Exposure of cultured hepatocytes to cyclic AMP enhances the vasopressin-mediated stimulation of inositol phosphate production

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Isolated rat hepatocytes in primary monolayer culture were maintained for 18–24 h in the presence of 10 % (v/v) serum and [^3H]inositol. Vasopressin (100 nM) stimulated the production of inositol mono-, bis- and tris-phosphates (IP_1 , IP_2 and IP_3). Prior exposure of hepatocytes to 8-bromo cyclic AMP (8Br-cAMP; 100 μM), but not 8-bromo cyclic GMP, enhanced the vasopressin-mediated stimulation of inositol phosphate accumulation, but had no significant effect on their formation in the absence of vasopressin. The effect of the cyclic AMP analogue was mimicked by glucagon (10 nM), and was seen whether cyclic AMP or glucagon was added 5 min or 12 h before the addition of vasopressin. An 8 h incubation with dexamethasone (100 nM) enhanced the accumulation of IP_3 , but not that of IP_2 or IP_1 , in the presence of 8Br-cAMP and vasopressin. Cycloheximide or actinomycin D had little effect on the vasopressin stimulation of inositol phosphate accumulation, after an 8 h incubation in the presence or absence of 8Br-cAMP.

INTRODUCTION

Vasopressin and other Ca^{2+} -mobilizing hormones stimulate the breakdown of PIP_2 to yield IP_3 , which subsequently mobilizes Ca^{2+} from intracellular stores such as the endoplasmic reticulum (Nishizuka, 1986; Berridge, 1987). Glucagon or cyclic AMP analogues can also increase $[\text{Ca}^{2+}]_i$ (Poggioli *et al.*, 1986; Mauger & Claret, 1986; Combettes *et al.*, 1986). The effects of glucagon and vasopressin appear to be additive in this respect (Altin & Bygrave, 1987; Whipps *et al.*, 1987; Poggioli *et al.*, 1986; Combettes *et al.*, 1986; Blackmore & Exton, 1986). The mechanism by which glucagon may be acting is not known, and there is a lack of agreement as to whether glucagon by itself, or in combination with vasopressin, stimulates PIP_2 hydrolysis to produce IP_3 . In this report we show that cyclic AMP or glucagon had no significant effect on basal inositol phosphate synthesis, but enhanced the effects of vasopressin.

MATERIALS AND METHODS

Preparation and incubation of hepatocytes

Hepatocytes were prepared as described by Pittner *et al.* (1985). The hepatocytes were attached to 25 mm Falcon multi-well tissue-culture plates at a density of approx. 1.25×10^6 cells/well for 1 h in 1.5 ml of a modified Liebowitz L15 medium containing 10 % (v/v) newborn-calf serum. They were subsequently incubated for 18–24 h in medium containing 5 μCi of [^3H]inositol/ml.

Measurement of inositol phosphate production

At 5 min before the end of the appropriate incubations, a small portion of LiCl was added to the medium to give a final concentration of 20 mM. 8Br-cAMP or other agents were added either at this point or at earlier time

points. This medium was then removed and replaced with fresh unlabelled serum-free medium which contained 0.2 % (w/v) bovine serum albumin and 20 mM-LiCl, with or without vasopressin as indicated. The hepatocytes were further incubated for 5 min at 37 °C, after which the medium was removed by aspiration and 1 ml of ice-cold methanol/2 M-HCl (9:1, v/v) was added, and the plates were replaced on ice. The cells were scraped and transferred to polypropylene tubes containing 1 ml of chloroform. The plates were additionally washed with 1 ml of distilled water. The phases were vortex-mixed and separated by centrifugation at 2300 g for 5 min. After neutralization with NH_3 , [^3H]inositol phosphates in the upper phase were separated on Dowex (formate form) columns as described by Downes & Michell (1981). Results are expressed as the total amount of IP_3 , IP_2 and IP_1 produced/plate. However, the IP_3 fraction also contains inositol tetrakisphosphates.

