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The labelling of polyphosphoinositides with [^{32}P]P_i and the accumulation of inositol phosphates in vasopressin-stimulated hepatocytes

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When hepatocytes were incubated with [^{32}P]P_i, the kinetics for the labelling of the monoester phosphate groups of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate were similar to each other and slightly slower than that for the labelling of the γ-phosphate of ATP. Analysis of the water-soluble ^{3}H-labelled materials derived from [^{3}H]inositol-labelled hepatocytes revealed that, in addition to inositol and its mono-, bis- and tris-phosphates (Ins, Ins$P$, InsP_2 and InsP_3), these cells contained two unidentified radioactive compounds which co-eluted with Ins$P$ on anion-exchange chromatography. When [^{3}H]inositol-labelled hepatocytes were stimulated with 0.23 μM-vasopressin in the presence of 10 mM-Li^+, there was an accumulation of radioactivity in Ins$P$, InsP_2 and InsP_3 but not in Ins or the two unidentified compounds. Further analysis of these inositol phosphates by h.p.l.c. revealed that vasopressin also stimulates the accumulation of inositol tetrakisphosphate (InsP_4) in these cells. Vasopressin-stimulated Ins$P$ and InsP_2 accumulations were maximal in the presence of 1–10 mM-Li^+ but InsP_3 accumulation continued to increase up to 50 mM-Li^+. Accumulated inositol phosphates were retained within the cell. Li^+ from 1 to 50 mM did not influence the extent of vasopressin-stimulated inositol lipid degradation in hepatocytes. In the absence of Li^+, radioactivity in vasopressin-stimulated hepatocytes accumulated almost entirely in free inositol. The vasopressin-stimulated accumulation of inositol phosphates in the presence of 10 mM-Li^+ was abolished by a V_1-vasopressin antagonist. Inositol phosphate accumulation was not influenced by ionophore A23187, dimethyl sulphoxide or indomethacin.

INTRODUCTION

Activation of cell-surface receptors for hormones, neurotransmitters and other ligands which use the Ca^{2+} ion as an intracellular messenger provokes the stimulation of inositol lipid metabolism in target cells (see Michell, 1975; Berridge, 1984, for reviews). In 1981 we reported that Ca^{2+}-mobilizing ligands cause a rapid depletion of PtdIns4P and PtdIns4,5P_2 in these cells (Kirk *et al.*, 1981*a*; Michell *et al.*, 1981). Polyphosphoinositide depletion has since been shown to be a widespread response to Ca^{2+}-mobilizing agonists in a variety of tissues (for reviews see Kirk *et al.*, 1984; Michell *et al.*, 1984; Berridge, 1984). Hormone-stimulated polyphosphoinositide depletion in hepatocytes is not mediated by an increase in intracellular [Ca^{2+}] and is closely coupled to receptor occupation (Creba *et al.*, 1983; Thomas *et al.*, 1983). These and other characteristics led to the proposal that receptor-mediated breakdown of PtdIns4,5P_2 (and maybe also of PtdIns4P) is an essential step in stimulus–response coupling at Ca^{2+}-mobilizing receptors (for reviews see Michell *et al.*, 1981; Downes & Michell, 1982, 1985; Kirk *et al.*, 1984; Michell *et al.*, 1984; Berridge, 1984).

Work in several laboratories has since shown that, as in other cells (see Berridge, 1984; Downes & Michell, 1985, for reviews), InsP_3, InsP_2 and Ins$P$ accumulate in stimulated hepatocytes, particularly when Li^+ ions are present to inhibit their dephosphorylation (Thomas *et al.*, 1984; Kirk *et al.*, 1984; Burgess *et al.*, 1984). Moreover, Ins1,4,5P_3 liberated from PtdIns4,5P_2 appears to act as the intracellular mediator for the hormone-stimulated release of Ca^{2+} from intracellular stores (see Berridge & Irvine, 1984, for review). Very recently, Batty *et al.* (1985) have reported the rapid accumulation of Ins1,3,4,5P_4 in rat brain slices following muscarinic stimulation, but the physiological role of this compound is not known.

In the present paper we provide confirmation of some of these observations, report additional studies on the rates of turnover of PtdIns4P and PtdIns4,5P_2 and on the characteristics of the accumulation of inositol phosphates that occurs in stimulated hepatocytes. Some of the results in this paper have been reported in preliminary form in Michell *et al.* (1984).

MATERIALS AND METHODS

Materials and animals were, unless otherwise noted, of the type and from the source specified previously (Creba *et al.*, 1983). Universal indicator was obtained from

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns4,5P_2, phosphatidylinositol 4,5-bisphosphate; Gro$P$Ins, glycerophosphoinositol; Gro$P$Ins4P, glycerophosphoinositol 4-phosphate; Gro$P$Ins4,5P_2, glycerophosphoinositol 4,5-bisphosphate; Ins$P$, inositol monophosphate; InsP_2, inositol bisphosphate; InsP_3, inositol trisphosphate; InsP_4, inositol tetrakisphosphate; Ins1P, inositol 1-phosphate; Ins1,4P_2, inositol 1,4-bisphosphate; Ins1,4,5P_3, inositol 1,4,5-trisphosphate; Ins1,3,4,5P_4, inositol 1,3,4,5-tetrakisphosphate; DMSO, dimethyl sulphoxide.

BDH. Dowex 1 (X10, 100–200 mesh, Cl^- form) was obtained from Fluka AG (Buchs, Switzerland). [1-(β-Mercapto-β,β-cyclopentamethylenepropionic acid),4-methyltyrosine, 8-arginine]vasopressin was a generous gift from Professor M. Manning, Medical College of Ohio, Toledo, OH, U.S.A.

[^{32}P]P_i and [^{14}C]Ins1*P* were purchased from Amersham International. *myo*-[2-^{3}H]Inositol was obtained from Amersham International or New England Nuclear. [^{3}H]Inositol from both sources was routinely passed through a 1 cm column of Dowex 1 (X10, 100–200 mesh, formate form) to remove ^{3}H-labelled contaminants. ^{32}P-labelled Ins1*P* and inositol cyclic 1,2-phosphate were prepared as described by Lapetina & Michell (1973) except that separation of the two phosphates was by high voltage electrophoresis, followed by autoradiography, elution with water, neutralization with NH_4OH and freeze-drying. ^{32}P-labelled Ins1,4P_2 and Ins1,4,5P_3 were prepared as described by Downes *et al.* (1982).

