Biochem. J. (1987) 248, 489–493 (Printed in Great Britain)

Turkey erythrocytes possess a membrane-associated inositol 1,4,5-trisphosphate 3-kinase that is activated by Ca^{2+} in the presence of calmodulin

Andrew J. MORRIS,* C. Peter DOWNES,†§ T. Kendall HARDEN‡ and Robert H. MICHELL*

*Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K., †Department of Cellular Pharmacology, Smith Kline & French Research Ltd., The Frythe, Welwyn, Herts. AL6 9AR, U.K., and ‡Department of Pharmacology, University of North Carolina, Chapel Hill, NC 27514, U.S.A.

Turkey erythrocytes contain soluble and particulate kinase activities which catalyse the ATP-dependent phosphorylation of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]. The particle-bound activity accounts for approximately one-quarter of the total cellular Ins(1,4,5)P_3 kinase, when assayed at a [Ca^{2+}] of 10 nM. The particle-bound Ins(1,4,5)P_3 kinase is not washed from the membrane by 0.6 M-KCl, yet may be solubilized by a variety of detergents. This suggests that it is an intrinsic membrane protein. The product of the membrane-bound Ins(1,4,5)P_3 kinase is inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4], identifying the enzyme as an Ins(1,4,5)P_3 3-kinase. In the presence of calmodulin, the membrane-associated Ins(1,4,5)P_3 3-kinase is activated as [Ca^{2+}] is increased over the range 0.2–1.0 μM. Under these conditions, the rates of dephosphorylation of Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 by phosphatases in the membrane fraction are unchanged.

INTRODUCTION

Activation of many cell-surface receptors stimulates a phosphoinositidase C which cleaves the plasma-membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, producing two molecules with second-messenger functions: Ins(1,4,5)P_3, which releases Ca^{2+} ions from an intracellular store; and *sn*-1,2-diacylglycerol, which activates protein kinase C (Berridge & Irvine, 1984; Nishizuka, 1984; Downes & Michell, 1985).

Ins(1,4,5)P_3 is metabolized by two pathways: 5-specific dephosphorylation (Downes *et al.*, 1982), or phosphorylation of the free 3-hydroxyl group (Irvine *et al.*, 1986), producing inositol 1,4-bisphosphate and Ins(1,3,4,5)P_4 respectively. The phosphorylation reaction is catalysed by an ATP-dependent Ins(1,4,5)P_3 3-kinase, present in a variety of mammalian tissues (Irvine *et al.*, 1986). In rat brain Ins(1,4,5)P_3 3-kinase is almost wholly soluble (Irvine *et al.*, 1986; A. J. Morris, K. J. Murray & C. P. Downes, unpublished work), and has a K_m for Ins(1,4,5)P_3 that is about two orders of magnitude lower than the K_m of Ins(1,4,5)P_3 5-phosphatase (Downes *et al.*, 1982; Connolly *et al.*, 1987). Observations of rapid increases in Ins(1,3,4,5)P_4 in cells after receptor stimulation and of its rapid metabolism after receptor blockade have led to speculation that it, too, may have some function as an intracellular signal (Batty *et al.*, 1985; Hawkins *et al.*, 1986). Irvine & Moor (1986) have presented evidence that Ins(1,3,4,5)P_4 plays a role in the raising of a fertilization membrane by sea-urchin eggs, a process dependent on the entry of Ca^{2+} into the cell.

Biden & Wollheim (1986) have demonstrated a role for Ca^{2+} in controlling the phosphorylation of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 in RINm5F insulinoma cells. Ca^{2+}-depleted cells produced less Ins(1,3,4,5)P_4 in response to stimulation by carbachol, and the phosphorylation of Ins(1,4,5)P_3 in broken cells was accelerated as [Ca^{2+}] was increased from 0.1 to 1.0 μM. By contrast, the enzyme purified from rat brain cytosol is modestly inhibited by Ca^{2+} in the range 0.1–1.0 μM (A. J. Morris, K. J. Murray, P. J. England, C. P. Downes & R. H. Michell, unpublished work). During recent investigations of guanine nucleotide-regulated phosphoinositidase C activity in turkey erythrocyte membranes, we have observed a membrane-associated Ins(1,4,5)P_3 3-kinase activity (Harden *et al.*, 1987). In the present paper, we describe the effect of changes in [Ca^{2+}] on the phosphorylation of Ins(1,4,5)P_3 catalysed by soluble and membrane-associated kinase activities of the turkey erythrocyte.