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

A more detailed separation of inositol phosphates was obtained by h.p.l.c. with a Whatman Partisil 10SAX column as described by Batty *et al.* (1985), with 1.7 M-ammonium formate, adjusted to pH 3.7 with H_3PO_4 , as the eluent. Samples were prepared as described above, and 1 ml was loaded on the column. The eluent concentration was increased from 0 to 0.05 M over 5 min to elute free inositol and glycerophosphoinositol. $\text{I}(1)\text{P}$ and $\text{I}(4)\text{P}$ were eluted by a linear gradient from 0.05 to 0.12 M over 12 min. $\text{I}(1,4)\text{P}_2$ was eluted by a linear gradient from 0.12 to 0.85 M over 5 min. $\text{I}(1,4,5)\text{P}_3$ was eluted by a linear gradient from 0.85 to 1.7 M over 5 min. Inositol polyphosphates were eluted by maintaining 1.7 M-ammonium formate for 3 minutes. Fraction volume was 0.5 ml. The various isomers were identified by using radioactive standards.

Abbreviations used: PIP_2 , phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-monophosphate; PI, phosphatidylinositol; IP_3 , IP_2 , IP_1 , *myo*-inositol tris-, bis- and mono-phosphates (with locants in parentheses where appropriate); $[\text{Ca}^{2+}]_i$, cytosolic free calcium; 8Br-cAMP, 8-bromo cyclic AMP; 8Br-cGMP, 8-bromo cyclic GMP.

Separation of phosphoinositides by t.l.c.

Lipids in the chloroform phase of hepatocyte extracts were dried down under vacuum and resuspended in 100 μ l of chloroform. Lipids were separated on polyester-backed Whatman silica G plates. The solvent used was chloroform/methanol/water/78% NH_3 (90:90:19:10, by vol.) as described by Schacht (1978). R_F values for PI, PIP and PIP_2 were approx. 0.75, 0.45 and 0.1 respectively, as determined by using radioactive standards.

Materials

$[^3\text{H}]\text{PIP}_2$, $[^3\text{H}]\text{PIP}$, $[^3\text{H}]\text{PI}$, *myo*-[2- ^3H]inositol, $[^3\text{H}]\text{-I(1)P}$, $[^3\text{H}]\text{-I(4)P}$ and $[^3\text{H}]\text{-I(1,4)P}_2$ were obtained from NEN Research Products; $[^3\text{H}]\text{-I(1,4,5)P}_3$ was from Amersham Radiolabelled Products; tissue-culture reagents were from Flow Laboratories; tissue-culture plates were from Falcon; bovine serum albumin was from Armour Pharmaceutical Co.; Dowex AG 1-X8 resin (100–200 mesh, formate form) was from Bio-Rad; collagenase and other reagents were from Sigma.

RESULTS AND DISCUSSION

Although both vasopressin and glucagon increase intracellular $[\text{Ca}^{2+}]$, the mechanism by which glucagon acts is unknown. One possibility is that glucagon directly or indirectly activates PIP_2 breakdown, releasing IP_3 , which in turn releases Ca^{2+} from internal stores such as the endoplasmic reticulum. Another possibility is that a Ca^{2+} gate located on the plasma membrane is directly regulated by a cyclic-AMP-dependent process allowing entry of external Ca^{2+} (Poggioli *et al.*, 1986). Wakelam *et al.* (1986) reported that glucagon at concentrations between 0.5 and 5 nM increased IP_3 synthesis by 20%, as did a glucagon analogue that did not affect cyclic AMP concentrations; they found no effect of glucagon at concentrations above 5 nM on inositol phosphate accumulation. However, Blackmore & Exton (1986) and Charest *et al.* (1985) reported 6–30% increases in IP_3 accumulation with glucagon at concentrations that were

found to be ineffective by Wakelam *et al.* (1986). In contrast, Poggioli *et al.* (1986) found no effect of glucagon on phosphoinositide metabolism. We also found no effect of glucagon alone on phosphoinositide breakdown, using cultured rat hepatocytes (Table 1).

