RESEARCH PAPER

Interactions of antagonists with subtypes of inositol 1,4,5-trisphosphate (IP3) receptor

Huma Saleem1, Stephen C Tovey1, Tadeusz F Molinski2* and Colin W Taylor1

1Department of Pharmacology, University of Cambridge, Cambridge, UK, and 2Department of Chemistry, University of California, Davis, CA, USA

BACKGROUND AND PURPOSE
Inositol 1,4,5-trisphosphate receptors (IP3Rs) are intracellular Ca2+ channels. Interactions of the commonly used antagonists of IP3Rs with IP3R subtypes are poorly understood.

EXPERIMENTAL APPROACH
IP3-evoked Ca2+ release from permeabilized DT40 cells stably expressing single subtypes of mammalian IP3R was measured using a luminal Ca2+ indicator. The effects of commonly used antagonists on IP3-evoked Ca2+ release and 3H-IP3 binding were characterized.

KEY RESULTS
Functional analyses showed that heparin was a competitive antagonist of all IP3R subtypes with different affinities for each (IP3R3 > IP3R1 ≥ IP3R2). This sequence did not match the affinities for heparin binding to the isolated N-terminal from each IP3R subtype. 2-aminoethoxydiphenyl borate (2-APB) and high concentrations of caffeine selectively inhibited IP3R1 without affecting IP3 binding. Neither Xestospongin C nor Xestospongin D effectively inhibited IP3-evoked Ca2+ release via any IP3R subtype.

CONCLUSIONS AND IMPLICATIONS
Heparin competes with IP3, but its access to the IP3-binding core is substantially hindered by additional IP3R residues. These interactions may contribute to its modest selectivity for IP3R3. Practicable concentrations of caffeine and 2-APB inhibit only IP3R1. Xestospongins do not appear to be effective antagonists of IP3Rs.

Abbreviations
2-APB, 2-aminoethoxydiphenyl borate; AdA, adenophostin A; CLM, cytosol-like medium; CPA, cyclopiazonic acid; ER, endoplasmic reticulum; HBS, HEPES-buffered saline; IBC, IP3-binding core (residues 224-604); IP3, inositol 1,4,5-trisphosphate; IPTG, isobutyl-β-D-thiogalactoside; NT1-3, residues 1-604 of IP3R1-3; SERCA, sarcoplasmic/endoplasmic reticulum Ca2+-ATPase; TEM, Tris-EDTA medium

*This article was amended (11 July 2014) after publication to correct the forename of this author.

Introduction
Inositol 1,4,5-trisphosphate receptors (IP3R) are intracellular Ca2+ channels expressed in the membranes of the endoplasmic reticulum (ER) in most eukaryotic cells (Berridge, 1993; Taylor et al., 1999; Foskett et al., 2007; nomenclature follows Alexander et al., 2013). IP3Rs are essential links between the many extracellular signals that stimulate PLC and initiation...
of cytosolic Ca\(^{2+}\) signals triggered by IP\(_{3}\)-evoked Ca\(^{2+}\) release from the ER. Three genes encode closely related IP\(_{3}\)R subunits in vertebrates, whereas invertebrates have only a single IP\(_{3}\)R gene (Taylor et al., 1999). Each of the three vertebrate IP\(_{3}\)R subtypes encodes a large polypeptide of about 2700 residues, and they share about 70% amino acid sequence identity (Foskett et al., 2007). Within each IP\(_{3}\)R subunit, IP\(_{3}\) binds to a clam-like IP\(_{3}\)-binding core (IBC; residues 224–604 in IP\(_{3}\)R1) (Bosanac et al., 2002) near the N-terminus. IP\(_{3}\) binding to the IBC re-orient its relationship with the associated suppressor domain (residues 1–223). That rearrangement disrupts interactions between adjacent subunits within the tetrameric IP\(_{3}\)R leading to gating of the Ca\(^{2+}\)-permeable channel (Seo et al., 2012). This central channel of each tetrameric IP\(_{3}\)R is formed by transmembrane helices and their associated re-entrant loops. These pore-forming structures lie towards the C-terminal of each subunit. How IP\(_{3}\)-evoked re-arrangement of N-terminal domains of the IP\(_{3}\)R leads to opening of the pore is not yet resolved, although it is likely to be conserved in all IP\(_{3}\)R subtypes and broadly similar for the other major family of intracellular Ca\(^{2+}\) channels, ryanodine receptors (Seo et al., 2012).

Most cells express mixtures of IP\(_{3}\)R subtypes, although tissues differ in which complements of IP\(_{3}\)R subunits they express (Taylor et al., 1999). Furthermore, the subunits assemble into both homo-tetrameric and hetero-tetrameric structures (Wojcikiewicz and He, 1995). Although all IP\(_{3}\)R subtypes are built to a common plan and they are all regulated by IP\(_{3}\) and Ca\(^{2+}\) (Foskett et al., 2007; Seo et al., 2012), the subtypes are subject to different modulatory influences (Patterson et al., 2004; Higo et al., 2005; Foskett et al., 2007; Betzenhauser et al., 2008; Wagner and Yule, 2012) and they are likely to fulfill different physiological roles (Matsumoto et al., 1996; Hattori et al., 2004; Futatsugi et al., 2005; Tovey et al., 2008; Wei et al., 2009). It is, however, difficult to disentangle the physiological roles of IP\(_{3}\)R subtypes in cells that typically express complex mixtures of homo- and hetero-tetrameric IP\(_{3}\)Rs. There are no ligands of IP\(_{3}\)Rs that usefully distinguish among IP\(_{3}\)R subtypes (Saleem et al., 2012; 2013) and nor are there effective antagonists that lack serious side effects (Michelangeli et al., 1995). Heparin (Ghosh et al., 1988), caffeine (Parker and Ivorra, 1991), 2-aminoethoxydiphenyl borate (2-APB) (Maruyama et al., 1997) and Xestospongin (Gafni et al., 1997) have all been widely used to inhibit IP\(_{3}\)-evoked Ca\(^{2+}\) release, but each has its limitations (see Results). Furthermore, the interactions of these antagonists with IP\(_{3}\)R subtypes have not been assessed. Peptides derived from myosin light-chain kinase (Nadif Kasri et al., 2006; Sun and Taylor, 2008), the N-terminal of IP\(_{3}\)R1 (Sun et al., 2013) or the BH4 domain of bcl-2 (Monaco et al., 2012) also inhibit IP\(_{3}\)-evoked Ca\(^{2+}\) release. These peptides are unlikely to provide routes to useful IP\(_{3}\)R antagonists because they are effective only at high concentrations and they need to be made membrane-permeable. A naturally occurring protein that inhibits IP\(_{3}\) binding to IP\(_{3}\)R, IBBIT (Ando et al., 2003), has the same limitations as an experimental tool, and it is effective only when phosphorylated. Many other drugs inhibit IP\(_{3}\)-evoked Ca\(^{2+}\) release, but none of these has found widespread use (see Michelangeli et al., 1995; Bultynck et al., 2003).

In the present study, we provide the first systematic analysis of the interactions between IP\(_{3}\)R subtypes and each of the commonly used antagonists. We use DT40 cell lines stably expressing only a single mammalian IP\(_{3}\)R subtype to define the effects of these antagonists on IP\(_{3}\)-evoked Ca\(^{2+}\) release via each IP\(_{3}\)R subtype.

## Methods

### Measurement of IP\(_{3}\)-evoked Ca\(^{2+}\) release

We used DT40 cells lacking endogenous IP\(_{3}\)Rs (Sugawara et al., 1997), but stably expressing rat IP\(_{3}\)R1 (GenBank accession number GQ233032.1; Pantazaka and Taylor, 2011), mouse IP\(_{3}\)R2 (GU980658.1; Tovey et al., 2010) or rat IP\(_{3}\)R3 (GQ233031.1; Rahman et al., 2009). Cells were grown in suspension in RPMI 1640 medium supplemented with 10% FBS, 1% heat-inactivated chicken serum, 2 mM glutamine and 50 μM 2-mercaptoethanol at 37°C in humidified air containing 5% CO\(_{2}\). Cells were used or passaged when they reached a density of ~1.5 × 10\(^{6}\) cells mL\(^{-1}\).

