0026-895X/93/040499-05$03.00/0

MOLECULAR PHARMACOLOGY, 43:499–503

ACCELERATED COMMUNICATION

Identification of Partial Agonists with Low Intrinsic Activity at the Inositol-1,4,5-Trisphosphate Receptor

S. T. SAFRANY, R. A. WILCOX, C. LIU, D. DUBREUIL,[1] B. V. L. POTTER, and S. R. NAHORSKI

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

Received December 17, 1992; Accepted February 4, 1993

SUMMARY

The interactions of synthetic L-*chiro*-inositol-2,3,5-trisphosphorothioate [L-ch-Ins(2,3,5)PS_3] and D-6-deoxy-*myo*-inositol-1,4,5-trisphosphorothioate [D-6-deoxy-Ins(1,4,5)PS_3] with D-*myo*-inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3] receptors have been examined using radioligand binding assays and Ca^{2+} mobilization from permeabilized SH-SY5Y cells. The ability of these analogues to compete with [^{3}H]Ins(1,4,5)P_3 for specific sites on adrenal cortical membranes indicated that, although weaker than Ins(1,4,5)P_3, both ligands competed fully for these sites [L-ch-Ins(2,3,5)PS_3, K_i = 0.5 μM; D-6-deoxy-Ins(1,4,5)PS_3, K_i = 5.3 μM]. However, in assays examining the amount of Ca^{2+} mobilized from the stores of permeabilized SH-SY5Y cells, both of these synthetic analogues displayed low intrinsic activity [L-ch-Ins(2,3,5)PS_3, 34%; D-6-deoxy-Ins(1,4,5)PS_3, 42% of that of Ins(1,4,5)P_3]. Moreover, L-ch-Ins(2,3,5)PS_3 and D-6-deoxy-Ins(1,4,5)PS_3 were able to inhibit the response to Ins(1,4,5)P_3 with K_i values (6 μM and 33 μM, respectively) virtually identical to their EC_{50} values for Ca^{2+} release. This is consistent with partial agonist behavior, because these compounds exhibit low maximal responses when the extent of Ca^{2+} release is examined. These compounds represent the first examples of inositol-based analogues with low intrinsic activity and may point the way towards the design of selective antagonists at Ins(1,4,5)P_3 receptors. It also seems probable that these may represent the first true affinity values of inositol phosphates at the active receptor.

Ins(1,4,5)P_3 (**1**, Fig. 1) is the second messenger that mobilizes Ca^{2+} from stores associated with the specialized components of the endoplasmic reticulum. It appears likely that this action underlies the complex Ca^{2+} signals evoked by many cell surface receptors linked to phosphoinositidase C and that interaction of Ins(1,4,5)P_3 with an intracellular receptor initiates Ca^{2+} release through an integral channel (1–3). After the initial cloning of the cerebellar Ins(1,4,5)P_3R, there is now evidence for multiple forms arising either from separate genes or from alternative mRNA splicing (4, 5). These new data have provoked much speculation on the possibility of a diversity of ligand affinities and/or regulation of the Ca^{2+} channel (5). However, clarification of the mechanisms regulating Ca^{2+} release by such intracellular receptors is still hampered by a lack of agents to manipulate their activity (6, 7). Indeed, the only Ins(1,4,5)P_3R antagonists available to date, heparin and decavanadate, are large complex molecules that are generally unsuitable because of their nonspecificity (4, 8), and the search for a specific ligand with low, or preferably no, intrinsic activity is underway.

Virtually all active inositol polyphosphates and analogues examined to date (synthetic and endogenous) appear to be full agonists with respect to Ins(1,4,5)P_3 when assessed by the extent to which they mobilize Ca^{2+} stores (4, 6). A possible exception to this general rule is inositol-1,3,4,6-tetrakisphosphate, which in permeabilized SH-SY5Y neuroblastoma cells releases 80–90% of the Ca^{2+} pool mobilized by Ins(1,4,5)P_3 (9).

Recent work from these laboratories has focused upon the synthesis of metabolically stable phosphorothioate analogues of Ins(1,4,5)P_3, such as Ins(1,4,5)PS_3 (**2**, Fig. 1) (6, 7, 10), and also ring-modified analogues, such as L-ch-Ins(2,3,5)P_3 (**3**, Fig. 1) (11, 12) and D-6-deoxy-Ins(1,4,5)P_3 (**5**, Fig. 1) (13, 14). Our synthesis of phosphorothioate derivatives of the latter two fully active Ins(1,4,5)P_3 analogues [L-ch-Ins(2,3,5)P_3 and D-6-deoxy-Ins(1,4,5)P_3] now provides the first evidence of ligands possessing low intrinsic activity, i.e., L-ch-Ins(2,3,5)PS_3 (**4**, Fig. 1) and D-6-deoxy-Ins(1,4,5)PS_3 (**6**, Fig. 1).

