Effect of cyclic AMP-dependent hormones and Ca^{2+} -mobilizing hormones on the Ca^{2+} influx and polyphosphoinositide metabolism in isolated rat hepatocytes

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The effect of the interaction between the Ca^{2+} -mobilizing hormone adrenaline, used as α -adrenergic agonist, and cyclic AMP-dependent hormones, including β -adrenergic agonists and glucagon, on the initial $^{45}\text{Ca}^{2+}$ uptake rate and polyphosphoinositide metabolism were investigated in isolated rat hepatocytes. Each hormone alone increased the initial $^{45}\text{Ca}^{2+}$ uptake rate. When adrenaline was added without inhibitor, it induced a rise in the initial $^{45}\text{Ca}^{2+}$ uptake rate larger than the sum of the rises elicited by its α and β components singly. Similarly, when adrenaline was used as an α -agonist and added together with glucagon, it enhanced the initial $^{45}\text{Ca}^{2+}$ uptake rate synergistically. Kinetic analysis of the initial $^{45}\text{Ca}^{2+}$ uptake rate measured at different Ca^{2+} concentrations suggested that the increased influx elicited by the combination of adrenaline as α -adrenergic agonist and glucagon reflects an activation of the rate of Ca^{2+} transport via a homogeneous population of Ca^{2+} channels or carriers. Dose–response curves for the α -adrenergic action of adrenaline or glucagon applied in the presence of increasing doses of glucagon or adrenaline showed that each hormone increases the maximal response to the other without affecting its ED_{50} . Measurement of polyphosphoinositide hydrolysis and of the inositol phosphates formed in the presence of adrenaline or vasopressin and/or glucagon showed that Ca^{2+} -mobilizing hormones and glucagon had no synergistic effects on inositol 1,4,5-trisphosphate production. It is therefore proposed that the synergistic action of glucagon and Ca^{2+} -mobilizing hormones on Ca^{2+} influx occurs at a step that takes place close to the Ca^{2+} channels or carriers themselves. The Ca^{2+} gating involved might be mainly controlled by two products, one of them arising from the polyphosphoinositide metabolism, and the other from the increase in internal cyclic AMP.

INTRODUCTION

In isolated hepatocytes, the Ca^{2+} influx is stimulated by noradrenaline via α_1 receptors, by angiotensin and vasopressin (Assimakopoulos-Jeannot *et al.*, 1977; Kepens *et al.*, 1977; Barritt *et al.*, 1981; De Witt & Putney, 1983, 1984; Berthon *et al.*, 1984; Blackmore *et al.*, 1984; Mauger *et al.*, 1984; Reinhart *et al.*, 1984; Binet *et al.*, 1985; Williamson *et al.*, 1985; but see also Blackmore *et al.*, 1982), as well as by glucagon and isoprenaline (Keppens *et al.*, 1977; Kraus-Friedmann, 1984; Mauger *et al.*, 1985; but see also Studer *et al.*, 1984). All these hormones act through different coupling mechanisms. Michell and his group (Michell, 1975; Michell *et al.*, 1977) first proposed that PI breakdown triggered by Ca^{2+} -mobilizing hormones (i.e. α -adrenergic agonists, angiotensin and vasopressin) might have a key role in control of cell-surface Ca^{2+} permeability. More recently it appeared that the coupling mechanism for these hormones induces a breakdown of PIP_2 , thus producing IP_3 and diacylglycerols (for review, see Kirk *et al.*, 1984; Nishizuka, 1984; Berridge & Irvine, 1984; Williamson *et al.*, 1985). Stimulation by cyclic AMP-dependent hormones such as β -adrenergic agonists and glucagon increases intracellular cyclic AMP by activating adenylate cyclase (for review, see Exton, 1985). However, neither

Ca^{2+} -mobilizing nor cyclic AMP-dependent hormones act independently on the Ca^{2+} influx, and evidence has shown that vasopressin, angiotensin and glucagon interact synergistically to increase the unidirectional Ca^{2+} influx (Mauger *et al.*, 1985).