A low-affinity Ca\(^{2+}\) indicator trapped within the ER of permeabilized DT40 cells was used to measure IP\(_{3}\)-evoked Ca\(^{2+}\) release (Tovey et al., 2006; Saleem et al., 2012). Briefly, the ER was loaded with indicator by incubating cells (~5 × 10\(^{6}\) mL\(^{-1}\)) in the dark with Mag-fluo-4AM (20 μM) in HEPES-buffered saline (HBS) containing 0.02% (v/v) Pluronic F127 for 1 h at 22°C. HBS had the following composition: 135 mM NaCl, 5.9 mM KCl, 11.6 mM HEPES, 1.5 mM CaCl\(_{2}\), 11.5 mM glucose, 1.2 mM MgCl\(_{2}\), pH 7.3. After permeabilization of the plasma membrane with saponin (10 μg·mL\(^{-1}\), 4 min, 37°C) in Ca\(^{2+}\)-free cytosol-like medium (CLM), permeabilized cells were washed (650 × g, 2 min) and resuspended (~10\(^{7}\) mL\(^{-1}\)) in Mg\(^{2+}\)-free CLM containing carbonyl cyanide 4-(trifluoromethoxy) phenyldihydrazide (FCCP, 10 μM) to inhibit mitochondria, and supplemented with CaCl\(_{2}\) to give a final free [Ca\(^{2+}\)] of 220 nM after addition of 1.5 mM MgATP. Ca\(^{2+}\)-free CLM had the following composition: 2 mM NaCl, 140 mM KCl, 1 mM EGTA, 20 mM PIPES, 2 mM MgCl\(_{2}\), pH 7.0. Permeabilized cells were then distributed into 96-well plates (50 μL, 5 × 10\(^{4}\) cells per well), centrifuged (300 × g, 2 min) and used for experiments at 20°C. Addition of MgATP (1.5 mM) allowed Ca\(^{2+}\) uptake by the ER, which was monitored at intervals of ~1 s using a FlexStation-3 plate reader (MDS Analytical Devices, Berkshire, UK; Tovey et al., 2006). After 2 min, when the ER had loaded to steady-state with Ca\(^{2+}\), IP\(_{3}\) was added with CPA (10 μM) to inhibit further Ca\(^{2+}\) uptake. IP\(_{3}\)-evoked Ca\(^{2+}\) release was expressed as a fraction of that released by ionomycin (1 μM; Tovey et al., 2006). Similar methods were used to measure IP\(_{3}\)-evoked Ca\(^{2+}\) release from intact or permeabilized HEK cells (Tovey et al., 2008). The timings of antagonist additions are described in the figure legends. The affinity of each competitive antagonist (pKD\(_{50}\)) was determined from the intercept on the abscissa of the Schild plot.

Concentration–effect relationships were fitted to Hill equations using Prism (version 5.0, GraphPad, San Diego, CA, USA), from which Hill coefficients (h), the fraction of the intracellular Ca\(^{2+}\) stores released by a maximally effective concentrations of IP\(_{3}\), and pEC\(_{50}\) values were calculated.
Expression of N-terminal fragments of IP₃ receptors

The plasmids used for bacterial expression of GST-tagged N-terminal fragments (NT, residues 1–604) of rat IP₃R1, mouse IP₃R2 and rat IP₃R3 have been described, and their coding sequences have been confirmed (Khan et al., 2013). Plasmids were transformed into BL21-CodonPlus (DE3)-RILP competent cells (Rossi and Taylor, 2011), and grown for 12 h at 37°C in 20 mL of Luria-Bertani (LB) medium containing carbenicillin (50 μg·mL⁻¹). The volume of medium was then increased to 1 L, and the incubation was continued at 37°C for 3–4 h until the OD₅₇₀ reached 1–1.5. Protein expression was induced by addition of IPTG (0.5 mM) for 20 h at 15°C. Bacteria were harvested (6000×g, 5 min), washed twice with cold PBS, and the pellet was suspended (~10⁹ cells·mL⁻¹) in 50 mL of Tris-EDTA medium (TEM: 50 mM Tris, 1 mM EDTA, pH 8.3) supplemented with 10% PopCulture, 1 mM 2-mercaptoethanol and protease inhibitor cocktail (Roche, Burgess Hill, West Sussex, UK; 1 tablet per 50 mL). After lysis by incubation with lysozyme (100 μg·mL⁻¹) and RNase (10 μg·mL⁻¹) for 30 min on ice and then sonication (Transsonic T420 water bath sonicator, Camlab, Cambridge, UK; sonicator, 50 Hz, 30 s), the supernatant was recovered (30,000×g, 60 min, 4°C). The supernatant was mixed with glutathione Sepharose 4B beads (50:1, v/v, lysate : beads) and incubated with gentle end-over-end rotation (6 rpm) for 45 min at 4°C. The beads were then loaded onto a PD-10 column and washed twice with PBS and twice with PreScission cleavage buffer (GE Healthcare) supplemented with 1 mM DTT. The column was then incubated with 0.5 mL of PreScission cleavage buffer containing 1 mM DTT and 80 units of GST-tagged PreScission protease for 12 h at 4°C using gentle end-over-end rotation. The PreScission protease cuts an engineered cleavage site to release the NT free of its GST tag. The eluted NT (~15 mg protein·mL⁻¹) was rapidly frozen and stored at ~80°C.

³H-IP₃ binding

Equilibrium competition binding assays were performed at 4°C in 500 μL of CLM (final free [Ca²⁺] = 220 nM) containing purified NT (30 μg) or cerebellar membranes (5 mg protein), ³H-IP₃ (1.5 nM) and appropriate concentrations of competing ligand. Reactions were terminated after 5 min by centrifugation (20,000×g, 5 min) for membranes, or by centrifugation after addition of poly(ethylene glycol)-8000 [30% (w/v), 500 μL] and γ-globulin (30 μL, 25 mg·mL⁻¹) for NT. The pellet was washed (500 μL of 15% PEG or CLM) and solubilized in 200 μL of CLM containing 1% (v/v) Triton-X-100 before liquid scintillation counting. Non-specific binding, whether determined by addition of 10 μM IP₃ or by extrapolation of competition curves to infinite IP₃ concentration, was <10% of total binding. Results were fitted to Hill equations using Prism, from which IC₅₀ values were calculated. Kᵦ (equilibrium dissociation constant) and pKᵦ (–logKᵦ) values were calculated from IC₅₀ values using the Cheng and Prusoff equation (Cheng and Prusoff, 1973).

Data analysis

Statistical comparisons used pEC₅₀ (or pKᵦ) values. For paired comparison of the effect of an antagonist, ΔpEC₅₀ values were calculated, where ΔpEC₅₀ = pEC₅₀ᵦ - pEC₅₀ᵦ-antagonist. Results are expressed as means ± SEM from n independent experiments. Statistical comparisons used paired Student’s t-test or ANOVA followed by Bonferroni’s test, with P < 0.05 considered significant.

Materials

Sources of many reagents were specified in earlier publications (Rossi et al., 2010a,b; Saleem et al., 2012). IP₃ was from Enzo Life Sciences (Exeter, UK). ³H-IP₃ (19.3 Ci·mmol⁻¹) was from PerkinElmer (Buckinghamshire, UK). Heparin (from porcine mucosa, M, 5000) and cyclopiazonic acid (CPA) were from Fisher Scientific (Loughborough, UK). Caffeine, 2-APB, lysozyme, RNase, γ-globulin and poly(ethylene glycol)-8000 were from Sigma-Aldrich (Dorset, UK). Xestospongins C and D were from Calbiochem ( Gibbstown, NJ, USA) or isolated and characterized as previously described (Gafni et al., 1997). PopCulture was from Novagen (Darmstadt, Germany). Simple Blue stain was from Invitrogen (Renfrewshire, Scotland). Dioxin-free isopropyl-β-D-thiogalactoside (IPTG), and Luria–Bertani agar and broth were from Formedium (Norfolk, UK). Glutathione Sepharose 4B beads and GST-tagged PreScission protease were from GE Healthcare (Buckinghamshire, UK). Carbenicillin was from Melford Laboratories (Suffolk, UK). BL21-CodonPlus (DE3)-RILP competent bacteria were from Agilent Technology (Berkshire, UK).

