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D-[^{35}S(U)]Inositol 1,4,5-trisphosphorothioate, a novel radioligand for the inositol 1,4,5-trisphosphate receptor

Complex binding to rat cerebellar membranes

R.A. John Challiss[1], Susan M. Smith[1], Barry V.L. Potter[2] and Stefan R. Nahorski[1]

[1] *Department of Pharmacology and Therapeutics, University of Leicester, Leicester, LE1 9HN, UK* and [2] *School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY, UK*

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D-[^{35}S(U)]*myo*-inositol 1,4,5-trisphosphorothioate ([^{35}S]InsPS$_3$), a synthetic, metabolically stable analogue of inositol 1,4,5-trisphosphate (InsP$_3$), binds with high affinity (K_d 58.6 ± 9.1 nM) to rat cerebellar membranes revealing a high density of specific binding sites (B_{max} 21.5 ± 2.1 pmol/mg of protein). Comparison with [^{3}H]InsP$_3$ binding reveals a higher density of sites labelled by [^{35}S]InsPS$_3$ and complex competition curves for displacement of specific [^{35}S]InsPS$_3$ by InsP$_3$. The results suggest that [^{35}S]InsPS$_3$ labels two sites in rat cerebellar membranes with equal affinity: the InsP$_3$ receptor and a site that displays low affinity for InsP$_3$.

Inositol 1,4,5-trisphosphate; Inositol 1,4,5-trisphosphorothioate; Inositol 1,4,5-trisphosphate receptor

1. INTRODUCTION

InsP$_3$ is the second messenger linking receptor-mediated activation of phosphoinositidase C to the release of Ca^{2+} from intracellular stores [1]. Substantial evidence that InsP$_3$ interacts with specific receptors has come from studies using radiolabelled InsP$_3$ [2–4], and the very high density of sites present in cerebellum [4,5] has allowed a careful analysis of the stereo- and positional specificity of these sites [4]. Moreover, the recent cloning and sequencing of the InsP$_3$ receptor from cerebellum [6], and its reconstitution into synthetic lipid bilayers and expression in the fibroblast L cell-line following gene transfection [7] have established that the recognition site is linked to Ca^{2+} channels in the endoplasmic reticulum.

Further studies on the relationship between InsP$_3$ recognition and channel opening have been hindered until recently by a lack of structurally-modified analogues. The first example of such a compound was inositol 1,4,5-tris-phosphorothioate (InsPS$_3$) [8] which binds to InsP$_3$ receptor sites in several tissues with only slightly decreased affinity compared to InsP$_3$. Moreover, it is a full and potent agonist for the release of intracellular Ca^{2+} and as expected from the properties of phosphorothioates, it is resistant to InsP$_3$-5-phosphatase, is not recognised with high affinity by InsP$_3$-3-kinase, and causes sustained Ca^{2+} release from intracellular stores in cells [9].

In view of these properties, a high specific activity ^{35}S-labelled InsPS$_3$ has been prepared as a novel radioligand for the InsP$_3$ receptor. The present data indicate, that this radioligand labels not only the InsP$_3$ receptor, as recognised by its natural ligand, but may also interact with another conformation of this receptor, and/or other sites in rat cerebellum.

Correspondence address: R.A.J. Challiss, Department of Pharmacology and Therapeutics, University of Leicester, P.O. Box 138, Medical Sciences Building, University Road, Leicester, LE1 9HN, UK

2. MATERIALS AND METHODS

2.1. *Materials*

[^{35}S(U)]InsPS$_3$ (25 Ci/mmol), [^{3}H]InsP$_3$ (17 Ci/mmol) and InsPS$_3$ were obtained as gifts from NEN DuPont (UK) Ltd. D-InsP$_3$ was purchased from RBI/SEMAT; D-Ins(2,4,5)P$_3$ and D-Ins(1,3,4,5)P$_4$ were purchased from Boehringer Mannheim; low m.wt. heparin was purchased from Sigma. L-InsP$_3$ was synthesized as in [8].