In our experiments, if hepatocytes were incubated with 100 μM -8Br-cAMP or 10 nM-glucagon for 5 min or 8 h in the presence of Li^+ , no significant change in net accumulation of labelled inositol phosphates was seen (Table 1). If vasopressin was added to hepatocytes that had been previously treated with 8Br-cAMP or glucagon for 5 min, an enhancement of the effects of vasopressin was seen (Table 1), in agreement with the findings of Blackmore & Exton (1986) and in contrast with those of Poggioli *et al.* (1986). Similar results were also seen if the hepatocytes had been incubated with 8Br-cAMP or glucagon for 8 h (Table 1) before the addition of vasopressin. There was no effect of vasopressin or 8Br-cAMP on the concentration of glycerophosphoinositol (results not shown).

8Br-cAMP did not alter the responsiveness of the hepatocytes to vasopressin (Fig. 1), since maximum effects were seen with 10 nM-vasopressin in either the presence or the absence of the cyclic AMP analogue. The enhancement was predominantly of IP_2 and IP_3 (+tetrakisphosphate) accumulation (Fig. 1). Increases in the vasopressin response required between 0.1 and 1 μM -8Br-cAMP (Fig. 2).

The maximum response to 8Br-cAMP was seen if the hepatocytes were preincubated for approx. 1 h before the addition of vasopressin. The effect was long lasting, as it was the same whether 8Br-cAMP was added 1 or 12 h before the addition of vasopressin (Fig. 3).

8Br-cGMP (100 μM) did not mimic the effects of 8Br-cAMP (Table 2). The effect of 100 μM -8Br-cGMP on accumulation of IP_3 even after 8 h was less than that noted with 0.1 μM -8Br-cAMP (Fig. 2). The only effect of 100 μM -Br-cGMP was a small enhancement, after 8 h incubation, of IP_1 accumulation in the presence of vasopressin (Table 2).

Whipps *et al.* (1987) reported an effect of a 10 min incubation with glucagon on labelling of PIP and PIP_2 in

Table 1. Effects of glucagon or 8Br-cAMP on inositol phosphate production

Hepatocytes were incubated with 8Br-cAMP (100 μM) or glucagon (10 nM) for either 5 min or 8 h and then incubated with or without vasopressin (100 nM) for 5 min. The effects of added agents are expressed as the % change of IP_1 , IP_2 and IP_3 from basal values and are means $\pm$ S.E.M. from four independent experiments. The significance of the effects of 8Br-cAMP or glucagon in the presence of vasopressin as compared with incubations containing vasopressin alone was calculated by a paired *t* test: ** $P < 0.001$; * $P < 0.005$; ††† $P < 0.01$; †† $P < 0.025$; † $P < 0.05$.

Additions	5 min incubation			8 h incubation		
	IP_1	IP_2	IP_3	IP_1	IP_2	IP_3
Basal (d.p.m.) . . .	2772 $\pm$ 764	140 $\pm$ 23	143 $\pm$ 9	2457 $\pm$ 318	130 $\pm$ 26	145 $\pm$ 14
8Br-cAMP (%)	0 $\pm$ 1	-1 $\pm$ 10	-1 $\pm$ 5	+4 $\pm$ 14	-5 $\pm$ 6	+5 $\pm$ 9
Glucagon (%)	-4 $\pm$ 3	+2 $\pm$ 19	-6 $\pm$ 3	-7 $\pm$ 4	+5 $\pm$ 7	+4 $\pm$ 8
Vasopressin (%)	+51 $\pm$ 22	+215 $\pm$ 79	+135 $\pm$ 40	+40 $\pm$ 9	+168 $\pm$ 63	+110 $\pm$ 23
Vasopressin + 8Br-cAMP (%)	+95 $\pm$ 36†	+397 $\pm$ 138†	+246 $\pm$ 59†††	+97 $\pm$ 17†††	+559 $\pm$ 112*	+306 $\pm$ 40†††
Vasopressin + glucagon (%)	+74 $\pm$ 40	+354 $\pm$ 120†	+233 $\pm$ 62††	+78 $\pm$ 18††	+391 $\pm$ 156†	+215 $\pm$ 54†

