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Synergistic stimulation of Ca^{2+} uptake by glucagon and Ca^{2+}-mobilizing hormones in the perfused rat liver

A role for mitochondria in long-term Ca^{2+} homoeostasis

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A perfused liver system incorporating a Ca^{2+}-sensitive electrode was used to study the long-term effects of glucagon and cyclic AMP on the mobilization of Ca^{2+} induced by phenylephrine, vasopressin and angiotensin. At 1.3 mM extracellular Ca^{2+} the co-administration of glucagon (10 nM) or cyclic AMP (0.2 mM) and a Ca^{2+}-mobilizing hormone led to a synergistic potentiation of Ca^{2+} uptake by the liver, to a degree which was dependent on the order of hormone administration. A maximum net amount of Ca^{2+} influx, corresponding to approx. 3800 nmol/g of liver (the maximum rate of influx was 400 nmol/min per g of liver), was induced when cyclic AMP or glucagon was administered about 4 min before vasopressin and angiotensin. These changes are over an order of magnitude greater than those induced by Ca^{2+}-mobilizing hormones alone [Altin & Bygrave (1985) Biochem. J. **232**, 911–917]. For a maximal response the influx of Ca^{2+} was transient and was essentially complete after about 20 min. Removal of the hormones was followed by a gradual efflux of Ca^{2+} from the liver over a period of 30–50 min; thereafter, a similar response could be obtained by a second administration of hormones. Dose–response measurements indicate that the potentiation of Ca^{2+} influx by glucagon occurs even at low (physiological) concentrations of the hormone. By comparison with phenylephrine, the stimulation of Ca^{2+} influx by vasopressin and angiotensin is more sensitive to low concentrations of glucagon and cyclic AMP, and can be correlated with a 20–50-fold increase in the calcium content of mitochondria. The reversible uptake of such large quantities of Ca^{2+} implicates the mitochondria in long-term cellular Ca^{2+} regulation.

INTRODUCTION

The movement of Ca^{2+} ions across cellular and organellar membranes appears to be an essential phenomenon that is associated with the action of many hormones, and other similar biological substances. Work on the mechanism of action of the Ca^{2+}-mobilizing hormones α_1-adrenergic agonists, vasopressin and angiotensin, has shown that the receptor-mediated breakdown of phosphatidylinositol 4,5-bisphosphate leads to the production of inositol 1,4,5-trisphosphate, which appears to be the signal that triggers the release of Ca^{2+} from the endoplasmic reticulum (Creba *et al.*, 1983; Berridge, 1984; Joseph *et al.*, 1984; Burgess *et al.*, 1984; Charest *et al.*, 1985). Although this model seems to account for the initial triggering of the physiological responses that are induced, little is known at present about the mechanism by which the hormones stimulate the influx of extracellular Ca^{2+} that is necessary in order to sustain the physiological responses that follow the depletion of the intracellular Ca^{2+} stores (Reinhart *et al.*, 1984*b*,*c*).

We previously reported (Altin & Bygrave, 1985) that the actions of phenylephrine, vasopressin and angiotensin differ in their ability to stimulate the net movement of Ca^{2+} into the perfused liver. In particular, we presented evidence which suggests that, unlike phenylephrine, both vasopressin and angiotensin each induce a spontaneous net uptake of Ca^{2+} even while the hormone is still being administered. In view of reports that glucagon and cyclic AMP can modulate the Ca^{2+} mobilization induced by Ca^{2+}-mobilizing hormones (Morgan *et al.*, 1983*a*,*b*; Cocks *et al.*, 1984), it seemed important to examine whether cyclic AMP might be involved in eliciting the different Ca^{2+} flux responses that we had observed (Altin & Bygrave, 1985). It also has been known for some years that glucagon or cyclic AMP alone is able to induce a redistribution of Ca^{2+} in the perfused rat liver (Friedmann & Park, 1968).