2.2. *Membrane preparation and [^{35}S]InsPS$_3$ and [^{3}H]InsP$_3$ binding studies*

A crude 'P$_2$' membrane fraction was prepared from rat cerebella in 20 mM $NaHCO_3$, 1 mM dithiothreitol, pH 8.0 as described previously [10,11]. Binding experiments were performed at 4°C in a final volume of 160 μl containing 25 mM Tris-HCl, 1 mM EDTA, pH 8.0. Isotopic dilution and competition experiments were performed in the presence of a single concentration of [^{35}S]InsPS$_3$ (3–6 nM) or [^{3}H]InsP$_3$ (2–3 nM) and various concentrations of competing ligands. Binding experiments were initiated by addition of 40–100 μg of cerebellar 'P$_2$' membrane protein and terminated after 30 min by centrifugation (12 000 × g, 4 min). The supernatant was carefully removed and the pellet solubilized before addition of scintillant for determination of radioactivity.

2.3. *Data analysis*

K_d and B_{max} values were derived by Scatchard transformation of the data obtained in isotope dilution experiments [10]. Competition isotherms were initially analysed by computer-assisted curve-fitting (ALLFIT; [12]) to obtain IC_{50} values. Curve-fitting routines were applied to compare one- and two-site modelling of the data-sets (see Table I).

3. RESULTS AND DISCUSSION

Although [^{35}S]InsPS$_3$ is resistant to both enzymic phosphorylation and dephosphorylation [10], under the present assay conditions there was no significant metabolism of either [^{3}H]InsP$_3$ or [^{35}S]InsPS$_3$. Specific binding of [^{3}H]InsP$_3$ to rat cerebellar membranes demonstrated characteristics virtually identical to those we have reported previously [4,11]. The high density of apparently homogenous binding sites was clearly stereo- and positionally specific and all competing ligands (including InsPS$_3$) displaced binding with slope factors close to unity (Fig. 1A, Table I). The properties of these sites are clearly compatible with those associated with the InsP$_3$ receptor linked to Ca^{2+} release from cerebellar microsomes [13] or those expressed in fibroblast L cells following gene transfection [7].

The properties of [^{35}S]InsPS$_3$ binding to cerebellar membranes under the same conditions appear to be more complex. Although the association rate of [^{35}S]InsPS$_3$ is rapid, and binding is reversible (data not shown), this radioligand labels a significantly higher number of cerebellar sites than [^{3}H]InsP$_3$ (Fig. 2). [^{35}S]InsPS$_3$ appears to label all its specific sites with an identical affinity (albeit 3-fold lower than [^{3}H]InsP$_3$). However, competition for these sites by a variety of inositol polyphosphates reveals a clear heterogeneity of the [^{35}S]InsPS$_3$-labelled sites. This is particularly evident for the InsP$_3$ interaction with these sites; the biphasic binding curve can be accurately described using computer-assisted curve fitting by an interaction with two sites possessing an almost 500-fold difference in affinity (Table I). However, all the other competing ligands displayed simple displacement curves which were better fitted by one-site compared to two-site models, although both Ins(2,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ displacement isotherms yielded slope factors significantly less than unity.

These data suggest therefore, that [^{35}S]InsPS$_3$ labels two populations of sites in cerebellar membranes with equal, or near-equal affinity. These sites are distinguished by their very different affinities for InsP$_3$. The site exhibiting high affinity for InsP$_3$ represents about 60% of the specific sites labelled with [^{35}S]InsPS$_3$ (Fig. 1B) and almost certainly is identical to the entire population labelled with [^{3}H]InsP$_3$. This is reflected in the excellent agreement of the differences in B_{max} values obtained between the radioligands (Fig. 2) and the proportion of sites labelled with [^{35}S]InsPS$_3$ and displaced with high affinity by InsP$_3$ (Fig. 1B).

The major question in the present study lies with the nature of the additional sites labelled with high affinity

