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The Ins(1,4,5)P_3 binding site of bovine adrenocortical microsomes: function and regulation

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Adrenocortical microsomes possess a single population of Ins(1,4,5)P_3-specific binding sites [IC_{50} 5.9 ± 0.9 nM; Palmer, Hughes, Lee & Wakelam (1988) Cell. Signalling **1**, 147–156]. Competition studies showed that Ins(1:2-cyclic,4,5)P_3 exhibits a 21-fold lower affinity for the site than Ins(1,4,5)P_3 (IC_{50} 124 ± 16 nM). The affinity of the binding sites for Ins(1,4,5)P_3 was not influenced by the non-hydrolysable GTP analogues GTPγS and Gpp[NH]p or by preincubation of the binding protein with a preparation of partially purified protein kinase C in the presence of ATP and TPA (12-*O*-tetradecanoylphorbol 13-acetate). These observations are discussed with reference to the identity and function of the Ins(1,4,5)P_3 binding site.

INTRODUCTION

Stimulation of phospholipase-C-mediated PtdIns-(4,5)P_2 hydrolysis by calcium-mobilizing agonists results in the production of Ins(1,4,5)P_3 (Berridge, 1984; Downes & Michell, 1985). Ins(1,4,5)P_3 acts as a second messenger to elevate cytosolic free Ca^{2+} concentrations by activating Ca^{2+} release from an intracellular store in the endoplasmic reticulum (Berridge & Irvine, 1984) or perhaps from specialized organelles termed calciosomes (Volpe *et al.*, 1988). The Ca^{2+}-release response of permeabilized cells exhibits structural specificity with respect to InsP_3 and thus it has been proposed that a specific intracellular receptor is involved (Burgess *et al.*, 1984). Evidence for the presence of high-affinity intracellular InsP_3 binding sites has been obtained in a number of systems (see Palmer *et al.*, 1988, for references) including bovine adrenal cortex homogenate (Baukal *et al.*, 1985) and adrenocortical microsomes (Guillemette *et al.*, 1987; Palmer *et al.*, 1988). We have recently defined some of the characteristics of the putative 'InsP_3 receptor' in adrenocortical microsomes, including its phosphate group positional specificity for Ins(1,4,5)P_3 (Palmer *et al.*, 1988). In addition, Willcocks *et al.* (1987) have demonstrated the stereospecificity of the InsP_3 binding site in rat cerebellar membranes. A bovine adrenocortical microsomal preparation has been incorporated into an Ins(1,4,5)P_3-specific binding assay and used to determine the intracellular concentration of Ins(1,4,5)P_3 in both unstimulated and vasopressin-stimulated rat hepatocytes (Palmer *et al.*, 1988). The data presented here further characterize the properties of the putative 'Ins(1,4,5)P_3 receptor' of a bovine adrenocortical microsomal preparation and demonstrate that: (i) Ins(1:2-cyclic,4,5)P_3 is unlikely to release intracellular stores of Ca^{2+} under physiological conditions; (ii) a G-protein is unlikely to be involved in Ins(1,4,5)P_3-stimulated Ca^{2+} release and (iii) protein kinase C activity does not affect Ins(1,4,5)P_3 binding to its putative receptor.

MATERIALS AND METHODS

Materials

[^{3}H]Ins(1,4,5)P_3 (specific activity 38–44 Ci/mmol) and [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) were obtained from Amersham International plc. Ins(1,4,5)P_3 and Ins(1:2 cyclic,4,5)P_3 were a generous gift from Dr. R. F. Irvine (A.F.R.C., Cambridge, U.K.). A crude protein kinase C preparation prepared from rat brain homogenate by the method of Niedel *et al.* (1983) was generously provided by Dr. Neil Thompson of the Wellcome Foundation, London, U.K. GTPγS and Gpp[NH]p were purchased from Boehringer Mannheim; histone III-S (isolated from calf thymus) was from Sigma. All other reagents were of the highest grade commercially available.

Radioligand binding studies

Aliquots of a bovine adrenocortical microsomal fraction containing 500–1000 μg of protein, prepared as described previously (Palmer *et al.*, 1988), were incubated in 25 mM-Tris (pH 9)/1 mM-EDTA/1 mM-EGTA/5 mM-$NaHCO_3$/0.25 mM-dithiothreitol/1 mg of bovine serum albumin (Fraction V)/ml. Incubations were performed for 15 min on ice in a final volume of 100 μl with [^{3}H]Ins(1,4,5)P_3 [approx. 4000–6000 c.p.m. = 82–123 fmol (0.82–1.23 nM)] and unlabelled Ins(1,4,5)P_3 as appropriate. Non-specific binding was determined in the presence of 1 μM-unlabelled Ins(1,4,5)P_3. Incubations were stopped by centrifugation (12000 *g*, 3 min, 4 °C) and subsequent removal of the supernatant. Particulate-bound radioactivity was analysed by liquid scintillation spectrometry.