Preparation and incubation of hepatocytes

Isolated hepatocytes were prepared from male Wistar rats (200–240 g) as described by Creba *et al.* (1983). In preliminary experiments we found, in agreement with Prpic *et al.* (1982*a*), that the inclusion of 0.1 mM-inositol during incubation of hepatocytes with [^{3}H]inositol led to optimal labelling of the inositol lipids. Hence this concentration of inositol, which is similar to that found in rat blood, was included in all perfusion and incubation media.

For the routine measurement of ligand-stimulated inositol phosphate accumulation, hepatocyte suspensions were incubated in bulk in bicarbonate-buffered saline (Krebs & Hensleit, 1932) containing 1.5 mM-Ca^{2+}, 10 mM-glucose and 0.1 mM-*myo*-[2-^{3}H]inositol (40 Ci/mol) for 60 min. Hepatocytes were sedimented (30 s, 500 $g_{av.}$), washed once and resuspended in unlabelled medium containing 0.1 mM-inositol. After a further 25 min incubation, 0.5 ml aliquots of the suspension were distributed into small plastic vials containing Li^+ and other additions as required. Hormones were added 10 min later and incubations were terminated by the addition of 0.2 ml of $HClO_4$ (10%, v/v) at the appropriate time. Total [^{3}H]inositol lipids were extracted from parallel incubations terminated by the addition of 3.75 vol. of chloroform/methanol/concn. HCl (40:80:1, by vol.). The lipid-soluble radioactivity of unstimulated hepatocytes which had been 'pulse-labelled' and chased as described above did not alter significantly over the subsequent 20 min during which hormone-stimulated events were monitored.

In some experiments, hepatic lipids were labelled with [^{3}H]inositol *in vivo* prior to the preparation of hepatocytes. For this purpose, 0.5 mCi of *myo*-[2-^{3}H]inositol was injected into the peritoneal cavity of a rat approx. 16 h before hepatocyte preparation.

In experiments to study the labelling of PtdIns4*P* and PtdIns4,5P_2 with [^{32}P]P_i and the hormone-stimulated depletion of these lipids, hepatocytes were incubated with [^{32}P]P_i as described by Creba *et al.* (1983) except that, where appropriate, Li^+ was added 10 min before the addition of vasopressin or other agents (Berridge *et al.*, 1982). These incubations were terminated with 1 vol. (0.5 ml) of trichloroacetic acid (20%, w/v).

All incubation vessels were flushed with O_2/CO_2 (19:1) on the addition of cell suspensions and at 15 min intervals thereafter.

Extraction procedures

Extraction of [^{3}H]inositol phosphates. $HClO_4$ extracts from [^{3}H]inositol-labelled hepatocytes were sedimented (2000 $g_{av.}$) and samples of the supernatants obtained were neutralized with 1.5 M-KOH containing 60 mM-Hepes in the presence of Universal Indicator. Neutralized acid extracts could be stored at −20 °C for several weeks without depletion of inositol phosphates. We have found that this method for inositol phosphate extraction is much more rapid than the trichloracetic acid precipitation followed by diethyl ether extraction used in a number of previous studies (e.g. Berridge *et al.*, 1983). Both procedures yielded similar recoveries of [^{3}H]inositol phosphates. In some experiments, hepatocytes were separated from their incubation medium, prior to acid quenching, by centrifugation through silicone oil (Shears & Kirk, 1984). This procedure permitted the separate detection of [^{3}H]inositol phosphates in intra- and extracellular compartments.

Lipid extraction. Total [^{3}H]inositol lipids were extracted from incubations parallel with those used for the determination of [^{3}H]inositol phosphate release as described by Downes & Wusteman (1983).

Experiments to study the labelling of PtdIns4*P* and PtdIns4,5P_2 with [^{32}P]P_i and their subsequent hormone-stimulated depletion were terminated with 1 vol. (0.5 ml) of trichloroacetic acid (20%, w/v) and the lipids were extracted and deacylated as described by Creba *et al.* (1983).

Separation techniques

Anion-exchange chromatography of glycerophosphoinositol esters and inositol phosphates. Anion-exchange columns of Dowex (X10, 100–200 mesh, formate form) were prepared as described by Creba *et al.* (1983). These were used for the separation of glycerophosphoinositol esters derived from extracted lipids (Creba *et al.*, 1983) and also for separating inositol phosphates in neutralized acid extracts from [^{3}H]inositol-labelled cells. In the latter case 0.6 ml samples of the neutralized cell extracts were loaded onto columns in the presence of 4.4 ml of disodium tetraborate/EDTA (5 mM/0.5 mM). Columns were eluted with 20 ml of water to remove free inositol, 20 ml of 60 mM-NH_4COOH/5 mM-$Na_2B_4O_7$ ('Gro*P*Ins fraction'), 22 ml of 150 mM-NH_4COOH/5 mM-$Na_2B_4O_7$ ('Ins*P* fraction'), 22 ml of 0.4 M-NH_4COOH/0.1 M-HCOOH ('InsP_2 fraction') and 22 ml of 1.0 M-NH_4COOH/0.1 M-HCOOH ('InsP_3 fraction'). The last eluant could be used directly, following elution of the 'Gro*P*Ins fraction', to elute total inositol phosphates. The radioactivity of 3 ml aliquots of these fractions was determined by liquid-scintillation spectrometry. The use of these columns was validated by using authentic radioactive samples of Ins, Ins1*P*, Ins1,4P_2 and Ins1,4,5P_3. Our standard elution conditions achieved complete separation of free inositol from the inositol phosphates and an almost complete separation of the individual inositol phosphates, but up to 13% of the Ins1,4P_2 was eluted in the 'InsP_3 fraction' (Kirk *et al.*, 1984). This procedure would not separate positional isomers of the inositol phosphates, so the radioactive

materials isolated from liver cells in this way have been designated simply as InsP_1, InsP_2 and InsP_3. Columns were routinely discarded after two separations.

