Glucagon and vasopressin interactions on Ca^{2+} movements in isolated hepatocytes

Laurent COMBETTES,* Brigitte BERTHON, Adrien BINET and Michel CLARET

Unité de Recherches de Physiologie et Pharmacologie Cellulaire, INSERM U-274, Université Paris-Sud, 91405 Orsay Cedex, France

The effects of glucagon and vasopressin, singly or together, on cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and on the $^{45}\text{Ca}^{2+}$ efflux were studied in isolated rat liver cells. (1) In the presence of 1 mM external Ca^{2+} , glucagon and vasopressin added singly induced sustained increases in $[\text{Ca}^{2+}]_i$. The rate of the initial fast phase of the $[\text{Ca}^{2+}]_i$ increase and the magnitude of the final plateau were dependent on the concentrations (50 pM–0.1 μM) of glucagon and vasopressin. Preincubating the cells with a low concentration of glucagon (0.1 nM) for 2 min markedly accelerated the fast phase and elevated the plateau of the $[\text{Ca}^{2+}]_i$ increase caused by vasopressin. (2) In the absence of external free Ca^{2+} , glucagon and vasopressin transiently increased $[\text{Ca}^{2+}]_i$ and stimulated the $^{45}\text{Ca}^{2+}$ efflux from the cells, indicating mobilization of Ca^{2+} from internal store(s). Preincubating the cells with 0.1 nM-glucagon accelerated the rate of the fast phase of the $[\text{Ca}^{2+}]_i$ rise caused by the subsequent addition of vasopressin. However, unlike what was observed in the presence of 1 mM- Ca^{2+} , glucagon no longer enhanced the maximal $[\text{Ca}^{2+}]_i$ response to vasopressin. (3) In the absence of external free Ca^{2+} , higher concentrations (1 nM–0.1 μM) of glucagon, which initiated larger increases in $[\text{Ca}^{2+}]_i$, drastically decreased the subsequent Ca^{2+} response to vasopressin (10 nM). At these concentrations, glucagon also decreased the vasopressin-stimulated $^{45}\text{Ca}^{2+}$ efflux from the cells. It is suggested that, in the liver, glucagon accelerates the fast phase and elevates the plateau of the vasopressin-mediated $[\text{Ca}^{2+}]_i$ increase respectively by (a) releasing Ca^{2+} from the same internal store as that permeabilized by vasopressin, probably the endoplasmic reticulum, and (2) potentiating the influx of extracellular Ca^{2+} caused by this hormone.

INTRODUCTION

Noradrenaline (via α_1 -adrenergic receptors), vasopressin and angiotensin act on hepatic metabolism (Exton, 1985; Williamson *et al.*, 1985) by using IP_3 (inositol 1,4,5-trisphosphate) as second intracellular messenger (Burgess *et al.*, 1984; Joseph *et al.*, 1984; Kirk *et al.*, 1984; Berridge & Irvine, 1984). These hormones raise $[\text{Ca}^{2+}]_i$, the cytosolic free Ca^{2+} concentration (Charest *et al.*, 1983; Berthon *et al.*, 1984), by mobilizing Ca^{2+} from an internal store and by increasing the influx of extracellular Ca^{2+} (Reinhart *et al.*, 1984; De Witt & Putney, 1984; Mauger *et al.*, 1984, 1985). Glucagon or the β -adrenergic agonists that increase cyclic AMP in liver have long been shown to interfere with the Ca^{2+} movements initiated by the α_1 -adrenergic agonists vasopressin or angiotensin (Kraus-Friedmann, 1984). This was first reported by Haylett & Jenkinson (1973) and Jenkinson & Koller (1977), who showed that combined addition of α_1 - and β -agonists synergistically increased the $^{45}\text{Ca}^{2+}$ efflux from guinea-pig liver slices. Since then, interactions between IP_3 -dependent and cyclic AMP-dependent hormones on cell Ca^{2+} fluxes have also been reported in perfused rat liver and isolated hepatocytes (Baddams *et al.*, 1983; Kimura *et al.*, 1982; Assimacopoulos-Jeannet *et al.*, 1982; Cocks *et al.*, 1984; Morgan *et al.*, 1984; Studer *et al.*, 1984). In the same line of research, Morgan *et al.* (1984) observed that pretreatment of isolated rat hepatocytes with glucagon potentiated the noradrenaline-mediated $[\text{Ca}^{2+}]_i$ increase.

The aim of the present work was to investigate the effects of glucagon on the $^{45}\text{Ca}^{2+}$ efflux and on the $[\text{Ca}^{2+}]_i$ increase initiated by vasopressin in isolated rat liver cells. The results show that glucagon facilitates the action of vasopressin on $[\text{Ca}^{2+}]_i$ (1) by accelerating the release of Ca^{2+} from the same internal store as vasopressin, probably the endoplasmic reticulum, and (2) by potentiating the vasopressin-mediated Ca^{2+} influx.

