Are there subtypes of the inositol 1,4,5-trisphosphate receptor?

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We have compared the properties of the [^3H]Ins(1,4,5) P_3 -binding sites from a number of tissues in an attempt to determine if heterogeneity exists within the Ins(1,4,5) P_3 -receptor family. The binding of Ins(1,4,5) P_3 was characterized in detail by using membranes prepared from human uterine smooth muscle and bovine adrenal cortex. Ins(1,4,5) P_3 exhibited an approx. 5 times greater affinity for the binding site in adrenal cortex ($K_D = 9.81 \pm 1.92$ nM) compared with uterine smooth muscle ($K_D = 37.1 \pm 1.8$ nM). The binding was dependent on pH in both tissues, with a maximum at pH 8.3; at this pH various inositol phosphates and nucleotides competed for the binding sites with similar potencies on both tissues. However, the binding of Ins(1,4,5) P_3 to the uterine smooth-muscle membranes was Ca^{2+} -sensitive, whereas that to the bovine adrenal cortex was not; furthermore, heparin displaced the binding of Ins(1,4,5) P_3 in the uterus with an IC_{50} value (concn. of displacer giving 50% inhibition of specific binding) of 3.9 $\mu\text{g}/\text{ml}$ (2.5, 6.4; lower, upper range), compared with a value of 22 (13, 30) $\mu\text{g}/\text{ml}$ in adrenal cortex. In view of the ability of Ins(1,4,5) P_3 and heparin to distinguish between these binding sites, their effect on other tissues was examined. Ins(1,4,5) P_3 showed a similar affinity for receptors located in the bovine cerebellum to those in the bovine adrenal cortex, but heparin displaced Ins(1,4,5) P_3 binding with a 5-fold greater affinity from the cerebellum. Ins(1,4,5) P_3 had a 2-fold greater affinity for its receptor with human platelets, as compared with human uterus, but heparin was unable to distinguish between these sites. In guinea-pig ileum, Ins(1,4,5) P_3 displayed a similar affinity for the receptors in the longitudinal muscle compared with the circular muscle, but heparin could distinguish between these sites. These data show that small differences exist between tissues, but no clear picture is apparent. It is possible that these results reflect tissue-dependent factors such as phosphorylation, the presence of calmodulin etc., rather than the presence of receptor subtypes or species difference.

INTRODUCTION

Inositol 1,4,5-trisphosphate [Ins(1,4,5) P_3] is now established as a second messenger mediating the release of intracellular Ca^{2+} from non-mitochondrial stores (Streb *et al.*, 1983; Berridge & Irvine, 1984; Prentki *et al.*, 1984). The existence of an Ins(1,4,5) P_3 receptor was first suggested from radioligand-binding experiments (Baukal *et al.*, 1985; Spät *et al.*, 1986; Guillemette *et al.*, 1987; Worley *et al.*, 1987). This was confirmed when an Ins(1,4,5) P_3 -binding site was purified to homogeneity from rat cerebellum (Supattapone *et al.*, 1988a), and later from mouse cerebellum, where it has been cloned, sequenced and expressed in the NG108-15 cell line (Furuichi *et al.*, 1989). In addition, reconstitution of this purified protein into planar lipid membranes has revealed that the Ins(1,4,5) P_3 receptor is a Ca^{2+} channel (Ferris *et al.*, 1989).

Although binding sites for Ins(1,4,5) P_3 have been described in many different tissues, variations exist in the reported affinity (K_D) values. For example, in rat cerebellum the K_D value for Ins(1,4,5) P_3 is approx. 40 nM (Worley *et al.*, 1987), compared with 5 nM in bovine adrenal cortex (Baukal *et al.*, 1985; Palmer *et al.*, 1988a). In addition, the mucopolysaccharide heparin has been shown to have different IC_{50} values (the concentration of displacer required to inhibit 50% of specific binding) for these two tissues, and these results have led to the suggestion of Ins(1,4,5) P_3 receptor subtypes (Willcocks & Nahorski, 1989).

Since the binding of Ins(1,4,5) P_3 to its receptor is sensitive to pH, the presence of bivalent cations and nucleotides, and possibly temperature, it is unclear whether these differences are real or represent differences in tissue preparation and experimental conditions. In the present study, therefore, we have characterized the binding of [^3H]Ins(1,4,5) P_3 to membranes from bovine adrenal cortex and human uterus prepared under identical conditions,

and show that Ins(1,4,5) P_3 has different K_D values for these two sites. In addition, these sites can be further distinguished by using heparin. The affinity of Ins(1,4,5) P_3 and the ability of heparin to displace Ins(1,4,5) P_3 binding were examined on other tissues such as bovine cerebellum, human platelets, guinea-pig ileum, rat cerebral cortex and rat cerebellum in an attempt to investigate whether a consistent pattern exists between the K_D of Ins(1,4,5) P_3 and the IC_{50} of heparin displacement.