Separation of inositol phosphates by high voltage electrophoresis and h.p.l.c. Neutralized acid extracts of hepatocytes were prepared for high voltage electrophoresis by the addition of excess EDTA to chelate divalent cations. These extracts were applied to paper together with standard samples of $[^{32}P]P_i$ and ^{32}P-labelled Ins1P, inositol cyclic 1,2-phosphate, Ins1,4,P_2 and Ins1,4,5P_3. Samples were separated at pH 3.5 and 60 V/cm for 80 min as described by Dawson & Clarke (1972). [^{3}H]Inositol phosphates were identified by their mobilities relative to P_i and the ^{32}P-labelled standards, and were eluted with water overnight. Recovery of total ^{3}H radioactivity from the paper was approx. 95%.

In some experiments a more detailed separation of inositol phosphates was achieved by h.p.l.c. on a Whatman Partisil 10SAX column (Batty *et al.*, 1985). After loading, the column was first eluted with water for 5 min to remove free inositol. The eluent was then increased linearly from 0 to 0.75 M-NH_4COOH buffered to pH 3.7 with orthophosphoric acid and held at this concentration for 2 min, during which Ins1P was eluted from the column. Ins1,4P_2 was eluted by increasing the eluent linearly to 1.0 M-NH_4COOH/phosphoric acid, pH 3.7, over a 6 min period. The eluent was held at this concentration for a further 5 min, during which Ins1,3,4P_3 and Ins1,4,5P_3 were separately eluted. Finally, the eluent was increased linearly to 1.7 M-NH_4COOH/phosphoric acid over the next 10 min and InsP_4 was eluted from the column towards the end of this period.

Other assays

The incorporation of $[^{32}P]P_i$ into the individual phosphate groups of PtdIns4P and PtdIns4,5,P_2 was determined following selective enzymic hydrolysis as described by Hawkins *et al.* (1984). The specific radioactivity of the γ-phosphate group of [^{32}P]ATP was determined as described by Hawkins *et al.* (1983).

RESULTS

Labelling of ATP, PtdIns4P and PtdIns45P_2 with $[^{32}P]P_i$

Our original measurements of polyphosphoinositide breakdown in hepatocytes were based upon the depletion of lipid-associated radioactivity in cells which had been incubated with $[^{32}P]P_i$ for 70 min so as to label the monoester phosphates of PtdIns4P and PtdIns4,5P_2 to equilibrium with the γ-phosphate of ATP. Since the radioactivity in polyphosphoinositides reaches a constant value within this period, the sustained loss of radioactivity from these lipids following stimulation has been assumed to reflect a reduction in their steady-state concentrations (Michell *et al.*, 1981; Creba *et al.*, 1983).

Fig. 1 compares the kinetics for the incorporation of $[^{32}P]P_i$ into the γ-phosphate of ATP and into the monoester phosphates of PtdIns4P and PtdIns4,5P_2. The time-courses for the labelling of the lipids were very similar to, but slightly slower than, that for ATP. Essentially all of the radioactivity of Gro$P$Ins4,5P_2, derived from the PtdIns4,5P_2 of hepatocytes that had been incubated with $[^{32}P]P_i$ for 20, 60 and 150 min, could be released by alkaline phosphatase: labelling under these conditions is therefore confined to the 4- and 5-positions.

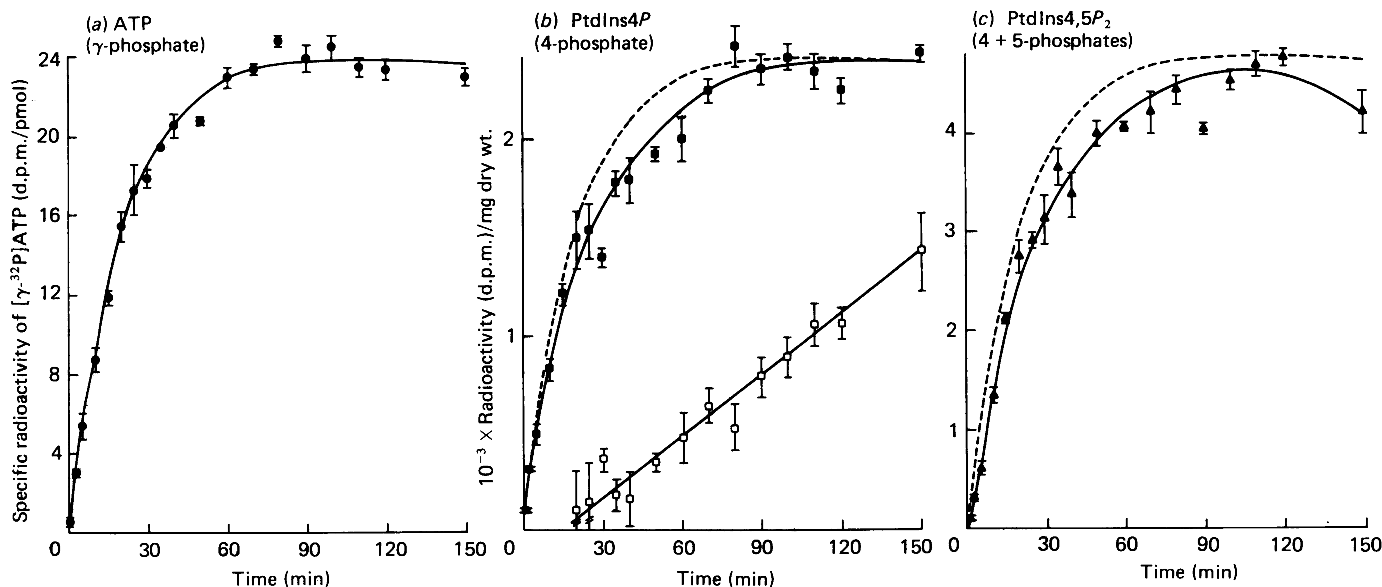

Fig. 1. Time course of the labelling with $[^{32}P]P_i$ of (*a*) the γ-phosphate of ATP, and of the monoester phosphate groups of (*b*) PtdIns4P and (*c*) PtdIns4,5P_2

Hepatocytes were prepared and incubated in 0.5 ml portions of inositol-free medium containing 60 μCi of $[^{32}P]P_i$ as described in the Materials and methods section. Incubations were terminated with $HClO_4$ (50 μl of 20%, v/v) and the specific radioactivity of the γ-phosphate of ^{32}P-labelled ATP (*a*) was determined as described by Hawkins *et al.* (1983). The radioactivity of the monoester phosphate groups of PtdIns4P (*b*) and PtdIns4,5P_2 (*c*) was determined after deacylation (Hawkins *et al.*, 1984). Radioactivity incorporated into an unknown contaminant of the Gro$P$Ins4P preparation was measured as that not released as $[^{32}P]P_i$ by alkaline phosphatase (*b*, □, see the text). The broken line in (*b*) and (*c*) corresponds to the labelling curve of the γ-phosphate of ATP, shown in (*a*). The results are means ± S.E.M. from three separate hepatocyte preparations.