The authors wish to thank The Wellcome Trust and The Science and Engineering Research Council (Molecular Recognition Initiative) for financial support and The Royal Society for an Exchange Fellowship (to D.D.). B.V.L.P. is a Lister Institute Fellow.

[1] Present address: Laboratoire de Synthese Organique, Faculte des Sciences et des Techniques, 2, Rue de la Housiniere, F 44072 Nantes, Cedex 03, France.

ABBREVIATIONS: Ins(1,4,5)P_3, D-*myo*-inositol-1,4,5-trisphosphate; Ins(1,4,5)PS_3, *myo*-inositol-1,4,5-trisphosphorothioate; L-ch-Ins(2,3,5)P_3, L-*chiro*-inositol-2,3,5-trisphosphate; D-6-deoxy-Ins(1,4,5)P_3, D-6-deoxy-*myo*-inositol-1,4,5-trisphosphate; L-ch-Ins(2,3,5)PS_3, L-*chiro*-inositol-2,3,5-trisphosphorothioate; D-6-deoxy-Ins(1,4,5)PS_3, D-6-deoxy-*myo*-inositol-1,4,5-trisphosphorothioate; Ins(1,4,5)P_3R, D-*myo*-inositol-1,4,5-trisphosphate receptor(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

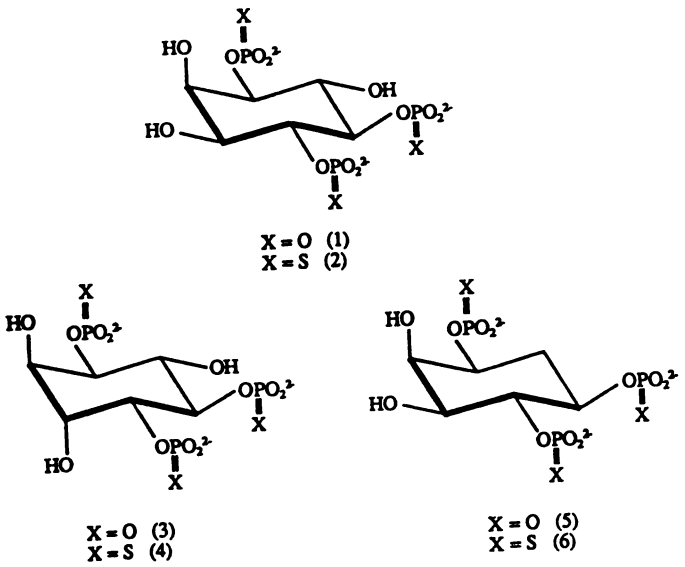

Fig. 1. Structures of Ins(1,4,5)P_3 (1), Ins(1,4,5)PS_3 (2), L-ch-Ins(2,3,5)P_3 (3), L-ch-Ins(2,3,5)PS_3 (4), D-6-deoxy-Ins(1,4,5)P_3 (5), and D-6-deoxy-Ins(1,4,5)PS_3 (6).

Materials and Methods

Synthesis of analogues. L-ch-Ins(2,3,5)PS_3 was synthesized from L-quebrachitol by a method similar to that used for L-ch-Ins(2,3,5)P_3 (12). Briefly, L-1,4,6-tri-*O*-benzoyl-*chiro*-inositol was phosphitylated with bis(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite tetrazole in anhydrous dichloromethane and the product was oxidized to the fully protected trisphosphorothioate with sulfur. D-6-Deoxy-Ins(1,4,5)PS_3 was synthesized, in a procedure similar to that used for D-6-deoxy-Ins(1,4,5)P_3, from D-2,3-*O*-cyclohexylidene-6-deoxy-*myo*-inositol via D-6-deoxy-*myo*-inositol-1,4,5-tris(dibenzylphosphorothioate) (14) by phosphitylation using *N*,*N*-diisopropylbisbenzyl phosphoramidite and tetrazole in anhydrous acetonitrile, followed by oxidation with sulfur. After complete deblocking of intermediates with sodium in liquid ammonia for L-ch-Ins(2,3,5)PS_3 and with sodium in liquid ammonia followed by Dowex H^+ ion exchange resin for D-6-deoxy-Ins(1,4,5)PS_3, the products were purified on a column of DEAE Sephadex A-25 resin, as described for Ins(1,4,5)PS_3 (15), using a gradient of triethylammonium bicarbonate. Evaporation of solvent from fractions containing product gave pure L-ch-Ins(2,3,5)PS_3 (4) and D-6-deoxy-Ins(1,4,5)PS_3 (6) as the triethylammonium salts, which were quantified using Briggs phosphate assay. Full chemical details will be published elsewhere.

