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Heterogeneity of [^{3}H]inositol 1,4,5-trisphosphate binding sites in adrenal-cortical membranes

Characterization and validation of a radioreceptor assay

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1. The characterization of a radioreceptor assay for determining Ins(1,4,5)P_3 concentration in tissue extracts is described which utilizes the binding of [^{3}H]Ins(1,4,5)P_3 to an adrenal-cortex membrane fraction. 2. Analysis of [^{3}H]Ins(1,4,5)P_3 binding by isotope dilution demonstrated an apparent single population of binding sites (K_D 3.65 ± 0.18 nM, $B_{max.}$ 872 ± 70 fmol/mg of protein). Specific binding of [^{3}H]Ins(1,4,5)P_3 was enhanced at alkaline pH values (maximum at pH 8.5), with complete loss of specific binding at pH < 6. These binding sites displayed strict stereo- and positional specificity for Ins(1,4,5)P_3, with L-Ins(1,4,5)P_3, Ins(1,3,4)P_3 and DL-Ins(1,3,4,5)P_4 causing 50% displacement of specific [^{3}H]Ins(1,4,5)P_3 binding (IC_{50} values) at concentrations of 14 ± 3 μM, 3.0 ± 0.3 μM and 0.53 ± 0.03 μM respectively. 3. Kinetic analysis of binding data, however, revealed a high-affinity [^{3}H]Ins(1,4,5)P_3 binding site (K_D 0.052 nM) in addition to the lower-affinity site (K_D 2.53 nM) already demonstrated in displacement studies. 4. It is shown that the presence of the high-affinity site can be exploited to increase the sensitivity of the [^{3}H]Ins(1,4,5)P_3 radioreceptor assay, allowing accurate detection of 20 fmol of Ins(1,4,5)P_3 in 300 μl of tissue extract. 5. Further validation of the specificity of the above assay for Ins(1,4,5)P_3 was provided by incubating tissue extracts with either a 5-phosphatase or 3-kinase preparation. It was shown that identical loss occurred of both Ins(1,4,5)P_3 mass and [^{3}H]Ins(1,4,5)P_3, added to parallel incubations. 6. The ability of the assay to measure basal and agonist-stimulated increases in Ins(1,4,5)P_3 concentration has been demonstrated with rat cerebral cortex and bovine tracheal smooth-muscle slices and a range of cultured and isolated cell preparations.

INTRODUCTION

Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], generated by receptor-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate, is known to play a central role in signal transduction in many cells, exerting its effects by interaction with specific intracellular receptors which cause Ca^{2+} release from intracellular organellar stores [1,2]. High-affinity binding sites for Ins(1,4,5)P_3 have been detected in permeabilized cells [3] and in both crude and microsome-rich membrane preparations of peripheral tissues and brain [4–8], and, in those systems in which the specificity of the site has been assessed, they are thought to represent the intracellular receptor for Ins(1,4,5)P_3 [9]. Autoradiographic mapping of the Ins-(1,4,5)P_3 receptor has been reported [10], and at a subcellular level these binding sites have been localized to the endoplasmic reticulum, including sub-plasmalemmal endoplasmic reticulum, Golgi apparatus, nuclear membrane and a specialized subcellular organelle termed the 'calciosome' [11,12].

Until recently, most studies investigating phosphoinositide metabolism have utilized radioisotopic prelabelling techniques which rely on the incorporation of radiolabelled substrates into the polyphosphoinositides and analysis of the labelled Ins$P$ products. Since true equilibrium labelling of the hormone-sensitive phosphoinositide pool(s) is rarely established, changes in specific radioactivity (e.g. as might be caused by agonist-stimulated phosphoinositide turnover) often give rise to changes in radioactivity of the Ins$P$s independent of changes in mass [13,14]. In addition, commonly used techniques to separate and quantify labelled inositol polyphosphate isomers, including Ins(1,4,5)P_3, are in general laborious, expensive and time-consuming, making them quite unsuitable for the routine assay of multiple samples. A few alternative methods have been described to measure Ins(1,4,5)P_3 mass (e.g. [15–17]), but these are likewise complex and expensive.

Recently, therefore, efforts have been directed at exploiting the properties of the Ins(1,4,5)P_3 binding site in various membrane preparations as the basis for a radioreceptor assay. Although the highest levels of Ins-(1,4,5)P_3 binding have been found in the cerebellum [5,6], this site has an approx. 10-fold higher K_D for Ins(1,4,5)P_3 than that found in many peripheral tissues, making the cerebellum preparation intrinsically less sensitive as the basis of such an assay. The first attempts to use this approach to measure changes in Ins(1,4,5)P_3 mass were made by Bradford & Rubin [18], using saponin-permeabilized neutrophils, with other groups subsequently describing similar assays using microsomal membrane preparations from adrenal cortex and cerebellum [14,19–21]. The present study, using a bovine adrenal-cortex membrane preparation, gives kinetic evidence for a second, very high-affinity, [^{3}H]Ins(1,4,5)P_3 binding site,

Abbreviations used: CC, cerebral cortex; BTSM, bovine tracheal smooth muscle.

