The influx of Ca^{2+} induced by the administration of glucagon and Ca^{2+} -mobilizing agents to the perfused rat liver could involve at least two separate pathways

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The Ca^{2+} -mobilizing actions of ADP, ATP and epidermal growth factor (EGF) and their interaction with glucagon were studied in a perfused liver system incorporating a Ca^{2+} -selective electrode. ADP (1–100 μM), ATP (1–100 μM) and EGF (10–50 nM) all induced a net efflux, followed by a net uptake of Ca^{2+} in the intact liver. The co-administration of glucagon (or of cyclic AMP) with these agents resulted in a synergistic potentiation of the Ca^{2+} uptake response in a way which resembles the synergism observed when glucagon is administered with phenylephrine, vasopressin or angiotensin [Altin & Bygrave (1986) *Biochem. J.* **238**, 653–661]. The inability of diltiazem, verapamil and nifedipine to inhibit the Ca^{2+} -influx response suggests that the stimulation of Ca^{2+} influx does not occur through voltage-sensitive Ca^{2+} channels. By contrast, the synergistic effects of glucagon in the stimulation of Ca^{2+} influx are inhibited by 10 mM-neomycin, and a lowering of the extracellular pH to 6.8. Simultaneous measurements of perfusate Ca^{2+} and pH changes suggest that the Ca^{2+} influx response is not mediated by a $\text{Ca}^{2+}/\text{H}^{+}$ exchange. The inability of neomycin and low extracellular pH to inhibit the refilling of the hormone-sensitive pool of Ca^{2+} , after the administration of Ca^{2+} -mobilizing agents alone, provides evidence for the existence in liver of at least two Ca^{2+} -influx pathways, or mechanisms for regulating Ca^{2+} influx.

INTRODUCTION

The mechanism by which Ca^{2+} -mobilizing hormones (α -adrenergic agonists, vasopressin and angiotensin) signal the mobilization of intracellular Ca^{2+} , and consequently a triggering of Ca^{2+} -dependent physiological responses in liver tissue, now can be explained in terms of a receptor-mediated breakdown of phosphoinositides in the plasma membrane. This event has been shown to generate the intracellular messenger inositol trisphosphate into the cytoplasm, and consequently a release of Ca^{2+} from the endoplasmic reticulum (Creba *et al.*, 1983; Berridge, 1984; Burgess *et al.*, 1984). It is also generally acknowledged that, once the intracellular hormone-sensitive stores of Ca^{2+} have been depleted, extracellular Ca^{2+} is required to sustain the hormone-induced physiological responses (Reinhart *et al.*, 1984a,c). This suggests that part of the action of the Ca^{2+} -mobilizing hormones is to stimulate an influx of Ca^{2+} into the cell (Barritt *et al.*, 1981; Reinhart *et al.*, 1984b). However, although the mechanism by which Ca^{2+} -mobilizing hormones stimulate the influx of Ca^{2+} has been the subject of much investigation in recent years (Mauger *et al.*, 1984, 1985; Poggioli *et al.*, 1985), hitherto, it seems that the mechanism by which this occurs remains to be clarified.

Among the suggested mechanisms for the way in which hormones stimulate Ca^{2+} influx is by inhibition of the Ca^{2+} -efflux pump, or in effect the Ca^{2+} -ATPase (Lin *et al.*, 1983; Prpic *et al.*, 1984; Lotersztajn *et al.*, 1985), and the activation of specific Ca^{2+} -influx channels, which

may be either voltage-sensitive (Glossmann *et al.*, 1982; Levitan *et al.*, 1983; Reuter, 1983; Nayler & Horowitz, 1983) or hormone-operated (Berridge, 1982). More recently, it has been shown that glucagon and its messenger cyclic AMP can act synergistically with phenylephrine, vasopressin and angiotensin in the stimulation of Ca^{2+} influx (Morgan *et al.*, 1983; Mauger *et al.*, 1985; Poggioli *et al.*, 1986; Altin & Bygrave, 1986). Since the magnitude of this influx is much greater, and therefore more readily measurable than that which occurs after the administration of Ca^{2+} -mobilizing agents alone, this has provided a useful system with which to study the properties of the Ca^{2+} influx mechanism.

In this work we have used the perfused rat liver incorporating a Ca^{2+} -selective electrode to study further the properties of the Ca^{2+} -influx pathway that is stimulated by the synergistic action of glucagon (or cyclic AMP) and Ca^{2+} -mobilizing agents in the perfused rat liver. More specifically, we have studied the effects of glucagon on the Ca^{2+} -mobilizing action of ADP, ATP and epidermal growth factor (EGF), to determine whether the synergism in stimulating Ca^{2+} influx that occurs with glucagon and phenylephrine, vasopressin and angiotensin (Altin & Bygrave, 1986) occurs also with other Ca^{2+} -mobilizing agents whose receptors are purportedly linked to the breakdown of phosphoinositides. We have examined also the effects of diltiazem, verapamil, nifedipine and neomycin, and a change in the extracellular pH, on the Ca^{2+} -influx response. Our results provide evidence for the existence of at least two

Abbreviation used: EGF, epidermal growth factor.