The labelling of the individual 4- and 5-phosphate groups of PtdIns4,5P_2 was determined by incubating samples of these [^{32}P]Gro$P$Ins4,5P_2 preparations with erythrocyte membrane Ins1,4,5P_3 5-phosphomonoesterase (Hawkins *et al.*, 1984). About 50% of the total ^{32}P radioactivity in each sample was released by such treatment, assuming that the first-order rate constant for this attack was similar to that when a standard sample of erythrocyte [^{32}P]Gro$P$Ins4,5P_2 was the substrate (Hawkins *et al.*, 1984).

Using our usual anion-exchange chromatography protocol for the separation of glycerophosphoinositol esters, we noted that [^{32}P]Gro$P$Ins4P co-eluted with another compound which was slowly labelled in the presence of [^{32}P]P_i. This unidentified contaminant was separated from Gro$P$Ins4P on high voltage electrophoresis by virtue of its relatively low mobility. Fig. 1(*b*) shows the time course for the incorporation of [^{32}P]P_i into Gro$P$Ins4P and into this less mobile contaminant. When Gro$P$Ins4P was purified by high voltage electrophoresis and treated with alkaline phosphatase, all of its radioactivity was released as [^{32}P]P_i, thus confirming that labelling was confined to the 4-phosphate. In contrast, the slowly labelling contaminant resisted alkaline phosphatase attack; it presumably contained diesterified ^{32}P.

Thus, when hepatocytes are incubated with [^{32}P]P_i the monoester phosphates of the polyphosphoinositides rapidly become labelled to a steady state in which they are at equilibrium with the cellular ATP pool. During a period of 120 min there is very little incorporation of [^{32}P]P_i into the diester phosphate of these lipids. Hormone-stimulated depletion of PtdIns4P and PtdIns4,5P_2 in hepatocytes labelled with ^{32}P for 70 min (Creba *et al.*, 1983; Thomas *et al.*, 1983) must, therefore, reflect a reduction in the steady-state concentration of these lipids.

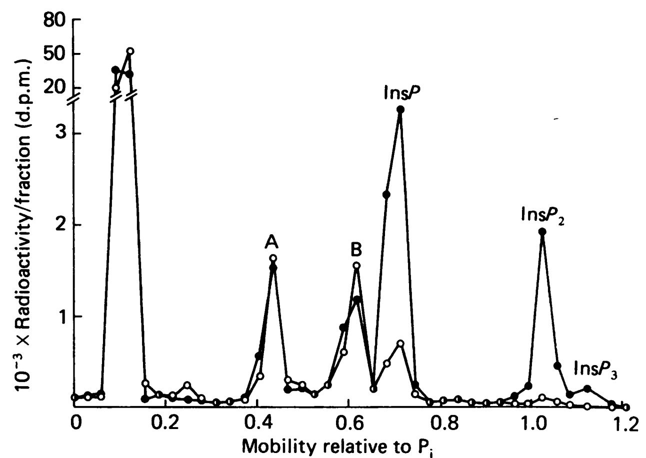

Fig. 2. High voltage electrophoresis of [^{3}H]inositol-labelled metabolites from vasopressin-stimulated hepatocytes

Hepatocytes were labelled with *myo*-[2-^{3}H]inositol (0.1 mM, 400 Ci/mol) and incubated with or without 0.25 μM-vasopressin in the presence of 10 mM-Li^+ as described in the Materials and methods section. Neutralized acid extracts were applied to Whatman no. 1 paper together with a reference sample of [^{32}P]P_i and separated at pH 3.5 and 60 V/cm for 80 min. Dried papers were autoradiographed to determine the position of [^{32}P]P_i and assayed for ^{3}H-labelled compounds as described in the Materials and methods section. Results are from a single hepatocyte preparation which was stimulated for 10 min and are typical of those obtained from four separate hepatocyte preparations.

Characterization of inositol phosphate accumulation in stimulated hepatocytes

Vasopressin-stimulated PtdIns depletion in hepatocytes is sustained for 5–15 min (Kirk *et al.*, 1981*b*), whereas the decrease in steady-state concentrations of PtdIns4P and PtdIns4,5P_2 is complete within 1 min (Kirk *et al.*, 1981*a*; Michell *et al.*, 1981; Creba *et al.*, 1983; Thomas *et al.*, 1983; Rhodes *et al.*, 1983). This led us to suggest that stimulated polyphosphoinositide hydrolysis is the primary stimulated event and that it is accompanied by polyphosphoinositide synthesis from PtdIns (Kirk *et al.*, 1981*a*; Michell *et al.*, 1981; Creba *et al.*, 1983). This idea may be tested if polyphosphoinositide breakdown is assayed directly by measuring the release of inositol phosphates from [^{3}H]inositol-labelled hepatocytes in the presence of Li^+ (Berridge *et al.*, 1983). We have characterized the water-soluble inositol metabolites in neutralized acid extracts from *myo*-[2-^{3}H]inositol-labelled hepatocytes by high-voltage electrophoresis. A typical profile appears in Fig. 2. In addition to free inositol and the three inositol phosphates, two additional ^{3}H-labelled materials (A and B) were detected that migrated more slowly than Ins1P. High voltage electrophoresis of the fractions separated by our routine anion-exchange chromatography procedure indicated that these two unidentified [^{3}H]inositol metabolites were largely eluted in the 'Ins$P$ fraction' in this system. We therefore estimate that up to 80% of the radioactivity in this fraction, isolated from unstimulated cells by anion-exchange chromatography, is associated with these unidentified metabolites. These compounds do not accumulate following vasopressin stimulation. Following 1 min stimulation with the hormone, the radioactivity associated with all three inositol phosphates was increased but the increase was most marked in InsP_2 (result not shown). After 10 min stimulation there was further accumulation of each of the inositol phosphates (Fig. 2). The amounts of radioactivity recovered in free inositol and in the two unidentified peaks were unchanged following vasopressin stimulation.