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describes and validates a radioreceptor assay for Ins(1,4,5)P_3, and reports its value in measuring both basal and agonist-stimulated changes in Ins(1,4,5)P_3 mass in a range of tissues.

MATERIALS AND METHODS

Materials

[^{3}H]Ins(1,4,5)P_3 (17–20 Ci/mmol) was generously donated by NEN–DuPont. [5-^{32}P]Ins(1,4,5)P_3 (1000 Ci/mmol) and Ins(1,4,5)P_3 were purchased from Amersham International. Synthetic DL-Ins(1,4,5)P_3 and DL-Ins(1,3,4,5)P_4 were generously provided by Dr. B. V. L. Potter (Department of Chemistry, University of Leicester, U.K.), and Ins(1,3,4)P_3 was a gift from Dr. R. F. Irvine (Department of Biochemistry, Babraham Research Institute, Cambridge, U.K.). All other reagents were obtained from commercial sources and were of analytical grade.

Preparation of Ins(1,4,5)P_3 binding protein

Sufficient fresh bovine adrenal glands were demedullated and decapsulated to obtain 60–80 g of cortex. Tissue was homogenized in 8 vol. of ice-cold 20 mM-$NaHCO_3$/1 mM-dithiothreitol, pH 8.0, in an Atomix blender, and the homogenate was centrifuged at 5000 ***g*** for 15 min at 4 °C. The supernatant was removed and the pellet re-homogenized in 4 vol. of $NaHCO_3$/dithiothreitol and re-centrifuged as before. The pooled supernatant fraction was centrifuged at 38000 ***g*** for 20 min at 4 °C, and the 'P_2' pellet obtained was washed with $NaHCO_3$/dithiothreitol and re-centrifuged as before. The pellet was resuspended in homogenization medium at a protein concentration of 20 mg/ml and frozen at −20 °C in 1 ml batches for subsequent use.

Ins(1,4,5)P_3 binding assay

Assays were routinely performed at 4 °C in a final volume of 120 μl. A 30 μl portion of sample or of trichloroacetic acid-extracted buffer containing standard amounts of Ins(1,4,5)P_3 (0.12–12 pmol) or DL-Ins(1,4,5)P_3 (0.3 nmol, to define non-specific binding) was added to 30 μl of 100 mM-Tris/HCl/4 mM-EDTA, pH 8.0, and 30 μl of [^{3}H]Ins(1,4,5)P_3 (~ 7000 d.p.m./assay). Then 30 μl (0.2–0.4 mg of protein) of the adrenal-cortical binding protein preparation was added, and samples were intermittently vortex-mixed for 30 min.

Bound and free [^{3}H]Ins(1,4,5)P_3 were separated by rapid filtration through Whatman GF/B glass-fibre filters with 4 × 3 ml washes of ice-cold 25 mM-Tris/HCl/5 mM-$NaHCO_3$/1 mM-EDTA, pH 8.0. Scintillant was added to the filter discs, and radioactivity determined after a 12 h extraction period by scintillation counting. Essentially identical data were obtained by using a centrifugation method to separate bound and free radioligand [12000 ***g*** for 4 min, followed by aspiration of the supernatant and dissolving the pellet in 100 μl of Lumasolve (Lumac, Landgraaf, The Netherlands)], although non-specific-binding values obtained by this method were considerably higher, resulting in a lower ratio of specific binding to total [^{3}H]Ins(1,4,5)P_3 binding.

Kinetic experiments

Association rates were determined by addition of the receptor preparation (~ 250 μg of protein) to 25 mM-Tris/HCl/1 mM-EDTA, pH 8.0, containing [^{3}H]-Ins(1,4,5)P_3 (~ 10000 d.p.m., 2 nM) at 4 °C. Incubations were terminated by rapid filtration and washing as described above. Non-specific binding was defined in the presence of 10 μM-DL-Ins(1,4,5)P_3. Dissociation rates were determined by addition of DL-Ins(1,4,5)P_3 (final concn. 10 μM) after equilibrium binding of [^{3}H]-Ins(1,4,5)P_3 had been achieved by incubation for 30 min at 4 °C.

Enhancement of assay sensitivity

To improve the detection limit of the Ins(1,4,5)P_3 radioreceptor assay (see the Results section) and to allow larger volumes of cell extract to be used, the assay volume was increased to 1.2 ml. A 300 μl portion of sample or of trichloroacetic acid-extracted buffer containing standard amounts of Ins(1,4,5)P_3 (0.036–36 pmol) or DL-Ins(1,4,5)P_3 (0.3 nmol, to define non-specific binding) was added to 300 μl of 100 mM-Tris/HCl/4 mM-EDTA, pH 8.0, and 300 μl of [^{3}H]Ins(1,4,5)P_3 diluted in distilled water (~ 7000 d.p.m./assay). The adrenal-cortical binding-protein preparation was diluted 1:9 in 20 mM-$NaHCO_3$/1 mM-dithiothreitol, pH 8.0, immediately before use, and 300 μl (0.2–0.4 mg of protein) was added to each sample. Incubation conditions and separation methods were identical with those described for the low-volume assay.