In the present work we have used the Ca^{2+}-selective electrode to study further the difference in the Ca^{2+}-flux responses induced by phenylephrine, vasopressin and angiotensin, and to examine the interaction of glucagon (and cyclic AMP) with these hormones in stimulating Ca^{2+} uptake by the perfused rat liver. Our results show that the interaction of phenylephrine, vasopressin or angiotensin with glucagon (or exogenous cyclic AMP) can have profound effects on the Ca^{2+}-flux changes that are induced. Furthermore, measurement of the total calcium content of subcellular fractions obtained by Percoll-density-gradient subfractionation of the liver after hormonal challenge show that the mitochondria are the sink for the Ca^{2+} uptake that occurs. We conclude that mitochondria play an important role in regulating long-term cellular and perhaps cytoplasmic Ca^{2+} concentrations. The physiological relevance of this in terms of liver metabolism is discussed.

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EXPERIMENTAL

Animals and perfusions

For all experiments male Wistar-strain albino rats weighing 280–350 g and having free access to food were used. 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 $CaCl_2$. All perfusions were conducted in a non-recirculating mode at a flow rate of 3.5 ml/min per g wet wt. of liver. All livers were pre-perfused for 15 min before the infusion of hormone. Other results are exactly as in Reinhart *et al.* (1982*b*).

Perfusate Ca^{2+} 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*b*). 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).

Liver subfractionation

This was carried out exactly as described by Reinhart *et al.* (1982*a*). Briefly, after termination of hormone infusion, the liver was perfused for 2 min with Krebs–Henseleit medium containing no added Ca^{2+} but instead 50 μM-EGTA, as indicated in the legend to Fig. 4. The liver was homogenized in a medium consisting of 210 mM-mannitol, 60 mM-sucrose, 10 mM-KCl, 20 μM-EGTA and 10 mM-Hepes/KOH buffer (pH 7.4). A portion of the resulting homogenate (2 ml) was layered on a discontinuous gradient of iso-osmotic Percoll, and centrifuged for 30 s at 39600 $g_{av.}$ in a Sorvall RC-5B refrigerated centrifuge fitted with an SS-34 rotor. In this way the homogenate was separated into ten distinct fractions within a total preparation time of 6 min. Fraction 8, and fractions 1 and 2, previously have been shown to be enriched in mitochondria and endoplasmic reticulum respectively (see Reinhart *et al.*, 1982*a*). The calcium content of these fractions isolated from control perfused rat livers (see Fig. 4) is closely related to estimations of the calcium content of mitochondria and endoplasmic reticulum *in vivo* (Reinhart *et al.*, 1982*a*, 1984*a*; Somlyo *et al.*, 1985). Immediately after isolation, a portion of each fraction was used for protein determination by the method of Lowry *et al.* (1951), and the remainder was extracted with ice-cold 2 M-$HClO_4$, and a suitable sample of the supernatant was analysed for calcium by atomic-absorption spectroscopy.

Chemicals and materials

Phenylephrine, [Arg^8]vasopressin, [Val^5]angiotensin and dibutyryl cyclic AMP were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Glucagon was obtained from Eli Lilly and Co., Indianapolis, IN, U.S.A. Percoll and density-marker beads were obtained from Pharmacia Fine Chemicals A.B., Uppsala, Sweden. Ca^{2+}-selectrode membranes (F2112) and filling solutions S43316 were obtained from Radiometer, Copenhagen, Denmark. Other chemicals used were of analytical grade.

Expression of data

All experiments were performed at least three times. Where indicated, data are expressed as means ± S.E.M. for the numbers of independent experiments described.

RESULTS

Effect of glucagon and cyclic AMP on Ca^{2+} mobilization induced by phenylephrine, vasopressin and angiotensin

Although some studies have been carried out on the influence of glucagon on the Ca^{2+} fluxes induced by Ca^{2+}-mobilizing hormones, it appears that much information still remains to be gained. The studies by Morgan *et al.* (1983*a*) and Mauger *et al.* (1985) have revealed some interesting aspects of the short-term effects of these hormones on the Ca^{2+} changes that are induced. However, our use of the perfused liver system, incorporating the Ca^{2+}-selective electrode, provides a means of monitoring these Ca^{2+}-flux changes over an extended period.