Results

Heparin is a competitive antagonist with different affinities for IP₃ receptor subtypes

Heparin is a competitive antagonist of IP₃-evoked Ca²⁺ release ( Ghosh et al., 1988), but it is membrane-impermeable and it has many additional effects. These include uncoupling of receptors from G-proteins (Willuweit and Aktories, 1988; Dasso and Taylor, 1991), stimulation of ryanodine receptors (Ehrlich et al., 1994) and inhibition of IP₃ 3-kinase (Guillemette et al., 1989). To assess the effects of heparin on each IP₃R subtype, permeabilized DT40 cells expressing each of the three IP₃R subtypes were incubated with heparin for 35 s. The effect of IP₃ on Ca²⁺ release from the intracellular stores was then assessed (Figure 1A). In permeabilized DT40-IP₃R1 cells, heparin caused parallel rightward shifts of the concentration–response relationship for IP₃-evoked Ca²⁺ release (Figure 1B). Schild plots, which had slopes of 0.95 ± 0.02 (mean ± SEM, n = 3), established that the equilibrium dissociation constant (Kᵦ) for heparin was 4.1 μg·mL⁻¹ (pKᵦ = 5.39 ± 0.00) (Figure 1C). Similar results were obtained when adenosphostin A (AdA), a high-affinity agonist of IP₃Rs (Rossi et al., 2010b; Saleem et al., 2013), was used to stimulate Ca²⁺ release. The Schild plots had slopes of 0.94 ± 0.03 (n = 3) and the Kᵦ for heparin was 6.9 μg·mL⁻¹ (pKᵦ = 5.16 ± 0.05) (Figure 1D and E; Table 1).

A similar analysis of the effects of heparin on IP₃-evoked Ca²⁺ release from permeabilized DT40-IP₃R2 cells was also consistent with competitive antagonism. The slope of the Schild plots was 0.97 ± 0.06 (n = 3) and the Kᵦ for heparin was 22 μg·mL⁻¹ (pKᵦ = 4.66 ± 0.07) (Figure 2A and B). IP₃R3 are less sensitive to IP₃ than the other subtypes (Iwai et al., 2007; Saleem et al., 2013) (Table 1). This made it difficult to add IP₃.
at concentrations sufficient to achieve maximal Ca\(^{2+}\) release in the presence of heparin concentrations greater than 5 \(\mu\)g·mL\(^{-1}\) (Figure 2C). Assuming the maximal response to IP\(_3\) was unaffected by heparin, we used the concentrations of IP\(_3\) that evoked release of 40% of the intracellular stores to construct Schild plots for IP\(_3\)R3. The results were consistent with competitive antagonism. The slope of the Schild plots was 1.14 ± 0.41 (n = 3) and the K\(_D\) for heparin was 2.8 \(\mu\)g·mL\(^{-1}\) (pK\(_D\) = 5.55 ± 0.09) (Figure 2D and Table 1). AdA has ∼10-fold higher affinity than IP\(_3\) for all three IP\(_3\)R subtypes (Table 1) (Rossi et al., 2010a; Saleem et al., 2013), and we have shown that the affinity of heparin for IP\(_3\)R1 is similar whether IP\(_3\) or AdA is used to evoke Ca\(^{2+}\) release (Figure 1B–E). To obtain an independent measure of the affinity of IP\(_3\)R3 for heparin, free of the problems associated with using IP\(_3\), we therefore repeated the Schild analysis using AdA to stimulate Ca\(^{2+}\) release. These conditions provided complete concentration–effect relationships for AdA at a wider range of heparin concentrations (Figure 2E). The Schild plots had a slope of 0.98 ± 0.04 (n = 6) and the K\(_D\) for heparin was 2.1 \(\mu\)g·mL\(^{-1}\) (pK\(_D\) = 5.68 ± 0.04) (Figure 2F and Table 1). The affinity of heparin for IP\(_3\)R3 was therefore similar whether measured using IP\(_3\) or AdA to evoke Ca\(^{2+}\) release.

These functional analyses establish that heparin is a competitive antagonist of IP\(_3\) at all three IP\(_3\)R subtypes, but with different affinities for each (IP\(_3\)R3 > IP\(_3\)R1 ≥ IP\(_3\)R2) (Table 1).
The results are consistent with an analysis of IP3 binding to mammalian IP3R expressed in Sf9 cells (Nerou et al., 2001), where the pKD values and rank order of heparin affinity (IP3R3 > IP3R1 ∼ IP3R2) were similar to those from the present functional analyses (Table 1).

Heparin binding is not solely determined by its interactions with the IP3-binding site

Activation of IP3Rs is initiated by binding of IP3 to the IP3-binding core (IBC, residues 224-604 of IP3R1) within the N-terminal region of each IP3R subunit (see Introduction) (Seo et al., 2012). The only contacts between IP3 and the IP3R are mediated by residues within the IBC (Bosanac et al., 2002), but interaction of the N-terminal suppressor domain (residues 1-223) with the IBC reduces its affinity for IP3. Hence, the IBCs from different IP3R subtypes bind IP3 with similar affinity, whereas the larger N-terminal regions (NT, residues 1-604) have lower affinities that differ between subtypes. The NTs bind IP3 with two- to threefold greater affinities than those of full-length IP3Rs, but the NTs and full-length IP3Rs have the same rank order of affinities for IP3, NT2 > NT1 > NT3 (Iwai et al., 2007; Rossi et al., 2009). The results shown in Figure 3A and B, which show IP3 binding to bacterially expressed NTs from each of the three IP3R subtypes (NT1-3), confirm previous results. Surprisingly, however, equilibrium-competition binding of heparin to NTs in medium that matches that used to measure IP3-evoked Ca2+ release was not consistent with the results obtained from functional analyses (Figure 3C). The affinity of the NT for heparin was up to 2000-fold greater than that measured in functional analyses, and the rank order of affinity for heparin was different for NTs (NT2 > NT1 > NT3) and full-length IP3Rs (IP3R3 > IP3R1 ≥ IP3R2) (Nerou et al., 2001; Tables 1 and 2).

IP3R1 is the major (>99%) subtype in cerebellar membranes (Wojcikiewicz, 1995). Equilibrium-competition binding of heparin to cerebellar membranes in CLM established that the affinity of IP3R1 for heparin (pK D = 5.61 ± 0.13, n = 3) was similar to that derived from Schild analysis of DT40-IP3R1 cells (pK D = 5.39 ± 0.00, n = 3) and similar to that reported for heparin binding to IP3R1 heterologously

### Table 1

| Functional analysis | IP3 or AdA | heparin | *Binding | pEC50(IP3)− pKD(heparin) |
|---------------------|------------|---------|----------|--------------------------|
| **IP3R1** | IP3 | 7.47 ± 0.02 | 5.39 ± 0.00 | 4.66 | 2.08 ± 0.02 |
| | AdA | 8.35 ± 0.03 | 5.16 ± 0.05 | – | – |
| **IP3R2** | IP3 | 6.82 ± 0.04 | 4.66 ± 0.07 | 4.62 | 2.16 ± 0.09* |
| **IP3R3** | IP3 | 6.66 ± 0.07 | 5.55 ± 0.09 | 5.34 | 1.11 ± 0.08* |
| | AdA | 7.71 ± 0.01 | 5.68 ± 0.04 | – | – |

The affinities for heparin determined from equilibrium-competition binding with 3H-IP3 to Sf9 membranes expressing IP3R1-3 are reproduced from (Nerou et al., 2001). The batch of heparin used for those binding studies was different from that used for the work reported here. The final column (derived from the results shown in Figures 1B,C and 2A–D) shows paired comparisons of pEC50(IP3) – pKD(heparin) as a means of reporting the relative effectiveness with which heparin might be expected to block IP3-evoked Ca2+ release via different IP3R subtypes. The results suggest that IP3R3 is likely to be substantially more susceptible to inhibition than IP3R1 or IP3R2.

*Denotes a value significantly different from IP3R1 in the final column (P < 0.05).

### Table 2

Heparin and IP3 binding to N-terminal fragments of IP3 receptor subtypes

| NT1  | NT2  | NT3  | IP3R1 |
|------|------|------|-------|
| IP3  | 7.76 ± 0.07 | 8.67 ± 0.15 | 7.39 ± 0.08 | 7.13 ± 0.08 |
| Heparin | 7.42 ± 0.09 | 7.95 ± 0.32 | 6.59 ± 0.09* | 5.61 ± 0.13 |

Equilibrium-competition binding with 3H-IP3 was used to measure pKD values for IP3 (as M) and heparin (as g mL−1) binding to purified NT1-3 and cerebellar membranes (IP3R1). Results are means ± SEM from three to six experiments.

*Denotes a significant difference from NT1 (P < 0.05) for pKDheparin.

From experiments similar to those shown in Figures 1 and 2, AdA or IP3-evoked Ca2+ release and their sensitivity to heparin were used to determine pEC50 (as M) and pKD (as sgm L−1) for DT40 cells expressing IP3R1, IP3R2 or IP3R3. Results are means ± SEM from three independent experiments (six for IP3R3).