Specific binding in the absence of unlabelled Ins-(1,4,5)P_3 varied between 35 and 45 % of total radioactivity in the incubation and was generally in excess of 2000 c.p.m. Non-specific binding was approx. 10 % of total radioactivity per incubation.

Abbreviations used: Ins(1:2-cyclic,4,5)P_3, D-*myo*-inositol 1:2-cyclic,4,5-trisphosphate; GTPγS, guanosine 5′-*O*-[3-thio]triphosphate; Gpp[NH]p, guanosine 5′-[βγ-imido]triphosphate; G-protein, guanine-nucleotide-binding regulatory protein; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

The Ins(1:2-cyclic,4,5)P_3 competition studies employed [^{3}H]Ins(1,4,5)P_3 alone and the appropriate concentration of Ins(1:2-cyclic,4,5)P_3. In studies to determine the effects of non-hydrolysable GTP analogues, GTPγS (100 μM final concn.) or Gpp[NH]p (100μM final concn.) were added to incubations prior to addition of the binding protein.

Protein kinase C studies

These experiments involved the preincubation of the binding protein with a crude protein kinase C preparation [containing 0.05% (w/v) Triton X-100]. The adrenocortical microsomal preparation was centrifuged (12000 *g*, 3 min, 4 °C), washed and resuspended in 25 mM-Tris (pH 7.5)/1 mM-$MgCl_2$/0.1 mM-$CaCl_2$/100 μM-ATP. Additions of either buffer, Triton X-100 [0.0025% (w/v) final] or the crude protein kinase C preparation [100 μg of protein/ml of binding protein; 0.0025% (w/v) Triton X-100] in the presence or absence of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (100 nM) were made before incubation at 20 °C for 20 min. An aliquot of each incubation was removed and assayed for protein kinase C activity (see below). An equal volume of ice-cold 100 mM-Tris (pH 9)/4 mM-EDTA/4 mM-EGTA/4 mg of bovine serum albumin (Fraction V)/ml was added to the remainder of the incubation. Chelation of divalent cations was employed to inhibit non-specific phosphatases and the protein kinase C. The binding proteins were washed twice and resuspended in 50 mM-Tris (pH 9)/2 mM-EDTA/2 mM-EGTA/2 mg of bovine serum albumin (Fraction V)/ml prior to assay for [^{3}H]Ins(1,4,5)P_3 binding activity as described above.

Protein kinase C activity, in the presence of binding protein, was determined by incubation of an aliquot of the above mixtures with 100 μg of histone III-S and 1 μCi of [γ-^{32}P]ATP at 20 °C for 15 min. Incubations were terminated by addition of 20 vol. of ice-cold 20% (w/v) trichloroacetic acid. Samples were centrifuged (12000 *g*, 3 min, 4 °C), the supernatant removed and the pellets washed once with a further 20 vol. of 20% (w/v) trichloroacetic acid. Radioactivity associated with the pellet minus that in the appropriate control was taken to represent protein kinase C-catalysed phosphorylation of histone III-S.

Statistical analysis was performed using Students *t*-test for unpaired data.

RESULTS AND DISCUSSION

Ins(1:2-cyclic,4,5)P_3 competition

Wilson *et al.* (1985) reported that the half-maximal concentration of Ins(1:2-cyclic,4,5)P_3 and Ins(1,4,5)P_3 required for the mobilization of Ca^{2+} from intracellular stores of saponin-permeabilized platelets were identical. A similar observation was made by Irvine *et al.* (1986) in saponin-permeabilized Swiss 3T3 cells. In addition, Ins(1,4,5)P_3-induced Ca^{2+} mobilization has been demonstrated in an adrenocortical microsomal fraction (Guillemette *et al.*, 1987). Therefore, if the Ins(1,4,5)P_3 binding site in this preparation is the putative Ins(1,4,5)P_3 receptor that is functionally related to Ca^{2+} release from intracellular stores, Ins(1:2-cyclic,4,5)P_3 and Ins(1,4,5)P_3 would be expected to exhibit similar binding characteristics.