MATERIALS AND METHODS

Preparation of inositol phosphates

[^{3}H]Ins(1,4,5)P_3 was purchased from Amersham International. [^{3}H]Ins(1,3,4,5)P_4 was prepared by phosphorylation of [^{3}H]Ins(1,4,5)P_3. Ins(1,4,5)P_3 3-kinase was partially purified from rat brain to a specific activity of 90 nmol/min per mg of protein (A. J. Morris, K. J. Murray, P. J. England, C. P. Downes & R. H. Michell, unpublished work); 10 μg of partially purified enzyme was incubated for 10 min with 250 μmol of [^{3}H]-Ins(1,4,5)P_3 in 200 μl of the assay buffer described below, to which was added 10 mM-ATP and 11 mM-$MgSO_4$. This incubation was terminated and neutralized as described in the section below. Ins(1,3,4,5)P_4 was purified by anion-exchange h.p.l.c. on Partisil 10 SAX and desalted by the method of Hawkins *et al.* (1986).

Abbreviations used: Ins(1,3,4)P_3, D-*myo*-inositol 1,3,4-trisphosphate; Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, D-*myo*-inositol 1,3,4,5-tetrakisphosphate.

§ To whom reprint requests should be addressed.

Assay of Ins(1,4,5)P_3 kinase, Ins(1,4,5)P_3 phosphatase and Ins(1,3,4,5)P_4 phosphatase

Most incubations were performed in a medium containing potassium glutamate (70 mM), Hepes, pH 7.2 (10 mM), NaCl (30 mM), EGTA (1 mM) and various total concentrations of $CaCl_2$. Incubations also contained ^{3}H-labelled inositol phosphates at concentrations of approx. 10 nM. In some experiments 70 mM-KCl replaced the potassium glutamate.

For kinase assays, 5 mM-ATP and 6 mM-$MgSO_4$ were added to the basal medium, whereas phosphatase incubations contained 3 mM-$MgSO_4$ and no ATP. Calmodulin, purified from rat testis, was added to some of the incubations at concentrations of up to 600 nM. Reactions were terminated by the addition of an equal volume of ice-cold 10% (w/v) $HClO_4$, cooled on ice, centrifuged to precipitate protein, and the acid was removed from the supernatant by the method of Sharpes & McCall (1979).

[^{3}H]Inositol phosphates were separated by anion-exchange chromatography on Bio-Rad AG1 (200–400 mesh; formate form) with ammonium formate/formic acid eluents (Downes *et al.*, 1986), and their radioactivities were determined by liquid-scintillation counting.

Determination of free Ca^{2+} concentrations in incubations

Ca^{2+}:EGTA ratios were calculated to give Ca^{2+} concentrations in the range 0–2 μM. The actual free Ca^{2+} concentration was then measured in a series of dummy incubations, containing 600 nM-calmodulin, by using the fluorescent indicator quin2 (Tsien *et al.*, 1982).

Preparation of turkey erythrocyte membranes, lysates, and cytosolic and particulate fractions

Fresh turkey blood was centrifuged at 1100 *g* for 5 min, the plasma and buffy coat were removed, and the packed erythrocytes were washed twice by resuspension in 4 vol. of ice-cold saline (150 mM-NaCl/1.5 mM-Hepes, pH 7.2) and further centrifugation.

The cells were lysed by resuspending them at a 15-fold dilution in 5 mM-NaH_2PO_4 (pH 7.4) / 5 mM-$MgCl_2$/ 1 mM-EGTA for 1 h on ice. The lysate was centrifuged at 17700 *g* for 5 min and the supernatant was removed. Unlysed cells, which formed a dense pellet at the bottom of the tube, were discarded. To prepare membranes, the total particulate fraction, obtained by centrifugation of the lysate, was washed four times by resuspension in lysis buffer and centrifugation at 8000 *g* for 5 min. During this process, a dense white pellet, presumably consisting predominantly of nuclear material, remained in the tube after the membranes had been resuspended by swirling, and was discarded. The membranes were finally resuspended in an equal volume of 10 mM-Hepes (pH 7.2)/ 2 mM-$MgSO_4$ and homogenized with ten strokes of a tight-fitting Potter homogenizer. If not assayed immediately, the membranes were stored in liquid N_2 until required.