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separate mechanisms for regulating Ca^{2+} influx. The possible significance of this in the mechanism of action of the Ca^{2+} -mobilizing agents is discussed.

EXPERIMENTAL

Animals and perfusions

For all experiments male Wistar-strain albino rats weighing 280–350 g and fed *ad libitum* were used. The rats were anaesthetized with sodium pentobarbitone (50 mg/kg body wt.), and the livers were perfused with Krebs–Henseleit (1932) bicarbonate buffer equilibrated with O_2/CO_2 (19:1) and containing 1.3 mM added CaCl_2 . The perfusions were conducted in a non-recirculating mode at a flow rate of 3.5 ml/min per g wet wt. of liver. For each experiment the liver was first pre-perfused for 15 min before the infusion of any hormone or Ca^{2+} -channel blocker, which was administered by a pump-driven infusion syringe. Owing to a significant change in the pH of the Krebs–Henseleit bicarbonate medium on addition of neomycin, the neomycin was not administered by infusion syringe, but was added directly to the medium and the pH was stabilized to 7.4 with NaOH before the experiment. Other details are exactly as described in Reinhart *et al.* (1982).

Perfusate Ca^{2+} and pH measurements

The perfusate Ca^{2+} concentration was monitored continuously with a Radiometer F2112 Ca^{2+} -selective electrode in a flow-through chamber placed on the outflow side of the liver (Reinhart *et al.*, 1982). The electrode was coupled to a Radiometer K801 reference electrode via an agarose–KCl salt-bridge, and the combined signals were fed via an Orion microprocessor ion-analyser to a SP4100 computing integrator for recording and analysis; for other details see Altin & Bygrave (1985). For some experiments the perfusate pH was also monitored. The pH electrode was inserted in a flow-through chamber and placed on the outflow side of the Ca^{2+} -electrode chamber; the pH signal was fed to a Radiometer PM84 pH-meter and a Rikadenki model B-341 chart recorder.

Chemicals and materials

Phenylephrine, [Arg⁸]vasopressin, [Val⁵]angiotensin, ATP, ADP, EGF, dibutylryl cyclic AMP, verapamil, diltiazem, nifedipine and neomycin sulphate were all obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Glucagon was obtained from Eli Lilly and Co., Indianapolis, IN, U.S.A. Except for nifedipine, which was dissolved in ethanol, all these agents were dissolved in Krebs–Henseleit buffer for infusion into the liver. The pH electrode (Gk2401c) and pH-meter model PM84, and Ca^{2+} -selective electrode membranes (F2112) and filling solutions (S43316), were obtained from Radiometer, Copenhagen, Denmark. Other chemicals used were of analytical grade.

Expression of data

All the traces presented are a representative from at least three independent experiments. Where indicated, data are expressed as means $\pm$ S.E.M. for the numbers of independent experiments described.

RESULTS

Effect of glucagon (and cyclic AMP) on the Ca^{2+} mobilization induced by ADP, ATP and EGF

We previously reported that, at physiological concentrations of extracellular Ca^{2+} (1.3 mM), the co-administration of glucagon (or cyclic AMP) and phenylephrine, vasopressin or angiotensin leads to a synergistic potentiation of Ca^{2+} uptake by the perfused liver, and consequently an increase in the calcium content of mitochondria (Altin & Bygrave, 1986). In order to characterize further the mechanism(s) involved in mediating this influx of Ca^{2+} , it seemed important to establish whether such synergism also occurs when glucagon (or cyclic AMP) is co-administered with other agents such as ADP, ATP and EGF, whose actions have been linked to the mobilization of Ca^{2+} in liver (Thor *et al.*, 1984; Staddon & McGivan, 1985; Charest *et al.*, 1985a; Taylor *et al.*, 1985a). Such information could indicate whether the synergism is specific for the actions of phenylephrine, vasopressin and angiotensin, or whether this is a more general property which is shared with other agents that induce a mobilization of cellular Ca^{2+} through a receptor-mediated breakdown of phosphoinositides, and a production of the second messengers inositol 1,4,5-trisphosphate and diacylglycerol (Berridge, 1984).