In the present work, we extended our study of the effects on the Ca^{2+} influx of the Ca^{2+} -mobilizing hormone vasopressin and the cyclic AMP-dependent hormone glucagon. This time the Ca^{2+} -mobilizing hormone explored in this connection was adrenaline, which was used as an α -adrenergic agonist in conjunction with the cyclic AMP-dependent hormones glucagon and adrenaline (in this case the latter was used as a β -adrenergic agonist). We attempted to establish whether or not synergistic interaction occurred on polyphosphoinositide metabolism. Because under certain conditions adrenaline and glucagon are released simultaneously into the blood (Eigler *et al.*, 1979), it seemed of physiological relevance to study in particular glucagon–adrenaline interaction when the latter was used as an α -adrenergic agonist. The results show that simultaneous stimulation of either the α - and β -adrenergic receptors or the α -adrenergic and glucagon receptors caused a synergistic increase in the Ca^{2+} influx. In contrast, no such effect was observed on polyphosphoinositide metabolism, indicating that the molecular event leading to the synergistic action on the

Abbreviations used: PIP_2 , phosphatidylinositol bisphosphate; PIP , phosphatidylinositol monophosphate; PI , phosphatidylinositol; IP_3 , *myo*-inositol trisphosphate; IP_2 , *myo*-inositol bisphosphate; IP , *myo*-inositol monophosphate.

Ca^{2+} influx by adrenaline and glucagon occurs at a step that takes place close to the channels or carriers themselves.

EXPERIMENTAL

Cell isolation and incubation procedures

Isolated hepatocytes from female Wistar rats weighing 200–250 g were prepared as previously described (Mauger *et al.*, 1985). The isolated cells were incubated at about 3×10^6 cells/ml at 37 °C in Eagle's medium containing: 116 mM-NaCl, 5.4 mM-KCl, 1.8 mM- CaCl_2 , 0.81 mM- MgCl_2 , 0.92 mM- NaH_2PO_4 , 25 mM- NaHCO_3 , 1 g of glucose/l, amino acids and vitamins supplemented with 1.5% gelatin (Difco) under an atmosphere of O_2/CO_2 (19:1).

$^{45}\text{Ca}^{2+}$ uptake measurement

The initial $^{45}\text{Ca}^{2+}$ uptake rate was determined as described by Mauger *et al.* (1984). Briefly, adrenaline, vasopressin or glucagon was added to the cells, and 30 s later these were incubated at 37 °C in Eagle's medium, pH 7.4, with 1 μCi of $^{45}\text{Ca}^{2+}$ /ml. Samples (100 μl) were taken at 15, 45, 75 and 105 s after the addition of $^{45}\text{Ca}^{2+}$. Each was diluted with 4 ml of ice-cold washing solution, containing, 144 mM-NaCl, 5 mM- CaCl_2 and 5 mM-Tris/HCl, pH 7.4, filtered through Whatman GF/C glass-fibre filters and washed with 3×4 ml of the washing solution. The radioactivity on the filter was counted with a liquid-scintillation spectrometer. When used, propranolol or phentolamine was added 2 min before adrenaline.

Phospholipid analysis

Isolated hepatocytes (3.5×10^6 – 4×10^6 cells/ml) were labelled to isotopic steady state by incubation for 90 min in Eagle's medium supplemented with 1.5% gelatin containing 11 μM -*myo*-[2- ^3H]inositol (10 μCi /ml). The cells were then washed three times to remove the extracellular label and resuspended at 2.5×10^6 cells/ml in modified inositol-free Eagle's medium containing 106 mM-NaCl and 10 mM-LiCl. The cell suspension was then preincubated for 10 min with LiCl, after which 0.6 ml samples of this suspension were further incubated for 30 s, with or without one of the hormones tested. A 0.5 ml portion of each sample was then transferred to Eppendorf microtubes containing 1 ml of ice-cold incubation medium, and quickly centrifuged for 1 s at 12000 g. The supernatant was discarded, the pellet was homogenized with 0.2 ml of water, and 0.75 ml of chloroform/methanol/HCl (100:50:1, by vol.) was added as described by Thomas *et al.* (1984).

