{2+} mobilization, functioning via the $Ins(1,4,5)P_3$ receptor-operated Ca^{2+} channel, in SH-SY5Y cells. Because similar $Ins(1,3,4,5)P_4$-induced Ca^{2+} mobilization responses have been observed in Swiss 3T3 fibroblasts and 1321N1 astrocytoma cells,[3] perhaps this suggests that the $Ins(1,4,5)P_3$ receptor may represent a potential physiological target for $Ins(1,3,4,5)P_4$. Current investigations with novel 3-position-modified $Ins(1,4,5)P_3$/$Ins(1,3,4,5)P_4$ analogues may allow us to probe inositol polyphosphate receptors and to further investigate alternative biological actions of $Ins(1,3,4,5)P_4$.

References

1. Berridge, M. J. Inositol trisphosphate and calcium signalling. *Nature (Lond.)* **361**:315–325 (1993).
2. Taylor, C. W., and A. Richardson. Structure and function of inositol trisphosphate receptors. *Pharmacol. Ther.* **51**:79–104 (1991).
3. Shears, S. B. Metabolism of inositol phosphates. *Adv. Second Messenger Res.* **26**:63–92 (1992).
4. Irvine, R. F., A. J. Letcher, D. J. Lander, and M. J. Berridge. Specificity of inositol phosphate-stimulated Ca^{2+} mobilization from Swiss-mouse 3T3 cells. *Biochem. J.* **240**:301–304 (1986).
5. Ehrlich, B. E., and J. Watras. Inositol 1,4,5-trisphosphate activates a channel from smooth muscle sarcoplasmic reticulum. *Nature (Lond.)* **336**:583–586 (1988).
6. Taylor, C. W., M. J. Berridge, A. M. Cooke, and B. V. L. Potter. Inositol 1,4,5-trisphosphorothioate, a stable analogue of inositol trisphosphate which mobilizes intracellular calcium. *Biochem. J.* **259**:645–650 (1989).
7. Changya, L., D. V. Gallacher, R. F. Irvine, B. V. L. Potter, and O. H. Petersen. Inositol 1,3,4,5-tetrakisphosphate is essential for sustained activation of the Ca^{2+}-dependent K^+ current in single internally perfused mouse lacrimal acinar cells. *J. Membr. Biol.* **109**:85–93 (1989).
8. Joseph, S. K., C. A. Hansen, and J. R. Williamson. Inositol tetrakisphosphate mobilizes calcium from cerebellum microsomes. *Mol. Pharmacol.* **36**:391–397 (1989).
9. Ely, J. A., L. Hunyady, A. J. Baukal, and K. J. Catt. Inositol 1,3,4,5-tetrakisphosphate stimulates Ca^{2+} release from bovine adrenal microsomes by a mechanism independent of the inositol 1,4,5-trisphosphate receptor. *Biochem. J.* **268**:333–338 (1990).
10. Parker, I., and I. Ivorra. Inositol tetrakisphosphate liberates stored Ca^{2+} in *Xenopus* oocytes and facilitates responses to inositol trisphosphate. *J. Physiol. (Lond.)* **433**:207–227 (1991).
11. Gawler, D. J., B. V. L. Potter, and S. R. Nahorski. Inositol 1,3,4,5-tetrakisphosphate-induced release of intracellular Ca^{2+} in SH-SY5Y neuroblastoma cells. *Biochem. J.* **272**:519–524 (1990).
12. Gawler, D. J., B. V. L. Potter, R. Gigg, and S. R. Nahorski. Interactions between inositol tris- and tetrakis-phosphates: effects on intracellular Ca^{2+} mobilization in SH-SY5Y cells. *Biochem. J.* **276**:163–167 (1991).
13. Bird, G. S. J., M. F. Rossier, A. R. Hughes, S. B. Shears, D. L. Armstrong, and J. W. Putney. Activation of Ca^{2+} entry into acinar cells by a non-phosphorylatable inositol trisphosphate. *Nature (Lond.)* **352**:162–165 (1991).
14. Cullen, P. J., R. F. Irvine, B. K. Drobak, and A. P. Dawson. Inositol 1,3,4,5-tetrakisphosphate causes release of Ca^{2+} from permeabilized mouse lymphoma L1210 cells by its conversion into 1,4,5-trisphosphate. *Biochem. J.* **259**:931–933 (1989).
15. Loomis-Husselbee, J. W., P. J. Cullen, R. F. Irvine, and A. P. Dawson. Electroporation can cause artifacts due to solubilization of cations from electrode plates: aluminium ions enchance conversion of inositol 1,3,4,5-tetrakisphosphate into inositol 1,4,5-trisphosphate in electroporated L1210 cells. *Biochem. J.* **277**:883–885 (1991).
16. Joseph, S. K., C. A. Hansen, and J. R. Williamson. Inositol 1,3,4,5-tetrakisphosphate increases the duration of the inositol 1,4,5-trisphosphate-mediated Ca^{2+} transient. *FEBS Lett.* **219**:125–129 (1987).