The data in Figs. 1(a), (b) and (c) represent Ca^{2+} -selective-electrode recordings of the perfusate Ca^{2+} concentration obtained when ADP, at concentrations of 1 μM , 10 μM and 100 μM respectively, was administered either alone, or co-administered after a 4 min pre-infusion of a maximal concentration of glucagon (10 nM). It is noteworthy that the administration of ADP alone induces a pattern of Ca^{2+} -flux changes which is dependent on the particular concentration used. The response induced by 100 μM -ADP (Fig. 1c), for example, represents a net efflux of approx. 60 nmol/g of liver during the first 1 min. This is followed by a net uptake of Ca^{2+} (approx. 350 nmol/g of liver) during the next 5 min of ADP administration, and an efflux of a similar amount of Ca^{2+} when the ADP is removed. This response is similar to that induced by 10 nM-vasopressin and 10 nM-angiotensin (see Altin & Bygrave, 1985). Moreover, the administration of glucagon 4 min before ADP results in a synergistic potentiation of Ca^{2+} uptake (approx. 4200 nmol/g of liver over a period of about 20 min) to an extent which is also similar to that observed after co-administration of glucagon with either vasopressin or angiotensin (see Altin & Bygrave, 1986). Similarly, the amount of Ca^{2+} that was influxed is also effluxed slowly over a period of about 30 min. A notable difference between the Ca^{2+} response induced by the co-administration of glucagon and ADP (100 μM), instead of glucagon and either vasopressin (10 nM) or angiotensin (10 nM), is that the maximum rate of Ca^{2+} efflux after the removal of the stimuli is achieved much more rapidly (approx. 1 min, by comparison with about 10 min; cf. Altin & Bygrave, 1986). Although slightly less effective at 1 μM and 10 μM concentrations, ATP administration induced essentially identical results (results not shown).

The data in Fig. 1(d) indicate firstly that the administration of EGF (10 nM) alone to the perfused liver is associated with relatively small Ca^{2+} -flux changes (5–10 nmol/g of liver), and secondly that the Ca^{2+} -influx phase of the response is also susceptible to potentiation

Fig. 1. Ca^{2+} response induced by the administration of ADP, ATP and EGF, and the potentiation of Ca^{2+} uptake by the administration of glucagon to the perfused rat liver

Rat livers were perfused with Krebs–Henseleit bicarbonate medium containing 1.3 mM-Ca^{2+} . A Ca^{2+} -selective electrode as outlined in the Experimental section was used to monitor continuously the Ca^{2+} concentration of the outflowing perfusate. After an equilibration period of about 15 min, ADP was infused into the portal vein by infusion syringe for the period indicated by the thin lines. After a subsequent rest period of about 15 min, glucagon (10 nM , bold line) was also infused for 4 min, and then both glucagon and ADP were co-administered by separate infusions for a period of 20 min. The Ca^{2+} -selective-electrode traces in (a), (b) and (c) represent separate experiments for which the ADP concentration was $1 \mu\text{M}$, $10 \mu\text{M}$ and $100 \mu\text{M}$ respectively. Though slightly less effective at $1 \mu\text{M}$ and $10 \mu\text{M}$ concentrations, essentially identical results were obtained when ATP was used instead of ADP. The trace in (d) shows the Ca^{2+} response for a similar experiment for which EGF (10 nM thin continuous line, or 50 nM thin broken line) was used instead of ADP. Each trace is a representative of at least three independent experiments.

by glucagon. At the highest concentration of EGF tested (50 nM), the co-administration of glucagon and EGF was associated with an efflux of approx. 10 nmol/g of liver during the first 1 min. This was followed by an influx of approx. 200 nmol/g of liver during the next 4 min, and an efflux of a similar amount of Ca^{2+} after removal of the stimuli. Although the response induced by glucagon and EGF (Fig. 1d) is much smaller than that induced by the other Ca^{2+} -mobilizing agents, the shape of the response, and its synergism with glucagon, are two features which are similar to those observed for ADP and ATP (Fig. 1a, 1b and 1c) and phenylephrine, vasopressin and angiotensin (see Fig. 1 in Altin & Bygrave, 1986).

Effect of diltiazem, verapamil and nifedipine on Ca^{2+} influx induced by glucagon and Ca^{2+} -mobilizing agents

There is much evidence to suggest that the organic compounds verapamil, diltiazem and nifedipine are

effective in inhibiting the flux of Ca^{2+} through voltage-sensitive Ca^{2+} channels in excitable tissues (see, e.g., Nayler & Horowitz, 1983; Towart & Schramm, 1984). Work by Taylor *et al.* (1985b) has provided some evidence for the existence of voltage-sensitive Ca^{2+} channels also in rat liver. Another report (Joseph *et al.*, 1985) describes an inhibition of hormone-induced influx of Ca^{2+} in hepatocytes by high concentrations ($500 \mu\text{M}$) of diltiazem. The use of these compounds in the perfused rat liver, coupled with the measurement of changes in the perfusate Ca^{2+} concentration with the Ca^{2+} -selective electrode, thus provides a convenient method to determine whether the Ca^{2+} -influx pathway synergistically stimulated by the co-administration of glucagon and a particular Ca^{2+} -mobilizing agent occurs through voltage-sensitive Ca^{2+} -channels.