After vortex-mixing and centrifugation for 2 min at 12000 g, a 300 μl sample of the upper aqueous phase of the chloroform/methanol extract was collected, diluted to 4 ml with water and neutralized. The [^3H]inositol phosphates were separated by anion-exchange chromatography on a Dowex 1X8 column (0.5 cm $\times$ 3 cm; formate form), as described by Downes & Michell (1981). Free inositol was not retained on the columns. [^3H]Inositol phosphates were eluted sequentially with 20 ml of the eluting solution for glycerophosphoinositol and 6 ml each of the eluting solutions for IP, IP₂ and IP₃ as described by Berridge *et al.* (1983). A 3 ml portion of each fraction was mixed with 12 ml of Lumagel (LUMAC) and counted for radioactivity by liquid scintillation. The [^3H]inositol lipids were isolated from the

lower phase of the cell extract. The dried lipid samples were dissolved in 0.5 ml of chloroform and deacylated by adding 0.1 ml of methanol/1 M-NaOH (19:1, v/v) and 0.1 ml of methanol. After 20 min at room temperature, 0.5 ml of chloroform, 0.3 ml of methanol and 0.3 ml of water were added, the preparation was mixed and centrifuged, and 0.5 ml of the upper phase was then withdrawn, neutralized with 0.5 M-boric acid and diluted to 5 ml with 0.22 M-ammonium formate/2 M-sodium tetraborate, as described by Creba *et al.* (1983). The resulting glycerophosphoryl esters were then separated by anion-exchange chromatography on columns identical with those used to separate the inositol phosphates. These esters were eluted sequentially by using 10 ml each of the elution solutions for glycerophosphoinositol, glycerophosphoinositol 4-phosphate and glycerophosphoinositol 4,5-bisphosphate, as described by Downes & Michell (1981). The ^3H content was determined by liquid-scintillation counting, as described above.

Materials

Collagenase was obtained from Boehringer, [arginine]-vasopressin, angiotensin, glucagon, (–)-adrenaline, ($\pm$)-propranolol, and (–)-isoprenaline were from Sigma, and phentolamine was a gift from Ciba-Geigy; Dowex 1X8 ion-exchange resin (formate form, 200–400 mesh) was from Bio-Rad. $^{45}\text{Ca}^{2+}$ was supplied by I.R.E., Fleurus, Belgium, and *myo*-[2- ^3H]inositol was from New England Nuclear.

RESULTS

Effect of Ca^{2+} -mobilizing hormones and cyclic AMP-dependent hormones on the initial $^{45}\text{Ca}^{2+}$ uptake rate

Adrenaline was applied in the presence either of the β -blocker propranolol and used as an α -adrenergic agonist, or of the α -blocker phentolamine and used as a β -adrenergic agonist. It was observed that the optimal conditions for measuring the $^{45}\text{Ca}^{2+}$ uptake rate were obtained when the hormones were added to the cell suspension either singly or together 30 s before the $^{45}\text{Ca}^{2+}$ (Mauger *et al.*, 1985; results not shown). As shown in Fig. 1, maximal doses of the Ca^{2+} -mobilizing hormones adrenaline (used as an α -agonist) and vasopressin, and of the cyclic AMP-dependent hormones adrenaline (used as a β -agonist), isoprenaline and glucagon, increased the initial $^{45}\text{Ca}^{2+}$ uptake rate compared with the controls (resting $^{45}\text{Ca}^{2+}$ uptake rate 0.24 ± 0.02 nmol/min per mg dry wt; $n = 13$). Among the Ca^{2+} -mobilizing hormones, adrenaline, when used as an α -agonist, was less effective than vasopressin in enhancing the $^{45}\text{Ca}^{2+}$ uptake rate [0.46 ± 0.02 nmol/min per mg dry wt. ($n = 14$) compared with 0.37 ± 0.01 ($n = 15$)]. Of cyclic AMP-dependent hormones, adrenaline, used as a β -adrenergic agonist, also proved less potent than the other β -adrenergic agonist, isoprenaline, and than glucagon [respective $^{45}\text{Ca}^{2+}$ uptake rates (nmol/min per mg dry wt.): 0.30 ± 0.02 ($n = 12$), 0.36 ± 0.04 ($n = 11$) and 0.41 ± 0.03 ($n = 15$)].