Fig. 1. Effects of 8 h exposure to 8Br-cAMP on vasopressin-mediated stimulation of inositol phosphate production

Hepatocytes were incubated in the absence (○) or the presence (●) of 8Br-cAMP (100 μ M) for 8 h and then exposed to various concentrations of vasopressin for 5 min, and inositol phosphate production was measured as described in the Materials and methods section. Results are expressed as the inositol phosphate production as a percentage of that of basal incubations in the absence of any added hormones, which were 1700 ± 315 , 112 ± 18 and 147 ± 33 d.p.m. for IP₁, IP₂ and IP₃ respectively, and are means $\pm$ ranges of two independent experiments.

Fig. 2. Dose/response curves for 8Br-cAMP effects on vasopressin stimulation of inositol phosphate production

Hepatocytes were incubated in the presence of various concentrations of 8Br-cAMP for 8 h and were then exposed to vasopressin (100 nM) for 5 min, and inositol phosphates were measured as described in the Materials and methods section. The increases in inositol phosphates produced by vasopressin alone were 885 ± 25 , 195 ± 72 and 165 ± 71 d.p.m. over basal incubations for IP₁, IP₂ and IP₃ respectively, and were normalized to 100%. The results are expressed as the inositol phosphate production as a percentage of that in incubations containing vasopressin alone and are means $\pm$ ranges from two independent experiments.

Table 2. Effects of 8Br-cAMP and 8Br-cGMP on inositol phosphate production

Hepatocytes were incubated with 8Br-cAMP (100 μ M) or 8Br-cGMP (100 μ M) for 5 min or 8 h and were then incubated for 5 min with vasopressin. Results are expressed as percentage changes in PI, PIP and PIP₂ and means $\pm$ S.E.M. from three independent experiments. The significance of differences was calculated as described in Table 1.

Additions	5 min incubation			8 h incubation		
	IP ₁	IP ₂	IP ₃	IP ₁	IP ₂	IP ₃
Basal (d.p.m.) . . .	2514 $\pm$ 1056	160 $\pm$ 5	138 $\pm$ 10	2380 $\pm$ 458	138 $\pm$ 35	149 $\pm$ 19
8Br-cAMP (%)	+0 $\pm$ 1	+5 $\pm$ 9	+2 $\pm$ 6	-7 $\pm$ 8	-5 $\pm$ 9	+1 $\pm$ 10
8Br-cGMP (%)	-2 $\pm$ 10	-3 $\pm$ 6	+0 $\pm$ 6	+3 $\pm$ 9	-2 $\pm$ 7	+8 $\pm$ 15
Vasopressin (%)	+62 $\pm$ 27	+267 $\pm$ 80	+158 $\pm$ 44	+45 $\pm$ 10	+197 $\pm$ 82	+126 $\pm$ 21
Vasopressin + 8Br-cAMP (%)	+99 $\pm$ 48	+481 $\pm$ 146	+275 $\pm$ 74†	+111 $\pm$ 8**	+617 $\pm$ 134††	+298 $\pm$ 58†
Vasopressin + 8Br-cGMP (%)	+67 $\pm$ 40	+292 $\pm$ 67	+164 $\pm$ 51	+88 $\pm$ 22†	+292 $\pm$ 166	+156 $\pm$ 36

Table 3. Effects of glucagon and 8Br-cAMP on phosphoinositide breakdown

PI, PIP and PIP₂ were measured as described in the Materials and methods section and are from the same experiments described in Table 1. Results are expressed as the percentage changes in PI, PIP and PIP₂ from basal values produced by added agents.