17. Wilcox, R. A., R. A. J. Challiss, G. Baudin, A. Vasella, B. V. L. Potter, and S. R. Nahorski. Stereoselectivity of $Ins(1,3,4,5)P_4$ recognition sites: implications for the mechanism of the $Ins(1,3,4,5)P_4$-induced Ca^{2+} mobilization. *Biochem. J.*. **294**:191–194 (1993).
18. Safrany, S. T., R. A. Wilcox, C. Liu, D. Dubreuil, B. V. L. Potter, and S. R. Nahorski. Identification of partial agonists with low intrinsic activity at the inositol 1,4,5-trisphosphate receptor. *Mol. Pharmacol.* **43**:499–503 (1993).
19. Wojcikiewicz, R. J. H., and S. R. Nahorski. Chronic muscarinic stimulation of SH-SY5Y neuroblastoma cells suppresses inositol 1,4,5-trisphosphate action: parallel inhibition of inositol 1,4,5-trisphosphate-induced Ca^{2+} mobilization and inositol 1,4,5-trisphosphate binding. *J. Biol. Chem.* **266**:22234–22241 (1992).
20. Liu, C.-H., S. R. Nahorski, and B. V. L. Potter. Total synthesis from L-quebrachitol of the *myo*-inositol 1,4,5-trisphosphate analogue, L-*chiro*-inositol 2,3,5-trisphosphate, a potent inositol 1,4,5-trisphosphate 5-phosphatase and 3-kinase inhibitor. *J. Chem. Soc. Chem. Commun.* 1014–1016 (1991).
21. Liu, C., S. R. Nahorski, and B. V. L. Potter. Synthesis from quebrachitol of 1L-*chiro*-$Ins(2,3,5)PS_3$, an inhibitor of the enzymes of 1D-*myo*-inositol 1,4,5-trisphosphate metabolism. *Carbohydrate Res.* **234**:107–115 (1992).
22. Safrany, S. T., R. A. Wilcox, C. Liu, B. V. L. Potter, and S. R. Nahorski. 3-Position modification of *myo*-inositol 1,4,5-triphosphate: consequences for intracellular Ca^{2+} mobilization and enzyme recognition. *Eur. J. Pharmacol.* **226**:265–272 (1992).
23. Liu, Y.-C., and C.-S. Chen. An efficient synthesis of optically active D-*myo*-inositol 1,4,5-trisphosphate. *Tetrahedron Lett.* **30**:1617–1620 (1989).
24. Gou, D.-M., and C.-S. Chen. An efficient route to D-*myo*-inositol-1,4,5-trisphosphate and D-*myo*-inositol-1,3,4,5-tetrakisphosphate. *Tetrahedron Lett.* **33**:721–724 (1992).
25. Lambert, D. G., A. S. Ghataorre, and S. R. Nahorski. Muscarinic receptor binding characteristics of a human neuroblastoma, SK-N-SH, and its clones SH-SY5Y and SH-EP1. *Eur. J. Pharmacol.* **165**:71–77 (1989).
26. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. *J. Biol. Chem.* **260**:3440–3449 (1985).
27. Batty, I. H., A. J. Letcher, and S. R. Nahorski. Accumulation of inositol polyphosphate isomers in agonist-stimulated cerebral-cortex slices. *Biochem. J.* **258**:23–32 (1989).
28. Safrany, S. T., C. Lui, B. V. L. Potter, and S. R. Nahorski. The evaluation of L-*chiro*-inositol trisphosphate analogues as selective inositol 1,4,5-trisphosphate 5-phosphatese inhibitors. *Br. J. Pharmacol.* **107**:221P (1992).
29. Challiss, R. A. J., A. L. Willcocks, B. Mulloy, B. V. L. Potter, and S. R. Nahorski. Characterization of inositol 1,4,5-trisphosphate- and inositol 1,3,4,5-tetrakisphosphate-binding sites in rat cerebellum. *Biochem. J.* **274**:861–867 (1991).
30. Challiss, R. A. J., S. T. Safrany, B. V. L. Potter, and S. R. Nahorski. Intracellular recognition sites for inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate. *Biochem. Soc. Trans.* **19**:888–893 (1991).
31. Ghosh, T. K., P. S. Eis, J. M. Mullaney, C. L. Ebert, and D. L. Gill. Competitive, reversible and potent antagonism of inositol 1,4,5-trisphosphate-activated calcium release by heparin. *J. Biol. Chem.* **263**:11075–11079 (1988).
32. Wojcikiewicz, R. J. H., S. Nakade, K. Mikoshiba, and S. R. Nahorski. Inositol 1,4,5-trisphosphate receptor immunoreactivity in SH-SY5Y human neuroblastoma cells is reduced by chronic muscarinic receptor activation. *J. Neurochem.* **59**:383–386 (1992).
33. Nahorski, S. R. Inositol polyphosphates and neuronal calcium homeostasis. *Trends Neurosci.* **11**:444–448 (1988).
34. Potter, B. V. L., and S. R. Nahorski. Synthesis and biology of inositol polyphosphate analogues. *Biochem. Soc. Trans.* **20**:434–442 (1992).
35. Ferris, C. D., R. L. Huganir, S. Supattapone, and S. H. Snyder. Purified inositol 1,4,5-trisphosphate receptor mediates calcium flux in reconstituted lipid vesicles. *Nature (Lond.)* **342**:87–89 (1989).
36. Miyawaki, A., T. Furuichi, N. Maeda, and K. Mikoshiba. Expressed cerebel-