Incubation techniques

Rat cerebral cortex (CC) and bovine tracheal smooth muscle (BTSM) slices (300 μm × 300 μm) were prepared as described previously [14,22]. After extensive washing, CC and BTSM slices were incubated for 60 min at 37 °C in Krebs–Henseleit bicarbonate buffer [KHB: 118 mM-NaCl, 4.7 mM-KCl, 1.3 mM-$CaCl_2$, 1.2 mM-KH_2PO_4, 1.2 mM-$MgSO_4$, 25 mM-$NaHCO_3$, 10 mM-glucose, pH 7.4, saturated with O_2/CO_2 (19:1)], with buffer replacement every 15 min. Slices were allowed to sediment, and 50 μl of packed slices was added to 250 μl of Krebs–Henseleit bicarbonate buffer, samples were gassed regularly, capped and incubated for 60 min. Carbachol was added to a final concentration of 0.1 mM (BTSM) or 1 mM (CC), and incubations were continued for the time periods given in the Results and discussion section.

All incubations were terminated by addition of 1 M-trichloroacetic acid (final concn. 0.5 M). Acidified samples were left on ice for 15 min, centrifuged (15 min, 3000 ***g***, 4 °C) and trichloroacetic acid was extracted with 5 × 2 vol. washes with water-saturated diethyl ether. Then 125 μl of 30 mM-EDTA and 125 μl of 60 mM-$NaHCO_3$ were added to 500 μl of tissue extract, and samples were taken for analysis. For all samples, the tissue pellet was washed, digested in 1 M-NaOH, and protein concentration was determined. Buffer samples were also taken through the acidification/extraction protocol to provide diluent for the Ins(1,4,5)P_3 assay standard curve.

Ins(1,4,5)P_3 5-phosphatase treatment

Neutralized extracts from control and carbachol-stimulated (0.1 mM, 5 s) BTSM slices were prepared as described above and adjusted to final concentrations of 30 mM-Hepes/4 mM-EGTA, pH 7.0. Samples were incubated in the absence or presence of authentic [^{3}H]-Ins(1,4,5)P_3 (~ 10000 d.p.m.) with 0.125 mg of protein of human erythrocyte ghosts (prepared as described in [23]), which contain an active Ins(1,4,5)P_3 5-phosphatase, for 0–60 min at 37 °C. Control experiments were carried

out with heat-inactivated (100 °C, 15 min) erythrocyte ghosts. Incubations were terminated by addition of 50 μl of 30 mM-Hepes/30 mM-EDTA (5 mM final concns.) and immediately centrifuged at 10000 g for 2 min. The resulting supernatants were then assayed for Ins(1,4,5)P_3 mass, as described above, or, for the samples containing [^{3}H]Ins(1,4,5)P_3, applied to Dowex AG1-X8 anion-exchange columns (formate form, 200–400 mesh), with elution of [^{3}H]InsP_{1-4} with ammonium formate/formic acid buffers [24].

Ins(1,4,5)P_3 3-kinase treatment

Extracts from control and carbachol-stimulated (1 mM, 10 s) CC slices were prepared as described above and adjusted to contain 10 mM-Tris/maleate, 1 mM-ATP, 2 mM-$MgCl_2$, 5 mM-phosphocreatine and 0.1 mg of creatine kinase/ml at pH 7.5. Samples were incubated in the absence or presence of authentic [^{3}H]Ins(1,4,5)P_3 ($\sim$ 10000 d.p.m.) with supernatant prepared from rat whole brain homogenate prepared as described in [25] (final concn. 0.3 %, w/v), which contains Ins(1,4,5)P_3 3-kinase activity, for 20 min at 37 °C. Control experiments were carried out with heat-inactivated (15 min, 100 °C) enzyme preparation. Incubations were terminated by addition of ice-cold 1 M-trichloroacetic acid, and the resulting supernatants were prepared for Ins(1,4,5)P_3 radioreceptor assay and separation of [^{3}H]Ins$P$s as described above.

RESULTS AND DISCUSSION

Preliminary experiments were performed to establish whether any metabolism of the [^{3}H]Ins(1,4,5)P_3 occurred under the radioreceptor-assay conditions described. After incubation of 2 nM-[^{3}H]Ins(1,4,5)P_3 with 200–600 μg of bovine adrenal-cortical membranes in the presence of 1 mM-EDTA, under the above assay conditions 97.2 ± 0.4 % ($n = 6$) of the added [^{3}H]Ins(1,4,5)P_3 was eluted in the InsP_3 fraction from Dowex AG1-X8 anion-exchange columns, and was shown to be [^{3}H]Ins(1,4,5)P_3 by separation of the trisphosphate isomers by h.p.l.c. Omission of EDTA resulted in significant metabolism of the [^{3}H]Ins(1,4,5)P_3, at least 80 % in the 30 min incubation period at 4 °C.