The data in Fig. 2 show that the infusion of diltiazem ($100 \mu\text{M}$) 8 min before the onset of the Ca^{2+} -influx response did not significantly affect the influx of Ca^{2+} associated with the re-filling of the hormone-sensitive

Fig. 2. Effect of the Ca^{2+} antagonist diltiazem on Ca^{2+} influx induced by the co-administration of glucagon and Ca^{2+} -mobilizing agents

Perfusions of rat livers and measurements of the perfusate Ca^{2+} concentrations were conducted as described in the legend to Fig. 1. After a pre-perfusion period of 14 min for equilibration, phenylephrine ($2 \mu\text{M}$) was administered for 4 min (thin broken line; Phe). Subsequently, at 20 min of perfusion, glucagon (10 nM , thick lines; Glu) and 4 min later vasopressin (10 nM , thin lines; Vaso) were co-administered by separate infusion syringes for the times indicated. The associated Ca^{2+} response is shown by the continuous trace (—). Trace (---) shows the Ca^{2+} response obtained from an experiment in which diltiazem ($100 \mu\text{M}$) was also infused at 10 min of perfusion. Each trace is a representative of three independent experiments. Similar results were obtained with verapamil ($10 \mu\text{M}$) and nifedipine ($5 \mu\text{M}$) instead of diltiazem. At these concentrations the three Ca^{2+} antagonists were also without significant effect in inhibiting the Ca^{2+} influx induced by the co-administration of glucagon with angiotensin (10 nM), ADP ($100 \mu\text{M}$), ATP ($100 \mu\text{M}$) or EGF (50 nM), instead of vasopressin. Diltiazem ($100 \mu\text{M}$) and verapamil ($10 \mu\text{M}$) inhibited the Ca^{2+} influx induced by the co-administration of glucagon and phenylephrine ($2 \mu\text{M}$) by approx. 50% (results not shown); it seems, however, that this may be due to an interaction of these antagonists with the α_1 -receptor (see, e.g., Nayler & Horowitz, 1983).

store(s) after the removal of phenylephrine ($2\ \mu\text{M}$). In addition, the influx of Ca^{2+} associated with a subsequent co-administration of glucagon and vasopressin was affected only slightly ($< 20\%$ inhibition, see Fig. 2). Similar results also were obtained when glucagon was co-administered with phenylephrine, angiotensin, ADP, ATP and EGF, instead of vasopressin (results not shown). Infusion of verapamil ($10\ \mu\text{M}$) or nifedipine ($5\ \mu\text{M}$) similarly did not influence Ca^{2+} influx (results not shown). Although the concentrations of the Ca^{2+} antagonists used were several times higher than those normally used to inhibit Ca^{2+} influx in excitable tissues, significant inhibition of the Ca^{2+} -influx response ($> 50\%$) could be achieved only by using very high concentrations of diltiazem ($250\ \mu\text{M}$), verapamil ($50\ \mu\text{M}$) and nifedipine ($40\ \mu\text{M}$). It seems unlikely, however, that this inhibition is due to some specific effect of these agents on the Ca^{2+} -influx pathway itself, but instead may be due to toxic effect(s) or interference with hormone-receptor binding. In support of this is the observation that the administration of such high concentrations of diltiazem and verapamil alone was accompanied by a stimulation of respiration, and that after a period of continued administration of these compounds (10–20 min) the liver became progressively less responsive to hormone stimulation, even after an equivalent 'rest' period. We conclude therefore that nominal concentrations of diltiazem, verapamil and nifedipine have little, if any, effect on Ca^{2+} influx in this system, and hence that the principal Ca^{2+} -influx mechanism(s) that is involved must be different from the voltage-sensitive Ca^{2+} channels that exist in excitable tissues.