METHODS

Preparation of hepatocytes

Hepatocytes were isolated by the conventional collagenase method from fed female Wistar rats as described previously (Binet *et al.*, 1985). After isolation, the cells (4 mg dry wt./ml) were equilibrated in Eagle's medium, containing 116 mM-NaCl, 5.4 mM-KCl, 1.0 mM- CaCl_2 , 0.81 mM- MgCl_2 , 0.92 mM- NaH_2PO_4 , 25 mM- NaHCO_3 , 1 mg of glucose/ml, and supplemented with 1.5% (w/v) gelatin, vitamins and amino acids and 1 mg of bacitracin/ml (see Binet *et al.*, 1985). The cell suspension was maintained under continuous gassing with O_2/CO_2 (19:1) at 37 °C.

$[\text{Ca}^{2+}]_i$ determination

The cells were loaded as described by Berthon *et al.* (1984). Routinely, 2 mg dry wt. of hepatocytes was incubated with 500 μl of 50 μM -quin2 tetra-acetoxymethyl ester for 150 s at 37 °C under continuous gassing. The

Abbreviations used: IP_3 , inositol 1,4,5-trisphosphate; PIP_2 , phosphatidylinositol biphosphate; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration.

* To whom reprint requests should be addressed.

Fig. 1. Hormone-mediated increases in $[Ca^{2+}]_i$ in quin2-loaded liver cells

Action of (a) vasopressin (VP; 10 nM) and (b) glucagon (Glu; 10 nM) in the absence and presence of 1 mM- Ca^{2+} . When used, EGTA (1.24 mM) was added to lower external free $[Ca^{2+}]$ to 1 μ M. (c) Action of isoprenaline (ISO; 1 μ M), dibutyryl cyclic AMP (dcAMP; 10 μ M) and forskolin (FO; 10 μ M) on $[Ca^{2+}]_i$. Equivalent volumes of the hormone or agent carriers (Eagle's solution for vasopressin, isoprenaline and dibutyryl cyclic AMP; 0.01 M-HCl for glucagon; 0.5% dimethyl sulphoxide for forskolin) had no effect on $[Ca^{2+}]_i$. Results of a typical experiment are shown.

cells were then washed twice in Eagle's medium containing no vitamins, amino acids, bacitracin or gelatin, and centrifuged at 50 *g* for 1 min. Finally, the cells were resuspended in the same medium at about 1 mg dry wt./ml and transferred into the cuvette of a Jobin-Yvon JY3D spectrofluorimeter, at 37 °C and under continuous magnetic agitation and gassing. Fluorescence (excitation at 342 nm and emission at 492 nm) was recorded on a potentiometric recorder. At the end of each experiment, $[\text{Ca}^{2+}]_i$ was calibrated and corrected for NAD(P)H fluorescence as indicated by Binet *et al.* (1985).

Time constant of the $[\text{Ca}^{2+}]_i$ increase

The time constant (s) of the $[\text{Ca}^{2+}]_i$ increase was used as an index of the initial rate of the rise initiated by the hormones. This initial fast phase could be reasonably fitted with a monoexponential component as a function of time. For instance, the correlation coefficients calculated by linear-regression analysis from the four initial points of the $[\text{Ca}^{2+}]_i$ increases caused by vasopressin (10 nM) in Fig. 5(a) ranged from 0.991 to 0.999 ($P < 0.01$). At low hormone concentrations (10 pM–0.1 nM), the small apparent delay in the $[\text{Ca}^{2+}]_i$ increase was included in the time constant.

$^{45}\text{Ca}^{2+}$ efflux

Isolated rat hepatocytes (approx. 6 mg dry wt./ml) were suspended in Eagle's medium containing 1 μCi of $^{45}\text{Ca}^{2+}$ /ml up to isotopic equilibrium (about 30 min). The $^{45}\text{Ca}^{2+}$ efflux was initiated by adding EGTA (1.24 mM, pH 7.40) to 1 ml samples to decrease the nominal external Ca^{2+} (1 mM) to 1 μM . Cell samples (200 μl) were removed 30, 60 and 90 s after adding EGTA and then filtered through Whatman GF/C glass filter and washed as indicated by Mauger *et al.* (1984). The radioactivity associated with the filter was then counted in a scintillation spectrometer after addition of the appropriate scintillant. The rate constant was calculated from the $^{45}\text{Ca}^{2+}$ efflux and the $^{45}\text{Ca}^{2+}$ content measured at 30 and 90 s. The hormones were added as indicated in Fig. 6.

Materials

The suppliers of the reagents are listed by Mauger *et al.* (1984, 1985).

RESULTS

Effects of vasopressin and glucagon on $[\text{Ca}^{2+}]_i$

The $[\text{Ca}^{2+}]_i$ increase arising from activation of vasopressin receptors reflects the mobilization of Ca^{2+} from an intracellular store and from an influx of extracellular Ca^{2+} . It is composed of an initial fast phase (5–10 s) followed by a second phase (10–30 s) that slowly raises $[\text{Ca}^{2+}]_i$ to a sustained plateau (Fig. 1a). When the cells are incubated in 1.8 mM- Ca^{2+} , we have estimated that the internal Ca^{2+} store accounts for 50–70% of the fast-initiated phase, the remaining fraction (and the second phase) resulting from the hormone-stimulated Ca^{2+} influx (Berthon *et al.*, 1984; Binet *et al.*, 1985). Other workers have reported that the fast phase reflects only the release of Ca^{2+} from the internal store and is insensitive to the presence of external Ca^{2+} (Charest *et al.*, 1983, 1985; Joseph *et al.*, 1985). As suggested previously (Binet *et al.*, 1985), this discrepancy results from the different external Ca^{2+} concentrations applied to the hepatocytes.