*The affinities for heparin determined from equilibrium-competition binding with 3H-IP3 to Sf9 membranes expressing IP3R1-3 are reproduced from (Nerou et al., 2001). The batch of heparin used for those binding studies was different from that used for the work reported here. The final column (derived from the results shown in Figures 1B,C and 2A–D) shows paired comparisons of pEC50(IP3) – pKD(heparin) as a means of reporting the relative effectiveness with which heparin might be expected to block IP3-evoked Ca2+ release via different IP3R subtypes. The results suggest that IP3R3 is likely to be substantially more susceptible to inhibition than IP3R1 or IP3R2.

*Denotes a value significantly different from IP3R1 in the final column (P < 0.05).
Heparin is a competitive antagonist with different affinities for types 2 and 3 IP$_3$ receptors. (A) Concentration-dependent release of Ca$^{2+}$ by IP$_3$ from the intracellular stores of DT40-IP$_3$R2 cells in the presence of the indicated concentrations of heparin added 35 s before IP$_3$. (B) Schild plot of the results. (C–F) Similar analyses of DT40-IP$_3$R3 cells stimulated with IP$_3$ (C, D) or AdA (E, F). For D, where maximal attainable concentrations of IP$_3$ were insufficient to evoke maximal responses in the presence of the highest concentrations of heparin, the Schild plot shows dose ratios calculated from IP$_3$ concentrations that evoked 40% Ca$^{2+}$ release. Results (A–F) are mean ± SEM from three experiments.
expressed in SF9 cells (Nerou et al., 2001), but very different to the heparin affinity of NT1 (pK\textsubscript{D} = 7.42 ± 0.09, n = 3) (Tables 1 and 2). These results demonstrate that the IBC is not the only determinant of competitive heparin binding to IP\textsubscript{3}Rs and suggest either that access of heparin to the IBC is influenced by additional interactions or that heparin binding to an additional site affects IP\textsubscript{3}R gating.

**2-APB selectively inhibits Ca\textsuperscript{2+} release via type 1 IP\textsubscript{3} receptors without affecting IP\textsubscript{3} binding**

2-APB is membrane-permeant and is often used to inhibit IP\textsubscript{3}-evoked Ca\textsuperscript{2+} release (Maruyama et al., 1997; Missiaen et al., 2001; Bilmen et al., 2002), but it has many additional effects. These include modulation of store-operated Ca\textsuperscript{2+} entry (Goto et al., 2010) and inhibition of the sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) that mediates Ca\textsuperscript{2+} sequestration by the ER (Missiaen et al., 2001; Bilmen et al., 2002; Bultynck et al., 2003). In permeabilized DT40-IP\textsubscript{3}R1 cells, 50 μM 2-APB had no effect on Ca\textsuperscript{2+} uptake by the ER, although higher concentrations reduced the steady-state Ca\textsuperscript{2+} content (Figure 4A and B). This is consistent with high concentrations of 2-APB causing inhibition of SERCA.

In permeabilized DT40-IP\textsubscript{3}R1 cells, 2-APB caused a concentration-dependent inhibition of IP\textsubscript{3}-evoked Ca\textsuperscript{2+} release (Figure 4C). With 50 μM 2-APB, the highest concentration

**Figure 3**

Heparin binding is not solely determined by its interactions with the IP\textsubscript{3}-binding core. (A) Immunoblots of purified NT1-3 (∼15 μL protein per lane) using an antiserum that recognizes a conserved sequence within all three IP\textsubscript{3}R subtypes (residues 62–75 in rat IP\textsubscript{3}R1). The positions of Mr, markers (kDa) are shown alongside each blot. (B, C) Equilibrium-competition binding of IP\textsubscript{3} (B) and heparin (C) to purified NT1-3 in CLM. (D, E) Similar analyses of binding to cerebellar membranes (IP\textsubscript{3}R1). Results (B–E) are means ± SEM from three to six experiments.
that avoids inhibition of Ca\(^{2+}\) uptake, there was an approximately sevenfold decrease in IP\(_3\) sensitivity (ΔpEC\(_{50}\) = 0.84 ± 0.12) with no effect on the maximal response to IP\(_3\) (Figure 4C). The same concentration of 2-APB (50 μM) had no significant effect on IP\(_3\)-evoked Ca\(^{2+}\) release from permeabilized DT40-IP\(_3\)R1-3 cells alone or with the indicated concentrations of 2-APB added 35 s before IP\(_3\). (F) Binding of \(^3\)H-IP\(_3\) (1.5 nM) to cerebellar membranes (IP\(_3\)R1), with 3 μM IP\(_3\) (non-specific) or with 2-APB. Results (B–F) are means ± SEM from three to nine experiments. *P < 0.05, significantly different from control.

Figure 4
2-APB selectively inhibits Ca\(^{2+}\) release via type 1 IP\(_3\) receptors. (A) Ca\(^{2+}\) uptake into the intracellular stores of permeabilized DT40-IP\(_3\)R1 cells is shown after addition of ATP in the presence of the indicated concentrations of 2-APB. Each trace is the average from two wells in a single plate. (B) Summary results show effects of 2-APB on Ca\(^{2+}\) contents measured 180 s after addition of ATP. (C–E) Concentration-dependent effects of IP\(_3\) on Ca\(^{2+}\) release from permeabilized DT40-IP\(_3\)R1-3 cells alone or with the indicated concentrations of 2-APB added 35 s before IP\(_3\). (F) Binding of \(^3\)H-IP\(_3\) (1.5 nM) to cerebellar membranes (IP\(_3\)R1), with 3 μM IP\(_3\) (non-specific) or with 2-APB. Results (B–F) are means ± SEM from three to nine experiments. *P < 0.05, significantly different from control.

Caffeine is a low-affinity antagonist of type 1 IP\(_3\) receptors
Caffeine is another membrane-permeant antagonist of IP\(_3\)-evoked Ca\(^{2+}\) release (Parker and Ivorra, 1991; Brown et al., 1992; Bultynck et al., 2003; Laude et al., 2005), but it is effective only at high (mM) concentrations and it has many additional effects (Michelangeli et al., 1995; Taylor and Tovey, 2010). These include stimulation of ryanodine receptors, inhibition of cyclic nucleotide phosphodiesterases, competitive antagonism of adenosine receptors, and effects on the fluorescence of some Ca\(^{2+}\) indicators (Brown et al., 1992; Ehrlich et al., 1994; Michelangeli et al., 1995; McKemy et al., 2000; Taylor and Tovey, 2010). High concentrations of caffeine (10–70 mM) inhibited Ca\(^{2+}\) release via IP\(_3\)R1 (Figure 5A) without affecting \(^3\)H-IP\(_3\) binding to cerebellar membranes.
Evoked Ca\(^{2+}\) affecting IP3 binding. Revealed a statistically significant (P < 0.025, one-tailed test), inhibition of IP3-evoked Ca\(^{2+}\) release by the antagonist. The results with Xestospongins C and D are pooled from experiments that included pre-incubation periods of 7 and 12 min (see Supporting Information Table S1). Results are means ± SEM from three to nine experiments.

* Denotes a value significantly greater than 0 (P < 0.025, one-tailed test).

ND, not determined.

**Xestospongins do not effectively inhibit IP3-evoked Ca\(^{2+}\) release**

Xestospongin C is membrane-permeant and was reported to inhibit IP3-evoked Ca\(^{2+}\) release from cerebellar microsomes (IC\(_{50}\) = 358 nM) without affecting IP3 binding (Gafni et al., 1997). Xestospongin D is less potent. Higher concentrations of Xestospongin C (10–20 μM) were required to inhibit IP3-evoked Ca\(^{2+}\) release in intact cells. We assessed the effects of Xestospongins C and D from different suppliers (see Materials) on Ca\(^{2+}\) release mediated by each of the three IP3R subtypes.

Pre-incubation of permeabilized DT40 cells with Xestospongin C (5–20 μM from either source) for 5–12 min before addition of IP3 had no significant effect on IP3-evoked Ca\(^{2+}\) release mediated by any of the three IP3R subtypes (Supporting Information Table S1). Figure 6A–C show IP3-evoked Ca\(^{2+}\) release after a 5 min pre-incubation with 5 μM purified Xestospongin C (Gafni et al., 1997). It had no significant effect on either the response to IP3 (Figure 6A–C) or the Ca\(^{2+}\) content of the stores (Figure 6D). Pooling all experiments with the highest concentration of Xestospongin C (20 μM, n = 6) revealed a statistically significant (P < 0.025, one-tailed test), but very small, inhibition of the maximal response from IP3R1, and an even smaller increase in pEC50 for IP3R1 and IP3R3 (Table 3 and Supporting Information Table S1).