Isotopic-dilution binding data for [^{3}H]Ins(1,4,5)P_3 are shown in Fig. 1. Non-specific binding, defined by inclusion of 10 μM-DL-Ins(1,4,5)P_3, was always < 10 % of total binding at 3 nM-Ins(1,4,5)P_3. Saturation analysis of these data demonstrated that Ins(1,4,5)P_3 bound to an apparently homogeneous receptor population with a K_D of 3.65 ± 0.18 nM and a $B_{max.}$ of 872 ± 70 fmol/mg of protein. This K_D value is in close agreement with those reported by Guillemette *et al.* [7] (1.26 ± 0.55 nM) and Palmer *et al.* [19] (6.82 ± 2.3 nM) in preparations of adrenal cortex. However, the 'P_2' membrane fraction used in the present studies yielded a greater number of receptor sites than previously reported.

The interaction of [^{3}H]Ins(1,4,5)P_3 with the adrenal-cortex binding site was very sensitive to pH, with maximal specific binding occurring at pH 8.5 and complete loss of binding observed at pH < 6. This is similar to that reported in rat cerebellum [5] and bovine parathyroid glands [26]; however, we have not been able to demonstrate any changes in the affinity of [^{3}H]Ins(1,4,5)P_3 binding with pH (results not shown) as reported by Bredt *et al.* [21] in cerebellar membranes.

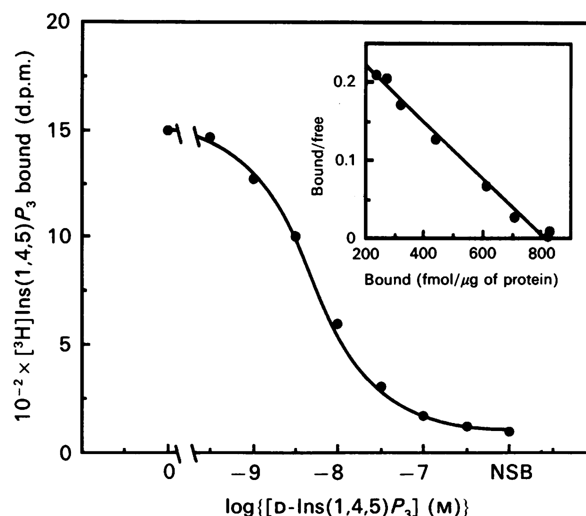

Fig. 1. Saturation analysis of [^{3}H]Ins(1,4,5)P_3 binding to adrenal-cortex membranes

Assays were performed with $\sim$ 1.5 nM-[^{3}H]Ins(1,4,5)P_3, 200–400 μg of adrenal-cortex 'P_2' membrane fraction and increasing concentrations of unlabelled Ins(1,4,5)P_3 in 25 mM-Tris/HCl/1 mM-EDTA buffer, pH 8.0 (final volume 120 μl). Incubations were performed for 30 min at 4 °C, with separation of bound and free radioligand by rapid filtration. Non-specific binding (NSB) was determined in the presence of 10 μM-DL-Ins(1,4,5)P_3. Scatchard transformation of isotope-dilution data is shown in the inset. Values represent means of duplicate determinations from a single experiment, with similar results obtained in a further four experiments.

The ability of various stereo- and positional inositol phosphate isomers to interact with the Ins(1,4,5)P_3 binding site is shown in Fig. 2. L-Ins-(1,4,5)P_3 causes 50 % displacement of specific [^{3}H]Ins(1,4,5)P_3 binding (IC_{50}) at a concentration of 14 ± 3 μM, indicating a 2400-fold selectivity for the D-isomer. DL-Ins(1,3,4,5)P_4 is the most potent competitor for Ins(1,4,5)P_3 binding sites, with an IC_{50} of 0.53 ± 0.03 μM, suggesting a greater than 50-fold selectivity for Ins(1,4,5)P_3 over Ins(1,3,4,5)P_4. This equates with a cross-reactivity for Ins(1,3,4,5)P_4 at the Ins(1,4,5)P_3 binding site of approx. 1.4 %, which does not support the recent statement that the Ins(1,4,5)P_3 binding site in the adrenal-cortex preparation may be less selective for Ins(1,4,5)P_3 over Ins(1,3,4,5)P_4 compared with cerebellar membranes [21]. The other major naturally occurring InsP_3 isomer, Ins(1,3,4)P_3, exhibited an approx. 500-fold lower affinity for the Ins(1,4,5)P_3 binding site (IC_{50} 3.0 ± 0.3 μM) (Fig. 2). These data are similar to those reported by Willcocks *et al.* [6] for a particulate preparation of rat cerebellum, except that D- and L-Ins(1,4,5)P_3, D-Ins(1,3,4)P_3 and DL-Ins(1,3,4,5)P_4 are all about 10 times more effective in displacing [^{3}H]Ins(1,4,5)P_3 specific binding in the adrenal-cortical preparation. The displacing activity of ATP was also investigated, since ATP is present at millimolar concentrations in cell extracts. The IC_{50} of 1.5 ± 0.4 mM indicates that ATP will not significantly interfere in the Ins(1,4,5)P_3 radioreceptor assay when the dilution involved in sample preparation is taken into consideration.

Kinetic evidence for the observed K_D, determined by analysis of equilibrium binding data, was also sought.