Fig. 2. Dose-response curves for the action of glucagon (Glu), vasopressin (VP), and glucagon plus vasopressin (both 0.1 nM) on the maximal value (a) and on the time constant of the fast phase (b) of the $[\text{Ca}^{2+}]_i$ increase

External $[\text{Ca}^{2+}] = 1$ mM. Control cells received equivalent volumes of the hormone carriers (0.01 M-HCl or Eagle's medium). Preincubating hepatocytes with glucagon (0.1 nM) for 2 min elevated the plateau and accelerated the rate of the $[\text{Ca}^{2+}]_i$ increase initiated by low doses of vasopressin. $[\text{Ca}^{2+}]_i$ is expressed as a percentage of the initial resting value. Results are means $\pm$ S.E.M. for 5 to 23 experiments.

In the present study, the contribution of the cell Ca^{2+} influx to the fast phase was slightly decreased by lowering external $[\text{Ca}^{2+}]$ to 1 mM. A further advantage of using a lower Ca^{2+} concentration for studies of this kind was that problems of indicator saturation were largely avoided. Under these conditions, the sustained $[\text{Ca}^{2+}]_i$ response to vasopressin (10 nM) (Fig. 1a) proved transient when the external $[\text{Ca}^{2+}]$ was decreased to 1 μM . The initial phase was maintained, confirming that the hormone releases Ca^{2+} from a rapidly exchangeable Ca^{2+} store.

Glucagon (10 nM) and isoprenaline (1 μM) also raised $[\text{Ca}^{2+}]_i$ although the initial rates and maximal values of

the increases were slightly lower than those induced by vasopressin (10 nM) (Fig. 1b). This was mimicked by external application of dibutyryl cyclic AMP (10 μ M) or the adenylate cyclase activator forskolin (2.5 μ M–0.1 mM), suggesting that the action of glucagon and isoprenaline was mediated by cyclic AMP (Fig. 1c). Glucagon also raised $[Ca^{2+}]_i$ in the absence of external Ca^{2+} . The response was transient and the fast component was maintained. These results confirm those reported by Charest *et al.* (1983) and Sistare *et al.* (1985). They indicate that glucagon mobilizes Ca^{2+} from a rapidly exchangeable internal store and from the extracellular medium. The dose–response curves for the action of the two hormones are shown in Fig. 2(a). Half-maximal stimulations were observed at 0.6 nM-vasopressin and 1 nM-glucagon.

The time constant of the $[Ca^{2+}]_i$ increase taken as an index of the rate of the fast phase (see the Methods section), was also dose-related to the concentration of vasopressin and glucagon added to the cells. For the lowest concentrations (0.05–0.1 nM for both hormones), the rise was very slow and required 100–120 s to attain a maximal value (Fig. 2b). It subsequently fell to 6 s and 15 s for 0.1 μ M-vasopressin and -glucagon respectively.

Effect of vasopressin plus glucagon on $[Ca^{2+}]_i$ in 1 mM- Ca^{2+}

Fig. 3(a) shows the effect of preincubating the cells for 2 min with glucagon (0.1 nM) on the $[Ca^{2+}]_i$ increase caused by vasopressin (0.1 nM). In agreement with Morgan *et al.* (1984), this low concentration of glucagon had two effects: it enhanced the initial rate and elevated

Fig. 3. Potentiating action of glucagon (Glu; 0.1 nM) on the $[Ca^{2+}]_i$ response to vasopressin (VP; 0.1 nM)

(a) In the presence of Ca^{2+} (1 mM), glucagon accelerated the rise and increased the maximal plateau of the Ca^{2+} response to vasopressin. (b) Lack of potentiating effect of vasopressin on the glucagon-mediated $[Ca^{2+}]_i$ rise. (c) In low-free- Ca^{2+} medium, glucagon accelerated the rise, but had little effect on the maximal peak of the vasopressin-mediated $[Ca^{2+}]_i$ increase. Control cells received equivalent amounts of hormone carriers.