Additions	5 min incubation			8 h incubation		
	PI	PIP	PIP ₂	PI	PIP	PIP ₂
Basal (d.p.m.) . . .	167451 $\pm$ 17789	3070 $\pm$ 1485	1037 $\pm$ 112	187765 $\pm$ 9757	3070 $\pm$ 1420	896 $\pm$ 113
8Br-cAMP (%)	+3 $\pm$ 5	-6 $\pm$ 16	-7 $\pm$ 9	-6 $\pm$ 2	+18 $\pm$ 14	+10 $\pm$ 5
Glucagon (%)	+3 $\pm$ 6	-5 $\pm$ 1	-13 $\pm$ 4	-5 $\pm$ 5	+9 $\pm$ 14	-4 $\pm$ 12
Vasopressin (%)	+2 $\pm$ 6	+4 $\pm$ 7	+9 $\pm$ 9	-13 $\pm$ 9	-2 $\pm$ 5	+14 $\pm$ 28
Vasopressin + 8Br-cAMP (%)	+6 $\pm$ 5	+23 $\pm$ 30	-7 $\pm$ 3	-10 $\pm$ 2	+8 $\pm$ 8	+4 $\pm$ 11
Vasopressin + glucagon (%)	+5 $\pm$ 7	+24 $\pm$ 34	-5 $\pm$ 11	-7 $\pm$ 2	-1 $\pm$ 12	+2 $\pm$ 5

isolated hepatocytes preincubated with [³²P]P_i for 60 min. However, in hepatocytes previously incubated with [³H]inositol for 18–24 h, exposure to glucagon or 8Br-cAMP for 5 min or 8 h had no significant effect on the amount of ³H-labelled PI, PIP or PIP₂ (Table 3). There was also no effect of vasopressin with or without 8Br-cAMP, which is hardly surprising, since a 5 min incubation with vasopressin even in the presence of 8Br-cAMP results in breakdown of less than 2% of labelled phosphoinositides (Tables 1 and 3).

We found that most of the basal inositol phosphates consisted of inositol 1-phosphate (Fig. 4). Vasopressin particularly increased the accumulation of label in I(4)P, I(1,4)P₂ and I(1,4,5)P₃. An 8 h incubation with 8Br-cAMP before the addition of vasopressin appeared simply to increase the accumulation of label in all the inositol phosphates, suggesting that the action of 8Br-cAMP is a mere amplification of the actions of vasopressin.

Over an 8 h period there is a marked induction of the enzymes phosphatidate phosphohydrolase and tyrosine

aminotransferase by dexamethasone (a synthetic glucocorticoid) or by cyclic AMP in cultured rat hepatocytes (Pittner *et al.*, 1985). The combination of both agents results in a greater stimulation of enzyme activity than was seen with either alone. Dexamethasone (100 nM) had little effect on basal or vasopressin-stimulated inositol phosphate formation (Table 4). However, accumulation of IP₃ owing to vasopressin in hepatocytes that had been incubated for 8 h with 8Br-cAMP and dexamethasone was 34% greater than in hepatocytes exposed to 8Br-cAMP alone (Table 4). The data show that the effect of dexamethasone was specific for IP₃ accumulation, suggesting an effect on the enzyme(s) responsible for IP₃ metabolism.

Actinomycin D (an inhibitor of mRNA synthesis) and cycloheximide (an inhibitor of protein synthesis) had surprisingly little effect on basal or vasopressin-stimulated inositol phosphate concentrations, even in the presence of 8Br-cAMP (Table 4). The data suggest that the long-term effect of 8Br-cAMP is not due to protein synthesis *de novo*, and indicate that the proteins involved

Fig. 3. Time course for the effects of 8Br-cAMP on vasopressin-mediated stimulation of inositol phosphate production

Hepatocytes were incubated with 100 μ M-8Br-cAMP for the times indicated before addition of vasopressin for 5 min. Zero preincubation represents simultaneous addition of 8Br-cAMP and vasopressin for 5 min. Inositol phosphate production was measured as described in the Materials and methods section. Results are expressed as the production of IP₁ ($\square$), IP₂ ($\triangle$) and IP₃ ($\circ$) compared with incubations containing vasopressin alone, which were normalized to 100 % as in Fig. 2, and are means from three independent experiments, except for the 15 min, 4 h and 12 h time points, which are means from two independent experiments; S.E.M. values and ranges have been omitted for the sake of clarity.