Fig. 1 illustrates the results of competition studies

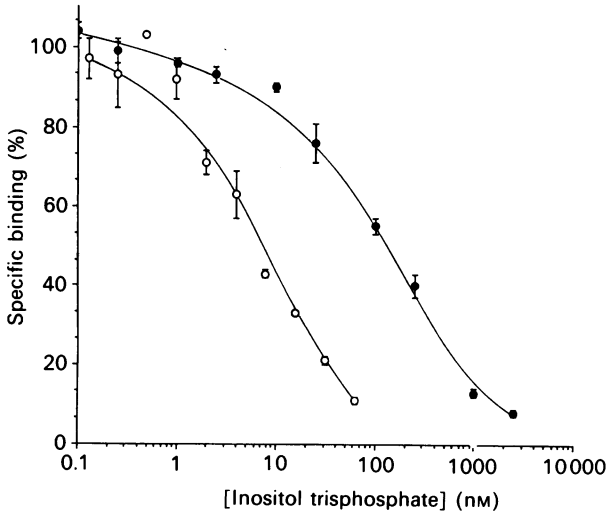

Fig. 1. Competition by Ins(1:2-cyclic,4,5)P_3 for Ins(1,4,5)P_3 binding sites

[^{3}H]Ins(1,4,5)P_3 binding to adrenocortical microsomes was determined in the presence of increasing concentrations of unlabelled Ins(1,4,5)P_3 or Ins(1:2-cyclic,4,5)P_3 as described in the Materials and methods section. ○, Ins(1,4,5)P_3; ●, Ins(1:2-cyclic,4,5)P_3. Results are means ± S.E.M. (n = 3) for a single experiment, typical of three.

performed using Ins(1:2-cyclic,4,5)P_3. These experiments demonstrated that Ins(1:2-cyclic,4,5)P_3 was able to compete for the Ins(1,4,5)P_3 binding site with an IC_{50} {concentration producing 50% inhibition of specific [^{3}H]Ins(1,4,5)P_3 binding} approx. 21-fold greater than that of Ins(1,4,5)P_3 [Ins(1,4,5)P_3, 5.9 ± 0.9 nM; Ins(1:2-cyclic,4,5)P_3, 124 ± 16 nM]. This observation is similar to that made by Willcocks *et al.* (1989) using rat cerebellar membranes which exhibit a lower affinity for Ins(1,4,5)P_3 [IC_{50} values: Ins(1,4,5)P_3, 55 ± 2.4 nM; Ins(1:2-cyclic,4,5)P_3, 2400 ± 140 nM; 44-fold lower affinity). Acid treatment of Ins(1:2-cyclic,4,5)P_3 prior to assay [incubation with an equal volume of 10% (w/w) $HClO_4$ for 1 h, then neutralization with KOH (1.5 M)/Hepes (60 mM)] resulted in a reduction of the radioactivity associated with the binding protein (742 ± 96 c.p.m., untreated; 244 ± 36 c.p.m., acid-treated; n = 3 from a single typical experiment). This probably reflected the hydrolysis of the cyclic moiety of Ins(1:2-cyclic,4,5)P_3 to produce the more competitive Ins(1,4,5)P_3.

These observations are apparently contradictory to the Ca^{2+} mobilization studies described above and suggest that the Ins(1,4,5)P_3 binding site of the preparation may not be the putative Ins(1,4,5)P_3 receptor linked to the intracellular Ca^{2+} store. However, it has recently been reported that Ins(1:2-cyclic,4,5)P_3 [< 5% contamination with Ins(1,4,5)P_3] has a lower potency (6-fold) than Ins(1,4,5)P_3 for the release of Ca^{2+} from intracellular stores in permeabilized Swiss 3T3 cells [Willcocks *et al.* (1989): EC_{50} for Ins(1,4,5)P_3, 3.56 μM; for Ins(1:2-cyclic,4,5)P_3, 21 μM]. In addition, Ins(1:2-cyclic,4,5)P_3 [at least 8% contamination with Ins(1,4,5)P_3] was 8-fold less potent in the activation of sea urchin eggs when microinjected [Crossley *et al.* (1988): EC_{50} for Ins(1,4,5)P_3, 2.5 μM, for Ins(1:2-cyclic,4,5)P_3, 19 μM]. Furthermore, Meyer *et al.* (1988) reported that whereas 75 nM-Ins(1,4,5)P_3 was able to release 30–70% of the intra-

cellular stored Ca^{2+} from permeabilized RBL cells, a similar concentration of Ins(1:2-cyclic,4,5)P_3 (of unspecified purity) was without effect. The apparent Ins(1:2-cyclic,4,5)P_3-induced Ca^{2+} mobilization observed in earlier studies may, therefore, have been due to contamination of the Ins(1:2-cyclic,4,5)P_3 preparation with Ins(1,4,5)P_3.