In order to explore the nature of the interaction between the membranes and their associated activities, the membranes were washed either with the assay incubation buffer (see above; this was designed to be isoionic with cytosol) or with 0.6 M-KCl/2 mM-$MgSO_4$/ 10 mM-Hepes, pH 7.2, by suspension in 10 vol. of these media and centrifugation at 8000 *g* for 5 min. They were then washed once more by resuspension in 10 mM-Hepes (pH 7.2)/2 mM-$MgSO_4$, followed by similar centrifugation. The packed membranes were diluted in an equal volume of the same Hepes/$MgSO_4$ before homogenization. To prevent resealing of membrane fragments, the lysate, particulate and membrane fractions were frozen and thawed before assay.

Membrane-associated activities were solubilized by treatment with a variety of detergents. For this, 1 vol. of packed membranes was added to 9 vol. of 10 mM-Hepes (pH 7.2)/2 mM-$MgSO_4$ containing various detergents, giving the following detergent (all from Sigma) concentrations (w/v): sodium cholate (purified by recrystallization), 1%; Triton X-100, 0.1%; Tween 20, 0.001%; Brij 58, 0.02%. The resuspended membranes were left on ice for 1 h with occasional mixing, followed by brief centrifugation (MSE micro-centrifuge, speed 10, 1 min) to precipitate material that had not been solubilized.

Protein assays

Protein was determined by the method of Bradford (1976). Particulate material was warmed in 0.1 M-NaOH before assay.

RESULTS AND DISCUSSION

Subcellular distributions of Ins(1,4,5)P_3 kinase, Ins(1,4,5)P_3 phosphatase and Ins(1,3,4,5)P_4 phosphatase activities

Turkey erythrocyte lysate, soluble and total particulate fractions were assayed for Ins(1,4,5)P_3 kinase, Ins(1,4,5)P_3 phosphatase and Ins(1,3,4,5)P_4 phosphatase. The incubations contained Ca^{2+} concentrations below 10 nM and ^{3}H-labelled inositol phosphates at concentrations of approx. 10 nM, considerably below the K_m values of the Ins(1,4,5)P_3 kinases and Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 phosphatases that have been studied previously (Downes *et al.*, 1982; Irvine *et al.*, 1986; Biden & Wollheim, 1986; Connolly *et al.*, 1987; A. J. Morris, K. J. Murray, P. J. England, R. H. Michell & C. P. Downes, unpublished work). Dephosphorylation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 displayed first-order kinetics. Although the kinetics of phosphorylation of Ins(1,4,5)P_3 are potentially more complex, owing to the presence of phosphatase activities against both substrate and product, the much greater activity of the kinase, when assayed under conditions of first-order kinetics, made the observed time courses apparently follow simple exponentials. The results presented in Table 1 are therefore given as first-order rate constants. The activities are corrected for dilution from the original volume of packed cells and are directly comparable.

These results show that, in the turkey erythrocyte, Ins(1,4,5)P_3 kinase is predominantly cytosolic. However, in contrast with the other tissues and cell types studied (Irvine *et al.*, 1986; Biden & Wollheim, 1986; Hansen *et al.*, 1986), approximately one-quarter of the total cellular Ins(1,4,5)P_3 kinase was particle-associated when assayed at a $[Ca^{2+}]$ of 10 nM.