MATERIALS AND METHODS

Materials

D-[^3H]Ins(1,4,5) P_3 (sp. radioactivity 34–52 Ci/mmol) was obtained from Amersham International. D-Ins(1,4,5) P_3 , DL-Ins(1,4,5) P_3 , ATP, GTP, UTP, TTP, CTP, ITP, XTP, heparin (pig; 181 USP units/mg) and other standard laboratory reagents were obtained from Sigma Chemical Co. Ins(1) P , Ins(4) P , Ins(1,3,4,5) P_4 and Ins(1,3,4,5,6) P_5 were kindly given by Dr. Robin Irvine (Babraham, Cambridge, U.K.) and Dr. Ian Ragan (Merck, Sharp and Dohme, Terlings Park, Harlow, Essex, U.K.). Dowex AG 1-X8 formate resin was obtained from Bio-Rad. Soluene-100 was from Packard Instrument Co., and Ready Value scintillant cocktail was from Beckman.

Tissue preparation

Myometrium was collected from the lower uterine segment of pregnant women, and the uterine corpus of non-pregnant women, in accordance with the requirements of the Central Oxfordshire Research Ethics Committee. The density and affinity of Ins(1,4,5) P_3 -binding sites were identical in tissue from both pregnant and non-pregnant subjects (Rivera *et al.*, 1990). Human platelets were obtained from platelet-rich plasma donated within the

Abbreviation used: IC_{50} , concn. of displacer required to inhibit 50% of specific binding.

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previous 24 h. Bovine adrenal cortex and bovine cerebellum were obtained fresh from the abattoir. Rat cerebellum was dissected from male Wistar rats (100–300 g). Guinea-pig ileum was taken from animals of the Dunkin–Hartley strain (350–360 g), and the longitudinal and circular muscle was dissected from it as described by Bitar & Makhlouf (1982).

Homogenates of the tissues (except platelets) were prepared essentially as described by Worley *et al.* (1987). Briefly, tissues were homogenized (Polytron, setting 9) in 10 vol. of ice-cold buffer A (20 mM-NaCl, 100 mM-KCl, 50 mM-Tris/HCl, 1 mM-EDTA, pH 8.3) and centrifuged at 1000 *g* for 15 min at 4 °C. The supernatants were pooled and centrifuged at 35000 *g* for 15 min at 4 °C, and the resulting pellets washed with a further 10 vol. of buffer and recentrifuged. The pellets were resuspended in buffer at a protein concentration of approx. 10 mg/ml and stored at –20 °C in 5 ml batches; activity was retained for at least 3 months. Platelets were suspended in buffer B (10 mM-Hepes, pH 7.4, containing 2 mM-EDTA) and disrupted by sonication (4 × 30 s) and centrifuged at 800 *g* for 10 min. The resultant pellet was again disrupted by sonication, and this was repeated up to five times, or until the pellet size remained constant. The supernatants were pooled and centrifuged, with washing with buffer A as described above.

[³H]Ins(1,4,5)P₃ binding

All experiments were performed at 4 °C in a final volume of 250 µl containing buffer A, approx. 1 nM-[³H]Ins(1,4,5)P₃, membrane suspensions containing 0.2–0.5 mg of protein, and various concentrations of displacing agents. Non-specific binding was determined in the presence of 10 µM-Ins(1,4,5)P₃. The contents of the assay were incubated for 5–15 min, and the reaction was stopped by centrifugation at 13000 *g* for 5 min. The supernatants were discarded and the pellets washed briefly, dissolved in Soluene-100, scintillant was added, and the radioactivity determined by liquid-scintillation counting. *K_D* and *B_{max}* values for [³H]Ins(1,4,5)P₃ binding were determined by Scatchard transformation of displacement data obtained by diluting with Ins(1,4,5)P₃ over the range 0.01 nM–3 µM.

In experiments designed to check the metabolism of [³H]-Ins(1,4,5)P₃, incubations were terminated at various time points by the addition of 750 µl of chloroform/methanol/HCl (50:100:1, by vol.), followed by 300 µl of chloroform and 300 µl of water to separate the aqueous and organic phases. Samples of the upper aqueous phase were diluted with water, and [³H]-Ins(1,4,5)P₃ was separated by anion-exchange chromatography as described by Berridge *et al.* (1982). Samples were added to glass columns containing 0.5 ml of Dowex AG 1-X8 formate resin (1 vol. of Dowex:1 vol. of water). Columns were eluted with 10 ml of water, followed by 16 ml of 400 mM-ammonium formate containing 0.1 M-formic acid. Finally, columns were eluted with 8 ml of 800 mM-ammonium formate/0.1 M-formic acid. This final fraction was collected in scintillation vials, scintillation fluid was added, and the vials were counted for radioactivity by liquid scintillation counting.