A typical profile, showing the h.p.l.c. separation of [^{3}H]inositol phosphates from control hepatocytes and those stimulated by vasopressin for 30 s, is shown in Fig. 3. The use of authentic radioactive standards enabled us to identify two of the peaks that are stimulated following vasopressin treatment as Ins1P and Ins1,4,5P_3 (see legend to Fig. 3). Two further peaks, which are centred around fraction nos. 75 and 94 and are enhanced in the presence of the hormone, have mobilities appropriate to Ins1,4P_2 and Ins1,3,4P_3 (Irvine *et al.*, 1985). We have not identified the peaks of radioactivity in the region of Ins1P (fraction 62) and Ins1,4P_2 (fraction 71) that are unaffected by vasopressin treatment but they may be the same materials as those we have previously observed on high voltage electrophoresis. The peak of radioactivity that appears in the region of fraction no. 145 following stimulation with vasopressin elutes identically with Ins1,3,4,5P_4 prepared from rat parotid slices according to the method of Batty *et al.* (1985). We therefore suggest that this material is Ins1,3,4,5P_4.

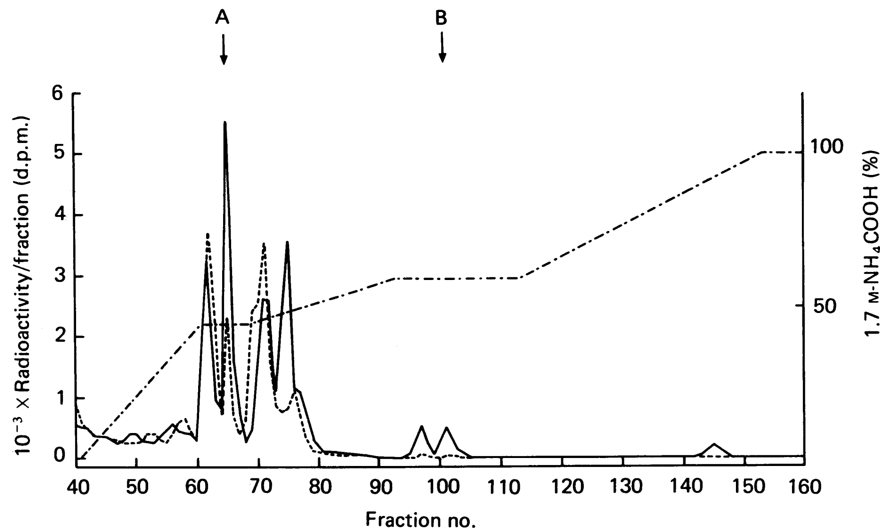

Fig. 3. Separation of inositol phosphates by h.p.l.c.

[^{3}H]Inositol-labelled hepatocytes were prepared as described in the legend to Fig. 2. After 10 min preincubation with 10 mM-Li^+, hepatocytes were exposed to vasopressin (——, 0.23 μM) or saline control (----) for 30 s, after which incubations were terminated with $HClO_4$ (10%, v/v). After neutralization with 1.5 M-KOH/50 mM-Hepes, this mixture was applied to the h.p.l.c. anion-exchange column and eluted as described in the Materials and methods section. Fractions (0.3 ml) were collected for the determination of radioactivity. Also shown is the percentage of 1.7 M-NH_4COOH (pH 3.7) present in the elution gradient. Ins1P (A) and Ins1,4,5P_3 (B) were identified with the aid of radioactive standards: [^{14}C]Ins1P was from Amersham International and [^{32}P]Ins1,4,5P_3 was prepared from human erythrocytes as described in the Materials and methods section. The results are from a single experiment representative of two.

The time course for the accumulation of total ^{3}H-labelled inositol phosphates, separated by ion exchange chromatography and expressed as a percentage of the total radioactivity in the inositol lipids, is shown in Fig. 4. There was a rapid accumulation of ^{3}H-labelled inositol phosphates in vasopressin-stimulated cells: a statistically significant accumulation of InsP_3 was observed after 5 s stimulation with vasopressin and a significant accumulation of InsP_2 was observed 5 s later; significant accumulation of Ins$P$ was first noted after 2 min stimulation (results not shown). Approx. 10% of the radioactivity initially present in inositol lipids appeared in the inositol phosphate fractions after 10 min stimulation with vasopressin. The radioactivity recovered in the inositol phosphate fractions from unstimulated

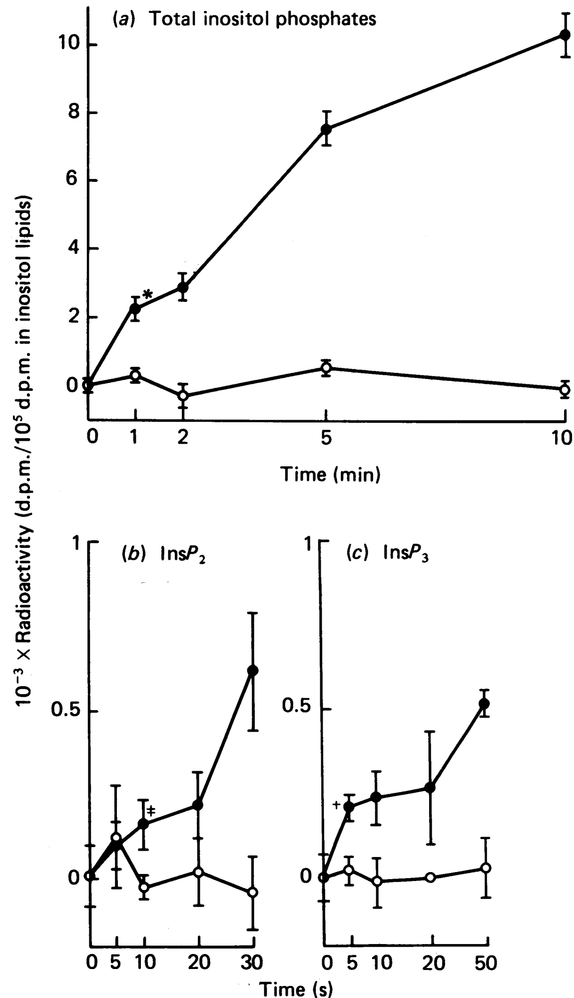

Fig. 4. Time course of vasopressin-stimulated inositol phosphate accumulation in hepatocytes