Similar treatments with Xestospongin D (10–20 μM from either source) for 5–12 min caused a modest, but statistically significant (P < 0.025, one-tailed test), inhibition of IP3-evoked Ca\(^{2+}\) release via IP3R1 (Supporting Information Table S1). Figure 6E–H show that a 5 min pre-incubation with 10 μM purified Xestospongin D (Gafni et al., 1997) had no effect on the Ca\(^{2+}\) content of the intracellular stores, but modestly inhibited IP3-evoked Ca\(^{2+}\) release via IP3R1 (P < 0.025, one-tailed test, Figure 6E). Pooling results with the highest concentration of Xestospongin D (20 μM, n = 6) revealed a statistically significant (P < 0.025, one-tailed test), but very small, inhibition of the maximal response from IP3R1 and IP3R2, and a tiny increase in the pEC50 for IP3R1 and IP3R3 (Table 3 and Supporting Information Table S1). These small inhibitory effects of Xestospongins C and D are not sufficient to be useful, and nor are they sufficient to reliably assess whether there is any subtype-selective interaction of Xestospongins with IP3Rs.

We also assessed the effects of Xestospongins on IP3-evoked Ca\(^{2+}\) release from intact and permeabilized HEK cells. IP3 caused a concentration-dependent release of Ca\(^{2+}\) from the intracellular stores of permeabilized HEK cells (Figure 7A and B). Pre-incubation of the permeabilized cells for 5 min with Xestospongin C (5 μM) or Xestospongin D (10 μM) had no effect on the Ca\(^{2+}\) content of the intracellular stores (Figure 7C) or the Ca\(^{2+}\) release evoked by IP3 (Figure 7A and B). Carbachol, via endogenous M\(_3\) muscarinic receptors of HEK cells, stimulates PLC and thereby IP3-evoked Ca\(^{2+}\) release. Pre-incubation of HEK cells with Xestospongin C or D (10 μM) for 30 min had no significant effect on the Ca\(^{2+}\) signals evoked by any concentration of carbachol (Figure 7D). This conflicts with published results from similar experiments, where Xestospongin C (10 μM for 30 min) caused substantial, though incomplete, inhibition of carbachol-evoked Ca\(^{2+}\) signals (Kurian et al., 2009). It is, however noteworthy, in

### Table 3

|            | IP3R1 |         | IP3R2 |         | IP3R3 |         |
|------------|-------|---------|-------|---------|-------|---------|
|            | ΔpEC50 (M) | ΔMax (%) | ΔpEC50 (M) | ΔMax (%) | ΔpEC50 (M) | ΔMax (%) |
| Heparin, 400 μg·mL\(^{-1}\) | 1.88 ± 0.05* | −7 ± 2 | ND | − | 2.34 ± 0.07* | −3 ± 3 |
| Heparin, 800 μg·mL\(^{-1}\) | ND | − | 1.49 ± 0.09* | −4 ± 2 | ND | − |
| Caffeine, 70 mM | 0.61 ± 0.07* | 12 ± 4 | −0.2 ± 0.07 | −1 ± 0 | −0.07 ± 0.08 | 0 ± 5 |
| 2-APB, 50 μM | 0.84 ± 0.12* | 0 ± 4 | −0.05 ± 0.10 | 0 ± 4 | 0.02 ± 0.09 | 8 ± 4 |
| Xestospongin C, 20 μM | 0.21 ± 0.10* | 6 ± 2* | −0.06 ± 0.04 | 1 ± 1 | 0.12 ± 0.03* | 1 ± 2 |
| Xestospongin D, 20 μM | 0.26 ± 0.09* | 18 ± 2* | −0.15 ± 0.05 | 8 ± 3* | 0.21 ± 0.10* | 2 ± 2 |

Summary of the functional analyses of antagonists on IP3-evoked Ca\(^{2+}\) release from permeabilized DT40-IP3R1-3 cells. The pEC50 values for IP3 and the maximal Ca\(^{2+}\) release are each expressed relative to the response evoked in paired controls without antagonist (Δ = control − response with antagonist). A positive Δ value demonstrates an inhibition of IP3-evoked Ca\(^{2+}\) release by the antagonist. The results with Xestospongins C and D are pooled from experiments that included pre-incubation periods of 7 and 12 min (see Supporting Information Table S1). Results are means ± SEM from three to nine experiments.

* Denotes a value significantly greater than 0 (P < 0.025, one-tailed test).

ND, not determined.
light of evidence that Xestospongins have been reported to inhibit Ca$^{2+}$ uptake into the ER (Castonguay and Robitaille, 2002; Solovyova et al., 2002), that in the experiments from Kurian et al. HEK cells were incubated with Xestospongin for 30 min in Ca$^{2+}$-free medium, while in our experiments extracellular free Ca$^{2+}$ was removed immediately before stimulation with carbachol. The discrepant results may, therefore, reflect an increased loss of Ca$^{2+}$ from intracellular stores during prolonged exposure to Xestospongin in Ca$^{2+}$-free medium.

Discussion

Acute analyses of IP$_3$-evoked Ca$^{2+}$ signalling are handicapped by lack of effective and selective antagonists (Michelangeli et al., 1995; Bultynck et al., 2003). Furthermore, the subtype-selectivity and in many cases the mechanism of action of the antagonists that are routinely used are not known. We have addressed these issues by examining the functional effects of the most widely used antagonists of IP$_3$R in cells expressing only a single IP$_3$R subtype.

Heparin is a competitive antagonist of IP$_3$ at cerebellar IP$_3$Rs (Ghosh et al., 1988), most likely because as a polyanion it may partially mimic the phosphate groups of IP$_3$. That is consistent with evidence that other polyanions, like decavanadate, ATP and dextran sulphate, can also competitively inhibit IP$_3$Rs (Bultynck et al., 2003). Our functional analyses establish that heparin is a competitive antagonist of all three IP$_3$R subtypes, but with modestly different affinities for each (IP$_3$R3 $>$ IP$_3$R1 $\geq$ IP$_3$R2) (Figures 1 and 2; Table 1). The affinities of IP$_3$R subtypes for heparin derived from functional analyses were similar to those determined from equilibrium-competition binding to native IP$_3$R1 (Figure 3E) or to heter-
ologously expressed IP₃R subtypes (Table 1). However, heparin bound to N-terminal fragments (NT) of IP₃Rs that include the IBC with an affinity that was up to 2000-fold greater than its affinity for the corresponding full-length IP₃R (Tables 1 and 2). Furthermore, the rank order of heparin affinity for IP₃R1-3 and NT1-3 was different. We conclude that heparin inhibits IP₃-evoked Ca²⁺ release by competing with IP₃, but its access to the IBC is substantially impaired in full-length IP₃Rs within native membranes. Phospholipids may contribute to the substantially lesser affinity of heparin for IP₃R in native membranes by electrostatically repelling the approach of polyanionic heparin to the membrane-bound IBC. In addition, we suggest that charged residues on the IP₃R surface may differentially influence heparin access to the IBC of each IP₃R subtype and thereby contribute to the modestly different affinities of heparin for IP₃R subtypes (Table 1). Our observations have more general significance for analyses of competitive antagonism. We have demonstrated that properties of either the

Figure 6

Xestospongins do not effectively inhibit IP₃ receptors. (A–C) IP₃-evoked Ca²⁺ release from permeabilized DT40-IP₃R1-3 cells is shown with or without 5 μM Xestospongin C (from Gafni et al., 1997) added 5 min before IP₃. (D) Effects of Xestospongin C (5–20 μM) added 5–12 min before ATP on the Ca²⁺ content of the intracellular stores (percentages of matched controls without Xestospongin). (E–H) Similar analyses using Xestospongin D (10 μM added 5 min before IP₃). Results (A–H) are means ± SEM from three experiments.
receptor or its environment that are remote from the ligand-binding site may significantly affect the apparent affinity of a receptor for a competitive antagonist.

Because heparin is a competitive antagonist of IP$_3$ (Figures 1 and 2), its experimental utility will depend on its affinity relative to IP$_3$ for each IP$_3$R subtype. Table 1 addresses this issue by comparing measured affinities for heparin with EC$_{50}$ values for IP$_3$ as an estimate of the relative affinity of each IP$_3$R subtype for IP$_3$. The analysis indicates that within native cells, responses of IP$_3$R3 to IP$_3$ are likely to be more susceptible to inhibition by heparin than the responses mediated by other IP$_3$R subtypes.