Cell culture and Ca^{2+} mobilization. Monolayers of SH-SY5Y human neuroblastoma cells (passages 80–90), initially a kind gift of Dr. J. L. Biedler (Sloane-Kettering Institute, New York, NY), were grown as described (16). Before use, the cells were harvested in 10 mM HEPES, 0.9% NaCl, 0.02% EDTA, pH 7.4, and were resuspended in a 'cytosol-like' buffer [120 mM KCl, 20 mM HEPES, 6 mM $MgCl_2$, 5 mM sodium succinate, 5 mM Na_2ATP, 2 mM KH_2PO_4, 10–30 μM EGTA (to reduce the free Ca^{2+} concentration to 70–300 nM), pH 6.9]. The cells were then exposed to three discharges of a 3-μF capacitor, centrifuged, and resuspended (0.5–1 mg of protein/ml) for 12–15 min at 18–20° in buffer supplemented with $^{45}Ca^{2+}$. Experimental incubations were started by addition of 30 μl of cell suspension to 20 μl of buffer containing stimulus. After 2 min (to obtain dose-response curves) or 30 sec to 20 min (to obtain time courses of $^{45}Ca^{2+}$ release and reuptake), 100 μl of a silicone oil mixture (Dow Corning 550/556, 9:11, v/v) were added and the tubes were centrifuged (16,000 × *g*, 3 min) to separate cells from buffer before assay of radioactivity of the pellet (17). Release of $^{45}Ca^{2+}$ in the presence of stimuli was calculated from the amount remaining in the cell pellet, expressed as a percentage of that sequestered after control incubations (50,000–100,000 cpm/tube).

Ins(1,4,5)P_3 binding assay. The preparation of bovine adrenal cortical membranes and [^{3}H]Ins(1,4,5)P_3 binding and displacement assays were performed essentially as described by Challiss *et al.* (18). Bound and free [^{3}H]Ins(1,4,5)P_3 were separated by rapid filtration through Whatman GF/B glass fiber filters. Specifically bound [^{3}H]Ins(1,4,5)P_3 (approximately 2500 dpm/assay) was displaced by Ins(1,4,5)P_3, L-ch-Ins(2,3,5)PS_3, or D-6-deoxy-Ins(1,4,5)PS_3 (0–300 μM). Nonspecific binding (approximately 150 dpm/assay) was defined by the addition of 10 μM Ins(1,4,5)P_3.

Data analysis. EC_{50} values for Ca^{2+} mobilization were derived using ALLFIT computer-assisted curve fitting (19). K_i values for enzyme inhibition were calculated according to the method of Cheng and Prusoff (20). K_d and K_i values from Ins(1,4,5)P_3 saturation and displacement experiments were calculated using EBDA (21) and LIGAND (22). Combined data from a number of independent experiments (n) are expressed as mean ± standard error where $n \geq 3$.

Results

The binding of [^{3}H]Ins(1,4,5)P_3 to adrenal cortical membranes, which has been fully characterized and shown to display strict stereospecificity and positional specificity, was inhibited in a concentration-dependent manner by Ins(1,4,5)P_3 (K_d = 6.5 nM), L-ch-Ins(2,3,5)PS_3 (K_i = 500 nM), and D-6-deoxy-Ins(1,4,5)PS_3 (K_i = 5300 nM) (Table 1; Fig. 2).

Both analogues also mobilized $^{45}Ca^{2+}$ from electrically permeabilized SH-SY5Y cells but were of lower potency than

TABLE 1
Effects of analogues on binding and calcium release

Ligand	Binding, K_i[a]	Calcium release	
		EC_{50}[b]	'K_i'[c]
	μM	μM	μM
Ins(1,4,5)P_3	0.0065 ± 0.0005	0.18 ± 0.02	
L-ch-Ins(2,3,5)PS_3	0.5 ± 0.02[d]	5.3 ± 0.8	6.1 ± 1.4
D-6-Deoxy-Ins(1,4,5)PS_3	5.3 ± 0.41[d]	16 ± 2	33 ± 7

[a] Binding affinity obtained by isotope dilution.
[b] EC_{50} for $^{45}Ca^{2+}$ release from permeabilized SH-SY5Y cells.
[c] K_i values obtained from IC_{50} values for the ability of L-ch-Ins(2,3,5)PS_3 and D-6-deoxy-Ins(1,4,5)PS_3 to inhibit Ca^{2+} mobilization induced by 0.7 μM Ins(1,4,5)P_3. K_i values were calculated according to the method of Cheng and Prusoff (20).
[d] Binding affinity obtained by displacement of [^{3}H]Ins(1,4,5)P_3 binding to bovine adrenal cortical membranes.