The data in Fig. 1 show typical Ca^{2+}-selective-electrode traces of the Ca^{2+}-flux changes that are induced when cyclic AMP is administered (*a*) after, (*b*) at the same time as, and (*c*) before, the infusion of phenylephrine, vasopressin and angiotensin to the perfused liver. Exogenous cyclic AMP was used instead of the cyclic AMP-producing hormone glucagon, in order to eliminate possible secondary effects of glucagon on the plasma membrane. The use of the Ca^{2+}-selective electrode to monitor the response continuously over a long period of time (approx. 1 h) reveals several important features of the modulation of the hormone-induced Ca^{2+} response by cyclic AMP, which were not revealed in the earlier work of Morgan *et al.* (1983*a*).

First, in each instance the stimulation of Ca^{2+} influx is transient and is dependent on the order of cyclic AMP and hormone administration (cf. Figs. 1*a*, 1*b* and 1*c*). Preliminary experiments showed that near-maximal stimulation of Ca^{2+} uptake by the liver occurred when the infusion of cyclic AMP (0.2 mM) preceded the infusion of hormone by about 4 min (results not shown). The maximum rate of Ca^{2+} uptake achieved by a 4 min pretreatment with 0.2 mM-cyclic AMP was 380–420 nmol/min per g of liver for both vasopressin and angiotensin, and 270–300 nmol/min per g of liver for phenylephrine (see Fig. 1*c* and Table 1). In each of these situations the maximum rate of Ca^{2+} uptake was achieved after about 90 s of hormone infusion. Furthermore, the uptake response was transient but nevertheless dependent on the co-administration of both cyclic AMP and hormone, as the removal of either agent resulted in a cessation of Ca^{2+} uptake within 2–5 min (results not shown). By contrast, the prolonged co-administration of cyclic AMP and hormone resulted in an uptake response which was nearly complete only after about 20 min. By this time the net amount of Ca^{2+} uptake by the liver was 3850 ± 330 nmol/g of liver for each of vasopressin and angiotensin, and 1250 ± 160 nmol/g of liver for phenylephrine. These Ca^{2+} changes are much greater than those induced by the administration of Ca^{2+}-mobilizing hormones alone, about 140 nmol/g of liver (see Altin & Bygrave, 1985). A 2 min administration of hormone (with 4 min pre-administration of cyclic AMP) resulted in an uptake of Ca^{2+} corresponding to only about 40% of these values (results not shown). The corresponding