The effect of Ca²⁺ and Mg²⁺ in displacing Ins(1,4,5)P₃ binding was investigated. Concentrations of free ions in Ca²⁺- or Mg²⁺-EDTA buffers were calculated by using the computer program 'Freecal2.1' kindly given by L. Brass (Philadelphia), and is based on the affinity constants derived by Fabiato & Fabiato (1979). For the required range of ion concentrations to be achieved, the pH of the buffer was lowered to the more physiological value of 7.1. With these binding experiments the incubation times were decreased to 5 min, to minimize Ins(1,4,5)P₃ metabolism.

For the comparison of Ins(1,4,5)P₃ and heparin binding on different tissues, *K_D* values of Ins(1,4,5)P₃ were obtained from

Scatchard plots, and IC₅₀ values for heparin were obtained from full displacement curves.

Data analysis

Displacement curves and saturation curves were analysed by non-linear regression by using an iterative curve-fitting program. Displacement curves were fitted to a Hill equation as originally described by Hill (1910), and saturation curves were fitted to a rectangular hyperbola. Both equations were fitted by using the computer program 'GraphPAD' (ISI Software).

RESULTS

Characterization of Ins(1,4,5)P₃ binding in bovine adrenal cortex and human uterus

Consistent with the results in other tissues, the binding of Ins(1,4,5)P₃ to membranes prepared from bovine adrenal cortex and uterine smooth muscle was extremely rapid and reached equilibrium within 2 min (Fig. 1). Similarly, the dissociation of Ins(1,4,5)P₃ was very rapid, with a half-life of approx. 10 s (Fig. 1), and because of this we considered it essential to use centrifugation as the method of separation of free Ins(1,4,5)P₃ from bound Ins(1,4,5)P₃. The breakdown of [³H]Ins(1,4,5)P₃ was investigated, and under our incubation conditions we could detect no more than a 5% metabolism of Ins(1,4,5)P₃.

Fig. 1. Association and dissociation of [³H]Ins(1,4,5)P₃ binding to membranes prepared from bovine adrenal cortex and human uterus

For association experiments (○), reactions were started by addition of the membranes, and stopped at various time points by centrifugation, as described in the Materials and methods section. For the dissociation (●), 10 µM-Ins(1,4,5)P₃ was added (arrow head). Each point shows the mean of values converted into percentage of maximal binding, performed in quintuplicate, and are plotted against time. Standard errors of each point were less than 10% of the mean values. (a) Association and dissociation for the bovine adrenal cortex; (b) results from human uterus.

Fig. 2. Effect of pH on $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding on bovine adrenal cortex and human uterus membranes

The graph shows specific binding (total minus non-specific binding), converted into percentage of maximal binding, against pH for bovine adrenal cortex (●) and human uterus (○). Data points show means from three or four experiments, each performed in triplicate. Standard errors of each point were less than 10% of the mean values.

The binding of $\text{Ins}(1,4,5)\text{P}_3$ was markedly altered by pH in both tissues, with specific binding increasing with pH up to a maximum at pH 8.3 (Fig. 2). Saturation analysis experiments performed at pH 8.3 revealed Hill coefficients (h) of 0.97 ± 0.10 for human uterus, and 0.95 ± 0.08 for bovine adrenal cortex. Scatchard transformations gave apparent K_D values of $37.1 \pm 1.8 \text{ nM}$ ($n = 36$) and $9.81 \pm 1.92 \text{ nM}$ ($n = 8$) in uterine smooth muscle and adrenal cortex respectively (Fig. 3). For both tissues, binding was to a single site with a density (B_{max}) of $1.47 \pm 0.4 \text{ pmol/mg}$ of protein and $2.91 \pm 0.51 \text{ pmol/mg}$ of protein for the human uterus and bovine adrenal cortex respectively. The density of sites for the bovine adrenal cortex is higher than reported by others (Baukal *et al.*, 1985; Guillemette *et al.*, 1989; Challiss *et al.*, 1990); however, this may be explained by a loss of the binding of $\text{Ins}(1,4,5)\text{P}_3$ by those authors who use filtration rather than centrifugation as the method of stopping the binding reaction, since in our hands stopping the reaction by filtration caused a decrease in $\text{Ins}(1,4,5)\text{P}_3$ binding.

The ability of several inositol phosphates to compete for the

Fig. 3. Scatchard plots of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding in bovine adrenal cortex and human uterus membranes

Bovine adrenal-cortex membranes (●) and human uterus membranes (○) were incubated with $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (approx. 1 nM) and various concentrations of unlabelled $\text{Ins}(1,4,5)\text{P}_3$. The displacement curves obtained were transformed into Scatchard plots, which are plotted as B/F [the ratio of specifically bound to free ligand, in fmol of $\text{Ins}(1,4,5)\text{P}_3$ $[\text{fmol} \cdot (\text{mg of protein})^{-1} \cdot \text{nM}^{-1}]$ against Bound [the amount of specifically bound ligand, in fmol of $\text{Ins}(1,4,5)\text{P}_3/\text{mg}$ of protein].