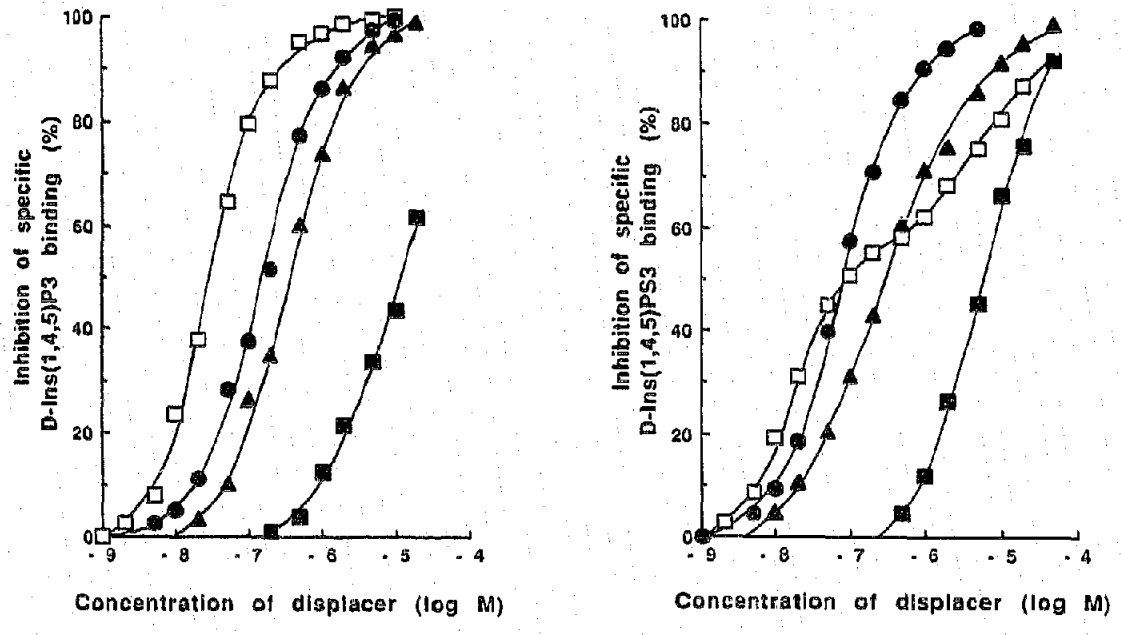

Fig. 1. Specificity of [^{3}H]InsP$_3$ and [^{35}S]InsPS$_3$ binding to cerebellar membranes. Assays were performed as described in section 2 with 2–3 nM [^{3}H]InsP$_3$ or 3–6 nM [^{35}S]InsPS$_3$ and the indicated concentrations of D-InsP$_3$ (□), L-InsP$_3$ (■), D-InsPS$_3$ (●) or D-Ins(2,4,5)P$_3$ (▲). Non-specific binding was defined by 50 μM DL-InsP$_3$ for [^{3}H]InsP$_3$, and 50 μM D-InsPS$_3$ for [^{35}S]InsPS$_3$ binding; similar non-specific binding values were obtained in the presence of 100 μg/ml low m.wt. heparin. Values are presented as means of at least 3 separate experiments performed in duplicate.

Table I

Specificity of [^{3}H]Ins(1,4,5)P_3 and [^{35}S]Ins(1,4,5)PS_3 binding to cerebellar membranes

	[^{3}H]InsP_3		[^{35}S]InsPS_3	
	IC_{50}	nH	IC_{50}	nH
D-Ins(1,4,5)P_3	26.6 ± 2.3	1.01 ± 0.04	K_H 21.9 ± 0.5 K_L 9700 ± 500	
L-Ins(1,4,5)P_3	11900 ± 900	0.95 ± 0.05	8730 ± 2440	1.04 ± 0.04
D-Ins(2,4,5)P_3	340 ± 22	0.98 ± 0.04	381 ± 15	0.76 ± 0.03
D-Ins(1,4,5)PS_3	141 ± 13	1.01 ± 0.03	68.1 ± 6.9	0.92 ± 0.02
D-Ins(1,3,4,5)P_4	681 ± 121	0.88 ± 0.06	584 ± 78	0.83 ± 0.05
Low m.wt. heparin*	1.8 ± 0.5	1.18 ± 0.09	1.6 ± 0.3	1.21 ± 0.10

Values are means ± S.E.M. for 3–6 separate experiments performed in duplicate using at least 10 concentrations of each displacing agent. All IC_{50} values are expressed as nM, except* μg/ml. nH is the slope factor. For Ins(1,4,5)P_3 displacement of specific [^{35}S]InsPS_3 binding the data were best fitted by a two-site model and K_H and K_L values are given.

by [^{35}S]InsPS_3, but not by [^{3}H]InsP_3. The rank order of inositol polyphosphate binding of this site (D-InsPS_3 > D-Ins(2,4,5)P_3 > D-InsP_3 > L-InsP_3) is dissimilar to that observed in Ca^{2+}-release experiments [9,14] and it is tempting to dismiss such sites as unrelated to the InsP_3 receptor linked to Ca^{2+} channels; however, alternative, low-capacity recognition sites for InsPS_3 do not come readily to mind.