Electron microscopy has revealed that turkey erythrocytes contain very few, if any, intracellular structures other than nuclei (see Beam *et al.*, 1979). In order to investigate the subcellular localization of the membrane-associated Ins(1,4,5)P_3 kinase, we next prepared membranes by repeated resuspension and gentle centrifugation of the total particulate fraction (see the

Table 1. Subcellular distribution of Ins(1,4,5)P_3 kinase in turkey erythrocytes: effects of Ca^{2+} and calmodulin

Various fractions were prepared and Ins(1,4,5)P_3 kinase activity was determined as described in the Materials and methods section. The results are expressed as first-order rate constants for the enzyme activity in each fraction or extract equivalent to 1 ml of packed cells. ND, not determined.

	Ins(1,4,5)P_3 kinase (first-order rate constant, $s^{-1} \cdot ml$ of packed cells^{-1})		Membrane-associated protein (mg/ml of packed membranes)
	$[Ca^{2+}] \approx 10$ nM	$[Ca^{2+}] = 1.8$ μM	
Lysate	6.67±0.11	8.23±0.23	ND
Soluble fraction	5.09±0.21	5.74±0.11	ND
Total particulate fraction	1.53±0.03	3.18±0.01	34.0
Membrane fraction	1.41±0.07	2.82±0.04	29.8
Membrane fraction washed with isoionic medium	1.37±0.03	2.88±0.08	
Membrane fraction washed with 0.6 M-KCl	1.13±0.01	2.60±0.08	19.9
Membrane-associated proteins solubilized with 0.02% Brij 58	0.59±0.02	ND	ND

Materials and methods section) as described by Beam *et al.* (1979), and assayed them for Ins(1,4,5)P_3 kinase. A small decrease in activity was observed during this process, possibly representing a loss of cytosolic activity. Membranes washed with 0.6 M-KCl, a process that removed about one-third of the total membrane-associated protein, retained their Ins(1,4,5)P_3 kinase activity (Table 1).

We also considered the possibility that Ins(1,4,5)P_3 kinase might be entrapped within the membrane preparation. This seemed unlikely, as the enzyme had access to its two polar substrates. When assayed in the presence of saponin at concentrations of up to 100 μg/ml, the activity of Ins(1,4,5)P_3 kinase in the membrane preparation was unchanged (results not shown).

Treatment of the membranes with a variety of ionic and non-ionic detergents, of which one, Brij 58, is illustrated in Table 1, resulted in solubilization, to different degrees, of the membrane-associated Ins(1,4,5)P_3 kinase. The persistence of Ins(1,4,5)P_3 kinase in the membrane fraction during salt washing and its susceptibility to solubilization in detergent suggest that this enzyme is an integral membrane protein.

As they could interfere with our Ins(1,4,5)P_3 kinase assays, we also investigated the subcellular distributions of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 phosphatases in the turkey erythrocyte. As reported for other cell types, Ins(1,4,5)P_3 phosphatase is predominantly particulate (Downes *et al.*, 1982; Storey *et al.*, 1984; Shears *et al.*, 1987). The less well studied Ins(1,3,4,5)P_4 phosphatase parallels Ins(1,4,5)P_3 phosphatase in its subcellular distribution (see Shears *et al.*, 1987). The particle-associated forms of these activities were retained during the isolation of membranes and were solubilized by incubation with detergents (results not shown).

Identity of the product of membrane-associated Ins(1,4,5)P_3 kinase

The soluble Ins(1,4,5)P_3 kinase of rat brain produces Ins(1,3,4,5)P_4 (Irvine *et al.*, 1986; Cerdan *et al.*, 1986). We performed a series of experiments similar to those described by Batty *et al.* (1985), using the methods of Stephens *et al.* (1987) to determine the structure of the InsP_4 produced on phosphorylation of Ins(1,4,5)P_3 by the membrane-associated Ins(1,4,5)P_3 kinase of turkey erythrocytes. The InsP_4 was not susceptible to periodate oxidation and, of the radioactivity recovered after alkaline phosphatase treatment, 93% ran as inositol on h.p.l.c. Incubation of this InsP_4 with turkey erythrocyte membranes, which, like mammalian erythrocytes, contain an Ins(1,3,4,5)P_4 5-phosphatase (A. J. Morris, unpublished work), produced an InsP_3 which yielded altritol on periodate oxidation and dephosphorylation (87% of total polyols recovered). The InsP_4 is therefore Ins(1,3,4,5)P_4.