Hepatocytes were labelled *in vitro* with *myo*-[2-^{3}H]inositol (40 Ci/mol) as described in the Materials and methods section. After exposure to 10 mM-Li^+ for 10 min ($t = 0$), incubations were continued with (●) or without (○) 0.23 μM-vasopressin for the periods indicated. Incubations were terminated with $HClO_4$ and neutralized acid extracts were applied to anion exchange columns. Total inositol phosphates (*a*), InsP_2 (*b*) and InsP_3 (*c*) were eluted as described in the Materials and methods section. Results, expressed as the radioactivity (d.p.m.) in each inositol phosphate fraction/10^5 d.p.m. in total inositol lipids, are means ± S.E.M. of 3–14 determinations from four separate hepatocyte preparations. The radioactivity recovered in inositol phosphates was 3450 ± 470 d.p.m. (Ins$P$), 710 ± 95 d.p.m. (InsP_2) and 90 ± 70 d.p.m. (InsP_3) at $t = 0$ ($n = 3$). The results in the Figure have been corrected for this incorporation. *$P < 0.02$, †$P < 0.01$, ‡$P < 0.001$ versus control incubations (paired t-test).

Table 1. Retention of accumulated inositol phosphates in the intracellular compartment of stimulated hepatocytes

Hepatocytes were labelled *in vitro* with *myo*-[2-^{3}H]inositol (0.1 mM, 400 Ci/mol) as described in the Materials and methods section. Li^+ (10 mM) was present for the final 20 min of incubation and vasopressin (0.23 μM) was added for the final 10 min as indicated. Hepatocytes were separated for their incubation medium by centrifugation through a silicone oil density barrier into 10% (v/v) $HClO_4$ (Shears & Kirk, 1983). Samples taken from above (extracellular phase) and below (intracellular phase) the oil were neutralized as required and loaded onto ion-exchange columns. These were sequentially eluted with 22 ml of water ('free inositol' fraction), 60 mM-NH_4COOH/5 mM-$Na_2H_3BO_7$ ('Gro*P*Ins' fraction) and 1.0 M-NH_4COOH/0.1 M-HCOOH ('total inositol phosphate' fraction). Results are means ±range ($n = 2$) from a single experiment typical of two.

Phase	[Vasopressin] (nM)	Radioactivity (d.p.m.) recovered in: 'Free inositol' fraction	'Gro*P*Ins' fraction	'Total inositol phosphates' fraction
Extracellular	0	$(1394 \pm 48) \times 10^3$	2612±2	9920±280
	230	$(1433 \pm 14) \times 10^3$	2555±379	13900±375
Intracellular	0	$(182 \pm 13) \times 10^3$	1855±21	9570±3536
	230	$(163 \pm 21) \times 10^3$	2055±64	83980±6760

hepatocytes did not change significantly during this period.

When [^{3}H]inositol-labelled hepatocytes were stimulated with vasopressin and separated from their incubation medium by centrifugation through a silicone oil density barrier, 95% of the accumulated inositol phosphates were found in the intracellular compartment (Table 1).

The stimulation of the accumulation of inositol phosphates in vasopressin-stimulated hepatocytes was prevented by a V_1-receptor antagonist. However, the ionophore A23187, which mimics the effects of vasopressin upon hepatic phosphorylase activity (Blackmore *et al.*, 1978), did not evoke any significant inositol phosphate accumulation in hepatocytes incubated with 10 μM extracellular free Ca^{2+}. Neither did the ionophore influence vasopressin-stimulated inositol phosphate production in these cells. DMSO, which we have previously shown to provoke PtdIns4,5P_2 depletion in hepatocytes (Creba *et al.*, 1983), and indomethacin also failed to cause any accumulation of inositol phosphates or to influence that observed in the presence of vasopressin (Table 2).

Influence of [Li^+] upon the accumulation of inositol phosphates in vasopressin-stimulated hepatocytes

In common with other workers (see Berridge & Irvine, 1984), we have routinely measured inositol phosphate accumulation in the presence of Li^+ to inhibit inositol phosphate phosphomonoesterase activity (Berridge *et al.*, 1982). We have verified the observation of Thomas *et al.* (1984) that greater concentrations of Li^+ are needed to maximize the accumulation of InsP_3 in stimulated cells (> 10 mM) than are required to maximize that of Ins*P* and InsP_2 accumulation (< 10 mM, results not shown). In order to check that [Li^+] had no striking effects upon vasopressin-stimulated polyphosphoinositide depletion in hepatocytes, cells were labelled with [^{32}P]P_i *in vitro* prior to stimulation for 60 s as in the studies of Creba *et al.* (1983). In these experiments vasopressin provoked a 15% depletion of [^{32}P]PtdIns4*P* and a 37% depletion of [^{32}P]PtdIns4,5P_2 and the extent of this polyphosphoinositide depletion did not significantly alter in the presence of up to 50 mM-Li^+. We have also verified that the presence of Li^+ did not influence the separation, by anion

Table 2. Influence of [Ca^{2+}], DMSO, indomethacin and a V_1 antagonist upon basal and vasopressin-stimulated inositol phosphate production

Hepatocytes were labelled *in vitro* with *myo*-[2-^{3}H]inositol (0.1 mM, 40 Ci/mol) and incubated with 10 mM-Li^+ and varying [Ca^{2+}] as described in the Materials and methods section. Where indicated, vasopressin was present for the final 10 min of incubation. The V_1 antagonist, DMSO, A23187, indomethacin and ethanol were added to incubations 10 min before hormone stimulation as required. Results are means ± S.E.M. of 10–22 determinations from six separate hepatocyte preparations. *$P < 0.001$ by paired *t*-test versus hormone-free control incubations; other differences are not significant.