When combined maximal doses of a Ca^{2+} -mobilizing hormone and a cyclic AMP-dependent hormone were applied, the response was greater than additive, i.e. the $^{45}\text{Ca}^{2+}$ uptake rate measured in the presence of both agonists was larger than the sum of each separate response (Fig. 1). This observation confirms and extends what was

Fig. 1. Effects of adrenaline + phentolamine, glucagon and isoprenaline on the initial $^{45}\text{Ca}^{2+}$ uptake rate in resting hepatocytes and in hepatocytes stimulated with adrenaline + propranolol or with vasopressin

Cells were preincubated for 3 min with 10 μM -propranolol (PRO), 10 μM -phentolamine (PHE) or vehicle. Then 1 μM -adrenaline (AD), 10 nM-glucagon (GLU), 10 μM -isoprenaline (ISO) and 10 nM-vasopressin (VP) were added singly or together, 30 s before the addition of $^{45}\text{Ca}^{2+}$. Values are means $\pm$ S.E.M. for 9 to 15 determinations on five different cell preparations.

reported previously about the combined effect of vasopressin and glucagon on the $^{45}\text{Ca}^{2+}$ uptake rate (Mauger *et al.*, 1985).

Do an α -adrenergic agonist and glucagon act on the same Ca^{2+} transport system?

In previous work (Mauger *et al.*, 1984, 1985) the Ca^{2+} -mobilizing hormones noradrenaline, vasopressin and angiotensin, the cyclic AMP-dependent hormone glucagon and the combination of vasopressin and glucagon were found to induce parallel increases in the apparent K_m and apparent $V_{\max}$ of Ca^{2+} transport in isolated rat liver cells. Whether the hormones were added singly or together, they were believed to enhance the rate of this transport through a fixed population of Ca^{2+} channels or carriers.

It was therefore expected here that, in the presence of a different combination of hormones, namely adrenaline and glucagon, the apparent K_m and $V_{\max}$ would increase. This was tested by determining the $^{45}\text{Ca}^{2+}$ uptake rate as a function of the external Ca^{2+} concentration, which varied from 160 μM to 4.8 mM both in the presence of 1 μM -adrenaline and 10 nM-glucagon, and under control conditions in the absence of hormones. Fig. 2 shows that the Hofstee plots of the above findings fitted straight lines, indicating that, as expected, Ca^{2+} entered the cells via a homogeneous population of Ca^{2+} channels or carriers under all the above conditions. Furthermore, in the presence of adrenaline and glucagon both the apparent K_m and $V_{\max}$ rose compared with the control values (K_m 2.20 $\pm$ 0.64 versus 0.57 $\pm$ 0.07 mM; $V_{\max}$ 0.80 $\pm$ 0.11 versus 0.27 $\pm$ 0.02 nmol/min per mg dry wt.; $n = 6$). The simplest interpretation of these rises

is that, as suggested previously (Mauger *et al.*, 1984), the combination of adrenaline and glucagon increases the rate of Ca^{2+} transport across the plasma membrane but does not increase either the number of channels or carriers, or their affinity for Ca^{2+} .