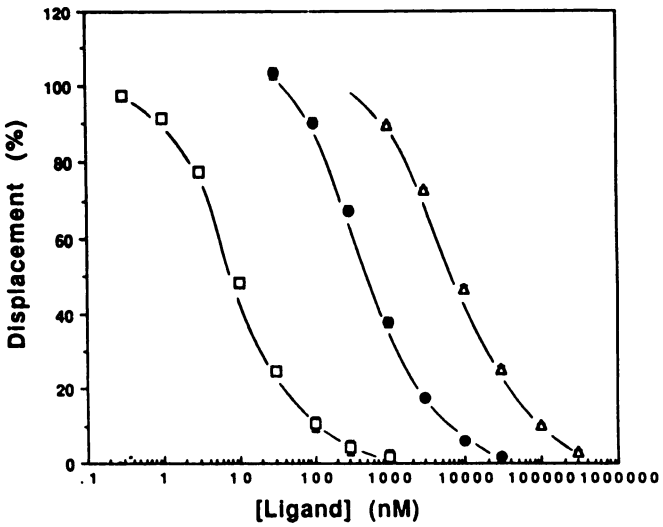

Fig. 2. Displacement of [^{3}H]Ins(1,4,5)P_3 binding by Ins(1,4,5)P_3 (□), L-ch-Ins(2,3,5)PS_3 (●), and D-6-deoxy-Ins(1,4,5)PS_3 (△) in bovine adrenal cortical membranes. [^{3}H]Ins(1,4,5)P_3 (approximately 1.84 nM) was incubated at 4° for 40 min with adrenal cortical membranes in the presence of increasing amounts of Ins(1,4,5)P_3 or its analogues. Data shown are mean values ± standard errors from at least three experiments.

Ins(1,4,5)P_3 itself. All three agents were maximally effective by 2 min (data not shown); this time was chosen for termination of all subsequent experiments. In agreement with binding studies, L-ch-Ins(2,3,5)PS_3 (EC_{50} = 5.3 μM, n_H = 1.9 ± 0.3, n = 8) was more potent than D-6-deoxy-Ins(1,4,5)PS_3 (EC_{50} = 16 μM, n_H = 2.2 ± 0.3, n = 7) but markedly less potent than Ins(1,4,5)P_3 (EC_{50} = 0.18 μM, n_H = 1.8 ± 0.1, n = 6) (Table 1; Figs. 3 and 4). It was clear, however, that neither L-ch-Ins(2,3,5)PS_3 nor D-6-deoxy-Ins(1,4,5)PS_3 fully mobilized the Ins(1,4,5)P_3-sensitive Ca^{2+} store, releasing only 34 and 42%, respectively, of that maximally mobilized by Ins(1,4,5)P_3. The apparently low intrinsic activity of these agents suggests that they are partial agonists and, therefore, can be characterized by their inhibitory properties against Ins(1,4,5)P_3.

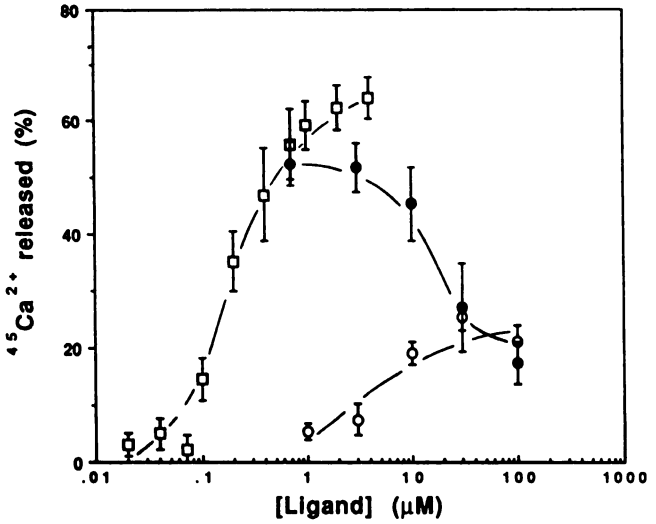

Fig. 3. Dose dependence of $^{45}Ca^{2+}$-releasing effects of Ins(1,4,5)P_3 (□) and L-ch-Ins(2,3,5)PS_3 (○) and inhibition of Ins(1,4,5)P_3 (0.7 μM) effect by L-ch-Ins(2,3,5)PS_3 (●) in permeabilized SH-SY5Y cells. Electrically permeabilized cells were incubated in the presence of $^{45}Ca^{2+}$ (loading of approximately 50,000 dpm, equivalent to 1–2 nmol/mg of protein) (37) and then challenged with Ins(1,4,5)P_3 or its analogues. Incubations, at 20°, were terminated after 2 min, at which time the amount of $^{45}Ca^{2+}$ released was assessed. Data shown are mean values ± standard errors from at least three experiments.