Effect of neomycin on the synergistic stimulation of Ca^{2+} influx by glucagon and Ca^{2+} -mobilizing agents

As a first step towards elucidating the mechanism by which the synergistic action of glucagon and Ca^{2+} -mobilizing agents stimulate Ca^{2+} influx into the liver cell, it seemed important to define more clearly the particular event (associated with the hormone-receptor interaction) that may be involved in supporting the stimulation of Ca^{2+} influx in the presence of glucagon or cyclic AMP (Mauger *et al.*, 1985; Altin & Bygrave, 1986). A crucial issue is thus to determine whether the interaction of a Ca^{2+} -mobilizing agent with its receptor, alone, without the action of phospholipase C, is sufficient to allow the Ca^{2+} -influx pathway to be stimulated by cyclic AMP. This information could indicate whether direct coupling exists between the hormone-receptor complex and the Ca^{2+} transporters themselves in the plasma membrane, or whether phosphoinositide breakdown or some metabolite that arises from this is required to activate the Ca^{2+} -influx pathway. Of potential relevance to this are the findings that neomycin, and related aminoglycoside antibiotics such as gentamicin, which are known to block neuromuscular transmission, are potent inhibitors of phosphoinositide hydrolysis in nerve and skeletal muscle (Van Rooijen & Agranoff, 1985; Vergara *et al.*, 1985; Shute & Smith, 1985). Although neomycin has been reported also to inhibit the action of phospholipase C in erythrocytes *in vitro* (Downes & Michell, 1981), it was decided to see whether this compound influences Ca^{2+} fluxes in the perfused rat liver.

The data in Fig. 3(b) show the effect of neomycin

(10 mM) on the Ca^{2+} response induced by the administration of vasopressin (10 nM), phenylephrine ($2\ \mu\text{M}$) and glucagon (10 nM) plus vasopressin. Relative to the control (Fig. 3a), the continuous administration of neomycin (commenced at 10 min of perfusion) inhibited the spontaneous uptake of Ca^{2+} that is normally associated with the administration of vasopressin alone. Moreover, the Ca^{2+} influx induced by a subsequent co-administration of glucagon and vasopressin was inhibited by approx. 90% (cf. Figs. 3a and 3b). At these concentrations neomycin did not inhibit, but instead slightly increased (by 20–40%), the Ca^{2+} efflux associated with the administration of phenylephrine alone, and the Ca^{2+} uptake that is induced following its removal (see Fig. 3; also Altin & Bygrave, 1985). The inhibition of the glucagon- and vasopressin-induced Ca^{2+} influx by neomycin is maintained only for as long as the infusion of neomycin is continued, as the removal of neomycin from the perfusion medium during such hormone administration is followed rapidly by a stimulation of Ca^{2+} uptake by the liver (results not shown). The dose-response curve for the inhibition of Ca^{2+} influx by neomycin is shown in Fig. 3(c); the half-maximal effective dose of neomycin was approx. 3 mM.

These results suggest that neomycin (10 mM) is effective in inhibiting the influx of Ca^{2+} induced by the administration of glucagon and vasopressin, apparently without any inhibition of the influx of Ca^{2+} that occurs to replenish the hormone-sensitive calcium store(s) when a Ca^{2+} -mobilizing agent (e.g. phenylephrine) is removed after a single hormone administration. The existence of at least two separate mechanisms for regulating Ca^{2+} influx is thus implied. At a concentration of 10 mM or below, the administration of neomycin was not followed by any significant mobilization of Ca^{2+} or stimulation of respiration; further, there were no signs of toxicity, as the liver remained responsive to Ca^{2+} -mobilizing agents for the duration of the experiment (around 80 min). A similar inhibition of Ca^{2+} influx by neomycin was observed when glucagon (or cyclic AMP, $200\ \mu\text{M}$) was co-administered with phenylephrine, angiotensin, ATP, ADP and EGF (results not shown).

Dependence of the Ca^{2+} influx on extracellular pH

The observation that agents which increase intracellular pH (e.g. nupercaine and NH_4^+) can lead to an increase in Ca^{2+} influx in the perfused rat liver (Friedmann, 1972), and that extracellular pH can affect the Ca^{2+} content of isolated hepatocytes after the separate administration of glucagon, vasopressin and angiotensin, has led to the suggestion that a $\text{Ca}^{2+}/\text{H}^+$ exchange mechanism may exist in liver (Friedmann, 1972; Kraus-Friedmann *et al.*, 1982; Blackmore *et al.*, 1984). In order to explore this possibility, it therefore seemed important to study the synergism that exists between glucagon and Ca^{2+} -mobilizing agents as a function of the extracellular pH and to monitor simultaneously the associated changes in both the Ca^{2+} concentration and the pH of the outflowing perfusate.

