Synergistic Stimulation of the Ca\(^{2+}\) Influx in Rat Hepatocytes by Glucagon and the Ca\(^{2+}\)-linked Hormones Vasopressin and Angiotensin II*

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Glucagon was added to isolated rat hepatocytes, either alone or together with vasopressin or angiotensin II, and the effects on the initial \(^{45}\text{Ca}^{2+}\) uptake rate were investigated. Addition of glucagon alone which increased cyclic AMP content of the cells slightly increased the initial \(^{45}\text{Ca}^{2+}\) uptake rate. When glucagon was added together with vasopressin or angiotensin II—both of which when added separately increased the initial \(^{45}\text{Ca}^{2+}\) uptake rate but did not affect the cellular content of cyclic AMP—the measured initial \(^{45}\text{Ca}^{2+}\) uptake rate was larger than the sum of that seen with each hormone alone. This indicates that glucagon and Ca\(^{2+}\)-linked hormones synergistically enhanced the Ca\(^{2+}\) influx in rat hepatocytes. These effects of glucagon can be mimicked by dibutyryl cyclic AMP or forskolin, suggesting that cyclic AMP augments both the resting Ca\(^{2+}\) and the vasopressin- or angiotensin II-stimulated influx.

Measurement of the initial \(^{45}\text{Ca}^{2+}\) uptake rate as a function of the extracellular Ca\(^{2+}\) concentration indicated that the increase in the Ca\(^{2+}\) influx resulting from single or combined glucagon and vasopressin administration occurred through a homogeneous population of Ca\(^{2+}\) gates. These hormones were found to raise both the apparent \(K_m\) for external Ca\(^{2+}\) and the apparent \(V_{\text{max}}\) of the Ca\(^{2+}\) influx. The maximal increase in these two parameters was observed when all the two hormones were added together. This suggests that glucagon and vasopressin synergistically stimulate the same Ca\(^{2+}\) gating mechanism.

The dose-response curves for the action of glucagon or vasopressin applied in the presence of increasing concentrations of vasopressin or glucagon, respectively, showed that each hormone increases the maximal response to the other without affecting its \(E_{\text{max}}\). It is proposed that glucagon and the Ca\(^{2+}\)-linked hormones control the cellular concentration of two intermediates which are both necessary to allow Ca\(^{2+}\) entry into the cells.

Convey the same type of response (for reviews see: Berridge, 1982, 1984; Rasmussen and Barrett, 1984). In some systems, such as platelets or smooth muscles, cyclic AMP has an inhibitory effect whereas that of calcium is stimulatory (Rasmussen, 1983). However, in others such as the secretory tissues (Berridge, 1982) or the liver (Exton, 1982), the action of both messengers is stimulatory.

In the mammalian liver, \(\beta\)-adrenergic agonists and glucagon increase the cell concentration of cyclic AMP which, by activating the cyclic AMP-dependent protein kinase, in turn stimulates glycogen phosphorylase activity (Exton, 1982). The \(\alpha\)-adrenergic agonists, angiotensin II, and vasopressin initiate membrane polyphosphoinositides degradation (Creba et al., 1983; Thomas et al., 1983, 1984; Seyford and Wells, 1984), raise the intracellular Ca\(^{2+}\) concentration (Charest et al., 1983; Berthon et al., 1984), and activate glycogen phosphorylase (Keppens et al., 1977; Exton, 1981; Williamson et al., 1981).

These three agents act on the intracellular Ca\(^{2+}\) concentration and glycogen phosphorylase partly by releasing Ca\(^{2+}\) from internal stores (Exton, 1981; Williamson et al., 1981; and Reinhart et al., 1984) and by augmenting the entry of extracellular Ca\(^{2+}\) (Assimacopoulos-Jeannet et al., 1977; Keppens et al., 1977; Berthon et al., 1984; Mauger et al., 1984; Reinhart et al., 1984; Blackmore et al., 1984; Binet et al., 1985).