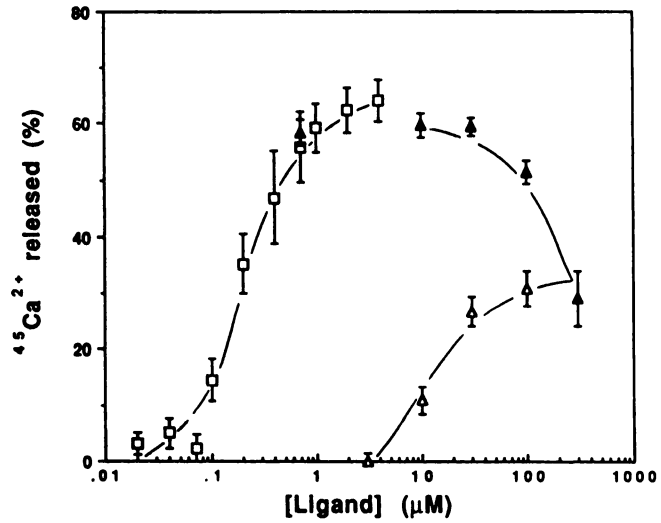

Fig. 4. Dose dependence of $^{45}Ca^{2+}$-releasing effects of Ins(1,4,5)P_3 (□) and D-6-deoxy-Ins(1,4,5)PS_3 (△) and inhibition of Ins(1,4,5)P_3 (0.7 μM) effect by D-6-deoxy-Ins(1,4,5)PS_3 (▲) in permeabilized SH-SY5Y cells. Data shown are mean values ± standard errors from at least three experiments. For experimental details see the legend to Fig. 3.

The ability of these agents, therefore, to inhibit the Ca^{2+}-mobilizing properties of Ins(1,4,5)P_3 was investigated (Table 1). L-ch-Ins(2,3,5)PS_3 was found to inhibit Ca^{2+} mobilization induced by Ins(1,4,5)P_3 (0.7 μM) in a dose-dependent manner (IC_{50} = 26 ± 6 μM; n = 3) (Fig. 3). Assuming this to be due to competition between Ins(1,4,5)P_3 and L-ch-Ins(2,3,5)PS_3 at the same site, an estimate of the K_i for L-ch-Ins(2,3,5)PS_3 of 6 μM can be obtained from the equation derived by Cheng and Prusoff (20); this is in good agreement with the EC_{50} obtained for Ca^{2+} release (5.3 μM). Similar inhibitory effects were observed with D-6-deoxy-Ins(1,4,5)PS_3 and also allow an apparent K_i to be calculated (Table 1; Fig. 4).

Discussion

The last few years have seen the synthesis of an increasing number of analogues of Ins(1,4,5)P_3 developed to probe the relationship between ligand structure, receptor occupation, and response (7, 10). Although this has been useful in determining the structural requirements for active ligands, our understanding of the occupation-response relationship at Ins(1,4,5)P_3R is still very limited. For example, it is still not clear what apparent binding affinity values represent in radioligand assays, because they are generally lower than EC_{50} values for Ca^{2+} release for most ligands (4). Although differences in assay conditions may partially underlie such discrepancies (23), it is also not clear whether the binding affinities could represent interaction with conformations other than the active Ins(1,4,5)P_3R. The binding of labeled Ins(1,4,5)P_3 to membranes reveals a high affinity, slowly dissociating site that is totally inconsistent with the kinetics estimated from Ca^{2+} flux experiments (see Ref. 4). Indeed, recent experiments in hepatocyte membranes have revealed that under conditions of low Ca^{2+} concentration a lower affinity, rapidly dissociating component of Ins(1,4,5)P_3 binding may represent labeling of the active form of the Ins(1,4,5)P_3R (24).

The complex nature of Ca^{2+} release through the Ins(1,4,5)P_3R channel appears to result from both extra- and intraluminal regulatory features (4). Thus, the ability of a Ca^{2+} store to respond rapidly, but transiently, to low concentrations of Ins(1,4,5)P_3, while maintaining its ability to release Ca^{2+} in response to higher concentrations, has been termed 'quantal' (25) or 'increment detection' (26) and complicates investigation of the relationship between Ins(1,4,5)P_3R occupation and Ca^{2+} release and discrimination between agonists of different efficacy. These complications have probably resulted in virtually all Ins(1,4,5)P_3 analogues examined to date displaying apparent full agonist properties, because even partial agonists may be able to deplete stores fully, given sufficient time. Differences in agonist efficacy should therefore be more readily revealed by measuring the initial rate rather than the final extent of release (27, 28). However, such rapid kinetic measurements are susceptible to regulation by released Ca^{2+} (29) and should be interpreted with caution unless stringent controls are performed.