Although Ins(1:2-cyclic,4,5)P_3 has been reported to be present in significant quantities in a number of tissues (see Palmer *et al.*, 1988 for references), it has been shown to constitute less than 5% of total inositol trisphosphates in others (Hawkins *et al.*, 1987; Wong *et al.*, 1988). Furthermore, the relatively low affinity of Ins(1:2-cyclic,4,5)P_3 for the 'Ins(1,4,5)P_3 receptor' and its apparent low potency for Ca^{2+} mobilization suggests that Ins(1:2-cyclic,4,5)P_3 has only a limited role, if any, in the acute regulation of intracellular Ca^{2+} concentrations.

G-protein involvement?

Dawson and coworkers (Dawson, 1985; Dawson *et al.*, 1986) observed that GTP, in the presence of poly(ethylene glycol) increased the amount of Ca^{2+} released from rat liver microsomes in response to Ins(1,4,5)P_3. However, this system appears unique, as GTP has been shown not to modulate Ins(1,4,5)P_3-induced Ca^{2+} release from intracellular stores in other cell types (N1E-115 microsomes, Ueda *et al.*, 1986; parotid gland heavy microsomes, Henne & Soling, 1986). Indeed, Henne *et al.* (1987) have isolated distinct GTP- and Ins(1,4,5)P_3-sensitive vesicles from a post-nuclear fraction prepared from guinea pig parotid gland. However, the question of G-protein involvement or otherwise at the putative Ins(1,4,5)P_3 receptor has not been assessed directly using classical receptor binding techniques. G-protein-linked receptors demonstrate a rightward shift in agonist-binding curves in the presence of GTP.

We have previously shown that GTP is a poor competitor for the Ins(1,4,5)P_3 binding site in bovine adrenocortical microsomes ($IC_{50} > 2.5 \times 10^{-4}$ M; Palmer *et al.*, 1988). A similar observation was made using rat cerebellar membranes ($IC_{50} = 4.3 \times 10^{-4}$ M; Willcocks *et al.*, 1987). We have extended this observation by investigating the influence of the non-hydrolysable GTP analogues, GTPγS and Gpp[NH]p, upon Ins(1,4,5)P_3 binding to adrenocortical microsomes (Table 1). The K_d of the binding site for Ins(1,4,5)P_3 was unchanged by incubation with either analogue. This suggests that there is no direct G-protein involvement at the putative Ins(1,4,5)P_3 receptor in this system. Ins(1,4,5)P_3-induced Ca^{2+} release from bovine adrenocortical microsomes, therefore, probably does not involve a signal transduction system in the classical sense. Rather, Ins(1,4,5)P_3-induced Ca^{2+} mobilization may involve the activation of an ion channel.

Influence of protein kinase C?

Agonist-induced mobilization of intracellular Ca^{2+} has been shown to be inhibited by activators of protein kinase C, for example TPA (Brock *et al.*, 1985; Misbahuddin *et al.*, 1985; Orellana *et al.*, 1985; Brown *et al.*, 1987). Brown *et al.* (1987) suggested a number of possible sites at which protein kinase C activation could inhibit Ca^{2+} efflux from intracellular stores, including the putative Ins(1,4,5)P_3 receptor.

Table 2 (column *a*) illustrates results obtained with adrenocortical microsomes that had been preincubated with or without partially purified rat brain protein kinase C. The results show that the characteristics of the Ins(1,4,5)P_3 binding site were not influenced by protein kinase C treatment. Protein kinase C was demonstrated to have been active under the conditions of the experiment, i.e. added histone IIIS was phosphorylated (Table 2, column *b*). Therefore, either the Ins(1,4,5)P_3 binding site is not a substrate for protein kinase C, or phosphorylation of the Ins(1,4,5)P_3 binding site by protein kinase C did not alter its affinity for Ins(1,4,5)P_3. Therefore, activation of protein kinase C probably does not inhibit agonist-induced Ca^{2+} release from intracellular stores at the level of Ins(1,4,5)P_3 binding.