However, the cyclic AMP and calcium messenger systems are to some extent interdependent in mammalian liver. Thus, the intracellular Ca\(^{2+}\) concentration was observed to be increased by glucagon (Charest et al., 1983; and Footnote 1, but see Studer et al., 1984) and the \(\beta\)-adrenergic agonist isopropyl terenol.

It has also been reported that glucagon can reverse the net movements of Ca\(^{2+}\) induced by the Ca\(^{2+}\)-linked hormones (Assimacopoulos-Jeannet et al., 1982; Morgan et al., 1983). Vasopressin and angiotensin II also inhibit the accumulation of cyclic AMP in isolated hepatocytes stimulated by glucagon (Crane et al., 1982; Keppens and De Wulf, 1984).

Finally, cyclic AMP-dependent and Ca\(^{2+}\)-linked hormones have been shown to act synergistically to induce hyperpolarization and effluxes of \(^{39}\text{K}^+\) and \(^{45}\text{Ca}^2+\) in guinea pig liver slices or hepatocytes (Jenkinson and Koller, 1977; Cocks et al., 1984).

These findings combined with the fact that glucagon, like vasopressin, angiotensin II, and phenylephrine, has long been known to increase \(^{45}\text{Ca}^{2+}\) uptake in rat hepatocytes (Keppens et al., 1977) prompted us to investigate the effect of glucagon on the resting Ca\(^{2+}\) influx in rat hepatocytes and on the influx stimulated by vasopressin and angiotensin II.

The results show that by raising the intracellular concentration of cyclic AMP, glucagon acts synergistically with the

Cyclic AMP and calcium, whose intracellular concentrations are regulated by different receptors, play a major role in the control of the cellular activity. They serve as intracellular messengers in the same types of tissue and both of them

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1 B. Berthon, unpublished observation.
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EXPERIMENTAL PROCEDURES

Cell Isolation—Hepatocytes were isolated from female Wistar rats (200-250 g) as described by Burgess et al. (1981) except that the collagenase concentration was lowered to 0.15 mg/ml and the perfusion of the liver and incubation of the disaggregated liver with the collagenase solution were reduced to 7 and 5 min, respectively. The isolated cells were incubated at a concentration of \(3 \times 10^6\) cells/ml in Eagle's medium containing in mM: NaCl, 116; KCl, 5.4; CaCl\textsubscript{2}, 1.8; MgCl\textsubscript{2}, 0.81; NaH\textsubscript{2}PO\textsubscript{4}, 0.92; NaHCO\textsubscript{3}, 25; 1 g/l of glucose, amino acids, and vitamins, supplemented with 1.5% gelatin (Difco) and gassed with 95% O\textsubscript{2}, 5% CO\textsubscript{2}. Gelatin was preferred to albumin as it allowed a better preservation of cell viability. Thirty min before the experiments began, the hepatocytes were resuspended in fresh solution of the same composition, supplemented with 1 mg/ml bacitracin to prevent degradation of the peptide hormones. Under these conditions, the cell viability, as judged by trypan blue exclusion, always exceeded 95% and remained stable until the end of the experiment, i.e. 4-5 h after the isolation.

Measurement of the Initial \(\text{Ca}^{2+}\) Uptake Rate—The initial \(\text{Ca}^{2+}\) uptake rate was determined as previously described (Mauger et al., 1984; Poggio\textit{li} et al., 1985). Briefly, cells were incubated in Eagle's medium with 1 \(\mu\)Ci/ml of \(\text{Ca}^{2+}\); 100-\(\mu\)l aliquots were taken at 15, 45, 75, and 105 s after adding the \(\text{Ca}^{2+}\). Each aliquot was diluted with 4 ml of ice-cold washing solution containing 144 mM NaCl, 5 mM CaCl\textsubscript{2}, and 5 mM Tris-HCl, pH 7.4, filtered through a Whatman GF/C glass fiber filter, and washed 3 times with 4 ml of washing solution. The radioactivity on the filter was counted with a liquid scintillation spectrometer. Unless otherwise indicated, the hormones tested were added 30 s before \(\text{Ca}^{2+}\).