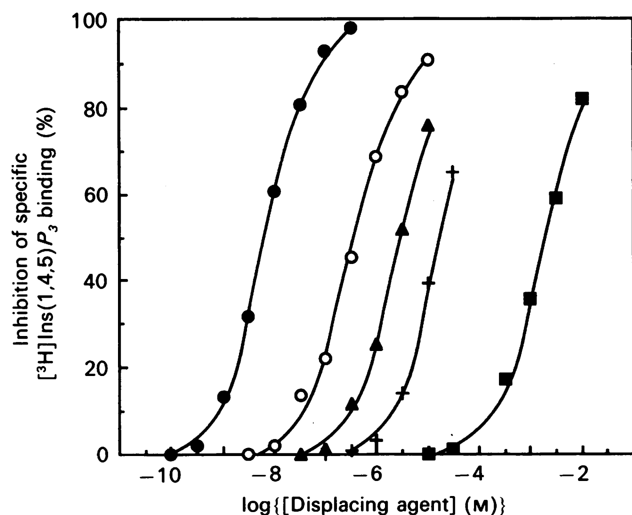

Fig. 2. Specificity of [^{3}H]Ins(1,4,5)P_3 binding to adrenal-cortex membranes

Incubation details were identical with those described in the legend to Fig. 1. Displacement of specific [^{3}H]-Ins(1,4,5)P_3 binding with increasing concentrations of D-Ins(1,4,5)P_3 (●), L-Ins(1,4,5)P_3 (+), DL-Ins(1,3,4,5)P_4 (○), D-Ins(1,3,4)P_3 (▲) and ATP (■) are shown. Data points represent means of 3–6 separate experiments, each performed in duplicate, with S.E.M. values less than 5% of mean in all cases.

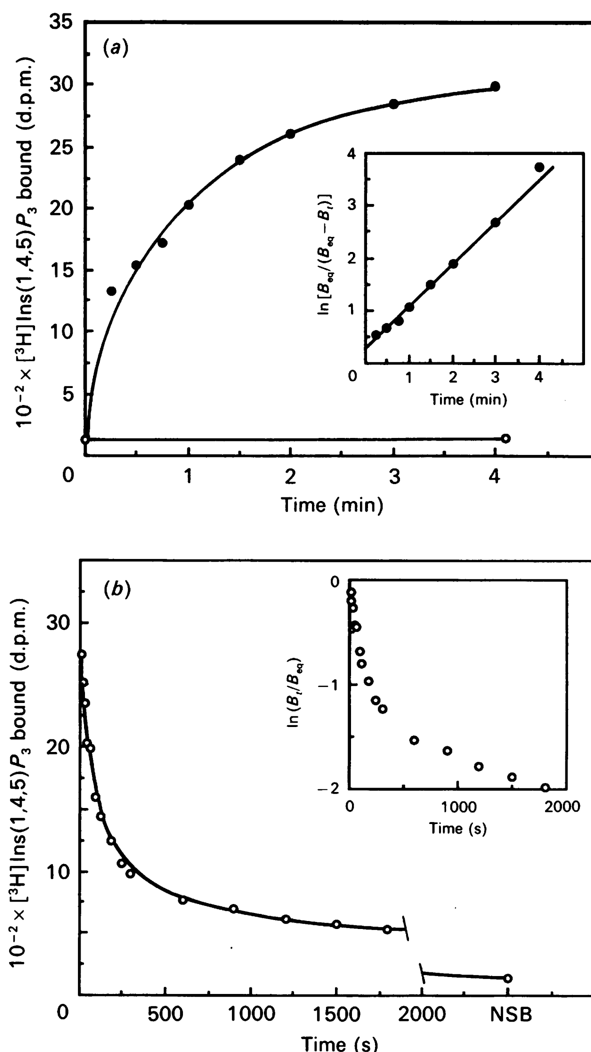

Fig. 3. Association (a) and dissociation (b) rates for [^{3}H]-Ins(1,4,5)P_3 binding to adrenal-cortex membranes

Assay conditions were identical with those described in the legend to Fig. 1. Assays were performed at 4 °C and commenced by the addition of 'P_2' membrane fraction. The dissociation curve was obtained by adding 10 μl of 120 μM unlabelled DL-Ins(1,4,5)P_3 (final concn. 10 μM) to adrenal-cortex membranes that had been incubated with ~ 2 nM-[^{3}H]Ins(1,4,5)P_3 for 30 min. Data points from single representative experiments (each of three) are shown, with semi-logarithmic transformations of these values shown as insets. B_{eq} and B_t are specific Ins(1,4,5)P_3 binding, respectively, at equilibrium of time t defined in the main Figures. Abbreviation: NSB, non-specific binding.