InsPS_3 is a potent inhibitor of InsP_3-5-phosphatase [15], but the discrepancy between the K_i value (6 μM for DL-InsPS_3) at this enzyme and the present binding site K_d (0.06 μM), coupled to the low capacity of this membrane site argues against the labelling of 5-phosphatase. Furthermore, the very high affinity of heparin for this site, and the inability of this glycosaminoglycan to inhibit the 5-phosphatase enzyme [16] virtually eliminates

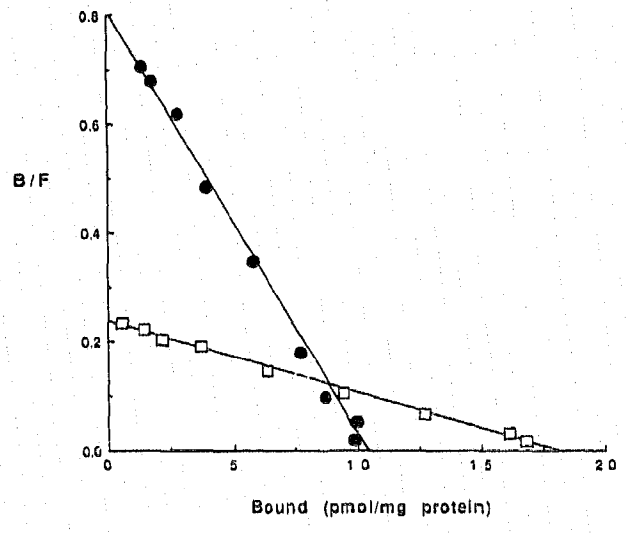

Fig. 2. Scatchard analysis of [^{3}H]InsP_3 and [^{35}S]InsPS_3 binding to cerebellar membranes. Data are presented for an isotope dilution experiment performed using [^{3}H]InsP_3 (●) or [^{35}S]InsPS_3 (□), using 9 concentrations of InsP_3 or InsPS_3 in duplicate, respectively. Scatchard analysis of this and 3–4 further data sets gave the following kinetic parameters for InsP_3 and InsPS_3 binding: K_d 19.7 ± 3.4 vs. 58.6 ± 9.1 nM, $P<0.01$; and B_{max} 12.8 ± 1.1 vs. 21.5 ± 2.1 pmol/mg protein, $P<0.01$.

this possibility. InsPS_3 possesses very low affinity for InsP_3-3-kinase [9], which is also a predominantly cytosolic enzyme, making the 3-kinase another unlikely candidate for the additional binding sites for [^{35}S]InsPS_3.

Since [^{35}S]InsPS_3 is the only available alternative radioligand for the InsP_3 receptor, the possibility of labelling another conformation of this receptor should be considered. The relationship between binding of InsP_3 to its receptor and Ca^{2+} channel opening appears to be complex [17]. Generally the binding affinity of InsP_3 is higher than its ability to release Ca^{2+} [3,7,16,18,19] (but see [14]). Indeed, recently it has been argued that the high-affinity sites may represent a desensitized form of the InsP_3 receptor [20]. Although InsPS_3 and Ins(2,4,5)P_3 are both agonists at the InsP_3 receptor [9], it is not known whether the relationship between binding, Ca^{2+} channel opening and desensitization is the same for these ligands as for InsP_3. Perhaps InsPS_3 and Ins(2,4,5)P_3 are less efficacious and bind with almost equal affinity to (and therefore stabilise) the proportions of two conformations of the receptor. Crucial to such an argument is the behaviour of heparin, which is a competitive antagonist at the InsP_3 receptor [16], and binds to both sites labelled with [^{35}S]InsPS_3 with equal affinity. Therefore, it could be argued that the antagonist heparin and the less efficacious agonists InsPS_3 and Ins(2,4,5)P_3 bind to both conformations of the InsP_3 receptor with equal affinities. In such a model only the natural second messenger InsP_3 (the most efficacious agonist) binds with higher affinity to one conformation of the receptor. Alternatively the extra sites specifically labelled by [^{35}S]InsPS_3 may represent quite separate entities. The recent observation by McCarren et al. [21] that InsPS_3, but not InsP_3 (even at high concentrations), could activate a novel K^+ conductance in hippocampal pyramidal cells may support this view.

Further investigation, including purification of these sites is required to distinguish between these possibilities. However, the present data represent the first results with an alternative radioligand to InsP_3 for

its receptor and reveals new information which may help in our understanding of the cellular roles of inositol polyphosphates.

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