[3] R. A. Wilcox, unpublished observations.

lar-type inositol 1,4,5-trisphosphate receptor, P_{400}, has calcium release activity in a fibroblast L cell line. *Neuron* **5**:11–18 (1990).
37. Parker, I., and R. Miledi. Nonlinearity and facilitation in phosphoinositide signalling studied by the use of caged inositol trisphosphate in *Xenopus* oocytes. *J. Neurosci.* **9**:4068–4077 (1989).
38. Parker, I., and I. Ivorra. Localized all-or-none calcium liberation by inositol trisphosphate. *Science (Washington D. C.)* **250**:977–979 (1990).
39. Bezprozvanny, I., J. Watras, and B. E. Ehrlich. Bell-shaped calcium-response curves of Ins(1,4,5)P_3- and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature (Lond.)* **351**:751–754 (1991).
40. Iino, M. Biphasic Ca^{2+} dependence of inositol 1,4,5-trisphosphate-induced Ca^{2+} release in smooth muscle cells of the guinea pig taenia caeci. *J. Gen. Physiol.* **95**:1103–1122 (1990).
41. Finch, E. A., T. J. Turner, and S. M. Goldin. Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. *Science (Washington D. C.)* **252**:443–446 (1991).
42. Cullen, P. J., R. F. Irvine, and A. P. Dawson. Synergistic control of Ca^{2+} mobilization in permeabilized mouse L1210 lymphoma cells by inositol 2,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate. *Biochem. J.* **271**:549–553 (1990).
43. Irvine, R. F., and R. M. Moor. Inositol 1,3,4,5-tetrakisphosphate-induced activation of sea urchin eggs requires the presence of inositol trisphosphate. *Biochem. Biophys. Res. Commun.* **146**:284–290 (1987).
44. Lambert, D. G., and S. R. Nahorski. Muscarinic-receptor-mediated changes in intracellular Ca^{2+} and inositol 1,4,5-trisphosphate mass in human neuroblastoma cell line, SH-SY5Y. *Biochem. J.* **265**:555–562 (1990).
45. Lambert, D. G., R. A. J. Challiss, and S. R. Nahorski. Accumulation and metabolism of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in muscarinic-receptor-stimulated SH-SY5Y neuroblastoma cells. *Biochem. J.* **273**:791–794 (1991).
46. Challiss, R. A. J., and S. R. Nahorski. Depolarization and agonist-stimulated changes in inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate mass accumulation in rat cerebral cortex. *J. Neurochem.* **57**:1042–1051 (1991).
47. Reiser, G., and F. Donié. Endothelin induces a rise of inositol 1,4,5-trisphosphate, inositol 1,3,4,5-tetrakisphosphate levels and of cytosolic Ca^{2+} activity in neural cell lines. *Eur. J. Neurosci.* **2**:769–775 (1990).
48. Donié, F., and G. Reiser. Mass measurements of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate in a neuronal cell line stimulated with bradykinin: inositol phosphate response shows desensitization. *Biochem. Biophys. Res. Commun.* **181**:997–1003 (1991).
49. Pittet, R., W. Schlegel, D. P. Lew, A. Monod, and G. W. Mayr. Mass changes in inositol tetrakis and pentakisphosphate isomers induced by chemotactic peptide stimulation in HL-60 cells. *J. Biol. Chem.* **264**:18489–18493 (1989).
50. Theibert, A. B., V. A. Estevez, R. J. Mourey, J. F. Marecek, R. K., G. D. Prestwich, and S. H. Snyder. Photoaffinity labeling and characterization of isolated inositol-1,3,4,5-tetrakisphosphate-binding proteins. *J. Biol. Chem.* **267**:9071–9079 (1992).
51. Irvine, R. F. Inositol tetrakisphosphate as a second messenger: confusions, contradictions, and a potential resolution. *BioEssays* **13**:419–428 (1991).
52. Lückhoff, A., and D. E. Clapham. Inositol 1,3,4,5-tetrakisphosphate activates an endothelial Ca^{2+}-permeable channel. *Nature (Lond.)* **355**:356–358 (1992).
53. Timerman, A. P., M. M. Mayrleitner, T. J. Lukas, C. C. Chadwick, A. Saito, D. M. Watterson, H. Schindler, and S. Fleischer. Inositol polyphosphate receptor and clathrin assembly protein AP-2 are related proteins that form potassium-selective ion channels in planar lipid bilayers. *Proc. Natl. Acad. Sci. USA* **89**:8976–8990 (1992).

Send reprint requests to: R. Wilcox, Department of Pharmacology and Therapeutics, University of Leicester, P.O. Box 138, Medical Sciences Building, University Road, Leicester, LE1 9HN, UK.