Ca\textsuperscript{2+}-linked hormones to augment the Ca\textsuperscript{2+} influx through a homogeneous population of gates located in the plasma membranes.

Cyclic AMP Content—The concentration of cyclic AMP in hepatocytes was determined by radioimmunoassay after deproteinization and acetylation of the samples.

Materials—Collagenase was obtained from Boehringer. (Arginine) vasopressin, angiotensin II, glucagon, and Bt2cAMP\textsuperscript{4} were obtained from Sigma. Forskolin was from Calbiochem. \(\text{Ca}^{2+}\) was from I.R.E., Fleurus Belgium. The cyclic AMP radioimmunoassay kit was from Institut Pasteur Production, Marnes-La-Coquette, France.

RESULTS

Effects of Glucagon and Ca\textsuperscript{2+}-linked Hormones on the Initial \(\text{Ca}^{2+}\) Uptake Rate—As shown in Fig. 1, maximal doses of glucagon and vasopressin raised the initial \(\text{Ca}^{2+}\) uptake rate by isolated liver cells. Vasopressin did so without affecting the intracellular content of cyclic AMP. Glucagon stimulated the initial \(\text{Ca}^{2+}\) uptake rate to a lesser extent than vasopressin and increased the intracellular cyclic AMP concentration about 8-fold. These effects of glucagon lasted for at least 2 min. When the hepatocytes were preincubated with vasopressin plus glucagon for 30 s prior to the addition of \(\text{Ca}^{2+}\), the initial \(\text{Ca}^{2+}\) uptake rate rose to a higher level than the sum of the rises observed with each hormone alone. The mean values obtained from 27 determinations on 9 different cell preparations were, in nmol/min/mg dry weight: 0.23 ± 0.01, 0.32 ± 0.02, 0.49 ± 0.02, and 0.99 ± 0.05 for control cells and cells stimulated with 10 nM glucagon, 10 nM vasopressin, or 10 nM glucagon plus 10 nM vasopressin, respectively. When vasopressin was added to the cells 1 min after glucagon, the

FIG. 1. Effect of glucagon plus vasopressin on the initial \(\text{Ca}^{2+}\) uptake rate and cyclic AMP content. Hepatocytes were preincubated for the period indicated with 10 nM glucagon (Glu) and/or 10 nM vasopressin (VP). Samples were then removed for determination of cyclic AMP (hatched columns) and \(\text{CaCl}_2\) was added to the remaining cell suspension for \(\text{Ca}^{2+}\) uptake rate measurements (open columns). Values are means ± S.E. of 3 determinations in one typical experiment.

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\textsuperscript{4} The abbreviation used is: Bt2cAMP, dibutyryl cyclic AMP.
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FIG. 2. Effects of glucagon, dibutyryl cyclic AMP, and forskolin on the initial 45Ca\textsuperscript{2+} uptake rate in resting cells and cells stimulated with vasopressin or angiotensin II. Hepatocytes were preincubated as indicated before determination of the 45Ca\textsuperscript{2+} uptake rate. Glucagon (Glu), vasopressin (VP), and angiotensin II (All) were added at 10 nM, 30 s before the addition of 45Ca\textsuperscript{2+}. Bt2cAMP (100 μM), forskolin (Fors) (100 μM), or ethanol (Eth) was added 1 min before 45Ca\textsuperscript{2+}. Values are means ± S.E. of 3 determinations in one typical experiment.

initial 45Ca\textsuperscript{2+} uptake rate was about the same as that seen when both hormones are added together. However, when vasopressin was added 1 min before glucagon, the initial 45Ca\textsuperscript{2+} uptake rate was stimulated to a lesser extent and the accumulation of cellular cyclic AMP was smaller. When the cells were preincubated for 2 min with both hormones, the initial 45Ca\textsuperscript{2+} uptake rate and the cyclic AMP concentration began to decrease slowly. These results indicated that the maximal synergistic effects of the hormones were observed when vasopressin was added after or together with glucagon. Consequently, in the experiments described below, the initial 45Ca\textsuperscript{2+} uptake rate was measured after the cells had been preincubated for 30 s with vasopressin or angiotensin II and glucagon.