$\text{Ins}(1,4,5)\text{P}_3$ -binding site was investigated. As shown in Table 1, all of the isomers studied were considerably less potent than $\text{Ins}(1,4,5)\text{P}_3$, and demonstrated similar profiles on both tissues. For example, $\text{Ins}(1,3,4,5)\text{P}_4$, which has been suggested to be a second messenger controlling the re-filling of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} pool (Irvine *et al.*, 1988), was 15-fold and 20-fold less potent than $\text{Ins}(1,4,5)\text{P}_3$ in uterine smooth muscle and bovine adrenal cortex respectively. Several nucleoside triphosphates were also investigated for their ability to displace the binding of $\text{Ins}(1,4,5)\text{P}_3$. GTP was the most potent displacer, with an IC_{50} of approx. $150 \mu\text{M}$ in both tissues, whereas ATP was 2–3 times less potent (Table 1). Other nucleotides, namely CTP, TTP, UTP, ITP and XTP, displaced the binding with IC_{50} values in the range $500\text{--}2000 \mu\text{M}$; values were almost identical in both tissues.

The $\text{Ins}(1,4,5)\text{P}_3$ -binding sites in both tissues could be distinguished by using heparin. Heparin displaced the binding of

Table 1. Effect of displacing agents on $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding in bovine adrenal cortex and human uterus membranes

Results for IC_{50} values were obtained from the curve-fitting computer program 'GraphPAD' and are expressed as the geometric mean (upper, lower range) from at least five separate experiments, each performed in quintuplicate. Hill coefficients (h) in each case were determined with the same computer program and are shown as means $\pm$ S.E.M. Experiments with those displacing agents which were not very potent at displacing $\text{Ins}(1,4,5)\text{P}_3$ were performed twice in quintuplicate, and therefore the mean IC_{50} value only is shown. All IC_{50} values are shown as μM , except heparin data, which are expressed as $\mu\text{g/ml}$. Abbreviation: GpIP_2 , L- α -glycerophospho-D-*myo*-inositol 4,5-bisphosphate.

Compound	Human uterus		Bovine adrenal cortex	
	IC_{50} (μM)	h	IC_{50} (μM)	h
$\text{Ins}(1,4,5)\text{P}_3$	0.034 (0.022, 0.052)	1.08 ± 0.11	0.0078 (0.0056, 0.011)	0.95 ± 0.06
$\text{Ins}(1)\text{P}_1$	24		30	
$\text{Ins}(4)\text{P}_1$	19		22	
$\text{Ins}(1,4)\text{P}_2$	22		30	
$\text{Ins}(1,3,4,5)\text{P}_4$	0.53 (0.38, 0.73)	0.92 ± 0.14	0.25 (0.19, 0.35)	0.90 ± 0.11
$\text{Ins}(1,3,4,5,6)\text{P}_5$	26		30	
InsP_6	20		16	
GpIP_2	0.083 (0.075, 0.115)	0.87 ± 0.15	0.095 (0.078, 0.123)	1.05 ± 0.10
ATP	312 (258, 377)	1.10 ± 0.15	429 (290, 646)	0.99 ± 0.08
GTP	171 (133, 218)	1.04 ± 0.17	130 (99, 168)	0.91 ± 0.12

Fig. 4. Displacement of [^3H]Ins(1,4,5) P_3 binding by heparin from bovine adrenal cortex and human uterus membranes

Bovine adrenal-cortex membranes (●) and human uterus membranes (○) were incubated with various concentrations of heparin (plotted in $\mu\text{g}/\text{ml}$). Inhibition curves were fitted by the curve-fitting program 'GraphPAD'. Points show means $\pm$ S.E.M. for between four and six experiments each performed in quintuplicate.

Ins(1,4,5) P_3 in adrenal cortex with an IC_{50} of 22 $\mu\text{g}/\text{ml}$ (13, 30 $\mu\text{g}/\text{ml}$; lower, upper range), compared with a value of 4.1 $\mu\text{g}/\text{ml}$ (2.5, 6.4) in uterine smooth muscle (Fig. 4). The Hill coefficients obtained from the inhibition curves were not significantly different from unity (Table 2). If these results are expressed relative to Ins(1,4,5) P_3 , heparin has an approx. 30 times greater potency for the Ins(1,4,5) P_3 -binding site in uterine smooth muscle than in adrenal cortex. Scatchard plots determined in the presence of a fixed concentration of heparin (10 $\mu\text{g}/\text{ml}$) suggest that, in the tissues studied (bovine adrenal cortex, human uterus and rat cerebellum), heparin acts as a competitive inhibitor at the same site as Ins(1,4,5) P_3 (Fig. 5).