Fig. 2. Hofstee plots of the effects of the extracellular Ca^{2+} concentration on the Ca^{2+} influx stimulated with adrenaline + propranolol and glucagon

Hepatocytes were preincubated for 3 min in a medium containing 10 μM -propranolol and CaCl_2 at the concentration required to give Ca^{2+} concentrations of 0.16–4.8 mM. Then 10 nM-glucagon + 1 μM -adrenaline or vehicle were added 30 s before the addition of $^{45}\text{Ca}^{2+}$. The mean apparent K_m values were 0.57 $\pm$ 0.07 and 2.20 $\pm$ 0.63 mM, and the mean apparent $V_{\max}$ values were 0.27 $\pm$ 0.02 and 0.80 $\pm$ 0.11 nmol/min per mg dry wt., in controls (●) and hormone-treated cells (○) respectively. Each point is the mean of six determinations on two cell preparations.

Dose-response curves for the action on the $^{45}\text{Ca}^{2+}$ uptake rate of adrenaline in the presence of increasing concentrations of glucagon, and of glucagon in the presence of increasing concentrations of adrenaline

To characterize further the synergistic action of adrenaline and glucagon, both hormones were added at increasing concentrations and the dose-response curves for the resulting effects were plotted. The data in Fig. 3(a) show that, at all the glucagon concentrations tested, adrenaline enhanced the initial $^{45}\text{Ca}^{2+}$ uptake rate without changing its ED_{50} . The same results were observed when the action of adrenaline was plotted at increasing concentrations of glucagon (Fig. 3b), which also augmented the response to adrenaline without changing its ED_{50} . These data suggest that the two hormones enhance each other's maximal effect.

Effect of Ca^{2+} -mobilizing hormones and cyclic AMP-dependent hormones on polyphosphoinositide degradation

In a separate set of experiments, we attempted to determine whether the synergistic action of glucagon and adrenaline on the Ca^{2+} influx resulted from potentiation of the polyphosphoinositide metabolism, either via an increase in phospholipase C activity or via the number of α_1 -adrenergic receptors, as suggested by Morgan *et al.* (1984).

The dose-response curve for vasopressin- or adrenaline-stimulated polyphosphoinositide degradation stays to the right of the curves for the physiological response (i.e. phosphorylase activation) and close to the curve for the binding of the agonists to their receptors (Creba *et al.*, 1983; Thomas *et al.*, 1984; Charest *et al.*, 1985). This is

why supramaximal doses of vasopressin, adrenaline and glucagon were used in the present experiments ($0.1 \mu\text{M}$, $10 \mu\text{M}$ and $0.1 \mu\text{M}$ respectively). It was checked that, as for combined doses of 10 nM -glucagon and adrenaline, these high doses increased the $^{45}\text{Ca}^{2+}$ uptake rate synergistically.

Vasopressin, adrenaline or glucagon were incubated with the cells, singly or together, for 30 s, a period long enough to obtain maximal stimulation of the Ca^{2+} influx and maximal PIP_2 degradation. These experiments were performed in the presence of 10 mM -LiCl, which blocks the enzyme hydrolysing the phosphomonoester bound in position 1 of inositol phosphate (Storey *et al.*, 1984). This blockage allows IP_3 to accumulate by displacing the chemical equilibrium between IP , IP_2 and IP_3 .

Incubation for 30 s with the hormones (either singly or together) did not significantly change the PI content of the cells. However, a significant decrease in cell PIP was initiated by vasopressin or vasopressin plus glucagon. The PIP_2 content was decreased by more than half by incubation with vasopressin, either alone or combined with glucagon. No significant change was elicited in any of the three phospholipids studied by either adrenaline or glucagon alone, or by adrenaline plus glucagon.

In the same experiments, the respective amounts of the inositol phosphates IP_3 , IP_2 and IP present in the cells were also determined. The IP content of the cells was not changed by any of the hormones tested. However, IP_3 production was always slightly increased by glucagon, and the production of IP_2 and IP_3 was enormously increased by both vasopressin and adrenaline, although vasopressin was twice as potent in this respect as adrenaline (Table 1). Neither vasopressin plus glucagon

Fig. 3. Dose-response relationship for the effects of adrenaline and glucagon on the initial $^{45}\text{Ca}^{2+}$ uptake rate