Additions	[Ca^{2+}]	Radioactivity in 'total inositol phosphates' fraction (d.p.m./10^5 d.p.m. in total inositol lipids): Control	+0.23 μM-Vasopressin
None	1.5 mM	1885±110	5585±295*
None	10 μM	2340±190	5230±290*
V_1-antagonist (1 μM)	1.5 mM	1920±100	1935±120
DMSO (2%, v/v)	1.5 mM	1950±100	4910±365*
Ethanol (2%, v/v)	10 μM	1940±150	4530±375*
A23187 (10 μM) +ethanol (2%, v/v)	10 μM	2165±130	4715±350*
Indomethacin (10 μM) +ethanol (0.2%, v/v)	10 μM	2125±165	5075±275*

Table 3. Accumulation of inositol phosphates in stimulated hepatocytes previously labelled *in vivo* with *myo*-[2-^{3}H]inositol

Rats were injected intraperitoneally with 0.5 mCi of *myo*-[2-^{3}H]inositol 16 h before the preparation of hepatocytes. Hepatocytes were incubated in 0.5 ml portions without added inositol and, where indicated, 50 mM-Li^+ was added 20 min before the termination of the experiment. After a further 10 min ($t = 0$ in Table), incubations received either 0.23 μM-vasopressin, an equal volume of vehicle or 0.1 ml of 20% (v/v) $HClO_4$ (zero time controls). Incubations were terminated and inositol phosphates were separated from neutralized acid extracts as described in the Materials and methods section. Results, expressed as the radioactivity recovered in each inositol phosphate fraction (d.p.m./10^5 d.p.m. in total inositol lipids) are means ± S.E.M. for eight determinations. $*P < 0.01$, $**P < 0.001$ versus unstimulated controls (paired t-test).

[Li^+] (mM)	Time (min)	[Vasopressin] (μM)	Radioactivity (d.p.m./10^5 d.p.m. in total inositol lipids) recovered in: 'Free inositol fraction'	'InsP_1 fraction'	'InsP_2 fraction'	'InsP_3 fraction'	Total inositol phosphates	Total water-soluble metabolites
0	0	0	19790±130	950±60	440±40	340±15	1740±120	21530±250
0	10	0	19720±100	950±30	510±70	355±30	1820±130	21540±230
0	10	0.23	22390±150**	1480±70*	780±70*	460±30*	2720±170*	25100±320**
50	0	0	20880±130	1260±70	550±75	370±30	2180±170	23060±310
50	10	0	19330±130	1040±70	435±60	300±30	1780±160	21120±280
50	10	0.23	19340±40	2520±40**	2265±70**	960±70**	5740±180**	25080±220**

exchange chromatography, of inositol phosphates in our samples (results not shown).

When hepatocytes were pulse-labelled *in vivo* by injection of *myo*-[2-^{3}H]inositol 16 h before death, allowing residual injected [^{3}H]inositol to be lost from intracellular precursor pools, vasopressin provoked a marked accumulation of radioactivity in the water-soluble products of inositol lipid breakdown (Table 3). In the presence of 50 mM-Li^+ this radioactiovity was distributed between the three inositol phosphate fractions, but there was no increase in the labelling of free inositol. In the absence of Li^+, vasopressin provoked an identical liberation of [^{3}H]inositol into water-soluble forms, but 73% of this radioactivity was recovered as free inositol. This result directly confirms the inhibitory effect of high [Li^+] upon the inositol phosphate phosphomonoesterases within intact hepatocytes.

DISCUSSION

Normal synthesis and metabolic turnover of PtdIns4P and PtdIns4,5P_2

The first important point to emerge from the results reported here is that labelling of the monoester phosphate groups of PtdIns4P and PtdIns4,5P_2 from added [^{32}P]P_i in unstimulated hepatocytes is detectably slower than the labelling of the γ-phosphate of ATP, its immediate metabolic precursor (Fig. 1). However, the time-lag which separates the labelling of ATP and of lipids is only a few minutes. This suggests that the half-times for metabolic renewal of the 4- and 5-phosphate groups of hepatic polyphosphoinositides in near steady-state are no more than about 5 min, and they may be substantially less. PtdIns4P and PtdIns4,5P_2 constitute 1–4% of the total inositol glycerolipids of liver cells (Michell *et al.*, 1970; Creba *et al.*, 1983; Rhodes *et al.*, 1983; Thomas *et al.*, 1983). Thus it seems likely that as much as 1% of the large PtdIns pool of the liver cell may flow into and out of the much smaller metabolic pools of the polyphosphoinositides every minute, simply as a result of this basal metabolic turnover. A rapid withdrawal of lipid from the PtdIns4,5P_2 pool, as is seen in intensely stimulated cells, could therefore lead naturally to depletion of cellular PtdIns at the observed rates of up to 1%/min (Kirk *et al.*, 1981*a*; Michell *et al.*, 1981; Creba *et al.*, 1983) although vasopressin may also activate PtdIns and PtdIns4P kinases. Thomas *et al.* (1983) have recently reported generally similar data on the rapid labelling of ATP and of polyphosphoinositides. However, they did not confine their analyses to the metabolically labile monoester phosphate groups of the lipids, and they analysed the time-courses of these processes in considerably less detail. An important technical point that emerges from our study is that an unidentified ^{32}P-labelled contaminant, which is a phosphodiester and turns over much more slowly than does PtdIns4P, contaminates the 'Gro$P$Ins4P fraction' that is isolated from the deacylation products of hepatic lipids by using small anion-exchange columns. This might be the glycerol-containing, but inositol-free, contaminant of such fractions that was first reported by Galliard *et al.* (1965); its parent lipid remains to be identified. We do not know whether the ^{32}P-labelled contaminant shown in Fig. 1(*b*) is related to the two unidentified [^{3}H]inositol metabolites detected by high voltage electro-

phoresis of the water soluble products of stimulated inositol lipid breakdown (Fig. 2).

The capacity of the liver cell continuously to synthesize PtdIns4P and PtdIns4,5P_2 at a high rate is further emphasized by the fact that, even when a hormonal stimulus causes PtdIns4P and PtdIns4,5P_2 depletion at rates of at least 1–3%/s (Kirk *et al.*, 1981*a*; Michell *et al.*, 1981; Creba *et al.*, 1983; Thomas *et al.*, 1983), the new steady-state levels of these lipids that are achieved within 15–60 s are still 50–80% of the levels maintained in unstimulated cells.

Inositol lipid hydrolysis is phosphodiesterase-catalysed

For many years, it is has been argued that inositol lipid breakdown in stimulated cells is phosphodiesterase-catalysed (for reviews, see Durell *et al.*, 1969; Michell, 1975), but proof has come only with the recent identification of 1,2-diacylglycerol and water-soluble inositol phosphates as the accumulated products of this type of response in various stimulated cells (for a review see Berridge, 1984). For stimulated liver cells, our observations of the accumulation of inositol phosphates confirm and complement those published from two other laboratories whilst this work was in progress (Burgess *et al.*, 1984; Thomas *et al.*, 1984). However, we have put more emphasis on relating the quantities of inositol phosphates accumulated to the quantitities of lipids degraded.