the plateau of the $[\text{Ca}^{2+}]_i$ increase. In contrast, preincubating the cells with vasopressin for 2 min had less effect on the subsequent addition of glucagon (Fig. 3b). A similar observation has been reported for the Ca^{2+} influx (Mauger *et al.*, 1985). One plausible explanation is that the IP_3 -dependent hormones substantially inhibit the β -adrenergic or glucagon-mediated cyclic AMP accumulation and adenylate cyclase activation (Jard *et al.*, 1981; Assimacopoulos-Jeannet *et al.*, 1982; Keppens & De Wulf, 1984). Figs. 2(a) and 2(b) shows the effect of glucagon (0.1 nM) on the final plateau and on the time constant of the $[\text{Ca}^{2+}]_i$ rise promoted by vasopressin. In the dose-response curve for the action of vasopressin in the presence of glucagon (Fig. 2a), the s.e.m. values were quite high, presumably because the variations in the response to the two hormones were cumulative. Nevertheless, the effect of glucagon plus vasopressin (0.05–0.1 nM) on the plateau of the $[\text{Ca}^{2+}]_i$ was significantly greater than the sum of the individual effect of each hormone. At higher vasopressin concentrations (10 nM and 0.1 μM), the apparent saturation of the glucagon effects, as shown in Fig. 2(a), may in part reflect a saturation of the indicator response. The enhancement of the vasopressin-induced $[\text{Ca}^{2+}]_i$ increase may therefore be somewhat underestimated in this part of the curve. Fig. 2(b) shows that the rise initiated by 0.05 nM- and 0.1 nM-vasopressin was much faster in the presence of glucagon. One possible explanation of these results is that glucagon accelerates the fast phase of the rise because it permeabilizes an internal Ca^{2+} store and elevates the plateau because it potentiates the vasopressin-mediated Ca^{2+} influx (Mauger *et al.*, 1985; Poggioli *et al.*, 1986).

Effect of glucagon plus vasopressin in low- Ca^{2+} medium

In order to distinguish between glucagon effects on vasopressin-induced Ca^{2+} release and Ca^{2+} influx, the next experiments were performed in low external $[\text{Ca}^{2+}]$. The Ca^{2+} influx was eliminated by adding EGTA to the bathing solution (external free $[\text{Ca}^{2+}] = 1 \mu\text{M}$).

Fig. 3(c) shows that glucagon (0.1 nM) always accelerated the vasopressin-mediated $[\text{Ca}^{2+}]_i$ rise. However, unlike what was observed in the presence of the Ca^{2+} influx, this low concentration of glucagon had little effect on the maximal amplitude of the Ca^{2+} response initiated by vasopressin (0.1 nM). The results of several experiments of this kind, performed at different vasopressin concentrations, are plotted in Fig. 4. In the presence of both glucagon and vasopressin the size of the final $[\text{Ca}^{2+}]_i$ peak did not differ from simple additivity (Fig. 4a). No potentiation of the type seen in normal- Ca^{2+} medium was found on the maximal $[\text{Ca}^{2+}]_i$ response to vasopressin. Fig. 4(b) shows that glucagon accelerated the rate of the $[\text{Ca}^{2+}]_i$ rise at 0.1 nM- and 0.5 nM-vasopressin. At submaximal and maximal concentrations of vasopressin, the effect of glucagon was no longer detected.

Another series of experiments in low- Ca^{2+} medium was carried out to determine whether the two hormones act on the same internal store. In these experiments, rising concentrations of glucagon (0.1 nM–0.1 μM) or of its vehicle (HCl; 0.01 M) were applied before a single test dose of 10 nM-vasopressin. The latter concentration was chosen to induce maximal release of the hormone-sensitive Ca^{2+} store. The Ca^{2+} chelator EGTA was added 30 s before glucagon to lower the external free $[\text{Ca}^{2+}]$ to below 1 μM . For clarity, the time scale of the Ca^{2+} response to vasopressin was extended (Fig. 5a). As expected from the

Fig. 4. Dose-response curves for the action of glucagon (Glu), vasopressin (VP) and glucagon plus vasopressin (both 0.1 nM) on the peak value (a) and on the time constant of the fast phase (b) of the $[\text{Ca}^{2+}]_i$ rise at 1 μM - Ca^{2+} .

The external free $[\text{Ca}^{2+}]$ was lowered to 1 μM by adding EGTA (1.24 mM). Control cells received equivalent amounts of the hormone carriers 0.1 mM-HCl or Eagle's medium. $[\text{Ca}^{2+}]_i$ is expressed as percentage of the initial resting value. Results are means $\pm$ s.e.m. for 5 to 32 experiments.

results found in low- Ca^{2+} medium, HCl or low doses of glucagon (0.1 nM) had little effect on the resting $[\text{Ca}^{2+}]_i$ and on the response to a submaximal dose of vasopressin. However, higher glucagon concentrations (1 and 10 nM) that initiated larger $[\text{Ca}^{2+}]_i$ increases proportionally decreased the response to the second hormone. The maximal amplitude of the $[\text{Ca}^{2+}]_i$ rise caused by vasopressin was inversely and linearly related to that initiated by glucagon (Fig. 5b). Fig. 5(c) shows the dose-response curve for the action of glucagon. A maximal concentration of glucagon (0.1 μM) totally abolished the vasopressin response. These results suggest that glucagon mobilizes the same store as that permeabilized by vasopressin via IP_3 , probably located in the endoplasmic reticulum. This finding is in complete

Fig. 5. Depleting effect of glucagon (Glu) on the internal store permeabilized by vasopressin (VP)