Hepatocytes were preincubated for 3 min with $10 \mu\text{M}$ -propranolol or vehicle and for 30 s with the indicated doses of glucagon and adrenaline before the addition of $^{45}\text{Ca}^{2+}$. (a) The dose-response curves for the action of glucagon were constructed in the absence (●) or presence of (○) 10 nM -, (△) $0.1 \mu\text{M}$ -, (■) $0.3 \mu\text{M}$ -, (▲) $1 \mu\text{M}$ - or (□) $10 \mu\text{M}$ -adrenaline. (b) Same data as in (a), but the dose-response curves show the action of adrenaline in the absence (●) or presence of (○) 10 pM -, (△) 0.1 nM -, (■) 0.3 nM -, (▲) 1 nM - or (□) 10 nM -glucagon.

nor adrenaline plus glucagon had any synergistic effects on inositol phosphate production, i.e. the increases in production induced by these two combinations of hormones were identical with those observed when vasopressin and adrenaline were added singly. This is clearly shown in Fig. 4, which gives the values for PIP_2 and IP_3 , expressed as percentages of their respective controls. These results suggest that the synergistic effect of adrenaline and glucagon on the Ca^{2+} influx is not due to any change in the coupling mechanism of the receptors, but rather to some event which occurs at a point closer to the Ca^{2+} channels than to the receptors themselves.

DISCUSSION

In vivo, the cells receive several simultaneous external signals mediating changes in the concentration of one or more intracellular second messengers. In liver, many hormones regulate cell metabolism by modulating the internal concentrations of cyclic AMP and Ca^{2+} . In the present work, it was shown that the initial $^{45}\text{Ca}^{2+}$ uptake rate was increased by glucagon, isoprenaline and adrenaline (when the α -blocker phentolamine was present), all of which increase cellular cyclic AMP. This enhancement of Ca^{2+} influx resulted from the rise in internal cyclic AMP, as observed in previous work (Mauger *et al.*, 1985); it could be mimicked by the permeant analogue dibutyryl cyclic AMP and by forskolin. The Ca^{2+} -mobilizing hormones adrenaline (in the presence of the β -blocker propranolol) and vasopres-

sin also raised the initial $^{45}\text{Ca}^{2+}$ uptake rate, as reported by Keppens *et al.* (1977), Barritt *et al.* (1981) and Mauger *et al.* (1984). Any combination of the two types of hormones induced a synergistic increase in the Ca^{2+} influx (Fig. 1). Combined addition to the cell preparations of certain cyclic AMP-dependent hormones and Ca^{2+} -mobilizing hormones has been reported to enhance K^+ permeability (Cocks *et al.*, 1984), Ca^{2+} permeability (Mauger *et al.*, 1985) and to modify carbohydrate metabolism (Assimakopoulos-Jeannet *et al.*, 1982).

To determine at what stage in the pathway for Ca^{2+} transport from the outside to the inside of the membrane such combinations promote a synergistic Ca^{2+} influx, measurements of the initial $^{45}\text{Ca}^{2+}$ uptake rate were performed as a function of the external Ca^{2+} concentration (Fig. 2). Results lead to the conclusion that adrenaline (plus propranolol) and glucagon activate a homogeneous population of Ca^{2+} channels or carriers. As this gating mechanism was identical with that previously described for Ca^{2+} -mobilizing hormones (Mauger *et al.*, 1984) and for the combination of glucagon and vasopressin (Mauger *et al.*, 1985), we suggest that under all the above conditions the rate of Ca^{2+} transport through the membrane rises once Ca^{2+} is bound to the outside of the channel or carrier.