Recent work from this laboratory in which we described the ability of naturally occurring inositol-1,3,4,6-tetrakisphosphate to release intracellular stores of Ca^{2+} from permeabilized SH-SY5Y cells gave a strong indication of partial agonist activity, in comparison with Ins(1,4,5)P_3 (9). However, this metabolite generally released >80% of the Ca^{2+} mobilized by Ins(1,4,5)P_3

in SH-SY5Y cells and behaves as a full agonist in other cells.[2] The synthetic Ins(1,4,5)P_3 analogues L-ch-Ins(2,3,5)P_3 and D-6-deoxy-Ins(1,4,5)P_3 were full agonists at the Ins(1,4,5)P_3R (11, 13) but were less potent than Ins(1,4,5)P_3. In the present experiments clear partial agonist activities were displayed by the synthetic phosphorothioates L-ch-Ins(2,3,5)PS_3 and D-6-deoxy-Ins(1,4,5)PS_3, as determined by the amount of Ca^{2+} released from permeabilized SH-SY5Y cells. This probably indicates that these agents possess very low intrinsic efficacy at the Ins(1,4,5)P_3R, providing only a small Ca^{2+} leak relative to the Ca^{2+} pump.

The ability of L-ch-Ins(2,3,5)PS_3 to compete fully with [^{3}H]Ins(1,4,5)P_3 binding sites on adrenal cortical membranes, yet release only 34% of the Ca^{2+} mobilized by a maximal concentration of Ins(1,4,5)P_3, is wholly consistent with partial agonist activity. However, the 10-fold discrepancy between the binding affinity and Ca^{2+}-mobilizing EC_{50} value of this analogue may highlight differences in the properties of the Ins(1,4,5)P_3R of the adrenal and SH-SY5Y membranes and/or the probability that radioligand affinities obtained in equilibrium assays in washed membranes may not represent binding to the active form of the receptor (see above). A more satisfactory result comes from the ability of L-ch-Ins(2,3,5)PS_3 to inhibit the ability of Ins(1,4,5)P_3 to release Ca^{2+} with an apparent K_i (6 μM) identical to the EC_{50} of the partial agonist. These calculations are based on the assumption that L-ch-Ins(2,3,5)PS_3 competes with Ins(1,4,5)P_3 at the same site, and this receives support from the ability of the partial agonists to shift to the right the Ins(1,4,5)P_3 dose-response curve for Ca^{2+} release.[3] We believe that this K_i represents the true affinity of this partial agonist for the active form of the Ins(1,4,5)P_3R and is the only value of its kind for any agonist at the Ins(1,4,5)P_3R.

It is premature to attempt to describe the properties of these partial agonists in terms of channel conductance and subconductance states at the Ins(1,4,5)P_3R (29), because of the uncertainties regarding possible cooperativity and regulation by both intra- and extraluminal Ca^{2+} (4, 30, 31). However, the data are very encouraging regarding our attempts to develop high affinity Ins(1,4,5)P_3 ligands with negligible efficacy. It seems very probable that the introduction of phosphorothioate groups would result in a substantial fall in agonist efficacy with only a small reduction in affinity. Ins(1,4,5)PS_3 has only a slightly lower affinity than Ins(1,4,5)P_3 at [^{3}H]Ins(1,4,5)P_3 binding sites (23, 32), but because [^{35}S]Ins(1,4,5)PS_3 labels two populations of sites with similar affinity that are dramatically distinguished with Ins(1,4,5)P_3 (33) we have argued that this would be consistent with a less efficacious agonist. Thus, although Ins(1,4,5)PS_3 can release the same maximal amount of Ca^{2+} as Ins(1,4,5)P_3 (17, 23), it may need to occupy a greater proportion of receptors to do so. Likewise, it can be surmised that L-ch-Ins(2,3,5)P_3 possesses relatively low efficacy at the Ins(1,4,5)P_3R, and its phosphorothioate derivative described here displays very low intrinsic activity even in the extent of Ca^{2+} release from permeabilized SH-SY5Y cells.

It seems certain that, unlike heparin and decavanadate, inositol-based Ins(1,4,5)P_3R antagonists will be much more specific, although L-ch-Ins(2,3,5)PS_3, like Ins(1,4,5)PS_3, is also a potent inhibitor of Ins(1,4,5)P_3 5-phosphatase (34). Of all the phosphorothioate analogues of inositol polyphosphates synthesized to date, these are the first to show other than full agonist activity. The reasons behind this are not yet clear, although it is obvious that phosphorothioate substitution leads to changes in molecular size, hydrophobicity, and charge distribution, any or all of which may contribute to producing a less than maximal response. It should be noted that for the second messenger cAMP phosphorothioate substitution has generated the only known competitive cAMP antagonists, R_p adenosine 3′,5′-cyclic monophosphorothioate (35) and R_p adenosine 3′,5′-cyclic monophosphorodithioate (36), of many hundreds of synthetic cAMP analogues.