The external free $[Ca^{2+}]$ was lowered to $1 \mu M$ by adding EGTA (1.24 mM) 30 s before the hormones. (a) Increasing concentrations of glucagon (first arrow) gradually abolished the $[Ca^{2+}]_i$ increase caused by the subsequent addition of a single dose (10 nM) of vasopressin (second arrow). Note that the time scale is extended for the action of vasopressin. (b) Peak amplitude of the $[Ca^{2+}]_i$ increase caused by 10 nM -vasopressin as a function of that initiated by previous addition of glucagon. (c) Dose-response curve for the inhibitory action of glucagon on the peak of the vasopressin (10 nM)-mediated $[Ca^{2+}]_i$ increase. $[Ca^{2+}]_i$ is expressed as a percentage of the initial $[Ca^{2+}]_i$. Results are means (a and b) or means $\pm$ S.E.M. (c) for four to nine experiments.

agreement with the results reported by Mauger & Claret (1986), showing that glucagon causes a net loss of $^{45}Ca^{2+}$ and abolishes that caused by vasopressin in isolated rat liver cells incubated in low- Ca^{2+} media.

Effect of glucagon plus vasopressin on the $^{45}Ca^{2+}$ efflux

A characteristic response of hepatocytes to hormones that increase $[Ca^{2+}]_i$ is the stimulation of the Ca^{2+} pump located in the plasma membrane. If the experiments are carried out in the absence of external Ca^{2+} , the stimulated Ca^{2+} efflux reflects uniquely the release of Ca^{2+} from internal stores. This was an alternative way of testing our finding that glucagon and vasopressin control a common internal Ca^{2+} store. For this purpose, the cells were first loaded to isotopic equilibrium with $^{45}Ca^{2+}$ and then

incubated in a low-free- Ca^{2+} medium. The $^{45}Ca^{2+}$ efflux was measured after the addition of glucagon or vasopressin, or glucagon plus vasopressin. At the end of the loading period, the $^{45}Ca^{2+}$ content of the hepatocytes was $1.93 \pm 0.03 \text{ nmol/mg cell dry wt.}$ ($n = 210$). Figs. 6(a) and 6(b) show the dose-dependent stimulation of the Ca^{2+} efflux by glucagon or vasopressin. The efflux was moderately increased, indicating that the Ca^{2+} pump was not saturated by the transient rise in $[Ca^{2+}]_i$. In agreement with the common-store hypothesis, incubating the cells with increasing doses of glucagon progressively decreased the ability of vasopressin to stimulate the $^{45}Ca^{2+}$ efflux. Fig. 6(c) shows that the amplitude of the efflux stimulated by 10 nM -vasopressin was inversely related to that caused by glucagon (10 pM – 10 nM). The dose-response curve for

Fig. 6. Inhibitory effect of glucagon (Glu) on the $^{45}\text{Ca}^{2+}$ efflux stimulated by vasopressin (VP)

(a) Dose-response curve for the action of glucagon. (b) Dose-response curves for vasopressin in the presence of increasing concentrations of glucagon. EGTA (1.24 mM) was added 30 s before glucagon. (c) Vasopressin (10 nM)-stimulated $^{45}\text{Ca}^{2+}$ efflux as a function of the efflux stimulated by previous addition of glucagon. (d) Inhibitory action of glucagon on the $^{45}\text{Ca}^{2+}$ efflux stimulated by 10 nM-vasopressin means $\pm$ S.E.M. for six to eight experiments. $^{45}\text{Ca}^{2+}$ efflux is expressed by its rate constant (a and b). In (c) and (d), the increment of the rate constant above the resting value (taken as zero) is shown.

the inhibitory action of glucagon on the $^{45}\text{Ca}^{2+}$ efflux stimulated by vasopressin is shown in Fig. 6(d). A glucagon-mediated decrease in the α_1 -adrenergic- or vasopressin-mediated net loss of total Ca^{2+} has sometimes been reported and interpreted as reflecting a glucagon-mediated redistribution of mitochondrial Ca^{2+} (Assimakopoulos-Jeannot *et al.*, 1982; Baddams *et al.*, 1983). The present results show that maximal doses of glucagon present results show that maximal doses of glucagon abolish the action of vasopressin on $[\text{Ca}^{2+}]_i$ and the Ca^{2+} efflux because it depletes the endoplasmic-reticulum Ca^{2+} store permeabilized by vasopressin.

Effect of returning to normal- Ca^{2+} medium on $[\text{Ca}^{2+}]_i$

The effects of glucagon via the Ca^{2+} influx and internal Ca^{2+} on the vasopressin-mediated $[\text{Ca}^{2+}]_i$ increase was

illustrated in a different way by the following experiments (Fig. 7): the external free $[\text{Ca}^{2+}]$ was lowered to $1 \mu\text{M}$, and glucagon and then vasopressin were successively applied at the same concentration (1 nM) that partially released Ca^{2+} from the internal store. In agreement with the data shown in Figs. 3 and 4, glucagon significantly accelerated the rate of the $[\text{Ca}^{2+}]_i$ rise, but had little effect on the peak initiated by vasopressin. Raising external free $[\text{Ca}^{2+}]$ to 2.24 mM then caused a rapid increase in $[\text{Ca}^{2+}]_i$. The maximal value of the final plateau was critically dependent on the hormone(s) added beforehand. The Ca^{2+} influx transported through the hormone-stimulated channels (or carriers) increased $[\text{Ca}^{2+}]_i$ to $767 \pm 240 \text{ nM}$ ($n = 5$) in vasopressin-treated cells and to $541 \pm 63 \text{ nM}$ ($n = 6$) in glucagon-treated cells. In the presence of glucagon plus vasopressin, the resulting Ca^{2+} influx

Fig. 7. Effects of vasopressin, glucagon and external $[Ca^{2+}]$ on $[Ca^{2+}]_i$.