Effect of Ca^{2+} and calmodulin on Ins(1,4,5)P_3 kinase activity

The effects of increasing $[Ca^{2+}]$ from 10 nM to 1.8 μM in the presence of 10 μg of calmodulin/ml (approx. 600 nM) on the rate of phosphorylation of Ins(1,4,5)P_3 catalysed by the soluble and membrane-associated activities of the turkey erythrocyte are recorded in Table 1. Ca^{2+} and calmodulin had no effect on the phosphatases (results not shown), but caused a significant increase in the rate of phosphorylation of Ins(1,4,5)P_3 by the activities present in the whole cell lysate. This activation was attributable solely to an activation of the particle-associated activity, which persisted during the preparation of membranes and remained on salt-washing. As shown in Table 1, the degree of activation of the membrane-bound Ins(1,4,5)P_3 kinase increased as the Ca^{2+}-insensitive kinase of the cytosol was removed.

Activation of the membrane-associated Ins(1,4,5)P_3 kinase by Ca^{2+}

Membranes were incubated with 600 nM-calmodulin and increasing $[Ca^{2+}]$, and the rate of phosphorylation of Ins(1,4,5)P_3 was determined. The rate of phosphorylation of Ins(1,4,5)P_3 increased sharply by about 2.5-fold as

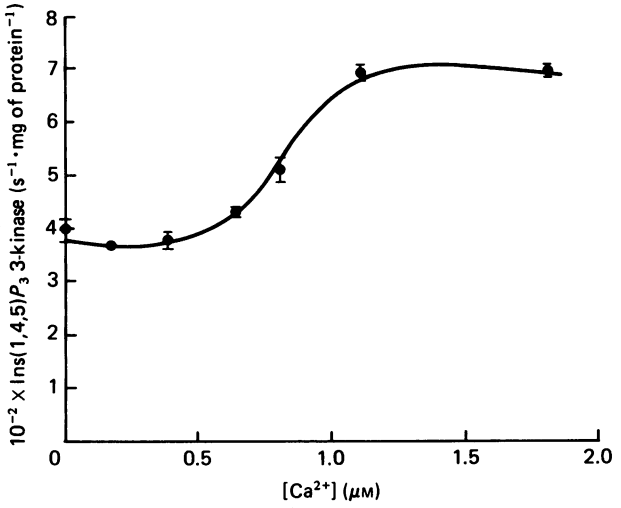

Fig. 1. Ca^{2+} activation of turkey erythrocyte membrane-associated Ins(1,4,5)P_3 3-kinase in the presence of 600 nM-calmodulin

Turkey erythrocyte membranes were incubated in the kinase assay buffer containing 600 nM-calmodulin and various Ca^{2+} concentrations. The actual Ca^{2+} concentration was measured in a series of parallel incubations with quin2, and these values are plotted against the first-order rate of phosphorylation of Ins(1,4,5)P_3 determinned as described in the Materials and methods section.

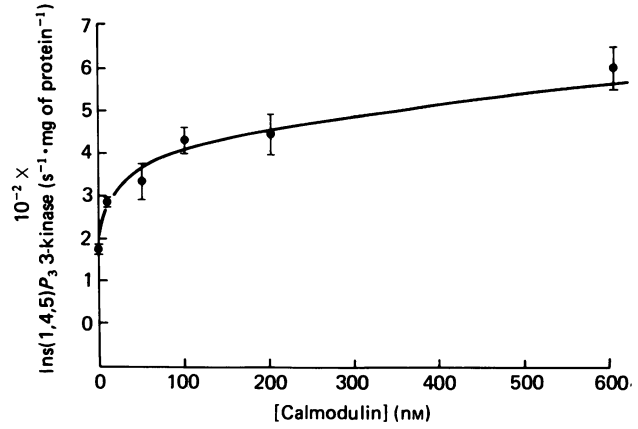

Fig. 2. Calmodulin-dependence of turkey erythrocyte membrane-associated Ins(1,4,5)P_3 3-kinase activation by 1.8 μM-Ca^{2+}

Turkey erythrocyte membranes were incubated in kinase assay buffer at a measured Ca^{2+} concentration of 1.8 μM and increasing concentrations of calmodulin. The first-order rate of phosphorylation of Ins(1,4,5)P_3, determined as described in the Materials and methods section, is plotted against calmodulin concentration.