Since glucagon increases both initial 45Ca\textsuperscript{2+} uptake rate and the cellular concentration of cyclic AMP, we tested the ability of the latter to accelerate the initial 45Ca\textsuperscript{2+} uptake rate and to potentiate the response of the Ca\textsuperscript{2+}-linked hormones. Fig. 2 shows that the permeant analog of cyclic AMP, Bt2cAMP, was able, like glucagon, to increase the initial 45Ca\textsuperscript{2+} uptake rate slightly and to potentiate the response of vasopressin and angiotensin II. Qualitatively similar results were obtained when the diterpene forskolin was used to activate the adenylate cyclase and increase the cellular concentration of cyclic AMP (Fig. 2).

Fig. 2 also shows that the stimulating effects of vasopressin and angiotensin II on the initial 45Ca\textsuperscript{2+} uptake rate were not additive. This indicates that the response induced by the two Ca\textsuperscript{2+}-linked hormones was not limited by the number of receptors activated. This might rule out the hypothesis that cyclic AMP potentiates the response to vasopressin and angiotensin II by increasing the number of available receptors for these hormones.

These findings indicated that cyclic AMP augments the Ca\textsuperscript{2+} influx in rat hepatocytes and potentiates the response of the Ca\textsuperscript{2+}-linked hormones.

Do Ca\textsuperscript{2+}-linked Hormones and Cyclic AMP Act on the Same Ca\textsuperscript{2+} Gating System?—The above findings suggest that vasopressin, angiotensin II, and cyclic AMP act on the same Ca\textsuperscript{2+} gating systems. We have previously demonstrated that both the resting Ca\textsuperscript{2+} influx and that stimulated by noradrenaline, vasopressin, and angiotensin II followed Michaelis-Menten kinetics when the initial 45Ca\textsuperscript{2+} uptake rate was measured as a function of external Ca\textsuperscript{2+} concentrations (Mauger et al., 1984). In the same report, it was suggested that the Ca\textsuperscript{2+}-linked hormones raised the rate of Ca\textsuperscript{2+} transport through the plasma membrane by the use of a single Ca\textsuperscript{2+} gating system. The same methodology was used in the present work to define the kinetic properties of the initial 45Ca\textsuperscript{2+} uptake rate in cells...
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**FIG. 3.** Hofstee plots of the effects of the extracellular Ca\textsuperscript{2+} concentration on the Ca\textsuperscript{2+} influx stimulated with vasopressin and/or glucagon. Hepatocytes were preincubated for 30 s in a medium containing 150 μM Ca\textsuperscript{2+} and the hormones before the addition of trace amounts of \textsuperscript{45}Ca\textsuperscript{2+} and of CaCl\textsubscript{2} at the concentration required to give Ca\textsuperscript{2+} concentrations of 0.15 to 4.8 mM. Cells were preincubated in the absence (closed circle) or presence (open circle) of 10 nM glucagon, without (A) or with 0.3 nM (B) or 10 nM (C) vasopressin. Each point is the mean of 9 determinations on 3 cell preparations (A and B) or of 6 determinations on 2 cell preparations (C). Mean values for $K_m$ and $V_{max}$ are given in Table I.