The external free $[Ca^{2+}]$ was lowered to $1 \mu M$ by adding EGTA (1.24 mM); 30 s later, the cells received submaximal doses of each hormone (1 nM) or equivalent volumes of their solvent, and then Ca^{2+} (free $[Ca^{2+}] = 2.24 \text{ mM}$) at the times indicated by the arrows: ●, H_1 , glucagon, H_2 , vasopressin, Ca^{2+} ; ○, H_1 , glucagon, H_2 , Eagle's medium, Ca^{2+} ; ▲, H_1 , 0.01 M-HCl , H_2 , vasopressin, Ca^{2+} . Note the ordinate logarithmic scale. Results of a typical experiment are shown.

caused $[Ca^{2+}]_i$ to increase to $2109 \pm 264 \text{ nM}$ ($n = 10$) in less than 1 min.

DISCUSSION

Hepatic cell Ca^{2+} is constantly exchanged with extracellular Ca^{2+} (Claret-Berthon *et al.*, 1977; Reinhart *et al.*, 1984; Williamson *et al.*, 1985). It enters the cells as a passive unidirectional influx driven by its high plasma concentration and negative membrane potential. In many types of mammalian cells, although not in the liver, this influx has been shown to be transported through single channels located in plasma membranes (Reuter, 1983). It is counterbalanced by an active unidirectional Ca^{2+} efflux mediated by the Ca^{2+} pump, which is activated by the submicromolar concentration of the cytosolic free Ca^{2+} (Lotersztajn *et al.*, 1981). In addition, Ca^{2+} is transported and accumulated by the endoplasmic-reticulum $Ca^{2+} + Mg^{2+}$ -ATPase and the mitochondrial potential-dependent Ca^{2+} uptake. These different pathways of transport and storage sites (Burgess *et al.*, 1983; Williamson *et al.*, 1985) have made difficult the study the action of hormones that alter Ca^{2+} metabolism in the liver. This may explain the apparently divergent effects of glucagon on cell $[Ca^{2+}]_i$ reported in the literature. For instance, it has been shown to decrease total Ca^{2+} (Assimakopoulos-Jeannet *et al.*, 1982; Studer *et al.*, 1984; Mauger & Claret, 1986), to have

little effect on $[Ca^{2+}]_i$ (Murphy *et al.*, 1980; Studer *et al.*, 1984) or to raise it (Charest *et al.*, 1983; Sistare *et al.*, 1985), to increase $^{45}Ca^{2+}$ uptake (Assimakopoulos-Jeannet *et al.*, 1977; Keppens *et al.*, 1977) and the $^{45}Ca^{2+}$ influx (Mauger *et al.*, 1985; Poggioli *et al.*, 1986), and to stimulate the net or unidirectional Ca^{2+} efflux (Friedmann & Park, 1968; Blackmore *et al.*, 1979; Kimura *et al.*, 1982; Baddams *et al.*, 1983; Cocks *et al.*, 1984).

The first part of the present results confirm and extend those reported by Morgan *et al.* (1984). Low doses of glucagon (0.1 nM) have two effects on the Ca^{2+} response of rat hepatocytes to vasopressin. First, it potentiates the maximal value of the $[Ca^{2+}]_i$ increase, but only in the presence of external Ca^{2+} . This is probably due to its synergistic effect on the vasopressin-mediated Ca^{2+} influx (Mauger *et al.*, 1985; Poggioli *et al.*, 1986). This was also illustrated by the time courses of the $[Ca^{2+}]_i$ rises initiated by returning Ca^{2+} to cells preincubated in low- Ca^{2+} medium and pretreated with vasopressin or glucagon, or both. In the latter case, the influx of Ca^{2+} transported through the hormone-potentiated Ca^{2+} channels increased $[Ca^{2+}]_i$ to values substantially higher than those reached with each hormone. Secondly, glucagon markedly accelerated the fast phase of the $[Ca^{2+}]_i$ rise initiated by vasopressin. The maintenance of the latter in low- Ca^{2+} medium indicated that it was fed by the release of Ca^{2+} from a rapidly exchangeable internal store.