The rate of association of [^{3}H]Ins(1,4,5)P_3 with the adrenal-cortical receptor is shown in Fig. 3(a). Semi-logarithmic transformation of the data set (Fig. 3a inset) indicated an observed association rate ($k_{obs.}$) of 0.85 ± 0.10 min^{-1}. In contrast, the semi-logarithmic transformation of the dissociation rate data (Fig. 3b) was clearly non-linear, with at least two distinct components being evident. Computer-assisted fitting of the data set revealed an initial rapid dissociation rate (k_{-1}) of 0.41 ± 0.12 min^{-1} and a slower dissociation rate (k_{-1}') of 0.016 ± 0.005 min^{-1}. From these data, K_D values of 2.53 nM and 52 pM could be calculated (Table 1). Despite obtaining linear Scatchard plots when [^{3}H]Ins(1,4,5)P_3 displacement data were analysed (Fig. 1) and Hill coefficients close to unity, the kinetic data, which provide a more sensitive method to detect receptor populations of differing affinity, thus indicated the presence of two receptor populations i.e. both high- (K_D 52 pM) and low- (K_D 2.53 nM) affinity binding sites. This apparent receptor heterogeneity does not affect the utility of the adrenal-cortex preparation in the radioreceptor binding assay, and indeed has been exploited to enable an increase in assay sensitivity. This was achieved by increasing the assay volume from 120 μl to 1.2 ml, while maintaining the same amount (but lower concentration) of [^{3}H]Ins(1,4,5)P_3 and membrane protein added (see the Materials and methods section). A comparison of displacement of 0.15 nM- or 1.5 nM-[^{3}H]Ins(1,4,5)P_3 by both D- and L-Ins(1,4,5)P_3 is shown in Fig. 4, and illustrates the increased sensitivity afforded to the assay by decreasing the radioligand concentration, while retaining identical stereospecificity between D- and L-Ins(1,4,5)P_3. This modification of the method allows accurate measurement of 20 fmol of Ins(1,4,5)P_3 in a 300 μl sample of tissue extract, compared with 70 fmol of Ins(1,4,5)P_3 in 30 μl in the lower-volume assay. This enhancement is a combination of the increased intrinsic sensitivity achieved by using the lower concentration of [^{3}H]Ins(1,4,5)P_3 and the increase in sample volume. Perhaps the improvement this affords is most clearly illustrated by stating that the concentration of Ins(1,4,5)P_3 detectable by each method is 2 nM for the low-volume assay and 70 pM for the high-volume assay.

To illustrate the value of the radioreceptor assay, Ins(1,4,5)P_3 concentrations determined under basal and

Table 1. Derivation of K_D values for [^{3}H]Ins(1,4,5)P_3 binding to adrenal-cortex membranes from kinetic experiments

k_{+1} was determined from the relationship: $k_{+1} = (k_{obs} - k_{-1})/L$, where L represents the radioligand concentration (2.73±0.37 nM, n = 3).

$k_{obs.}$ (min^{-1})	k_{-1} (min^{-1})	k_{+1} ($min^{-1} \cdot nM^{-1}$)	K_D (nM)
0.857±0.103	0.412±0.121	0.163	2.53
0.857±0.103	0.016±0.005	0.308	0.052

agonist-stimulated conditions are shown for a variety of cell types in Table 2. The standard assay (120 μl assay volume) was used for determinations in CC and BSTM tissue extracts and SH-SY5Y cells; however, it was necessary to use the larger-volume (1.2 ml assay volume) method for determination of Ins(1,4,5)P_3 concentration in extracts of cerebellar granule cells, eosinophils and platelets. Maximally effective concentrations of the muscarinic-cholinergic agonist carbachol caused 50%, 110%, 160% and 1500% increases in Ins(1,4,5)P_3 concentration in CC, BTSM, cerebellar granule cells and SH-SY5Y cells respectively. Similarly, 1 μM platelet-activating factor caused a 3-fold increase in Ins(1,4,5)P_3 concentration in eosinophils. The results for basal and thrombin-stimulated platelet Ins(1,4,5)P_3 concentrations, obtained in the present study, are directly comparable with values reported previously [16]. More detailed time courses of agonist-stimulated changes in Ins(1,4,5)P_3 concentration are shown in Fig. 5. In CC slices, the increase in Ins(1,4,5)P_3 concentration was maximal by 20 s and maintained for at least 5 min, whereas in BTSM there was a transient increase in Ins(1,4,5)P_3 concentration, which was maximal after 5 s exposure to agonist and then decreased to below basal values by 60 s.

A striking feature of these observations, particularly for the slice preparations, is the relatively high basal and stimulated Ins(1,4,5)P_3 concentrations present in these tissues, equivalent to an approximate cytosolic concentration of 3–4 μM under resting conditions. Since these concentrations of Ins(1,4,5)P_3 are higher than those reported to produce half-maximal stimulation of Ca^{2+} release in similar neuronal and airway smooth-muscle

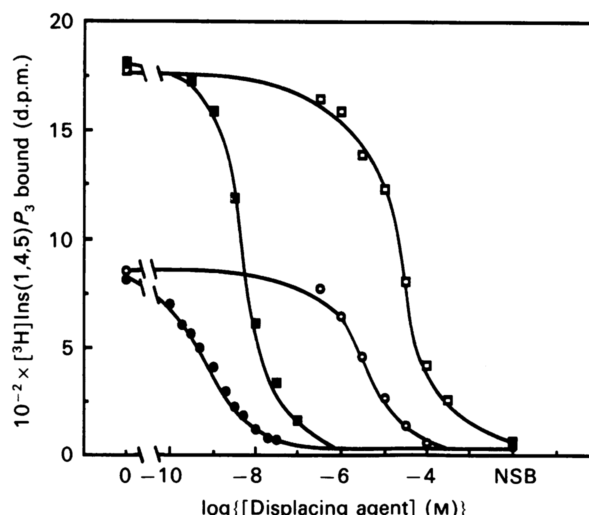

Fig. 4. Comparison of displacement of [^{3}H]Ins(1,4,5)P_3 at 0.15 nM and 1.5 nM by unlabelled Ins(1,4,5)P_3 in adrenal-cortex membranes