[Ca^{2+}] was increased over the range 380–1100 nM (Fig. 1). Activation across a narrow range of Ca^{2+} concentrations is a common feature of calmodulin-stimulated enzymes, resulting in part from the requirement of calmodulin to bind three or four Ca^{2+} ions to form an active complex (Cheung, 1980). For the turkey erythrocyte membrane Ins(1,4,5)P_3 kinase, half-maximal activation was at 703 ± 65 nM-Ca^{2+} (means ± S.E.M. for three experiments on different membrane preparations). This is almost identical with the value of 800 nM obtained for a soluble calmodulin-sensitive Ins(1,4,5)P_3 kinase from the insulin-secreting cell line RINm5F (Biden *et al.*, 1987), although it should be pointed out that the actual Ca^{2+}-sensitivity of the kinase in intact cells will depend on the available calmodulin concentration. However, the physiological significance of this sensitivity to Ca^{2+} has been demonstrated in intact RINm5F cells, in which the synthesis of Ins(1,3,4,5)P_4 and its breakdown product Ins(1,3,4)P_3 appear to be accelerated by the Ca^{2+} signal resulting from stimulation of the cells with a muscarinic agonist (Biden & Wollheim, 1986). Whether turkey erythrocytes utilize a rise in [Ca^{2+}] as an intracellular signal in response to activation of cell-surface receptors is not known at present.

Ca^{2+}/calmodulin-dependence of turkey erythrocyte Ins(1,4,5)P_3 kinase

The particulate Ins(1,4,5)P_3 kinase showed a high affinity for Ca^{2+}/calmodulin, which was estimated to be half-maximal at around 70 nM-calmodulin when the free Ca^{2+} concentration was buffered at 1.8 μM (Fig. 2). The data obtained did not fit a simple mass-action relationship, possibly owing to the presence of other calmodulin-binding sites in the membrane preparation.

Concluding remarks

These results suggest a tissue-specific heterogeneity both in the subcellular distribution of Ins(1,4,5)P_3 3-kinase and in the mechanisms involved in its regulation. Our observations of Ins(1,4,5)P_3 kinase activation by Ca^{2+}/calmodulin were made at low substrate concentrations, at which the enzyme displayed first-order kinetics. The existence of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 phosphatases in the membrane preparation precluded the more detailed kinetic analysis of the enzyme which would be necessary to predict how the rate of phosphorylation of Ins(1,4,5)P_3 might change as both its concentration and the intracellular [Ca^{2+}] increase in cells after activation of phosphoinositidase C.

Neither we nor Irvine *et al.* (1986) have observed any activation by Ca^{2+} of the soluble Ins(1,4,5)P_3 3-kinase of rat brain, when assayed either in a high-speed supernatant or as a partially purified preparation to which calmodulin was added (A. J. Morris, K. J. Murray, P. J. England, R. H. Michell & C. P. Downes, unpublished work). In contrast, Biden & Wollheim (1986) showed that, in the range 0.1–1.0 μM, Ca^{2+} activated a soluble Ins(1,4,5)P_3 kinase, which appeared to contain negligible particle-associated activity in RINm5F insulinoma cells. We have recently learned that the soluble kinase of RINm5F cells is also regulated by Ca^{2+} in a calmodulin-dependent manner (Biden *et al.*, 1987).

We thank Dr. Ken Murray for the purified calmodulin, Dr. Trevor Hallam for assistance with the [Ca^{2+}] measurements using quin2, Dr. Paul England, Dr. Phil Hawkins and Dr. Len Stephens for many helpful discussions and Jane Willacy for preparation of the manuscript. We thank the Medical Research Council (U.K.) for their continued support. A.J.M. was funded by a S.E.R.C. C.A.S.E. studentship. T.K.H. was on sabbatical leave from the Department of Pharmacology, University of North Carolina, Chapel Hill, NC, U.S.A.

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Received 17 July 1987/1 September 1987; accepted 16 September 1987