The data in Figs. 4(a), 4(b) and 4(c) show the perfusate Ca^{2+} and pH changes that occur after the co-administration of glucagon and vasopressin to livers perfused with media of pH 6.8, 7.4 and 8.0 respectively. Also, Fig. 5 shows the corresponding amount of Ca^{2+} uptake by the liver that is induced by this administration

Fig. 3. Effect of neomycin on Ca^{2+} influx induced by the co-administration of glucagon and Ca^{2+} -mobilizing agents

Livers were perfused with Krebs–Henseleit bicarbonate medium containing 1.3 mM- Ca^{2+} , and the Ca^{2+} concentration of the perfusate was continuously monitored with a Ca^{2+} -selective electrode as previously described. At 15 min and 35 min of perfusion, respectively, maximal doses of vasopressin (10 nM, thin line; V) and phenylephrine (2 μM , thin broken line; P) were each separately infused for a period of 5 min, as indicated. At 55 min of perfusion, after an intervening 'rest' period, glucagon (10 nM, thick line; G) and 4 min later, vasopressin (10 nM; V) were co-administered for a period of 20 min. Trace (a) shows the associated Ca^{2+} response induced by the administration of the hormones. The result for a similar experiment in which neomycin (10 mM, thick broken line) was continuously infused commencing at 10 min of perfusion is shown in (b). The dose-response curve for the inhibition of Ca^{2+} influx by neomycin is shown in (c). Each point represents the Ca^{2+} uptake that occurs when glucagon and vasopressin are co-administered for a period of 20 min. Each trace in (a) and (b) is a representative of three independent experiments; each point in (c) represents the mean $\pm$ S.E.M. of between three and five independent experiments. The infusion of neomycin resulted in a similar inhibition of Ca^{2+} influx when glucagon was co-administered with phenylephrine, angiotensin, ADP, ATP and EGF.

of hormones when the extracellular pH is varied from 6.8 to 8.0. The pH of the perfusion medium was altered by varying the concentration of bicarbonate in the range 7–75 mM. By comparison with the Ca^{2+} influx that occurs at the physiological pH of 7.4, at extracellular pH below 7.4 the synergistic effects of the hormones on Ca^{2+} influx were inhibited, whereas at extracellular pH above 7.4 the synergism was enhanced (see Figs. 4 and 5). Also, when the extracellular pH was below 7.80 ± 0.05 , no significant net uptake of Ca^{2+} (< 20 nmol/g of liver) was induced by the administration of glucagon (or cyclic AMP) alone. However, at extracellular pH above 7.80, administration of glucagon (or exogenous cyclic AMP) alone was sufficient to stimulate extensive Ca^{2+} uptake by the liver; the co-administration of vasopressin further potentiated this effect (see Fig. 4c). These differences in hormone action (i.e. synergism versus glucagon alone) are highlighted in Fig. 5. The reason for the abrupt change in Ca^{2+} influx induced by glucagon around pH 7.8 was not pursued further in this study. In contrast with the pattern displayed by the synergistic effect of glucagon, the Ca^{2+} influx induced by the removal of a Ca^{2+} -mobilizing agent (e.g. phenylephrine, when administered alone) was not significantly affected by a lowering of the extracellular pH to 6.8 (see Fig. 4a). Also, by comparison with the Ca^{2+} efflux observed at an extracellular pH of 7.4 (Fig. 4b), vasopressin and phenylephrine administered alone induced a significantly greater amount of Ca^{2+} efflux (approx. 100% increase) at extracellular pH 6.8,

but induced a smaller amount of Ca^{2+} efflux (40% decrease) at an extracellular pH of 8.0 (cf Figs. 4a, 4b, and 4c).

Another important feature of the data in Fig. 4 is that, at the extracellular pH of 6.8, 7.4 and 8.0, the total decrease in perfusate pH that occurs after the co-administration of glucagon and vasopressin is no larger than the decrease that occurs after the administration of vasopressin alone. This result suggests that the influx of Ca^{2+} is not associated with any increase in the efflux of H^{+} . Similar results could be obtained if the pH of the perfusion medium was adjusted by using HCl instead of by altering the bicarbonate concentration. In addition, the Ca^{2+} influx and pH responses induced by the co-administration of glucagon and phenylephrine, angiotensin, ADP, ATP or EGF displayed a similar sensitivity to changes in extracellular pH (results not shown).

DISCUSSION

The first major observation revealed in the present work concerns the ability of ATP, ADP and EGF to induce net Ca^{2+} changes in the intact liver. Although ADP and ATP (Charest *et al.*, 1985a; Sistare *et al.*, 1985; Staddon & McGivan, 1985) and EGF (Moolenaar *et al.*, 1984, 1986) have been reported to increase cytosolic Ca^{2+} concentration in isolated cells, with techniques using quin2, to our knowledge no detailed studies of the effects of these agents have been conducted in the intact tissue.