A striking finding was that, in the absence of external Ca^{2+} , higher concentrations (1 nM – $0.1 \mu M$) of glucagon producing significant increases in $[Ca^{2+}]_i$ caused a gradual decrease in the cell response to 10 nM -vasopressin. A maximal concentration of $0.1 \mu M$ -glucagon totally abolished the $[Ca^{2+}]_i$ increase mediated by vasopressin. In the same context, it was found that pretreatment of the cells with glucagon concentrations stimulating the Ca^{2+} pump decreased in a dose-dependent manner the ability of vasopressin to stimulate the carrier. It appears thus that glucagon via cyclic AMP and vasopressin via IP_3 mobilize Ca^{2+} from a common store. Additional evidence for this is the finding that maximal concentrations of glucagon (10 nM) and vasopressin (10 nM) promote non-additive net losses of total Ca^{2+} from isolated rat hepatocytes incubated in low- Ca^{2+} medium (Mauger & Claret, 1986). The present results do not indicate the source of this store. However, hormones such as vasopressin initiate the degradation of plasma-membrane PIP_2 , leading to an enhancement of cell IP_3 content (Creba *et al.*, 1983; Thomas *et al.*, 1984). IP_3 in turn releases Ca^{2+} from the endoplasmic reticulum when added to permeabilized hepatocytes (Burgess *et al.*, 1984; Joseph *et al.*, 1984) or isolated microsomal fractions (Dawson & Irvine, 1984). Consequently, our results suggest that, in rat liver, glucagon releases Ca^{2+} not from mitochondria but from the endoplasmic reticulum. This conclusion is reinforced by the observations reported by Denton & McCormack (1980) and McCormack (1985a,b). In an important work on the role of Ca^{2+} and hormones on mitochondrial deshydrogenases, these authors showed that adrenaline and glucagon activate the Ca^{2+} -sensitive deshydrogenases by increasing the intramitochondrial Ca^{2+} concentration (for references, see McCormack, 1985b).

There is little to indicate the mechanism by which cytosolic cyclic AMP induces this net loss of Ca^{2+} from the endoplasmic reticulum. The first possibility is that cyclic AMP could increase the IP_3 content of the cells. Glucagon, which increases cell cyclic AMP content, does

not seem to induce measurable degradation of PIP_2 or IP_3 production in rat hepatocytes (Creba *et al.*, 1983; Charest *et al.*, 1985; Poggioli *et al.*, 1986). Nevertheless, we cannot exclude the possibility that cyclic AMP produced an amount of IP_3 too small to be chemically detected but sufficient to induce Ca^{2+} release from the endoplasmic reticulum. For instance, this is the case for the action of vasopressin in the liver. Negligible degradation of PIP_2 or accumulation of IP_3 with 1 nM-vasopressin is associated with maximal increases in $[\text{Ca}^{2+}]_i$ and phosphorylase *a* activity (Creba *et al.*, 1983; Charest *et al.*, 1985; but see Thomas *et al.*, 1984). The second hypothesis is that cyclic AMP might inhibit the ability of Ca^{2+} to concentrate in the endoplasmic reticulum. However, this is at variance with the studies showing that glucagon or cyclic AMP analogues enhance Ca^{2+} uptake by the microsomal fractions or enriched reticulum fractions from pretreated rat liver (Andia-Waltenbaugh & Friedmann, 1978; Taylor *et al.*, 1979). A third hypothesis is that cyclic AMP increases the efflux of Ca^{2+} and depletes the Ca^{2+} pool in the same way as IP_3 . Such an effect of cyclic AMP been described, for example, in sarcoplasmic reticulum (Katz, 1982). According to this, cyclic AMP might therefore stimulate the endoplasmic-reticulum permeability to Ca^{2+} either independently of IP_3 or indirectly by facilitating the action of IP_3 on the internal Ca^{2+} store.

To sum up, the present results show that low doses of glucagon, acting via cyclic AMP, potentiate the action of vasopressin on $[\text{Ca}^{2+}]_i$ by increasing the vasopressin-mediated Ca^{2+} influx and accelerating the release of Ca^{2+} from the same internal store as that permeabilized by vasopressin, probably the endoplasmic reticulum. In contrast, at high concentration and in the absence of external Ca^{2+} , glucagon may abolish the Ca^{2+} response to vasopressin by depleting the hormone-sensitive Ca^{2+} store. The molecular mechanism used by cyclic AMP is still unclear and requires further studies before the effects of its interaction with IP_3 on the Ca^{2+} movements in the liver can be elucidated.