Ca^{2+} produced an inhibition of the binding of Ins(1,4,5) P_3 in uterine smooth muscle at the intracellular pH of 7.1, with an IC_{50} of approx. 100 μM (Fig. 6), but had no effect on Ins(1,4,5) P_3 binding in adrenal cortex. Mg^{2+} was found to inhibit the binding of Ins(1,4,5) P_3 in both tissues at pH 7.1, with an IC_{50} value of 100 μM . Since these ions are known to stimulate metabolism of Ins(1,4,5) P_3 (Storey *et al.*, 1984; Biden & Wollheim, 1986), the breakdown of [^3H]Ins(1,4,5) P_3 was investigated. Under our

Fig. 5. Effect of heparin on Scatchard transformations of [^3H]Ins(1,4,5) P_3 binding in bovine adrenal-cortex and rat cerebellum membranes

Displacement curves were performed as described in Fig. 3, except that one was performed in the absence (●) and one in the presence (○) of 10 μg of heparin/ml. (a) Results from bovine adrenal cortex, (b) results from rat cerebellum, from one typical experiment, representative of three separate experiments.

incubation conditions, we could detect no more than a 10% decrease in [^3H]Ins(1,4,5) P_3 in the presence of 10 μM free Ca^{2+} , 0.3 mM free Mg^{2+} , and with other agents which decreased Ins(1,4,5) P_3 binding, including nucleotides and heparin (results not shown).

Table 2. Ins(1,4,5) P_3 binding and heparin displacement in different tissues

K_D values for Ins(1,4,5) P_3 were calculated from Scatchard transformations of displacement curves, and are shown as the means $\pm$ S.E.M. for between 3 and 36 experiments, expressed in nM. B_{max} values are shown as means $\pm$ S.E.M. and expressed as pmol/mg of protein. IC_{50} values for heparin displacement curves were calculated with the computer program 'GraphPAD', and are shown as the geometric mean (upper, lower range) for between three and eight experiments, expressed in $\mu\text{g}/\text{ml}$. Corresponding Hill coefficients (h) are shown as means $\pm$ S.E.M. Abbreviation: GPI, guinea-pig ileum.

Tissue	Ins P_3		Heparin	
	K_D (nM)	B_{max} (pmol/mg of protein)	IC_{50} ($\mu\text{g}/\text{ml}$)	h
Bovine adrenal cortex	9.81 $\pm$ 1.92	2.91 $\pm$ 0.51	19.8 (13, 30)	0.96 $\pm$ 0.08
Bovine cerebellum	11.0 $\pm$ 0.6	1.05 $\pm$ 0.30	3.8 (2.4, 6)	0.91 $\pm$ 0.18
Human uterus	37.1 $\pm$ 1.8	1.47 $\pm$ 0.41	3.9 (2.5, 6.4)	1.08 $\pm$ 0.11
Human platelets	20.8 $\pm$ 1.8	0.64 $\pm$ 0.24	4.1 (2.5, 6.5)	1.15 $\pm$ 0.21
GPI longitudinal	4.20 $\pm$ 0.90	0.44 $\pm$ 0.08	4.3 (2.4, 7.5)	0.94 $\pm$ 0.15
GPI circular	6.88 $\pm$ 1.18	0.30 $\pm$ 0.05	52.1 (32, 85)	0.88 $\pm$ 0.14
Rat cerebellum	36.5 $\pm$ 4.5	16.4 $\pm$ 1.6	6.54 (3.9, 11.1)	1.08 $\pm$ 0.12
Rat cerebral cortex	22.8 $\pm$ 2.6	1.61 $\pm$ 0.26	6.18 (4.1, 9.6)	0.85 $\pm$ 0.15

Fig. 6. Effect of Ca^{2+} on $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding in membranes prepared from bovine adrenal cortex and human uterus

$[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding was performed at pH 7.1 on bovine adrenal-cortex (●) and human uterus (○) membranes in the presence of various concentrations of Ca^{2+} . Free Ca^{2+} concentrations were calculated with the computer program 'FreeCalc' (see the Materials and methods section) and are plotted against the specific binding, expressed as a percentage of maximal binding. Points represent means $\pm$ S.E.M. for at least three experiments performed in quintuplicate.

Ins(1,4,5) P_3 -binding sites in different tissues

Ins(1,4,5) P_3 binding was examined in a number of other tissue types in an attempt to see if a pattern could be determined between K_D values for Ins(1,4,5) P_3 and IC_{50} values of heparin displacement. For example, it is possible that the difference in the affinity of heparin between the human uterus and the bovine adrenal cortex is the result of a species difference.