Fig. 4. Dependence of Ca^{2+} influx on extracellular pH

Livers were perfused with Krebs–Henseleit bicarbonate medium containing 1.3 mM-Ca^{2+} for the first 10 min. Thereafter, the perfusions were conducted either with the same medium (bicarbonate concentration 28 mM , pH 7.4), or with medium for which the bicarbonate concentration was changed to some pre-determined value between 7 and 75 mM , and the corresponding buffered pH of the medium was in the range 6.8 – 8.0 . The perfusate was continuously monitored for changes in Ca^{2+} concentration and pH with a Ca^{2+} -selective electrode and a pH-electrode respectively, as detailed in the Experimental section. After an additional equilibration period of 5 min to allow stabilization of the pH, vasopressin (10 nM , thin line; V), phenylephrine ($2 \text{ }\mu\text{M}$, thin broken line; P), and glucagon (10 nM , bold line; G) plus vasopressin, were separately infused as indicated. The traces in (a), (b) and (c) represent the Ca^{2+} responses induced by the administration of these hormones when the pH of the perfusion medium was 6.8 , 7.4 and 8.0 respectively. In each instance the broken trace shows the associated hormone-induced changes in perfusate pH. Each Ca^{2+} and pH trace is a representative of three or four independent experiments conducted at the indicated pH.

In addition, hitherto, there has been no evidence to suggest that glucagon (or cyclic AMP) can interact synergistically with these agents in the stimulation of Ca^{2+} influx. Thus, unlike the findings of Charest *et al.* (1985a), which suggest that ADP and ATP stimulate Ca^{2+} efflux only, our data demonstrate that ADP, ATP and EGF all induce a net efflux, followed by a net influx, of Ca^{2+} (albeit small for low concentrations of these agents; see Fig. 1). Further, Thor *et al.* (1984) observed no effect of 1 mM-ADP on the accumulation of Ca^{2+} in hepatocytes. Our data also show that glucagon (and cyclic AMP) can

Fig. 5. Effect of extracellular pH on the net uptake of Ca^{2+} induced by the co-administration of glucagon with vasopressin

Rat livers were perfused with Krebs–Henseleit bicarbonate medium for the first 10 min. Thereafter, perfusions were conducted either with the same medium ($28 \text{ mM-bicarbonate}$, pH 7.4), or with a medium of different pH in the range 6.8 – 8.0 . This was achieved by changing the bicarbonate concentration exactly as described in the legend to Fig. 4. Maximal doses of glucagon (Glu; 10 nM) and vasopressin (Vaso; 10 nM) were separately infused at 15 and 19 min of perfusion respectively, and both hormones were then co-administered for a period of 20 min . ●, Total net amount of Ca^{2+} uptake by the liver that is induced during this period in an experiment conducted at the indicated pH; ○, net amount of Ca^{2+} uptake that is induced in a similar experiment for which glucagon, alone, was administered for the 24 min period. Each point is the mean $\pm \text{S.E.M.}$ of three to five experiments performed independently.

interact synergistically with ADP, ATP and EGF in the stimulation of Ca^{2+} influx. This potentiation closely resembles the synergism observed by the co-administration of glucagon with phenylephrine, vasopressin or angiotensin (cf. Fig. 1 of present work with Fig. 1 of Altin & Bygrave, 1986).

Work by Charest *et al.* (1985a) suggests that the action of ATP and ADP in hepatocytes is linked with the breakdown of phosphoinositides, probably through interaction with P_2 -purinergic receptors. Although there are suggestions that EGF receptors may also be linked to phosphoinositide breakdown in some tissues (Sawyer & Cohen, 1981; Berridge, 1984), work by Taylor *et al.* (1985a) was unable to detect an EGF-linked breakdown of phosphoinositides in liver. In this regard it is noteworthy that the present results show that, even at concentrations of 50 nM (270 ng/ml), EGF was the least effective of the agonists tested in stimulating Ca^{2+} influx when co-administered with glucagon (see Fig. 1d). Also,

phenylephrine, which has been shown to be less effective than either vasopressin or angiotensin in inducing phosphoinositide breakdown and accumulation of inositol trisphosphate (Lynch *et al.*, 1985; Charest *et al.*, 1985b), has been shown also to be less effective in inducing Ca^{2+} influx when co-administered with cyclic AMP to the perfused rat liver (see Altin & Bygrave, 1986). It therefore appears that there is some association of the ability of a Ca^{2+} -mobilizing agent to induce phosphoinositide breakdown with its ability to interact synergistically with glucagon (or cyclic AMP) in the stimulation of Ca^{2+} influx.

It was of considerable interest to observe that neomycin (10 mM) can inhibit the synergistic effects of glucagon and Ca^{2+} -mobilizing agents stimulating Ca^{2+} influx (see Fig. 2), but not the uptake of Ca^{2+} associated with the re-filling of the hormone-sensitive pool of Ca^{2+} . The precise site(s) of action of neomycin were not established in this work, but there are suggestions that neomycin may inhibit the action of phospholipase C by binding to the phosphoinositides in the plasma membrane (Schibeci & Schacht, 1977; Downes & Michell, 1981). Further, a direct effect of neomycin on the Ca^{2+} -influx pathway cannot be eliminated. That neomycin at these concentrations does not significantly interfere with hormone-receptor binding is suggested by the observation that neomycin had a similar effect on the Ca^{2+} influx induced by all the Ca^{2+} -mobilizing agents tested, and that its presence did not significantly alter the Ca^{2+} -efflux response or a stimulation of whole-tissue respiration, an associated physiological response (results not shown).