REFERENCES

- Andia-Waltenbaugh, A. M. & Friedmann, N. (1978) *Biochem. Biophys. Res. Commun.* **82**, 603–608
- Assimakopoulos-Jeannet, F. D., Blackmore, P. F. & Exton, J. H. (1977) *J. Biol. Chem.* **252**, 2662–2669
- Assimakopoulos-Jeannet, F. D., Blackmore, P. F. & Exton, J. H. (1982) *J. Biol. Chem.* **257**, 3759–3765
- Baddams, H. M., Chang, L. B. F. & Barritt, G. J. (1983) *Biochem. J.* **210**, 73–77
- Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Berthon, B., Binet, A., Mauger, J.-L. & Claret, M. (1984) *FEBS Lett.* **167**, 19–24
- Binet, A., Berthon, B. & Claret, M. (1985) *Biochem. J.* **228**, 565–574
- Blackmore, P. F., Assimakopoulos-Jeannet, F. D., Chang, T. M. & Exton, J. H. (1979) *J. Biol. Chem.* **254**, 2818–2834
- Burgess, G. M., McKinney, J. S., Fabiato, A., Leslie, B. A. & Putney, J. W. (1983) *J. Biol. Chem.* **258**, 15336–15345
- Burgess, G. M., Godfrey, P. P., McKinney, J. S., Berridge, M. J., Irvine, R. F. & Putney, J. W. (1984) *Nature (London)* **309**, 63–66
- Charest, R., Blackmore, P. F., Berthon, B. & Exton, J. H. (1983) *J. Biol. Chem.* **258**, 8769–8773
- Charest, R., Prpic, V., Exton, J. H. & Blackmore, P. F. (1985) *Biochem. J.* **227**, 79–90
- Claret-Berthon, B., Claret, M. & Mazet, J.-L. (1977) *J. Physiol. (London)* **272**, 529–552
- Cocks, T. M., Jenkinson, D. H. & Koller, K. (1984) *Br. J. Pharmacol.* **83**, 281–291
- Creba, J. A., Downes, C. P., Hawkins, P. T., Brewster, G., Michell, R. H. & Kirk, C. J. (1983) *Biochem. J.* **212**, 733–747
- Dawson, A. P. & Irvine, R. F. (1984) *Biochem. Biophys. Res. Commun.* **120**, 858–864
- De Witt, L. M. & Putney, J. W. (1984) *J. Physiol. (London)* **346**, 395–407
- Denton, R. M. & McCormack, J. G. (1980) *FEBS Lett.* **119**, 1–8
- Exton, J. H. (1985) *Am. J. Physiol.* E633–E647
- Friedmann, N. & Park, C. R. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **61**, 504–508
- Haylett, D. G. & Jenkinson, D. H. (1973) in *Drugs Receptors* (Rang, H. P., ed.), pp. 15–25, MacMillan, London
- Jard, S., Cantau, B. & Jacobs, K. H. (1981) *J. Biol. Chem.* **256**, 2603–2606
- Jenkinson, D. H. & Koller, K. (1977) *Br. J. Pharmacol.* **59**, 163–175
- Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F. & Williamson, J. R. (1984) *J. Biol. Chem.* **259**, 3077–3081
- Joseph, S. K., Coll, K. E., Thomas, A. P., Rubin, R. & Williamson, J. R. (1985) *J. Biol. Chem.* **260**, 12508–12515
- Katz, A. M. (1982) *Handb. Exp. Pharmacol.* **58**, part II, 347–364
- Keppens, S. & De Wulf, H. (1984) *Biochem. J.* **222**, 277–280
- Keppens, S., Vandenheede, J. R. & De Wulf, H. (1977) *Biochim. Biophys. Acta* **496**, 448–457
- Kimura, S., Kugai, N., Tada, R., Kojima, I., Abe, K. & Ogata, E. (1982) *Horm. Metab. Res.* **14**, 133–138
- Kirk, C. J., Bone, E. A., Palmer, S. & Michell, R. H. (1984) *J. Recept. Res.* **4**, 489–504
- Kraus-Friedmann, N. (1984) *Physiol. Rev.* **64**, 170–259
- Lotersztajn, S., Hanoune, J. & Pecker, F. (1981) *J. Biol. Chem.* **256**, 2603–2606
- Mauger, J.-P. & Claret, M. (1986) *FEBS Lett.* **195**, 106–110
- Mauger, J.-P., Poggioli, J., Guesdon, F. & Claret, M. (1984) *Biochem. J.* **221**, 121–127
- Mauger, J.-P., Poggioli, J. & Claret, M. (1985) *J. Biol. Chem.* **260**, 11635–11642
- McCormack, J. G. (1985a) *Biochem. J.* **231**, 581–595
- McCormack, J. G. (1985b) *Biochem. J.* **231**, 597–608
- Morgan, N. G., Charest, R., Blackmore, P. F. & Exton, J. H. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4208–4212
- Murphy, E., Coll, K., Rich, T. & Williamson, J. R. (1980) *J. Biol. Chem.* **255**, 6600–6608
- Poggioli, J., Mauger, J.-P. & Claret, M. (1986) *Biochem. J.* **235**, 663–669
- Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. (1984) *Biochem. J.* **223**, 1–13
- Reuter, H. (1983) *Nature (London)* **301**, 569–574
- Sistare, F. D., Picking, R. A. & Haynes, R. C., Jr. (1985) *J. Biol. Chem.* **260**, 12744–12747
- Studer, R. K., Snowdowne, K. W. & Borle, A. B. (1984) *J. Biol. Chem.* **259**, 3596–3604
- Taylor, W. M., Reinhart, P., Hunt, N. H. & Bygrave, F. L. (1979) *FEBS Lett.* **112**, 92–96
- Thomas, A. P., Alexander, J. & Williamson, J. R. (1984) *J. Biol. Chem.* **259**, 5574–5584
- Williamson, J. R., Cooper, R. H., Joseph, S. K. & Thomas, A. (1985) *Am. J. Physiol.* **258**, C203–C216