Table 1. Influence of non-hydrolysable GTP analogues on the K_d of the Ins(1,4,5)P_3 binding site

Binding of [^{3}H]Ins(1,4,5)P_3 to adrenal microsomes was determined in the presence and absence of GTPγS or Gpp[NH]p as described in the Materials and methods section. K_d values were obtained by analysing data by computer-assisted curve fitting. Values are means ± S.E.M. ($n = 9$; combined data from three separate experiments).

Compound	Concentration	K_d(nM)
No analogue	—	8.35 ± 0.99
GTPγS	100 μM	6.87 ± 1.52
Gpp[NH]p	100 μM	7.50 ± 2.26

Table 2. Influence of protein kinase C on the K_d of the Ins(1,4,5)P_3 binding site

(*a*) Binding protein was preincubated in the presence or absence of partially purified rat brain protein kinase C (PKC) prior to the determination of the binding affinity of the Ins(1,4,5)P_3 binding site by competition of [^{3}H]Ins(1,4,5)P_3 with increasing concentrations of unlabelled Ins(1,4,5)P_3. Scatchard data were analysed by computer-assisted curve fitting. Values are means ± S.E.M. ($n = 9$; combined data from three separate experiments). (*b*) Binding protein, after treatment as described in (*a*), was incubated in the presence of [γ-^{32}P]ATP and 100 μg of histone IIIS (see the Materials and methods section). Incubations were terminated by the addition of 20 vol. of ice-cold 20% (w/v) trichloroacetic acid. The pellets were washed once and their radioactivity determined. Results are means ± S.E.M. from a single representative experiment ($n = 3$). *$P < 0.02$.

Treatment	(*a*) K_d (nM)	(*b*) Radioactivity associated with pellet (c.p.m.)
None	7.29 ± 1.55	—
Resuspension in PKC incubation buffer	5.04 ± 1.64	6197 ± 139
PKC incubation buffer + 0.0025% (w/w) Triton X-100	5.67 ± 2.66	—
PKC incubation buffer + 100 μg of PKC	5.30 ± 2.33	8005 ± 114*
PKC incubation buffer + 100 μg of PKC + 100 nM-TPA	5.97 ± 3.15	—

Identity of the putative Ins(1,4,5)P_3 receptor

The identity of the Ins(1,4,5)P_3 binding site of adrenocortical microsomes remains unclear. However, it is unlikely to be Ins(1,4,5)P_3 5-phosphatase, since this enzyme exhibits a relatively low affinity for its substrate (K_m = 17 μM in adrenal cortex). Although the Ins(1,4,5)P_3 3-kinase is predominantly soluble in those tissues where its distribution has been studied (see Shears, 1989 for references), the possibility that a membrane-bound form could be the binding site for Ins(1,4,5)P_3 cannot be excluded. Ins(1:2-cyclic,4,5)P_3 and Ins(2,4,5)P_3, which are relatively poor substrates for the kinase (Irvine & Moor, 1986) also have reduced affinities for the Ins(1,4,5)P_3 binding site (see Fig. 1; Palmer *et al.*, 1988; Wilcocks *et al.*, 1989). Protein kinase C activation has been reported to result in persistently increased activity of Ins(1,4,5)P_3 3-kinase, presumably by covalent modification (Imboden & Pattison, 1987; Biden *et al.*, 1988*a*, *b*). In the present study, protein kinase C treatment of the binding protein was without effect on its affinity for Ins(1,4,5)P_3, although the phosphorylation state of the Ins(1,4,5)P_3 binding site was not determined. In addition, Supattapone *et al.* (1988*b*) have reported that the purified Ins(1,4,5)P_3 receptor of rat brain (Supattapone *et al.*, 1988*a*) is not a substrate for protein kinase C. Furthermore, there is good correlation between the relative affinities of Ins(1,4,5)P_3 and Ins(1:2-cyclic,4,5)P_3 for the binding site in adrenocortical microsomes and rat cerebellar membranes and their abilities to mobilize Ca^{2+} in a variety of tissues (Fig. 1; Crossley *et al.*, 1988; Meyer *et al.*, 1988; Willcocks *et al.*, 1989). This suggests that the Ins(1,4,5)P_3 binding site observed in a number of tissues, including bovine adrenocortical microsomes, could be that functionally linked to Ca^{2+} mobilization. However, as yet there is no definitive evidence for the true identity of the Ins(1,4,5)P_3 binding site.

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