The question arises then as to the identity of the intermediate involved in the synergistic effect of glucagon and adrenaline on the Ca^{2+} influx. Ca^{2+} -mobilizing hormones have been reported to inhibit the cyclic AMP accumulation induced by glucagon (Assimakopoulos-Jeannet *et al.*, 1982; Morgan *et al.*, 1983; Keppens &

Table 1. Effect of glucagon on the cellular content of the different phosphoinositides and inositol phosphates in control hepatocytes and in hepatocytes stimulated with vasopressin or adrenaline

Hepatocytes were labelled with 10 μCi of *myo*- ^3H inositol/ml for 90 min, washed and resuspended for 10 min in a medium containing 10 mM-LiCl and 10 μM -propranolol. Then 0.1 μM -vasopressin (VP), 10 μM -adrenaline (AD) and 0.1 μM -glucagon (GLU) were added singly or together, and 30 s later the incubation was terminated by adding chloroform/methanol/HCl (100:50:1, by vol.). The water-soluble ^3H inositol phosphates were extracted as described in the Experimental section and separated by anion-exchange chromatography. They were eluted sequentially with 5 mM-sodium tetraborate/60 mM-ammonium formate (for glycerophosphoinositol), 5 mM-sodium tetraborate/0.15 M-ammonium formate (for inositol 1-phosphate), 0.4 M-ammonium formate/0.1 M-formic acid (for inositol 1,4-bisphosphate) and 1 M-ammonium formate/0.1 M-formic acid (for inositol 1,4,5-trisphosphate). The ^3H inositol lipids contained in the chloroform/methanol extracts of the cells were identified after deacylation and separation of the resulting glycerophosphoryl esters on the same ion-exchange columns as were used to separate the water-soluble compounds. The deacylated phospholipids were eluted sequentially with 5 mM-sodium tetraborate/0.18 M-ammonium formate (for glycerophosphoinositol), 0.1 M-formic acid/0.3 M-ammonium formate (for glycerophosphoinositol 4-phosphate) and 0.1 M-formic acid/0.75 M-ammonium formate (for glycerophosphoinositol 4,5-bisphosphate). The ^3H content of each sample was determined by liquid-scintillation counting as outlined in the Experimental section. Each value is the mean $\pm$ S.E.M. for *n* determinations; * $P < 0.001$ for difference from control.

Addition . . .	0.9% NaCl	VP	AD	GLU	VP+GLU	AD+GLU
PI (c.p.m./mg of protein)	6938 $\pm$ 945	6711 $\pm$ 854	6412 $\pm$ 567	6523 $\pm$ 775	5224 $\pm$ 550	6475 $\pm$ 885
<i>n</i>	9	6	6	8	6	9
PIP (c.p.m./mg of protein)	399 $\pm$ 20	263 $\pm$ 21*	410 $\pm$ 26	442 $\pm$ 34	260 $\pm$ 22*	386 $\pm$ 25
<i>n</i>	9	6	6	8	6	9
PIP_2 (c.p.m./mg of protein)	516 $\pm$ 26	229 $\pm$ 24*	467 $\pm$ 30	513 $\pm$ 28	209 $\pm$ 19*	453 $\pm$ 29
<i>n</i>	9	6	6	8	6	9
IP (c.p.m./mg of protein)	588 $\pm$ 94	782 $\pm$ 177	659 $\pm$ 110	552 $\pm$ 74	785 $\pm$ 158	720 $\pm$ 110
<i>n</i>	11	6	7	11	6	7
IP_2 (c.p.m./mg of protein)	128 $\pm$ 13	556 $\pm$ 51*	252 $\pm$ 22*	127 $\pm$ 14	640 $\pm$ 87*	257 $\pm$ 25*
<i>n</i>	11	6	7	11	6	7
IP_3 (c.p.m./mg of protein)	63 $\pm$ 7	307 $\pm$ 22*	165 $\pm$ 22*	73 $\pm$ 7	320 $\pm$ 20*	171 $\pm$ 21*
<i>n</i>	11	6	7	11	6	7

Fig. 4. Effect of glucagon on the phosphatidylinositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate contents in resting hepatocytes and in hepatocytes stimulated with vasopressin, adrenaline (+ propranolol) and/or glucagon