**TABLE 1**

| Addition | Concentration | $K_m$ (mM) | $V_{max}$ (nmol/min/mg dry wt) |
|----------|---------------|------------|-------------------------------|
| None     |               | 0.40 ± 0.08| 0.26 ± 0.03                   |
| Glucagon | 10            | 0.64 ± 0.03| 0.46 ± 0.07                   |
| Vasopressin | 0.3     | 0.62 ± 0.02| 0.33 ± 0.08                   |
| Vasopressin | 10     | 1.7        | 1.2                           |
| Glucagon + | 10     | 2.8 ± 1.1  | 1.1 ± 0.4                     |
| Glucagon + + | 0.3    | 5.4        | 3.5                           |

incubated with vasopressin and/or glucagon. Fig. 3 shows the Hofstee plots obtained from the initial \textsuperscript{45}Ca\textsuperscript{2+} uptake rate measured in the presence of external Ca\textsuperscript{2+} concentrations ranging from 0.15 to 4.8 mM in control cells or in cells stimulated with 0.3 or 10 nM vasopressin in the presence of 10 nM glucagon or in its absence. For all the conditions tested, the Hofstee plots of the data fitted straight lines. This indicates that in each case Ca\textsuperscript{2+} always entered the cells through a homogeneous population of gates. In addition, it was found that glucagon, like vasopressin, increased both the apparent $K_m$ and the apparent $V_{max}$ for the Ca\textsuperscript{2+} influx, determined from Fig. 3, and that these increases were larger when maximal doses of the two hormones were added together (Table I). The observation of parallel rises in $K_m$ and $V_{max}$ suggests that the 2 hormones act synergistically to stimulate the Ca\textsuperscript{2+} influx in rat hepatocytes by raising the rate of Ca\textsuperscript{2+} transport through the plasma membrane, using the same gating system.

**Stimulation of the Initial \textsuperscript{45}Ca\textsuperscript{2+} Uptake Rate As a Function of the Glucagon and Vasopressin Concentrations**—To further characterize the synergistic action of the two hormones, dose-response curves for the action of each one were performed in the presence of increasing concentrations of the other. The data in Fig. 4A show that at all the vasopressin concentrations tested, glucagon increased the rise in the initial \textsuperscript{45}Ca\textsuperscript{2+} uptake rate which occurred in responses to vasopressin without affecting its $ED_{50}$. Conversely, the dose-response curves for the action of glucagon plotted in the presence of increasing doses of vasopressin also show that vasopressin augments the re-
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**Fig. 4.** Dose-response relationship for the effects of glucagon and vasopressin on initial $\text{Ca}^{2+}$ uptake rate. Hepatocytes were preincubated for 30 s with the indicated doses of glucagon and vasopressin before the addition of $\text{Ca}^{2+}$. A, dose-response curves for the action of vasopressin were done in the absence (●) or presence of $10^{-11} \text{ M} (\bigcirc), 10^{-10} \text{ M} (\blacksquare), 3 \times 10^{-10} \text{ M} (\bigcirc), 10^{-9} \text{ M} (\blacktriangle),$ or $10^{-8} \text{ M} (\blacktriangleleft)$ glucagon. B, same data as in A but showing the dose-response curves for the action of glucagon drawn in the absence (●) or presence of $10^{-11} \text{ M} (\bigcirc), 10^{-10} \text{ M} (\blacksquare), 3 \times 10^{-10} \text{ M} (\bigcirc), 10^{-9} \text{ M} (\blacktriangle),$ or $10^{-8} \text{ M} (\blacktriangleleft)$ vasopressin. Each point is the mean of 3 experiments.

These data suggest that the two hormones enhance each other's maximal effects. This was more obvious when an alternative plot was used to express the results (Fig. 5, A and B). In Fig. 5A the initial $\text{Ca}^{2+}$ uptake rate measured in the presence of vasopressin plus glucagon is plotted as a function of the rate measured in the presence of vasopressin alone, each point being determined in the presence of one concentration of vasopressin. From the figure, which, for greater clarity, only includes 3 curves, it can be seen that the data fit straight lines whose slope is increased by glucagon. This indicates that glucagon enhances the maximal response (apparent $J_{\text{max}}$) induced by vasopressin with stimulating factors being determined by the slopes of the lines in Fig. 5A. The dose-response curve for the stimulating effect of glucagon on the apparent $J_{\text{max}}$ of the vasopressin response is shown in the inset of Fig. 5A. Symmetrical results were observed for the effect of vasopressin on the glucagon response, i.e. vasopressin increases in a dose-dependent manner the apparent $J_{\text{max}}$ of the glucagon response (Fig. 5B and inset). Note that each hormone increased the apparent $J_{\text{max}}$ of the other with an ED$_{50}$ quite similar to that observed for stimulating $\text{Ca}^{2+}$ uptake rate.