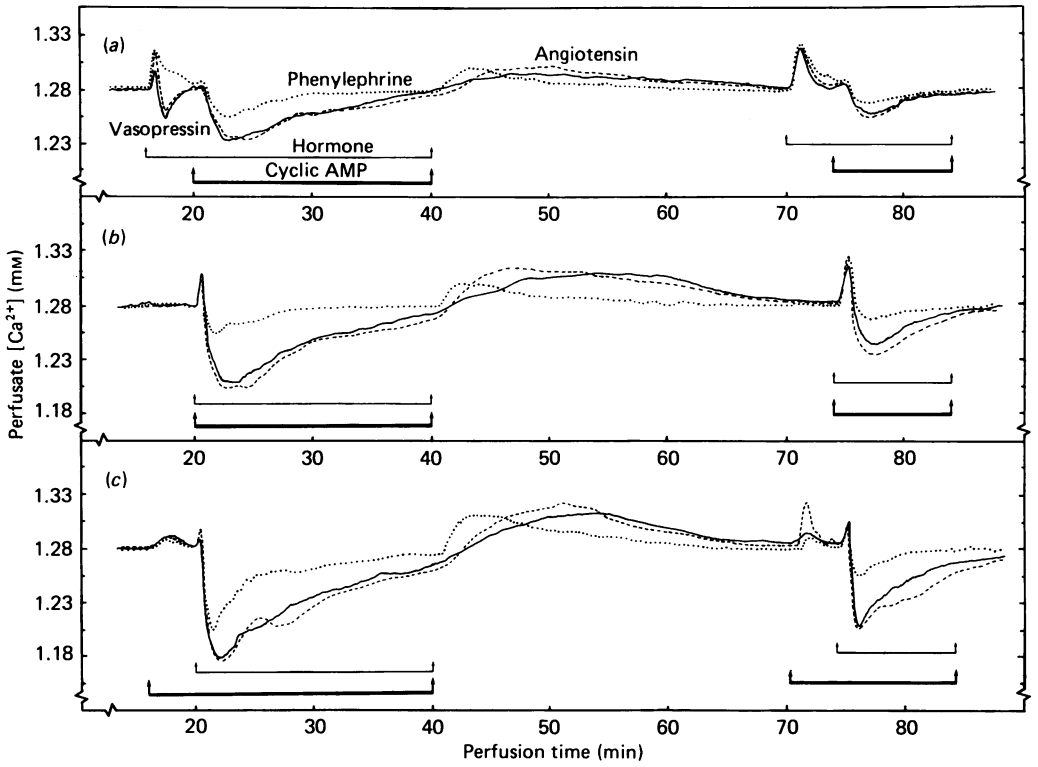

Fig. 1. Effect of cyclic AMP on the Ca^{2+} response induced by the administration of Ca^{2+}-mobilizing hormones to the perfused liver

Livers of fed rats were perfused with Krebs–Henseleit bicarbonate medium containing 1.3 mM-Ca^{2+}. The Ca^{2+} concentration of the effluent perfusate was monitored continuously with a Ca^{2+}-selective electrode. See the Experimental section for further details. After an equilibration period of about 15 min, both cyclic AMP (0.2 mM) and a Ca^{2+}-mobilizing hormone were co-administered by separate infusion syringes for the times indicated by the bold and the thin arrowed bars respectively. The traces show the Ca^{2+} response that is induced by the co-administration of cyclic AMP with vasopressin (10 nM) (——), angiotensin (10 nM) (------) or phenylephrine (2 μM) (· · · ·). The traces in (*a*), (*b*) and (*c*) represent experiments for which the infusion of vasopressin, angiotensin or phenylephrine was commenced 4 min before, at the same time as, or 4 min after, the infusion of cyclic AMP, respectively. Because a non-recirculating perfusion system was used, pen deflection above an imaginary baseline at 1.28 mM-Ca^{2+} represents Ca^{2+} efflux, whereas deflection of the pen below the baseline represents Ca^{2+} influx. Each trace is a representative of three to five experiments performed independently. Differences between the Ca^{2+} influx induced by phenylephrine and that induced by vasopressin and angiotensin became less obvious, whereas the Ca^{2+} response induced by vasopressin and angiotensin was essentially unchanged, when glucagon (10 nM) was used instead of cyclic AMP.

Table 1. Maximum rates of Ca^{2+} influx and Ca^{2+} efflux, and the corresponding net amounts of Ca^{2+} influx and Ca^{2+} efflux, induced by the co-administration of cyclic AMP (0.2 mM) and maximal doses of vasopressin, angiotensin and phenylephrine

Liver perfusions were conducted exactly as described in the legend to Fig. 1. The Table gives the magnitude of the Ca^{2+}-flux changes that are induced after the first cycle of hormone administration and hormone removal in each Ca^{2+}-selective-electrode trace in Figs. 1(*a*), 1(*b*) and 1(*c*). The maximum rate of Ca^{2+} influx or Ca^{2+} efflux was calculated from the maximum deflection of the pen from the baseline, and the maximum net amount of Ca^{2+} influx and Ca^{2+} efflux was computed from the areas above or below the relevant section of curve, respectively. Values are means ± S.E.M. for three to five independent experiments. Values in parentheses show the maximum rates, in nmol/min per g of liver; the S.E.M. is omitted for clarity, but was always less than 10%.