Cells were labelled by incubation for 90 min in a medium containing 11 μ M free inositol and 10 μ Ci of *myo*-[2-³H]inositol/ml. Cells were washed and preincubated for 10 min in the presence of 10 mM-LiCl and 10 μ M-propranolol. Experiments were initiated by adding 0.1 μ M-glucagon (GLU), 0.1 μ M-vasopressin (VP) or 10 μ M-adrenaline (AD) either singly or together, and 30 s later the incubations were stopped by adding chloroform/methanol/HCl (100:50:1, by vol.). The water-soluble [³H]inositol phosphates were extracted and separated by anion-exchange chromatography. The [³H]phosphoinositides were extracted and deacylated. The water-soluble deacylation products were applied to anion-exchange columns and sequentially eluted as described in the Experimental section and in the legend to Table 1. The release of [³H]IP₃ and the change in amounts of [³H]PIP₂ were expressed as percentages of their respective control values, determined in the absence of hormones (63 $\pm$ 7 and 516 $\pm$ 26 c.p.m./mg of protein for IP₃ and PIP₂ respectively). Each point represents the mean $\pm$ S.E.M. for six to nine determinations on two to four cell preparations.

De Wulf, 1984), and it therefore seemed very unlikely that the synergistic action of the α -adrenergic agonist adrenaline and of glucagon on the Ca²⁺ influx resulted from changes in the internal cyclic AMP concentration. Nevertheless, it was possible that the synergistic effect of these two hormones on the Ca²⁺ influx resulted from the potentiation of the polyphosphoinositide metabolism. However, this was improbable, as we showed that cell IP₃ content rose to the same value in the presence of maximal doses of vasopressin, whether it was used alone or with glucagon. This apparent lack of potentiation of PIP₂ hydrolysis might have been due to saturation of the mechanism coupling the receptors and phospholipase C, but this was not the case, since glucagon had no synergistic effect on IP₃ formation in the presence of the α -adrenergic agonist adrenaline, i.e. under conditions where IP₃ production could have been further enhanced. If there is a linear relationship between IP₃ production and the number of receptors occupied (Lynch *et al.*, 1985), the present data suggest that glucagon does not unmask receptors efficiently coupled to phospholipase C.

The mechanism of action of the messengers generated after the binding of glucagon and adrenaline to their respective receptors should be discussed in the light of a previous study of the effect of vasopressin and glucagon on the Ca²⁺ influx, which showed that each hormone increases the maximal response to the other without affecting its ED₅₀ (Mauger *et al.*, 1985). In the present work, the same observation was made for adrenaline and glucagon (Fig. 3). By analogy with an enzymic reaction involving two substrates, a model was proposed in which

two intermediates, one arising from the polyphosphoinositide metabolism and the other from changes in the internal cyclic AMP concentration, are thought to be necessary for the Ca²⁺ transport system. The present data are in good agreement with this model.

One attractive possibility would be that the Ca²⁺ gates need multiple phosphorylations to be activated. Thus, in heart cells, it has been proposed that phosphorylation of a channel protein is a prerequisite for channel opening after depolarization (Osterrieder *et al.*, 1982; Rinaldi *et al.*, 1981), and that under β -adrenergic stimulation cyclic AMP-dependent phosphorylation increases either the number of functional phosphorylated channels (Bean *et al.*, 1984) or the probability that pre-existing Ca²⁺ channels will open (Brum *et al.*, 1984). In support of this hypothesis, glucagon has been shown to induce the phosphorylation of membrane proteins in liver (Shlitz & Marinetti, 1971; Vargas *et al.*, 1982). The mechanism of the action of the Ca²⁺-mobilizing hormones on Ca²⁺ gating remains speculative. At present, any product arising from the action of phospholipase C on the phosphoinositides and/or from the action of phospholipase A₂ or diglyceride kinase on the diacylglycerols should be considered a possible agent responsible for opening the Ca²⁺ channels or activating the carriers.

In conclusion, the present results indicate that, in rat hepatocytes, the combination of the α -adrenergic agonist adrenaline and glucagon increases the Ca²⁺ influx synergistically, but it has no such effect on IP₃ production. It is therefore proposed that this effect occurs at a step subsequent to phospholipase C activation, and

at a site closer to the Ca^{2+} channels or carriers than to the receptors.

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