The development of a low intrinsic activity partial agonist is clearly a significant development in delineating structure-activity relationships at the Ins(1,4,5)P_3R and could prove useful in revealing the mechanisms of complex Ca^{2+} signaling in cells and in the development of more effective pharmacological tools to probe the polyphosphoinositide pathway of signal transduction.

Acknowledgments

We thank Dr. J. Cleophax for synthesis of D-2,3-*O*-cyclohexylidene-6-deoxy-*myo*-inositol.

References

1. Berridge, M. J., and R. F. Irvine. Inositol phosphates and cell signalling. *Nature (Lond.)* **341**:197–202 (1989).
2. Berridge, M. J. Calcium oscillations. *J. Biol. Chem.* **265**:9583–9586 (1990).
3. Jacob, R. Calcium oscillations in electrically non-excitable cells. *Biochim. Biophys. Acta* **1052**:427–438 (1990).
4. Taylor, C. W., and A. Richardson. Structure and function of inositol trisphosphate receptors. *Pharmacol. Ther.* **51**:97–137 (1991).
5. Ferris, C. D., and S. H. Snyder. Inositol phosphate receptors and calcium disposition in the brain. *J. Neurosci.* **12**:1567–1574 (1992).
6. Nahorski, S. R., and B. V. L. Potter. Molecular recognition of inositol polyphosphates by intracellular receptors and metabolic enzymes. *Trends Pharmacol. Sci.* **10**:139–144 (1989).
7. Potter, B. V. L. Recent advances in the chemistry and biochemistry of inositol phosphates of biological interest. *Nat. Prod. Rep.* **7**:1–24 (1990).
8. Strupish, J., R. J. H. Wojcikiewicz, R. A. J. Challiss, S. T. Safrany, A. L. Willcocks, B. V. L. Potter, and S. R. Nahorski. Decavanadate interacts with inositol polyphosphate binding sites and is a competitive antagonist at the inositol 1,4,5-trisphosphate receptor. *Mol. Neuropharmacol.* **1**:111–116 (1991).
9. Gawler, D. J., B. V. L. Potter, 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).
10. Potter, B. V. L., and S. R. Nahorski. Synthesis and biology of inositol polyphosphate analogues. *Biochem. Soc. Trans.* **20**:434–442 (1992).
11. 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-trisphosphate: consequences for intracellular Ca^{2+} mobilisation and enzyme recognition. *Eur. J. Pharmacol.* **226**:265–272 (1992).
12. Liu, C., S. R. Nahorski, and B. V. L. Potter. Synthesis from quebrachitol of 1L-*chiro*-inositol 2,3,5-trisphosphate, an inhibitor of the enzymes of 1D-*myo*-inositol 1,4,5-trisphosphate metabolism. *Carbohydr. Res.* **234**:107–115 (1992).
13. Safrany, S. T., R. J. H. Wojcikiewicz, J. Strupish, S. R. Nahorski, D. Dubreuil, J. Cleophax, S. D. Gero, and B. V. L. Potter. Interaction of synthetic D-6-deoxy-*myo*-inositol 1,4,5-trisphosphate with the Ca^{2+}-releasing D-*myo*-inositol 1,4,5-trisphosphate receptor, and the metabolic enzymes 5-phosphatase and 3-kinase. *FEBS Lett.* **278**:252–256 (1991).
14. Noble, N. J., D. Dubreuil, and B. V. L. Potter. Total synthesis of *myo*-inositol 1-phosphate 4,5-pyrophosphate, a novel second messenger analogue, via *myo*-inositol 1-phosphate 4,5-bisphosphorothioate. *Bioorg. Med. Chem. Lett.* **2**:471–476 (1992).
15. Cooke, A. M., R. Gigg, and B. V. L. Potter. *myo*-Inositol 1,4,5-trisphosphorothioate: a novel analogue of a biological second messenger. *J. Chem. Soc. Chem. Commun.* 1525–1526 (1987).
16. 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).
17. Strupish, J., A. M. Cooke, B. V. L. Potter, R. Gigg, and S. R. Nahorski. Stereospecific mobilization of intracellular calcium by inositol (1,4,5)trisphosphate: comparison with inositol (1,4,5)trisphosphorothioate and inositol (1,3,4)trisphosphate. *Biochem. J.* **253**:901–905 (1988).
18. Challiss, R. A. J., E. J. Chilvers, A. L. Willcocks, and S. R. Nahorski. Heterogeneity of [^{3}H]inositol 1,4,5-trisphosphate binding sites in adrenal-cortical membranes. *Biochem. J.* **265**:421–427 (1990).
19. DeLean, A., P. J. Munson, and D. Rodbard. Simultaneous analysis of families