Ins(1,4,5) P_3 bound to membranes prepared from human platelets with a K_D value of 21 ± 2 nM, compared with 37 ± 1.8 nM for the human uterus, but the IC_{50} of heparin was almost identical on both of these tissues, with a value of approx. $4 \mu\text{g}/\text{ml}$ (Table 2). However, with bovine tissue, Ins(1,4,5) P_3 showed a similar affinity for receptors located in the bovine cerebellum to those in the bovine adrenal cortex (9.81 ± 1.92 and 11.0 ± 0.51 nM respectively), but heparin displaced Ins(1,4,5) P_3 binding with a 5-fold greater potency on the cerebellum [$\text{IC}_{50} = 3.8$ (2.4, 6) $\mu\text{g}/\text{ml}$] compared with the adrenal cortex [$\text{IC}_{50} = 19.8$ (13, 30) $\mu\text{g}/\text{ml}$]. In the guinea-pig ileum, the affinity of Ins(1,4,5) P_3 for its receptor in the longitudinal muscle was similar to that obtained in the circular muscle (4.2 ± 0.9 nM and 6.9 ± 1.2 nM respectively). Heparin, however, distinguished between these tissues, since it was approx. 10-fold more active in displacing Ins(1,4,5) P_3 from the longitudinal muscle compared with the circular muscle, with IC_{50} values of 4.3 (2.4, 7.5) $\mu\text{g}/\text{ml}$ and 52 (32, 85) $\mu\text{g}/\text{ml}$ respectively. K_D values for Ins(1,4,5) P_3 were different for the rat cerebellum and the rat cerebral cortex; Ins(1,4,5) P_3 showed a greater affinity for receptors located in the cerebral cortex than for those of the cerebellum (23 ± 3 nM and 37 ± 5 nM respectively), but heparin did not distinguish between these two tissue preparations [6.54 (3.9, 11.1) $\mu\text{g}/\text{ml}$ for cerebellum, and 6.18 (4.1, 9.6) $\mu\text{g}/\text{ml}$ for cortex]. In all of these tissue preparations the binding of Ins(1,4,5) P_3 was to a single site, and Hill coefficients of heparin displacement curves were not significantly different from unity (Table 2).

DISCUSSION

Differences in Ins(1,4,5) P_3 -binding sites

The ability of inositol phosphates to compete for the Ins-

(1,4,5) P_3 -binding site in bovine adrenal cortex and human uterus parallels closely their ability to stimulate the release of Ca^{2+} , suggesting that the binding is to the Ins(1,4,5) P_3 receptor rather than Ins(1,4,5) P_3 3-kinase or 5-phosphatase (Nahorski, 1988). In addition, heparin, which inhibits Ins(1,4,5) P_3 -induced Ca^{2+} release in a number of tissues, displaces Ins(1,4,5) P_3 binding with an affinity of the same order as that for inhibition of Ca^{2+} release in bovine adrenal cortex (Guillemette *et al.*, 1989).

However, small differences in the affinity of Ins(1,4,5) P_3 and heparin for Ins(1,4,5) P_3 -binding sites have been reported in the literature (see the Introduction), and these may reflect the existence of receptor subtypes (Willcocks & Nahorski, 1989). It is unclear, however, whether these differences are real or represent differences in tissue preparation and experimental conditions, since the binding of Ins(1,4,5) P_3 is sensitive to pH, bivalent cations and nucleotides. Care has therefore been taken to ensure that in the present study tissue preparation and binding experiments have been carried out under identical conditions to allow a direct comparison of the results. The characteristics of the binding site in human uterus are almost identical with those described for the Ins(1,4,5) P_3 receptor in rat cerebellum (Willcocks & Nahorski, 1989), and can be distinguished from the binding site in the bovine adrenal cortex by the affinity of heparin and Ins(1,4,5) P_3 .

The results of Willcocks & Nahorski (1989), which show differences in Ins(1,4,5) P_3 -binding characteristics between rat cerebellum and bovine adrenal cortex, could be attributed to species variation. We therefore chose to examine Ins(1,4,5) P_3 -binding sites from tissues obtained from the same species and have compared these results with each other, and between species. We have found that small differences in K_D values for Ins(1,4,5) P_3 are present in tissues from the same species. For example, the K_D of Ins(1,4,5) P_3 shows about a 2-fold difference between human platelets and human uterus, or between rat cerebral cortex and rat cerebellum. In addition, K_D values for bovine adrenal cortex and bovine cerebellum are similar, as are those obtained on the guinea-pig ileum longitudinal and circular muscles. This suggests, for example, that the difference in the K_D of Ins(1,4,5) P_3 between bovine adrenal cortex and human uterus, or rat cerebellum and bovine adrenal cortex (Willcocks & Nahorski, 1989), is probably an inter-species difference. It is possible that the small differences in the affinity of Ins(1,4,5) P_3 within a species may be attributed to factors such as variable phosphorylation of Ins(1,4,5) P_3 receptors. Indeed, it has been reported that the Ins(1,4,5) P_3 receptor in rat and mouse cerebellum can be phosphorylated by a cyclic-AMP-dependent protein kinase (Supattapone *et al.*, 1988b; Furuichi *et al.*, 1989).