Incubation conditions for displacement of specific binding of 1.5 nM-[^{3}H]Ins(1,4,5)P_3 by D- (■) and L- (□) Ins(1,4,5)P_3 were as detailed in the legend to Fig. 1 (final volume 120 μl). The displacement of specific binding of 0.15 nM-[^{3}H]Ins(1,4,5)P_3 by D- (●) and L- (○) Ins(1,4,5)P_3 was performed in parallel incubations, by increasing the assay volume 10-fold to 1.2 ml while maintaining the same buffer composition and adding an identical amount of [^{3}H]Ins(1,4,5)P_3 and adrenal-cortex membrane protein as used above. Bound and free radioligand was separated in both instances by rapid filtration after incubation for 30 min at 4 °C. Data from a single representative experiment are shown, with similar results obtained in a further four experiments. Abbreviation: NSB, non-specific binding.

Table 2. Mass measurement of basal and agonist-stimulated Ins(1,4,5)P_3 in tissue and cell preparations

Results are expressed as means±S.E.M. for 3–5 experiments, each performed in duplicate or triplicate. Cerebellar granule cells were prepared from 7–8-day-old rats [27]; experiments were performed after cell culture for 7 days. Human neuroblastoma SH-SY5Y cells were cultured as described in [28]. Cells were washed in Krebs–Henseleit bicarbonate and incubated as described for tissue-slice incubations in the Materials and methods section. Eosinophils were obtained by lavage of polymyxin B-treated guinea pigs [29] and purified as described in [30]. Platelets were prepared by gel filtration from freshly drawn human blood and incubated as described in [31].

	Stimulus	Ins(1,4,5)P_3 concn. (pmol/mg of protein or * pmol/10^8 cells) Basal	Stimulated
Cerebral cortex	Carbachol (1 mM, 20 s)	17.9±0.7	26.9±0.9
Tracheal smooth muscle	Carbachol (0.1 mM, 5 s)	12.9±0.8	27.1±1.5
Cerebellar-granule cells	Carbachol (1 mM, 10 s)	6.6±1.7	17.1±0.8
SH-SY5Y human neuroblastoma cells	Carbachol (1 mM, 10 s)	29.4±7.8	466±142
Eosinophils*	Platelet-activating factor (1 μM, 5 s)	3.7±0.1	13.7±2.3
Platelets*	Thrombin (1 unit/ml, 15 s)	0.44±0.09	1.18±0.11

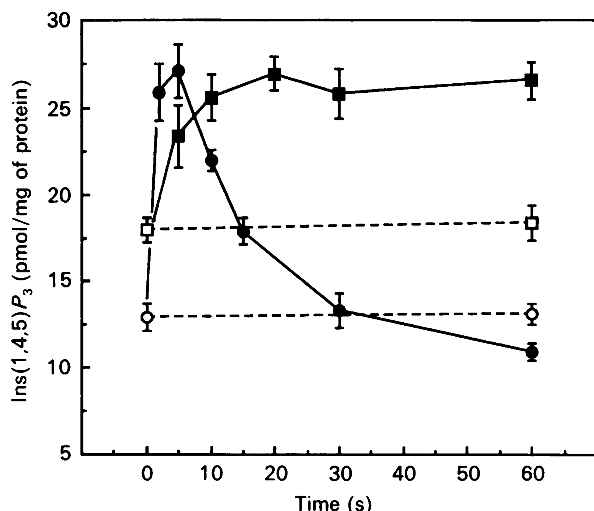

Fig. 5. Time course for carbachol-stimulated accumulation of Ins(1,4,5)P_3 in cerebral cortex and tracheal smooth-muscle slices

Rat CC (□, ■) and BTSM (○, ●) slices were prepared as described previously [14,22], and after preincubation in Krebs–Henseleit buffer were stimulated with 1 mM- and 0.1 mM-carbachol (■, ●) respectively or buffer (□, ○). Neutralized trichloroacetic acid extracts were obtained and Ins(1,4,5)P_3 mass was determined as described in the Materials and methods section.