Both 2-APB and caffeine selectively inhibited IP$_3$-evoked Ca$^{2+}$ release via IP$_3$R1, without affecting IP$_3$ binding (Figures 4 and 5; Table 3). Higher concentrations of 2-APB caused some inhibition of IP$_3$R3, but this was accompanied by inhibition of ER Ca$^{2+}$ uptake (Figure 4). The highest concentration of caffeine used (70 mM) also inhibited Ca$^{2+}$ sequestration by the ER, but without significantly affecting the sensitivity to IP$_3$ of IP$_3$R2 or IP$_3$R3, or the fraction of the remaining Ca$^{2+}$ stores released via them by a maximally effective concentration of IP$_3$ (Figure 5). Previous analyses of cells expressing different mixtures of native IP$_3$R subtypes have also suggested that IP$_3$R2 may be resistant to inhibition by 2-APB (Gregory et al., 2001; Hauser et al., 2001; Kukkonen et al., 2001; Bootman et al., 2002; Soulsby and Wojcikiewicz, 2002) and caffeine (Kang et al., 2010). The mechanism of action of 2-APB is unresolved, but for IP$_3$R1 caffeine appears to compete with ATP for the site through which ATP potentiates IP$_3$-evoked Ca$^{2+}$ release (Missiaen et al., 1994; Maes et al., 2001). This mechanism appears not to explain the actions of 2-APB (Missiaen et al., 2001). ATP potentiates IP$_3$-evoked Ca$^{2+}$ release via all three IP$_3$R subtypes (Smith et al., 1985; Mak et al., 1999; Maes et al., 2001; Tu et al., 2005; Betzenhauser et al., 2008), but the mechanisms and ATP-binding sites differ (Betzenhauser et al., 2008; 2009; Betzenhauser and Yule, 2010). Work from Yule and his colleagues suggests that IP$_3$R2 is most sensitive to ATP and for it, but not other IP$_3$R subtypes, an ATPB site within each IP$_3$R subunit mediates the potentiating effect of ATP (Betzenhauser and Yule, 2010). It is, therefore, tempting to speculate that the different sensitivities of IP$_3$R subtypes to inhibition by caffeine (Figure 5) may be related to their different modes of regulation by ATP.

Xestospongins were initially shown to inhibit IP$_3$-evoked Ca$^{2+}$ release selectively (Gafni et al., 1997), and numerous subsequent analyses of their effects on intact cells are consistent with inhibition of IP$_3$Rs (e.g. Bishara et al., 2002; Duncan et al., 2007; Oka et al., 2002; Ozaki et al., 2002; Rosado and Sage, 2000; Schafer et al., 2001; Yuan et al., 2005), but few of these later analyses directly addressed the effects of Xestospongins on IP$_3$Rs (e.g. Oka et al., 2002; Ozaki et al., 2002). The latter is important because Xestospongins have

Figure 7
Xestospongins do not inhibit IP$_3$-evoked Ca$^{2+}$ signals in HEK cells. (A–C) Permeabilized HEK cells were incubated with Xestospongins C (5 μM) or Xestospongins D (10 μM) for 5 min before addition of IP$_3$. Both Xestospongins were prepared as described (Gafni et al., 1997). Results show IP$_3$-evoked Ca$^{2+}$ release (A, B) or the steady-state Ca$^{2+}$ content of the intracellular stores (C, as a percentage of matched controls without Xestospongins). (D) Concentration-dependent effects of carbachol on the increase in intracellular free Ca$^{2+}$ concentration [Ca$^{2+}$]$_i$ of intact fluo-4-loaded HEK cells after treatment with Xestospongins C or D (10 μM for 30 min). pEC$_{50}$ (M) values for the carbachol-evoked Ca$^{2+}$ signals were 4.99 ± 0.13, 4.92 ± 0.23 and 4.70 ± 0.11 for control cells and cells treated with Xestospongins C and D respectively. Results (A–D) are means ± SEM, from three experiments.
additional effects that include inhibition of SERCA (De Smet et al., 1999; Castonguay and Robitaille, 2002; Solovyova et al., 2002), store-operated Ca\(^{2+}\) entry (Bishara et al., 2002), L-type Ca\(^{2+}\) channels and Ca\(^{2+}\)-activated K\(^{+}\) channels (Ozaki et al., 2002), and modulation of ryanodine receptors (Ta et al., 2006). The potencies of Xestospongins also differ between studies and some reports challenge whether they effectively inhibit IP\(_3\)Rs (Solovyova et al., 2002; Duncan et al., 2007; Govindan and Taylor, 2012). We used two sources of Xestospongins C and D, A range of concentrations and incubation periods, two different cell types (see also Govindan and Taylor, 2012), and both intact and permeabilized cells. Although the Xestospongins caused some inhibition of IP\(_3\)-evoked Ca\(^{2+}\) release, none of our analyses succeeded in demonstrating that attainable (≤20 μM) concentrations of Xestospongins substantially inhibited any IP\(_3\)R subtype (Figures 6 and 7; Table 3; Supporting Information Table S1).

We conclude that none of the commonly used antagonists of IP\(_3\)Rs is free of pitfalls. Heparin is perhaps the most reliable, it is competitive with IP\(_3\), but it is membrane-impermeant, and its binding to the IBC of IP\(_3\)Rs is influenced by more distant residues that cause it to bind with different affinity to each IP\(_3\)R subtype (Figures 1–3). Caffeine and 2-APB are membrane-permeant, they do not compete with IP\(_3\), but neither achieves effective inhibition of IP\(_3\)Rs without affecting other Ca\(^{2+}\)-regulating proteins, and both show selectivity for IP\(_3\)R1 (Figures 4 and 5). Xestospongins are membrane-permeant and reported to inhibit IP\(_3\)-evoked Ca\(^{2+}\) release without affecting IP\(_3\) binding (Gafni et al., 1997), but in our hands they do not inhibit any IP\(_3\)R subtype (Figures 6 and 7).

**Acknowledgements**

Supported by the Wellcome Trust (101844), Biotechnology and Biological Sciences Research Council (BB/H009736) and a studentship from the Jameel Family Trust to Huma Saleem. We thank Dr Ana Rossi for providing plasmids.

**Author contributions**

HS performed and analysed experiments. TFM provided reagents. CWT and SCT supervised the project and contributed to analysis. CWT wrote the paper. All authors reviewed the paper.

**Conflict of interest**

None

**References**

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Catterall WA et al. (2013). The Concise Guide to PHARMACOLOGY 2013/14: Ligand-Gated Ion Channels. Br J Pharmacol 170: 1582–1606.

Ando H, Mizutani A, Matsu-ura T, Mikoshiba K (2003). IRBIT, a novel inositol 1,4,5-trisphosphate (IP\(_3\)) receptor-binding protein, is released from the IP\(_3\) receptor upon IP\(_3\) binding to the receptor. J Biol Chem 278: 10602–10612.

Berridge MJ (1993). Inositol trisphosphate and calcium signalling. Nature 361: 315–325.

Betzenhauser MJ, Yule DI (2010). Regulation of inositol 1,4,5-trisphosphate receptors by phosphorylation and adenine nucleotides. Curr Top Membr 66: 273–298.

Betzenhauser MJ, Wagner LE 2nd, Iwai M, Michikawa T, Mikoshiba K, Yule DI (2008). ATP modulation of Ca\(^{2+}\) release by type-2 and type-3 InsP\(_3\)-R: differing ATP sensitivities and molecular determinants of action. J Biol Chem 283: 21579–21587.

Betzenhauser MJ, Wagner LE 2nd, Park HS, Yule DI (2009). ATP regulation of type-1 inositol 1,4,5-trisphosphate receptor activity does not require walker A-type ATP-binding motifs. J Biol Chem 284: 16156–16163.

Bilmen JG, Wootton LL, Godfrey RE, Smart OS, Michelangeli F (2002). Inhibition of SERCA Ca\(^{2+}\) pumps by 2-aminoethoxydiphenyl borate (2-APB). 2-APB reduces both Ca\(^{2+}\) binding and phosphoryl transfer from ATP, by interfering with the pathway leading to the Ca\(^{2+}\)-binding sites. Eur J Biochem 269: 3678–3687.

Bishara NB, Murphy TV, Hill MA (2002). Capacitative Ca\(^{2+}\) entry in vascular endothelial cells is mediated via pathways sensitive to 2 aminoethoxydiphenylborate and xestospongin C. Br J Pharmacol 135: 119–128.