In an earlier study, Prpic *et al.* (1982*b*) showed that free inositol, derived from degraded lipids, is liberated by liver cells stimulated by Ca^{2+}-mobilizing hormones. Our experiments with liver cells labelled with [^{3}H]inositol *in vivo* (Table 3) demonstrate that in the presence of Li^+, which inhibits inositol monophosphate phosphatase, all of this inositol accumulates as inositol phosphates rather than free inositol. Berridge *et al.* (1982) first reported that Li^+ prevents inositol release from stimulated blowfly salivary glands and we have now shown that the inositol phosphates produced in hepatocytes following receptor stimulation are largely retained within the cells. Surprisingly, the latter observation appears not to have been made before, despite the fact that there is already a wide acceptance of the idea that Ins1,4,5P_3 liberated from PtdIns4,5P_2 acts as a diffusible intracellular second messenger molecule (for review, see Berridge & Irvine, 1984). Some 12% of the accumulated inositol phosphates in vasopressin-stimulated hepatocytes were recovered in the extracellular compartment (Table 2). This may be attributable to the release of these compounds from damaged cells, since lactate dehydrogenase leakage from hepatocytes is typically in the range 5–12%. However, it seems unlikely that damaged cells would retain full sensitivity to the hormone and the presence of inositol phosphates in the extracellular phase may, therefore, reflect a slight release of these compounds from intact cells.

In our previous studies, we established that the depletion of total inositol lipids during intense stimulation of liver cells with vasopressin (e.g. with 0.25 μM-vasopressin as in the present study) amounts to 8–15% during 10–15 min (Kirk *et al.*, 1981*b*). In the present study we demonstrate that the rate of accumulation of inositol phosphates is sufficient to account for degradation of about 1% of the total cellular inositol lipids/min for at least 15 min. No accumulation of any inositol-containing products other than Ins$P$, InsP_2, InsP_3 and InsP_4 was observed at any time in cells stimulated in the presence of Li^+, so it seems that receptor-controlled inositol lipid degradation proceeds via phosphodiesterase-catalysed hydrolysis of one or more of the inositol glycerolipids.

Using anion-exchange chromatography to separate the water-soluble products of inositol lipid degradation, we detected a statistically significant accumulation of InsP_3 and InsP_2 after 5 and 10 s stimulation respectively. Ins$P$ accumulation was a much later event. Previous studies, with guinea-pig (Burgess *et al.*, 1984) and rat (Thomas *et al.*, 1984) hepatocytes, have indicated that InsP_3 accumulates very quickly in stimulated liver cells. However, the present results are the first to indicate a significant and quantitatively greater accumulation of InsP_3 compared with that of InsP_2 following brief periods of hormonal stimulation. Hence, these results are compatible both with the suggestion that phosphodiesterase-catalysed hydrolysis of PtdIns4,5P_2 is the reaction in inositol lipid metabolism that is controlled by receptors (Kirk *et al.*, 1981*a*; Michell *et al.*, 1981; Creba *et al.*, 1983) and with the suggested function of liberated Ins1,4,5P_3 as an intracellular second messenger (Berridge & Irvine, 1984).

The separation of [^{3}H]inositol phosphates by h.p.l.c. revealed two further characteristics of vasopressin-stimulated inositol phosphate release in hepatocytes. Firstly, within 30 s of stimulation, the radioactivity associated with InsP_3 was roughly equally divided between Ins1,4,5P_3 and Ins1,3,4P_3. This observation is in general agreement with a recent report of Burgess *et al.* (1985). Secondly, an inositol phosphate, which behaved identically with Ins1,3,4,5P_4 on h.p.l.c., also accumulates as a rapid consequence of vasopressin stimulation in these cells. Enhanced accumulation of this compound was first observed within 10 s of hormone stimulation (results not shown). Ins1,3,4,5P_4 has recently been reported in carbachol-stimulated rat brain slices (Batty *et al.*, 1985). It seems likely that Ins1,3,4,5P_4 is the metabolic precursor of Ins1,3,4P_3 in stimulated cells (Batty *et al.*, 1985) but it is not yet clear whether either of these molecules may, as has been suggested for Ins1,4,5P_3, serve as intracellular second messengers in their own right.

In confirmation of the report by Thomas *et al.* (1984), we observed that a larger proportion of the accumulated inositol phosphates was harvested as InsP_3 when high concentrations of Li^+ were present. As in their experiments, there appeared to be a graded effect of Li^+ addition with maximum accumulation of Ins$P$ and InsP_2 at 10 mM-Li^+ but a continued increase in InsP_3 accumulation at higher Li^+ concentrations. The effect of Li^+ upon Ins$P$ and InsP_2 accumulation is in accord with our studies of the degradation of individual inositol phosphates by cell-free preparations from rat liver in which Li^+ effectively inhibited the dephosphorylation of Ins1P and Ins1,4P_2 (Storey *et al.*, 1984). However, we have not observed any effect of Li^+ upon the activity of Ins1,4,5P_3 5-phosphatase. Taken together, these results suggest that two or more separate enzymes are responsible for the dephosphorylation of the three inositol phosphates.

When, in an earlier study (Creba *et al.*, 1983), we observed that DMSO caused a decrease in the steady-state concentration of PtdIns4,5P_2 in liver cells, we did not know whether its effect was similar to that of

receptor stimulation. A hint that DMSO might act via a separate mechanism was provided by its lack of effect on hepatic PtdIns4*P*. The results in the present paper show that DMSO, at a concentration which provokes PtdIns4,5P_2 depletion, does not cause accumulation of any inositol phosphates and that it does not change the yield of inositol phosphates in response to vasopressin. It therefore seems that DMSO may shift the equilibrium of the PtdIns4*P*–PtdIns4,5P_2 cycle in the direction of PtdIns4*P*, but further experiments will be necessary to determine whether the target of this effect is PtdIns4*P* kinase or PtdIns4,5P_2 5-phosphatase. It is, however, particularly interesting that DMSO, a chemical capable of committing a variety of stem cells to differentiation, should reduce the cellular concentration of PtdIns4,5P_2, an essential component of a signalling system crucial to the control of cell proliferation (Michell, 1982, 1984; Berridge, 1984).

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