**Correlation between the Initial $\text{Ca}^{2+}$ Uptake Rate and the Cellular Level of Cyclic AMP**—The results shown in Figs. 1 and 2 suggest that the effects of glucagon on the $\text{Ca}^{2+}$ influx were mediated by cellular cyclic AMP. The data shown in Fig. 6, obtained in the same series of experiments as those described in Figs. 4 and 5, give the initial $\text{Ca}^{2+}$ uptake rate as a function of the cellular concentration of cyclic AMP measured in the presence of increasing doses of glucagon or in its absence and in the presence of 0.3 or 10 nM vasopressin or in its absence. Under the 3 conditions, initial $\text{Ca}^{2+}$ uptake rates increased with the concentrations of cyclic AMP, but reached a plateau at the highest concentrations. Vasopressin increased the maximal effect obtained in the presence of high cyclic AMP concentrations.

$\text{Ca}^{2+}$ uptake rates increased directly as the concentrations of cyclic AMP were raised above the basal level by the addition of glucagon. This might mean that the basal level of cyclic AMP is sufficient to allow the resting $\text{Ca}^{2+}$ influx, as well as the stimulating effects of vasopressin and angiotensin II.

**DISCUSSION**

Two types of receptor lead to an increase in the glycogen breakdown in mammalian liver by stimulating cyclic AMP- or $\text{Ca}^{2+}$-dependent pathways, which converge to activate phosphorylase kinase. The results of the present work indicate that the two pathways are not independent. We have shown (Figs. 1 and 2) that in the rat hepatocytes glucagon acts synergistically with vasopressin and angiotensin II to increase the $\text{Ca}^{2+}$ influx. This effect of glucagon can be mimicked by...
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**Fig. 5.** Effects of glucagon and vasopressin on the maximal responses induced by vasopressin and glucagon. A, the \( \text{Ca}^{2+} \) uptake rate measured in the presence of glucagon (GLU) plus vasopressin (VP) is represented as a function of the \( \text{Ca}^{2+} \) uptake rate measured in the presence of the corresponding concentration of vasopressin alone. At 10\(^{-11}\) M (○), 3 \times 10\(^{-10}\) M (□), and 10\(^{-9}\) M (△), glucagon increased the slope of the straight line. The inset shows the dose dependence of this slope for increasing concentrations of glucagon. B, the \( \text{Ca}^{2+} \) uptake rate measured in the presence of vasopressin plus glucagon is represented as a function of the \( \text{Ca}^{2+} \) uptake rate measured in the presence of the corresponding concentration of glucagon alone. At 10\(^{-11}\) M (○), 3 \times 10\(^{-10}\) M (□), and 10\(^{-9}\) M (△), vasopressin increased the slope of the straight line. The inset shows the dose dependence of this slope for increasing concentrations of vasopressin. Each point is the mean from 3 separate experiments.

B\(_t\)cAMP or forskolin, which suggests that glucagon stimulates the \( \text{Ca}^{2+} \) influx by raising the cellular cyclic AMP concentration. The dose-response curves in Fig. 4 indicated that the enhancement of the \( \text{Ca}^{2+} \) influx by glucagon and vasopressin even occurs at concentrations as low as 0.1 nM. This concentration is in the range of the concentrations of glucagon and vasopressin found under physiological conditions in the plasma. Consequently, cyclic AMP-dependent and \( \text{Ca}^{2+} \)-linked hormones may be involved in regulating cellular \( \text{Ca}^{2+} \) in the mammalian liver in vivo.