Bootsman MD, Collins TJ, Mackenzie I, Roderick HL, Berridge MJ, Peppiatt CM (2002). 2-aminoethoxydiphenylborate (2-APB) is a reliable blocker of store-operated Ca\(^{2+}\) entry but an inconsistent inhibitor of InsP\(_3\)-induced Ca\(^{2+}\) release. FASEB J 16: 1145–1150.

Bosanac I, Alattia J-R, Mai TK, Chan J, Talarico S, Tong FK et al. (2002). Structure of the inositol 1,4,5-trisphosphate receptor binding core in complex with its ligand. Nature 420: 696–700.

Brown GR, Sayers LG, Kirk CJ, Mitchell RH, Michelangeli F (1992). ATP modulation of Ca\(^{2+}\) release and regulator of K channels. Trends Cell Biol 2: 119–128.

Bultynck G, Sienaert I, Parys JB, Callewaert G, De Smedt H, Boens N et al. (2003). Pharmacology of inositol trisphosphate receptor pumps. Pflugers Arch 445: 629–642.

Castonguay A, Robitaille R (2002). Xestospongin C is a potent inhibitor of SERCA at a vertebrate synapse. Cell Calcium 32: 39–47.

Cheng Y-C, Prusoff WH (1973). Relationship between the inhibition constant (K\(_i\)) and the concentration of inhibitor causing 50% inhibition (IC\(_{50}\)) of an enzymatic reaction. Biochem Pharmacol 22: 3099–3108.

Dasso LIT, Taylor CW (1991). Heparin and other polyanions uncouple α\(_1\)-adrenoceptors from G-proteins. Biochem J 280: 791–795.

De Smet P, Parys JB, Callewaert G, Weidema AF, Hill E, De Smedt H et al. (1999). Xestospongin C is an equally potent inhibitor of the inositol 1,4,5-trisphosphate receptor and the endoplasmic-reticulum Ca\(^{2+}\) pumps. Cell Calcium 26: 9–13.

Duncan RS, Hwang SY, Koulen P (2007). Differential inositol 1,4,5-trisphosphate receptor signaling in a neuronal cell line. Int J Biochem Cell Biol 39: 1852–1862.

Ehrlich BE, Kaftan E, Bezprozvanny S, Bezprozvanny I (1994). The pharmacology of intracellular Ca\(^{2+}\)-release channels. Trends Pharmacol Sci 15: 145–149.
Foskett JK, White C, Cheung KH, Mak DO (2007). Inositol trisphosphate receptor Ca^{2+} release channels. Physiol Rev 87: 593–658.

Futatsugi A, Nakamura T, Yamada MK, Ebisui E, Nakamura K, Uchida K et al. (2005). IP_3 receptor types 2 and 3 mediate exocrine secretion underlying energy metabolism. Science 309: 2232–2234.

Gaffen J, Munsch JA, Lam TH, Catlin MC, Costa LG, Molinski TF et al. (1997). Xestospongin: potent membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor. Neuron 19: 723–733.

Ghosh TK, Eis PS, Mullaney JM, Ebert CL, Gill DL (1988). Competitive, reversible, and potent antagonism of inositol 1,4,5-trisphosphate-activated calcium release by heparin. J Biol Chem 263: 11075–11079.

Goto J, Suzuki AZ, Ozaki S, Matsumoto N, Nakamura T, Ebisui E et al. (2010). Two novel 2-aminoethyl diphenylborinate (2-APB) analogues differentially activate and inhibit store-operated Ca^{2+} entry via STIM proteins. Cell Calcium 47: 1–10.

Govindan S, Taylor CW (2012). P2Y receptor subtypes evoke different Ca^{2+} signals in cultured aortic smooth muscle cells. Purinergic Signal 8: 763–777.

Gregory RB, Rychkov G, Barratt GJ (2001). Evidence that 2-aminoethyl diphenylborate is a novel inhibitor of store-operated Ca^{2+} channels in liver cells, and acts through a mechanism which does not involve inositol trisphosphate receptors. Biochem J 354: 285–290.

Guillemette G, Lamontagne S, Boulay G, Mouillac B (1989). Differential effects of heparin on inositol 1,4,5-trisphosphate binding, metabolism, and calcium release activity in the bovine adrenal cortex. Mol Pharmacol 35: 339–344.

Hattori M, Suzuki AZ, Higo T, Miyauchi H, Michikawa T, Nakamura T et al. (2004). Distinct roles of inositol 1,4,5-trisphosphate receptor types 1 and 3 in Ca^{2+} signaling. J Biol Chem 279: 11967–11975.

Hauser CJ, Fekete Z, Adams JM, Garced M, Livingston DH, Deitch EA (2001). PAF-mediated Ca^{2+} influx in human neutrophils occurs via store-operated mechanisms. J Leukocyte Biol 69: 63–68.

Higo T, Hattori M, Nakamura T, Natsume T, Michikawa T, Mikoshiba K (2005). Subtype-specific and ER luminal environment-dependent regulation of inositol 1,4,5-trisphosphate receptor type 1 by ERp44. Cell 120: 85–98.

Iwai M, Michikawa T, Bosanac I, Ikura M, Mikoshiba K (2005). Molecular basis of the isoform-specific ligand-binding affinity of inositol 1,4,5-trisphosphate receptors. J Biol Chem 282: 12755–12764.

Kang SS, Han KS, Ku BM, Lee YK, Hong J, Shin HY et al. (2010). Caffeine-mediated inhibition of calcium release channel inositol 1,4,5-trisphosphate receptor subtype 3 blocks gobliatoma invasion and extends survival. Cancer Res 70: 1173–1183.

Khan SA, Rossi AM, Riley AM, Potter BV, Taylor CW (2013). Subtype-selective regulation of IP_3 receptors by thimerosal via cystine residues within the IP_3-binding core and suppressor domain. Biochem J 451: 177–184.

Kukkonen JP, Lund PE, Akerman KE (2001). 2-aminoethoxydiphenyl borate reveals heterogeneity in receptor-activated Ca^{2+} discharge and store-operated Ca^{2+} influx. Cell Calcium 30: 117–129.

Kurian N, Hall CJ, Wilkinson GF, Sullivan M, Tobin AB, Willars GB (2009). Full and partial agonists of muscarinic M_3 receptors reveal single and oscillatory Ca^{2+} responses by b_2-adrenoceptors. J Pharmacol Exp Ther 330: 502–512.

Laude AJ, Tovey SC, Dedos S, Potter BVL, Lumsis SCR, Taylor CW (2005). Rapid functional assays of recombinant IP_3 receptors. Cell Calcium 38: 45–51.

Maes K, Missiaen L, Parys JB, De Smet P, Sienaert I, Waelkens E et al. (2001). Mapping of the ATP-binding sites on inositol 1,4,5-trisphosphate receptor type 1 and type 3 homotetramers by controlled proteolysis and photoaffinity labelling. J Biol Chem 276: 3492–3497.

Mak D-OD, McBride S, Foskett JK (1999). ATP regulation of type 1 inositol 1,4,5-trisphosphate receptor channel gating by allosteric tuning of Ca^{2+} activation. J Biol Chem 274: 22231–22237.

Maruyama T, Kanaji T, Nakade S, Kanno T, Mikoshiba K (1997). 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P_3-induced Ca^{2+} release. J Biochem 122: 498–505.

Matsumoto M, Nakagawa T, Inoue T, Nagata E, Tanaka K, Takano H et al. (1996). Ataxia and epileptic seizures in mice lacking type 1 inositol 1,4,5-trisphosphate receptor. Nature 379: 168–171.

McKemy DD, Welch W, Airey JA, Sutko JL (2000). Concentrations of caffeine greater than 20 mM increase the indo-1 fluorescence ratio in a Ca^{2+}-independent manner. Cell Calcium 27: 117–124.

Michelangeli F, Mezna M, Tovey S, Sayers LG (1995). Pharmacological modulators of the inositol 1,4,5-trisphosphate receptor. Neuropharmacology 34: 1111–1122.

Missiaen L, Parys JB, De Smedt H, Casteels R (1994). Inhibition of inositol trisphosphate-induced calcium release by caffeine is prevented by ATP. Biochem J 300: 81–84.

Missiaen L, Callewaert G, De Smedt H, Parys JB (2001). 2-Aminoethoxydiphenyl borate affects the inositol 1,4,5-trisphosphate receptor, the intracellular Ca^{2+} pump and the non-specific Ca^{2+} leak from the non-mitochondrial Ca^{2+} stores of permeabilized A7r5 cells. Cell Calcium 29: 111–116.