Heparin acts as an antagonist at the Ins(1,4,5) P_3 receptor (Worley *et al.*, 1987), and also inhibits the Ins(1,4,5) P_3 3-kinase and the Ins(1,4,5) P_3 5-phosphatase, but with different affinities (Guillemette *et al.*, 1989). The results obtained with heparin agree with those of Ghosh *et al.* (1988), and show that heparin competes for Ins(1,4,5) P_3 binding in a competitive manner. In addition, we find that heparin displaces Ins(1,4,5) P_3 binding with different affinities between tissues. However, it is noteworthy that in some tissues, where Ins(1,4,5) P_3 had equal affinity, heparin could distinguish between receptors. In other tissues, where the affinity of Ins(1,4,5) P_3 differed, heparin did not distinguish between receptors. For example, human platelets and human uterus show a 2-fold difference in their affinity for Ins(1,4,5) P_3 , but heparin appears to displace Ins(1,4,5) P_3 binding with equal affinities from both tissues. Conversely, in the bovine adrenal cortex and the bovine cerebellum, similar K_D values for Ins(1,4,5) P_3 exist, but heparin shows a 5-fold difference in its ability to displace Ins(1,4,5) P_3 binding. It appears that the affinity of heparin is not necessarily correlated with the affinity of Ins-

(1,4,5) P_3 , and is not species specific. However, it should be noted that heparin is a considerably larger molecule than Ins(1,4,5) P_3 , and thus differences in the affinity of heparin between tissues may simply be due to the access difficulties associated with the larger molecule. The different K_D values of Ins(1,4,5) P_3 and IC_{50} values of heparin between different tissues cannot be attributed to variable metabolism.

We therefore consider there to be Ins(1,4,5) P_3 receptors present in the same species, and between species, with different affinities for Ins(1,4,5) P_3 and heparin. However, although these affinities vary slightly between tissues, there is no consistent pattern to suggest distinct subtypes of the Ins(1,4,5) P_3 receptor. The small changes in affinity may therefore be due to different forms of post-translational modifications such as phosphorylation or differences in the lipid environment, e.g. it has been shown that the affinity of ligands for muscarinic receptors is modulated by the lipid environment (Berstein *et al.*, 1989).

It has recently been reported that Ins(1,4,5) P_3 receptors in the rat liver can exist in two states which display different affinities for Ins(1,4,5) P_3 , and that the balance between these two states can be altered by Ins(1,4,5) P_3 -dependent hormones (Mauger *et al.*, 1989). However, in all our preparations only a single affinity for the Ins(1,4,5) P_3 -binding site could be detected (but see Challiss *et al.*, 1990). The possibility of one state of these receptors being lost under our experimental conditions requires investigation.

Physiological relevance of displacement of Ins(1,4,5) P_3 binding by pH, ATP, Ca^{2+} and Mg^{2+}

The observation that intracellular concentrations of Ca^{2+} ions inhibit the binding of Ins(1,4,5) P_3 in the uterus is in agreement with results from rat cerebellum (Worley *et al.*, 1987). Those authors ascribed the inhibitory action of Ca^{2+} to an allosteric effect mediated by the Ca^{2+} -binding protein calmodin, loosely associated with the receptor. It is unclear as to why the binding in the bovine adrenals (and other peripheral tissues that we have studied) is unaffected by Ca^{2+} ; whether this is due to the absence of calmodin from these tissues, or because the Ins(1,4,5) P_3 receptors in these tissues are insensitive to calmodin, remains to be seen.

Ins(1,4,5) P_3 binding was dependent on pH, and it has been suggested that this may be of physiological relevance, since intracellular pH is increased by growth factors and hormones (Worley *et al.*, 1987). Further, nucleotides such as ATP and GTP also compete for Ins(1,4,5) P_3 -binding sites at concentrations close to their intracellular levels, suggesting that they may also regulate the binding of Ins(1,4,5) P_3 . However, it is not known whether the nucleotides retain their ability to bind to the Ins(1,4,5) P_3 receptor when complexed with Mg^{2+} ions, the predominant species found within cells. Interestingly, Mg^{2+} ions also displaced the binding of Ins(1,4,5) P_3 , possibly as a consequence of binding directly to Ins(1,4,5) P_3 , at a concentration at least one order of magnitude less than that found within cells (intracellular free concentration ≈ 1 mM; Rink *et al.*, 1982).