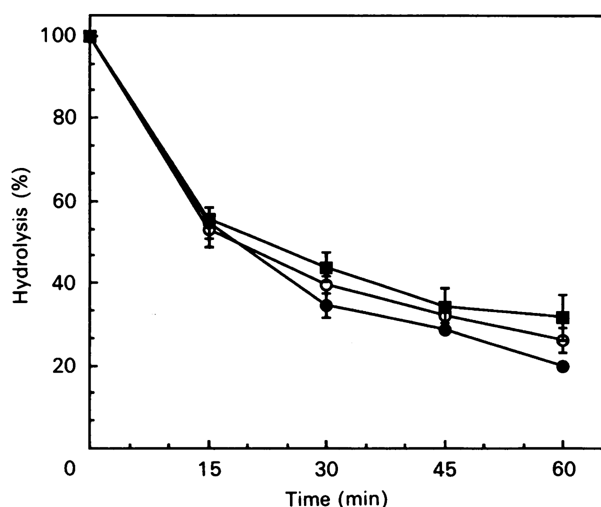

Fig. 6. Metabolism of Ins(1,4,5)P_3 and [^{3}H]Ins(1,4,5)P_3 in tissue extracts by a 5-phosphatase preparation

Time course for hydrolysis of Ins(1,4,5)P_3 (●, ■) and 'spiked' [^{3}H]Ins(1,4,5)P_3 (○) in extracts from control (●,○) and carbachol-stimulated (■) BTSM slices by human red-blood-cell ghosts (0.5 mg/ml). Loss of Ins(1,4,5)P_3 mass was assessed by the Ins(1,4,5)P_3 radioreceptor assay, and metabolism of [^{3}H]Ins(1,4,5)P_3 was measured by Dowex AG1-X8 anion-exchange resin as described in the Materials and methods section. Details of the incubation conditions used for the enzyme treatment are in the Materials and methods section. Data are shown as means ± S.E.M. of three experiments each performed in duplicate.

preparations [32–34], it was clearly important to demonstrate that the values obtained were a true measurement of cellular Ins(1,4,5)P_3 concentration. This question was addressed by enzymically removing Ins(1,4,5)P_3 from tissue extracts, either by conversion into Ins(1,4)P_2 by 5-phosphatase treatment, or by metabolism to Ins(1,3,4,5)P_4 by 3-kinase treatment, and comparing the rate and degree of loss of Ins(1,4,5)P_3 mass in these samples with loss of authentic [^{3}H]Ins(1,4,5)P_3 'spiked' into identically treated samples. The time course of Ins(1,4,5)P_3 and [^{3}H]Ins(1,4,5)P_3 metabolism after addition of the erythrocyte 5-phosphatase preparation to basal and carbachol-stimulated extracts from BTSM slices is shown in Fig. 6. The similar time course of changes in Ins(1,4,5)P_3 concentration and [^{3}H]Ins(1,4,5)P_3 (added to samples of tissue extract immediately before addition of the 5-phosphatase preparation) is clearly illustrated. Although only 70–80 % of the Ins(1,4,5)P_3 mass and [^{3}H]Ins(1,4,5)P_3 were metabolized in the 60 min incubation period, preliminary studies using a higher concentration of erythrocyte ghosts (1 mg of protein/ml) had demonstrated that over 95 % metabolism of Ins(1,4,5)P_3 could be achieved for both basal and carbachol-stimulated tissue extracts.

Similar data were obtained by incubating tissue extracts from rat CC slices with a crude 3-kinase preparation in the presence of ATP. Enzyme treatment resulted in 75–80 % loss of both [^{3}H]Ins(1,4,5)P_3 (converted exclusively into [^{3}H]InsP_4) and Ins(1,4,5)P_3 mass from basal and carbachol-stimulated tissue extracts. Complete removal of the Ins(1,4,5)P_3 was not possible, as the high concentrations of ATP (or phosphocreatine added as a component of the ATP-regenerating system), required to maintain kinase activity, interfere with the Ins(1,4,5)P_3 radioreceptor assay (see Fig. 2). Thus these experiments demonstrate that metabolism of Ins(1,4,5)P_3 to InsP_2 or InsP_4 results in a strict correlation between the rate and extent of [^{3}H]-Ins(1,4,5)P_3 metabolism and the loss of binding in the radioreceptor assay, implying that the apparent concentrations measured in tissue extracts represent measurement of authentic Ins(1,4,5)P_3 and that, at concentrations present in tissue extracts, the immediate metabolites of Ins(1,4,5)P_3 do not interfere significantly with the mass determination.

Since the values for Ins(1,4,5)P_3 concentration within brain and tracheal smooth-muscle tissue appear to be in the low micromolar range, this suggests either compartmentation of this second messenger in cells, or the presence of a 3-kinase/5-phosphatase-sensitive soluble inhibitor of [^{3}H]Ins(1,4,5)P_3 binding present in these tissue extracts other than Ins(1,3,4,5)P_4, Ins(1,3,4)P_3 or ATP. The possible influence of Ca^{2+} on the specific binding of [^{3}H]Ins(1,4,5)P_3 (as has been demonstrated in neuronal tissue [35]) has been excluded by the presence of 1 mM-EDTA in the assay. This idea of possible compartmentation of Ins(1,4,5)P_3 within cells was also proposed by Horstman *et al.* [36] on the basis of studies in AR42J pancreatoma cells, where basal and substance P-stimulated Ins(1,4,5)P_3 concentrations were 2 μM and 25 μM respectively. From estimates of basal Ins(1,4,5)P_3 turnover they suggested that approx. 95 % of the Ins(1,4,5)P_3 present in these cells may be sequestered in a non-cytosolic compartment.

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