The rise in the \( \text{Ca}^{2+} \) influx induced by the synergistic effect of glucagon and \( \text{Ca}^{2+} \)-linked hormones might explain the observation that the combination of the two types of hormones induces the accumulation of calcium in the hepatocytes, whereas a net loss of calcium occurs if these hormones are added separately (Assimacopoulos-Jeannet et al., 1982; Morgan et al., 1983). A larger \( \text{Ca}^{2+} \) influx could counterbalance the increase in the unidirectional \( \text{Ca}^{2+} \) efflux resulting from the release of \( \text{Ca}^{2+} \) from internal stores induced by the hormones.

A synergistic effect has also been observed in guinea pig liver cells where simultaneous treatment with a cyclic AMP-dependent and a \( \text{Ca}^{2+} \)-linked hormone caused a much larger loss of K\(^+\) than the sum of the loss observed with each hormone alone (Cocks et al., 1984). Assuming that the loss of K\(^+\) is induced by a rise in the intracellular \( \text{Ca}^{2+} \) concentration (Cocks et al., 1984), the exerting of a synergistic effect by the two types of hormones on the \( \text{Ca}^{2+} \) influx might help to increase this loss.

The question arises as to the stage at which the cyclic AMP and the \( \text{Ca}^{2+} \)-dependent pathways give rise to a stimulated \( \text{Ca}^{2+} \) influx. Measurements of the initial \( \text{Ca}^{2+} \) uptake rate as a function of the extracellular \( \text{Ca}^{2+} \) concentration (Fig. 3) lead to the conclusion that glucagon and vasopressin activate the same \( \text{Ca}^{2+} \) entry mechanism via the same \( \text{Ca}^{2+} \) gating system. We previously proposed that the \( \text{Ca}^{2+} \)-linked hormones increase the rate constant of the reaction or reactions by which the \( \text{Ca}^{2+} \) ion is transported into the cell after being bound on its recognition site (Mauger et al., 1984).

As regards the respective intermediates involved in the coupling between the receptor and the \( \text{Ca}^{2+} \) gate, we showed here that glucagon acted by raising the intracellular cyclic AMP concentration, which probably in turn stimulated cyclic AMP-dependent protein kinase activity. It has been suggested that cyclic AMP can modulate the \( \alpha_1 \)-adrenergic receptor properties in rat liver cells (Morgan et al., 1984). However, this mechanism does not seem to be involved in stimulation of the \( \text{Ca}^{2+} \) influx. This conclusion is based on the observation...
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Fig. 6. Relationship between the cyclic AMP content of the cells and the Ca\(^{2+}\) influx: effect of vasopressin. The cyclic AMP content of the cells and the \(^{45}\)Ca\(^{2+}\) uptake rate were determined in the same series of experiments as those described in Fig. 4. Each point was determined in the absence or presence of the doses of glucagon indicated on Fig. 4A, without (o) or with 3 × 10\(^{-8}\) M (□) or 10\(^{-7}\) M (△) vasopressin. Each point is the mean of 3 separate experiments.

that the responses to vasopressin and angiotensin II are not additive, indicating that the number of receptors activated is not the limiting step in the response to individual hormone. As regards the response to combined glucagon and Ca\(^{2+}\)-linked hormone administration, it seems probable that cyclic AMP acts either on the coupling mechanism leading from the Ca\(^{2+}\)-linked hormone receptor to the increased Ca\(^{2+}\) influx or on the Ca\(^{2+}\) gate itself. In agreement with this hypothesis are the observations that, in several cell types, protein phosphorylation regulates ion channels such as those of K\(^{+}\) and Ca\(^{2+}\) (see, for example, Levitan et al., 1983). Injection into neurones or cardiac myocytes of cyclic AMP or of the catalytic subunit of cyclic AMP-dependent protein kinase has been shown to induce Ca\(^{2+}\) currents (see, for example, Reuter, 1983).