Table 4. Effects of dexamethasone, actinomycin D and cycloheximide on inositol phosphate production

Hepatocytes were incubated with dexamethasone (100 nM), actinomycin D (1 μ g/ml) or cycloheximide (5 μ g/ml) for 8 h in the presence (c) or the absence (a and b) of 8Br-cAMP (100 μ M) and were then incubated with vasopressin for 5 min (b and c). Results are expressed as the percentage changes in d.p.m. in IP₁, IP₂ and IP₃ (a) due to agents alone, (b) in the presence of vasopressin and (c) in the presence of 8Br-cAMP and vasopressin, and are means $\pm$ S.E.M. from three independent experiments. The significance of difference was determined by paired analysis, and the symbols for significance are as described in Table 1.

Additions	IP ₁	IP ₂	IP ₃
(a) Basal (d.p.m.)...	2380 $\pm$ 458	139 $\pm$ 35	149 $\pm$ 19
Dexamethasone (%)	-11 $\pm$ 2	+3 $\pm$ 20	-2 $\pm$ 6
Actinomycin D (%)	-14 $\pm$ 15	-3 $\pm$ 17	-6 $\pm$ 13
Cycloheximide (%)	-18 $\pm$ 18	-6 $\pm$ 18	-12 $\pm$ 9
(b) + Vasopressin (d.p.m.)...	3739 $\pm$ 1313	409 $\pm$ 146	340 $\pm$ 65
Dexamethasone (%)	+23 $\pm$ 24	0 $\pm$ 6	+21 $\pm$ 18
Actinomycin D (%)	+89 $\pm$ 48	+28 $\pm$ 21	+38 $\pm$ 37
Cycloheximide (%)	+22 $\pm$ 60	+9 $\pm$ 16	-7 $\pm$ 4
(c) + Vasopressin + 8Br-cAMP (d.p.m.)...	5037 $\pm$ 1062	958 $\pm$ 232	608 $\pm$ 170
Dexamethasone (%)	+4 $\pm$ 32	-16 $\pm$ 13	+34 $\pm$ 8††
Actinomycin D (%)	-29 $\pm$ 26	+10 $\pm$ 21	-3 $\pm$ 9
Cycloheximide (%)	-48 $\pm$ 27	-26 $\pm$ 9	-27 $\pm$ 13

Fig. 4. Separation of inositol phosphates by h.p.l.c.

Extracts of [³H]inositol-labelled hepatocytes were prepared from incubations described in Table 1, and applied to a SAX h.p.l.c. column as described in the Materials and methods section. The percentage of 1.7 M-ammonium formate (pH 3.7) present in the elution gradient (·····) is shown in panel (a), and the relative positions of I(1)P, I(4)P, I(1,4)P₂ and I(1,4,5)P₃ identified by using radioactive standards are noted in panel (b). Panel (a) shows the elution profile of [³H]inositol phosphates in hepatocytes from basal incubations ($\circ$), (b) shows that from hepatocytes incubated with 100 nM-vasopressin ($\triangle$) for 5 min, and (c) shows that from hepatocytes incubated for 8 h with 100 μ M-8Br-cAMP before the addition of 100 nM-vasopressin ($\square$) for 5 min. The fraction size was 0.5 ml. Results are from a representative experiment.

in the response of hepatocytes to both cyclic AMP and vasopressin with respect to phosphoinositide turnover are relatively stable.

The results demonstrate that in cultured rat hepatocytes there is a marked synergistic effect of cyclic AMP on vasopressin stimulation of phosphoinositide breakdown. Since cyclic AMP reproduces the effects of glucagon, we do not need to invoke any mechanisms involving glucagon effects independent of cyclic AMP formation. There was no effect of cyclic AMP or glucagon alone, even after 8 h, on phosphoinositide breakdown.

The site of cyclic AMP action remains to be established. The three most likely possibilities are phosphorylation of the vasopressin receptor, the putative 'Gp' protein as suggested by Blackmore & Exton (1986), or the phospholipase C enzyme itself.

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