The level of Ins(1,4,5) P_3 found within unstimulated cells is low-micromolar (Challis *et al.*, 1988; Chilvers *et al.*, 1989; Palmer *et al.*, 1988b), a result confirmed by ourselves in cerebral cortex, platelets and smooth muscle (M. A. Varney & S. P. Watson, unpublished work), and this concentration would be expected to stimulate Ca^{2+} release maximally. It is possible, however, that the high level of Ins(1,4,5) P_3 found within cells

reflects interference with binding to its receptor through a combination of the above factors.

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REFERENCES

- Baukal, A. J., Guillemette, G., Rubin, R., Spät, A. & Catt, K. J. (1985) *Biochem. Biophys. Res. Commun.* **133**, 532–538
- Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Berridge, M. J., Downes, C. P. & Hanley, M. R. (1982) *Biochem. J.* **206**, 587–595
- Berstein, G., Haga, T. & Ichiyama, A. (1989) *Mol. Pharmacol.* **36**, 601–607
- Biden, T. J. & Wollheim, C. B. (1986) *J. Biol. Chem.* **261**, 11931–11934
- Bitar, K. N. & Makhoul, G. M. (1982) *Am. J. Physiol.* **242**, G400–G407
- Challis, R. A. J., Batty, I. H. & Nahorski, S. R. (1988) *Biochem. Biophys. Res. Commun.* **157**, 684–691
- Challis, R. A. J., Chilvers, E. R., Willcocks, A. L. & Nahorski, S. R. (1990) *Biochem. J.* **265**, 421–427
- Chilvers, E. R., Challis, R. A. J., Barnes, P. J. & Nahorski, S. R. (1989) *Eur. J. Pharmacol.* **164**, 587–590
- Fabiato, A. & Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 433–501
- Ferris, C. D., Hagan, R. L., Supattapone, S. & Snyder, S. H. (1989) *Nature (London)* **342**, 87–89
- Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. & Mikoshiba, K. (1989) *Nature (London)* **342**, 32–38
- Ghosh, T. K., Eis, P. S., Mullery, J. M., Ebert, C. L. & Gill, D. L. (1988) *J. Biol. Chem.* **263**, 11075–11079
- Guillemette, G., Balla, T., Baukal, A. J., Spät, A. & Catt, K. J. (1987) *J. Biol. Chem.* **262**, 1010–1015
- Guillemette, G., Lamontagne, S., Boulay, G. & Mouillac, B. (1989) *Mol. Pharmacol.* **35**, 339–344
- Hill, A. V. (1910) *J. Physiol. (London)* **382**, 523–535
- Irvine, R. F., Moor, R. M., Pollock, K., Smith, P. M. & Wreggett, K. A. (1988) *Philos. Trans. R. Soc. London B* **320**, 281–297
- Mauger, J.-P., Claret, M., Pietri, F. & Hilly, M. (1989) *J. Biol. Chem.* **264**, 8821–8826
- Nahorski, S. R. (1988) *Trends Neurosci.* **11**, 444–448
- Palmer, S., Hughes, K. T., Lee, D. Y. & Wakelam, M. J. O. (1988a) *Cell. Signalling* **1**, 147–156
- Palmer, S., Hughes, K. T., Lee, D. Y. & Wakelam, M. J. O. (1988b) *Biochem. Soc. Trans.* **16**, 991–992
- Prentki, M., Biden, T. J., Janjic, D., Irvine, R. F., Berridge, M. J., Wollheim, C. B. & Lew, P. D. (1984) *Nature (London)* **309**, 562–564
- Rink, T. J., Tsien, R. Y. & Pozzan, T. (1982) *J. Cell Biol.* **95**, 189–196
- Rivera, J., Varney, M. A., Watson, S. P. & Lopez Bernal, A. (1990) *Endocrinology (Baltimore)*, in the press
- Spät, A., Fabiato, A. & Rubin, R. P. (1986) *Biochem. J.* **233**, 929–932
- Streb, H., Irvine, R. F., Berridge, M. J. & Shultz, I. (1983) *Nature (London)* **306**, 67–68
- Storey, D. J., Shears, S. B., Kirk, C. J. & Michell, R. H. (1984) *Nature (London)* **312**, 374–376
- Supattapone, S., Worley, P. F., Baraban, J. M. & Snyder, S. H. (1988a) *J. Biol. Chem.* **263**, 1530–1534
- Supattapone, S., Danoff, S. K., Thiebert, A., Joseph, S. K., Steiner, J. & Snyder, S. H. (1988b) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8747–8750
- Willcocks, A. L. & Nahorski, S. R. (1989) *Brit. J. Pharmacol.* **96**, 364P
- Worley, P. F., Baraban, J. M., Supattapone, S., Wilson, V. S. & Snyder, S. H. (1987) *J. Biol. Chem.* **262**, 12132–12136