[2] R. A. Wilcox, B. V. L. Potter, and S. R. Nahorski, unpublished observations.
[3] S. T. Safrany, R. A. Wilcox, C. Liu, B. V. L. Potter, and S. R. Nahorski, unpublished observations.

of sigmoidal curves: applications to bioassay, radioligand assay and physiological dose-response curves. *Am. J. Physiol.* **235**:E97-E102 (1978).
20. Cheng, Y. C., and W. H. Prusoff. Relationship between the inhibitor constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibiton (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
21. McPherson, G. A. A practical computer-based approach to the analysis of radioligand binding experiments. *Comput. Prog. Biomed.* **17**:107–114 (1983).
22. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for the characterization of ligand binding systems. *Anal. Biochem.* **107**:220–239 (1980).
23. Nunn, D., and C. W. Taylor. Liver inositol 1,4,5-trisphosphate-binding sites are the Ca^{2+}-mobilizing receptor. *Biochem. J.* **270**:227–232 (1990).
24. Rouxel, F. P., M. Hilly, and J.-P. Mauger. Characterization of a rapidly dissociating inositol (1,4,5)trisphosphate binding site in liver membranes. *J. Biol. Chem.* **267**:20017–20023 (1992).
25. Muallem, S., S. J. Pandol, and T. G. Beeker. Hormone-evoked calcium release from intracellular stores is a quantal process. *J. Biol. Chem.* **264**:205–212 (1989).
26. Meyer, T., and L. Stryer. Transient calcium release induced by successive increments of inositol 1,4,5-trisphosphate. *Proc. Natl. Acad. Sci. USA* **85**:3841–3845 (1990).
27. Champeil, P., L. Combettes, B. Berthon, E. Doucet, S. Orlowski, and M. Claret. Fast kinetics of calcium release induced by *myo*-inositol trisphosphate in permeabilized rat hepatocytes. *J. Biol. Chem.* **264**:17665–17673 (1989).
28. Meyer, T., T. Wensel, and L. Stryer. Kinetics of calcium channel opening by inositol 1,4,5-trisphosphate. *Biochemistry* **29**:32–37 (1990).
29. 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).
30. Irvine, R. F. 'Quantal' Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates: a possible mechanism. *FEBS Lett.* **263**:5–9 (1991).
31. Watras, J., I. Bezprozvanny, and B. E. Ehrlich. Inositol 1,4,5-trisphosphate-gated channels in cerebellum: presence of multiple conductance states. *J. Neurosci.* **11**:3239–3245 (1991).
32. Willcocks, A. L., B. V. L. Potter, A. M. Cooke, and S. R. Nahorski. *myo*-Inositol (1,4,5)-trisphosphorothioate binds to specific inositol (1,4,5)-trisphosphate sites in rat cerebellum and is resistant to 5-phosphatase. *Eur. J. Pharmacol.* **155**:181–183 (1988).
33. Challiss, R. A. J., S. M. Smith, B. V. L. Potter, and S. R. Nahorski. D-[^{35}S(U)] Inositol 1,4,5-trisphosphorothioate, a novel radioligand for the inositol 1,4,5-trisphosphate receptor. *FEBS Lett.* **281**:101–104 (1991).
34. Safrany, S. T., C. Liu, B. V. L. Potter, and S. R. Nahorski. The evaluation of L-*chiro* inositol trisphosphate analogues as selective inositol 1,4,5-trisphosphate 5-phosphatase inhibitors. *Br. J. Pharmacol.* **107**:221P (1992).
35. Parker Bothelo, L. H., J. D. Rothermel, R. V. Coombs, and B. Jastorff. cAMP analog antagonists of cAMP action. *Methods Enzymol.* **159**:159–172 (1988).
36. Parker Bothelo, L. H., J. W. Baraniak, and W. J. Stec. Inhibition of cAMP-dependent protein kinase by adenosine cyclic 3′,5′-phosphorothioate, a second cAMP antagonist. *J. Biol. Chem.* **263**:5301–5305 (1988).
37. Wojcikiewicz, R. J. H., and S. R. Nahorski. Chronic muscarinic stimulation of SH-SY5Y neuroblastoma cells suppresses inositol 1,4,5-trisphosphate action. *J. Biol. Chem.* **266**:22234–22241 (1991).

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