Monaco G, Decrock E, Aki H, Ponsaerts R, Vervliet T, Luyten T et al. (2012). Selective regulation of IP_3-receptor-mediated Ca^{2+} signaling and apoptosis by the BH4 domain of Bcl-2 versus Bcl-X_L. Cell Death Differ 19: 295–309.

Nadif Kasri N, Torok K, Galione A, Garnham C, Callewaert G, Missiaen L et al. (2006). Endogenously bound calmodulin is essential for the function of the inositol 1,4,5-trisphosphate receptor. J Biol Chem 281: 8332–8338.

Nerou EP, Riley AM, Potter BVL, Taylor CW (2001). Selective recognition of inositol phosphates by subtypes of inositol trisphosphate receptor. Biochem J 355: 59–69.

Oka T, Sato K, Hori M, Ozaki H, Karaki H (2002). Xestospongin C, a novel blocker of IP_3 receptors, attenuates the increase in cytosolic calcium level and degranulation that is induced by antigen in RBL-2H3 mast cells. Br J Pharmacol 135: 1959–1966.

Ozaki H, Hori M, Kim YS, Kwon SC, Ahn DS, Nakazawa H et al. (2002). Inhibitory mechanism of xestospongin-C on contraction and ion channels in the intestinal smooth muscle. Br J Pharmacol 137: 1207–1212.

Pantazaka E, Taylor CW (2011). Differential distribution, clustering and lateral diffusion of subtypes of inositol 1,4,5-trisphosphate receptor. J Biol Chem 286: 23378–23387.

Parker I, Iovra I (1991). Caffeine inhibits inositol trisphosphate-mediated liberation of intracellular calcium in Xenopus oocytes. J Physiol 433: 229–240.

Patterson RL, Boehning D, Snyder SH (2004). Inositol 1,4,5-trisphosphate receptors as signal integrators. Annu Rev Biochem 73: 437–465.

Rahman TU, Skupin A, Falcke M, Taylor CW (2009). Clustering of IP_3 receptors by IP_3 retunes their regulation by IP_3 and Ca^{2+}. Nature 458: 655–659.
Rosado JA, Sage SO (2000). Coupling between inositol 1,4,5-trisphosphate receptors and human transient receptor potential channel 1 when intracellular Ca\(^{2+}\) stores are depleted. Biochem J 350: 631–635.

Rossi AM, Taylor CW (2011). Analysis of protein-ligand interactions by fluorescence polarization. Nat Prot 6: 365–387.

Rossi AM, Riley AM, Tovey SC, Rahman T, Deliss O, Taylor EJA et al. (2009). Synthetic partial agonists reveal key steps in IP\(_3\) receptor activation. Nat Chem Biol 5: 631–639.

Rossi AM, Riley AM, Potter BVL, Taylor CW (2010a). Adenophostins: high-affinity agonists of IP\(_3\) receptors. Curr Top Membr 66: 209–233.

Rossi AM, Sureshan KM, Riley AM, Potter BVL, Taylor CW (2010b). Selective determinants of inositol 1,4,5-trisphosphate and adenophostin A interactions with type 1 inositol 1,4,5-trisphosphate receptors. Br J Pharmacol 161: 1070–1085.

Saleem H, Tovey SC, Rahman T, Riley AM, Potter BVL, Taylor CW (2012). Stimulation of inositol 1,4,5-trisphosphate (IP\(_3\)) receptor subtypes by analogues of IP\(_3\). PLoS ONE 8: e54877.

Saleem H, Tovey SC, Riley AM, Potter BVL, Taylor CW (2013). Stimulation of inositol 1,4,5-trisphosphate (IP\(_3\)) receptor subtypes by adenophostin A and its analogues. PLoS ONE 8: e58027.

Schafer M, Bahde D, Bosche B, Ladilov Y, Schafer C, Piper HM et al. (2001). Modulation of early [Ca\(^{2+}\)], rise in metabolically inhibited endothelial cells by xestospongin C. Am J Physiol 280: H1002–H1010.

Seo M-D, Velamakanni S, Ishiyama N, Stathopulos PB, Rossi AM, Khan SA et al. (2012). Structural and functional conservation of key domains in InsP\(_3\) and ryanodine receptors. Nature 483: 108–112.

Smith JB, Smith L, Higgins BL (1985). Temperature and nucleotide dependence of calcium release by myo-inositol 1,4,5-trisphosphate in cultured vascular smooth muscle cells. J Biol Chem 260: 14413–14416.

Soloyova N, Fenyhough P, Glazner G, Verkhratsky A (2002). Xestospongic C empties the ER calcium store but does not inhibit InsP\(_3\)-induced Ca\(^{2+}\) release in cultured dorsal root ganglia neurones. Cell Calcium 32: 49–52.

Soulsby MD, Wojcikiewicz RJ (2002). 2-Aminoethoxydiphenyl borate inhibits inositol 1,4,5-trisphosphate receptor function, ubiquitination and downregulation, but acts with variable characteristics in different cell types. Cell Calcium 32: 175–181.

Sugawara H, Kurosaki M, Takata M, Kurosaki T (1997). Genetic evidence for involvement of type 1, type 2 and type 3 inositol 1,4,5-trisphosphate receptors in signal transduction through the B-cell antigen receptor. EMBO J 16: 3078–3088.

Sun Y, Taylor CW (2008). A calmodulin antagonist reveals a calmodulin-independent interdomain interaction essential for activation of inositol 1,4,5-trisphosphate receptors. Biochem J 416: 243–253.

Sun Y, Rossi AM, Rahman T, Taylor CW (2013). Activation of IP\(_3\) receptors requires an endogenous 1-8-14 calmodulin-binding motif. Biochem J 449: 39–49.

Ta TA, Feng W, Molinski TF, Pesah IN (2006). Hydroxylated xestospongins block inositol-1,4,5-trisphosphate-induced Ca\(^{2+}\) release and sensitize Ca\(^{2+}\)-induced Ca\(^{2+}\) release mediated by ryanodine receptors. Mol Pharmaco 69: 532–538.

Taylor CW, Genazzani AA, Morris SA (1999). Expression of inositol trisphosphate receptors. Cell Calcium 26: 237–251.

Tovey SC, Sun Y, Taylor CW (2006). Rapid functional assays of intracellular Ca\(^{2+}\) channels. Nat Prot 1: 259–263.

Tovey SC, Dedos SG, Taylor EJA, Church JE, Taylor CW (2008). Selective coupling of type 6 adenyl cyclase with type 2 IP\(_3\) receptors mediates a direct sensitization of IP\(_3\) receptors by cAMP. J Cell Biol 183: 297–311.

Tovey SC, Dedos SG, Rahman T, Taylor EJA, Pantazaka E, Taylor CW (2010). Regulation of inositol 1,4,5-trisphosphate receptors by cAMP independent of cAMP-dependent protein kinase. J Biol Chem 285: 12979–12989.

Tu H, Wang Z, Nosyrev E, De Smeth H, Bezprozvanny I (2005). Functional characterization of mammalian inositol 1,4,5-trisphosphate receptor isoforms. Biophys J 88: 1046–1055.

Wagner LE 2nd, Yule DI (2012). Differential regulation of the InsP\(_3\) receptor type-1 and -2 single channel properties by InsP\(_3\), Ca\(^{2+}\) and ATP. J Physiol 590: 3245–3259.

Wei C, Wang X, Chen M, Ouyang K, Song LS, Cheng H (2009). Calcium flickers steer cell migration. Nature 457: 901–905.

Willuweit B, Aktories K (1988). Heparin uncouples \(\alpha\)-adrenoceptors from the G-protein in membranes of human platelets. Biochem J 249: 857–863.

Wojcikiewicz RJH (1995). Type I, II and III inositol 1,4,5-trisphosphate receptors are uniquely susceptible to down-regulation and are expressed in markedly different proportions in different cell types. J Biol Chem 270: 11678–11683.

Wojcikiewicz RJH, He Y (1995). Type I, II and III inositol 1,4,5-trisphosphate receptor co-immunoprecipitation as evidence for the existence heterotetrameric receptor complexes. Biochem Biophys Res Commun 213: 334–341.

Yuan Z, Cai T, Tian J, Ivanov AV, Giovannucci DR, Xie Z (2005). Na/K-ATPase tethers phospholipase C and IP\(_3\) receptor into a calcium-regulatory complex. Mol Biol Cell 16: 4034–4045.

**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12685

**Table S1** Xestospongins ineffectively inhibit IP\(_3\)-evoked Ca\(^{2+}\) release.