Our knowledge of the mechanisms involved in the action of the Ca\(^{2+}\)-linked hormones has recently progressed (see Nishisuka, 1984; Berridge and Irvine, 1984). In liver, as in other tissues, these hormones trigger the degradation of membrane polyphosphoinositides (Rhodes et al., 1983; Creba et al., 1983; Litosch et al., 1983; Seyfred and Wells, 1984). This leads to: 1) diminution of the concentrations of the phosphatidylinositol 4-phosphate and the phosphatidylinositol 4,5-bisphosphate; 2) the production of inositol triphosphate, which releases intracellular Ca\(^{2+}\) (Burgess et al., 1984; Thomas et al., 1984; and Joseph et al., 1984); and 3) the production of diacylglycerol which helps to activate protein kinase C (Hughes et al., 1984; Nishisuka, 1984). Although the breakdown or turnover of the polyphosphoinositides has been suggested to control Ca\(^{2+}\) gating (Michell, 1975, 1982) we do not know which of the above three features of this complex messenger system leads to the increased Ca\(^{2+}\) influx.

In conclusion, the present results indicate that in rat hepatocytes, cyclic AMP- and Ca\(^{2+}\)-linked hormones increase synergistically the Ca\(^{2+}\) influx by activating the same Ca\(^{2+}\) gating system. It is suggested that both the intermediates, respectively, arising from the increase in cellular cyclic AMP content and degradation of the polyphosphoinositides are substrates necessary to allow resting and stimulated Ca\(^{2+}\) entry into the cells.

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Kaibuchi et al., 1982) and does not lead to the disappearance of polyphosphoinositides (Creba et al., 1983). Consequently, cyclic AMP does not seem to potentiate the response of the Ca\(^{2+}\)-linked hormones by increasing hydrolysis of phosphatidylinositol 4,5-bisphosphate, but more probably works with one of the intermediates produced by the degradation of phosphatidylinositol 4,5-bisphosphate to trigger the Ca\(^{2+}\) influx.

The analysis of Figs. 4 and 5 may provide information about the mode of the interaction between cyclic AMP and the inositol lipid-dependent messengers. The results in these figures show that each hormone increases, in a dose-dependent manner, the maximal response to the other without affecting its ED\(_{50}\). If G is the intermediate involved in the glucagon response (possibly cyclic AMP) and V, the intermediate involved in the vasopressin response, each intermediate can influence the maximal response induced by the other according to the following equations:

For the glucagon response:

\[
J_{\text{max}} = \frac{J_{\text{max}}(V)}{K_v + (V)}
\]

and for the vasopressin response:

\[
J_{\text{max}} = \frac{J_{\text{max}}(G)}{K_v + (G)}
\]

Where \(J_{\text{max}}\) is the maximal response obtained in the presence of infinite \(G\) and \(V\) concentrations; \(J_{\text{max},G}\) and \(J_{\text{max},V}\) are the maximal responses obtained with infinite concentrations of \(G\) and \(V\) in the presence of given concentrations of \(V\) and \(G\), respectively, and \(K_v\) and \(K_G\) are the apparent dissociation constants for \(V\) and \(G\), respectively. The Ca\(^{2+}\) influx \((J_{\text{Ca}})\) will then follow the equation:

\[
J_{\text{Ca}} = J_{\text{max}} \frac{(G)}{K_v + (G)} \frac{(V)}{K_v + (V)}
\]

This equation (3) is typical of reactions involving two substrates which form complexes with an enzyme. This model, which accounts for the data in Figs. 4 and 5 implies that there is a Ca\(^{2+}\) influx, provided that minimal concentrations of both \(G\) and \(V\) are present in the cytosol. The data in Fig. 6 suggest that the basal cyclic AMP concentration, which is about 2 pmol/mg dry weight or 1 μM is sufficient to allow basal and vasopressin-stimulated Ca\(^{2+}\) influxes. This also implies that even in the absence of a Ca\(^{2+}\)-linked hormone, there is a polyphosphoinositides turnover that gives rise to an intracellular concentration of the intermediate called \(V\) in our model sufficient to account for both the resting and cyclic AMP-stimulated Ca\(^{2+}\) influxes.
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