Because we have yet to establish that in the perfused rat liver the main effect of neomycin is to inhibit the action of phospholipase C, we cannot argue too strongly at present for an involvement of the second messengers inositol trisphosphate and diacylglycerol (or some metabolite produced from these) in the regulation of Ca^{2+} influx. However, the possibility for such an involvement had previously been proposed by Michell (1975), who studied the effects of Ca^{2+} -mobilizing hormones on phosphoinositol hydrolysis.

Another important finding made in the present work relates to the inability of nominal concentrations of diltiazem, verapamil and nifedipine to inhibit the Ca^{2+} influx that occurs after the administration of Ca^{2+} -mobilizing agents with or without glucagon pre-treatment, or the influx associated with the refilling of the hormone-sensitive pool of Ca^{2+} after the removal of agents such as phenylephrine, ADP (100 μM) or ADP (100 μM) (which induce a more pronounced uptake of Ca^{2+}). This suggests that voltage-sensitive Ca^{2+} channels of the type known to exist in excitable tissues (e.g. heart) are unlikely to be involved in mediating either of the two separate components of Ca^{2+} influx that are observed. Although this is inconsistent with the findings of Blackmore *et al.* (1984) and Joseph *et al.* (1985), we argue that in their experiments these workers used concentrations of these agents which in some cases was more than an order of magnitude higher than the established effective doses in excitable tissues; in our hands toxic effects of these agents became apparent at much lower concentrations than were used.

Finally, the present work shows that the Ca^{2+} -influx mechanism that is synergistically stimulated by the co-administration of glucagon and Ca^{2+} -mobilizing

agents is pH-sensitive: inhibited by low extracellular pH (pH 6.8), but potentiated by extracellular pH above 7.4. This agrees with the pH-dependence of hormonally induced Ca^{2+} influx previously reported by Blackmore *et al.* (1984). Moreover, our observation that the decrease in perfusate pH that occurs after the co-administration of glucagon and vasopressin was slightly less at pH 6.8, where no net Ca^{2+} influx was apparent (see Fig. 4), could argue for some $\text{Ca}^{2+}/\text{H}^{+}$ exchange mechanism. However, since the total decrease in pH was less than that induced by the administration of vasopressin alone, it seems that one would need to postulate that glucagon (or cyclic AMP) is able to couple the extrusion of H^{+} with the influx of Ca^{2+} . Taking into account the magnitude of the perfusate Ca^{2+} and H^{+} changes that occur (and taking into consideration that the pH change is buffered), and assuming that the stoichiometry of the putative exchange mechanism to be one Ca^{2+} for two H^{+} ions, our calculations suggest that the component of pH change that may be coupled to Ca^{2+} influx can account for only about 20–30% of the changes in the perfusate Ca^{2+} concentration that are observed. We therefore conclude that under these conditions the principal component of the Ca^{2+} influx that is induced by the administration of these hormones must occur by a mechanism which is different from a simple $\text{Ca}^{2+}/\text{H}^{+}$ exchange.

Thus, although we cannot as yet speculate as to the nature of the Ca^{2+} -influx pathway that is stimulated by the action of glucagon and Ca^{2+} -mobilizing agents, the observations made in this work imply that at least two separate mechanisms exist for regulating Ca^{2+} influx in liver. The one that is neomycin- and pH-sensitive appears not to involve a simple $\text{Ca}^{2+}/\text{H}^{+}$ exchange, but may be regulated by a common mechanism which is dependent on cyclic AMP (perhaps involving cyclic AMP-dependent phosphorylation), phosphoinositide breakdown and intracellular pH. The other Ca^{2+} -influx mechanism, which is neomycin-, pH- and voltage-insensitive, appears to regulate the uptake of Ca^{2+} associated with the refilling of the intracellular hormone-sensitive stores of Ca^{2+} after the removal of Ca^{2+} -mobilizing agents. Although it may be possible that this may reflect Ca^{2+} uptake by the endoplasmic reticulum from the passive entry of Ca^{2+} into the cell, it is clear that a better understanding of this and the pH-sensitive cyclic AMP-dependent regulatory mechanism described, and a characterization of the Ca^{2+} transporters themselves, require further investigation.

This work was supported by a Ph.D. Scholarship to J. G. A. and a grant to F. L. B. from the National Health and Medical Research Council of Australia.

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Received 30 July 1986/19 September 1986; accepted 16 October 1986