Order of administration of agents	Influx (nmol of Ca^{2+}/g of liver)	Efflux (nmol of Ca^{2+}/g of liver)
Cyclic AMP before vasopressin	3850 ± 330 (395)	1470 ± 250 (112)
Vasopressin with cyclic AMP	2350 ± 240 (250)	1300 ± 180 (76)
Vasopressin before cyclic AMP	1170 ± 150 (160)	690 ± 90 (52)
Cyclic AMP before angiotensin	3985 ± 350 (408)	2150 ± 210 (140)
Angiotensin with cyclic AMP	2755 ± 235 (286)	1790 ± 105 (113)
Angiotensin before cyclic AMP	1860 ± 214 (190)	1160 ± 120 (95)
Cyclic AMP before phenylephrine	1250 ± 160 (284)	1330 ± 145 (90)
Phenylephrine with cyclic AMP	540 ± 38 (66)	312 ± 26 (51)
Phenylephrine before cyclic AMP	307 ± 26 (86)	180 ± 18 (43)

average maximum change in perfusate Ca^{2+} concentrations was approx. 120 μM for vasopressin and angiotensin. These changes are greater than those described in the experiments of Morgan *et al.* (1983*a*), where the average change in perfusate Ca^{2+} concentration was measured to be 70 μM.

As shown in Figs. 1(*b*) and 1(*c*) and Table 1, both the maximum rates and the net amounts of Ca^{2+} uptake were considerably decreased, to about 70% and 40%, when cyclic AMP was administered at the same time as, or 4 min after, the hormones, respectively. It is relevant that administration of cyclic AMP alone induced only a relatively small amount of Ca^{2+} efflux (< 50 nmol/g of liver), sometimes followed by a smaller amount of Ca^{2+} influx (< 15 nmol/g of liver). Similar results were obtained by using glucagon (10 nM) instead of cyclic AMP; however, with glucagon both the net amount and the maximum rate of Ca^{2+} influx tended to be 10–15% greater, probably owing to the higher intracellular cyclic AMP concentrations that are generated (results not shown). These data thus suggest that glucagon and its second messenger, cyclic AMP, interact synergistically with Ca^{2+}-mobilizing hormones in stimulating Ca^{2+} uptake by the liver. A similar conclusion was reached by Mauger *et al.* (1985), who studied the stimulation of $^{45}Ca^{2+}$ uptake by hepatocytes. Further, our results clearly demonstrate that the degree of stimulation is dependent on the temporal relationship between the hormone–receptor interaction and the cyclic-AMP-triggered events that may be involved. Also, the ability of cyclic AMP plus phenylephrine to stimulate Ca^{2+} influx is only about 30–40% of that of cyclic AMP plus vasopressin, and cyclic AMP plus angiotensin, under otherwise identical experimental conditions (see Fig. 1 and Table 1).

A second important feature of the Ca^{2+} response induced by the co-administration of cyclic AMP with phenylephrine, vasopressin or angiotensin is that the Ca^{2+}-uptake phase of the response (which lasts for about 20 min for a prolonged administration of hormones) is followed by a more gradual efflux of Ca^{2+} from the liver once the hormones are removed. Both the time over which the Ca^{2+} effluxes, and the maximum rate at which the efflux occurs, appear to be dependent on the amount of Ca^{2+} previously accumulated. For a prolonged stimulation by cyclic AMP plus hormone (20 min), which results in a large uptake of Ca^{2+} (approx. 3850 nmol/g of liver with vasopressin and angiotensin, for example), the maximum rate of Ca^{2+} efflux is 90–140 nmol/min per g of liver (see Fig. 1 and Table 1). This decreases to less than 20 nmol/min per g of liver 30 min after removal of the stimulus. At this time a similar Ca^{2+}-uptake response again can be induced by hormone administration, suggesting that, insofar as Ca^{2+} uptake is concerned, no permanent liver damage has occurred (see Fig. 1). It is also noteworthy that the time taken to reach a maximum rate of Ca^{2+} efflux after the removal of stimulation differs with each hormone: about 2 min for cyclic AMP plus phenylephrine, 6–10 min for cyclic AMP plus angiotensin, and 10–15 min for cyclic AMP plus vasopressin. This suggests that the characteristic effect of each hormone continues to be registered by the liver for some considerable time after the removal of hormone stimulation.

Despite the characteristic differences in the time

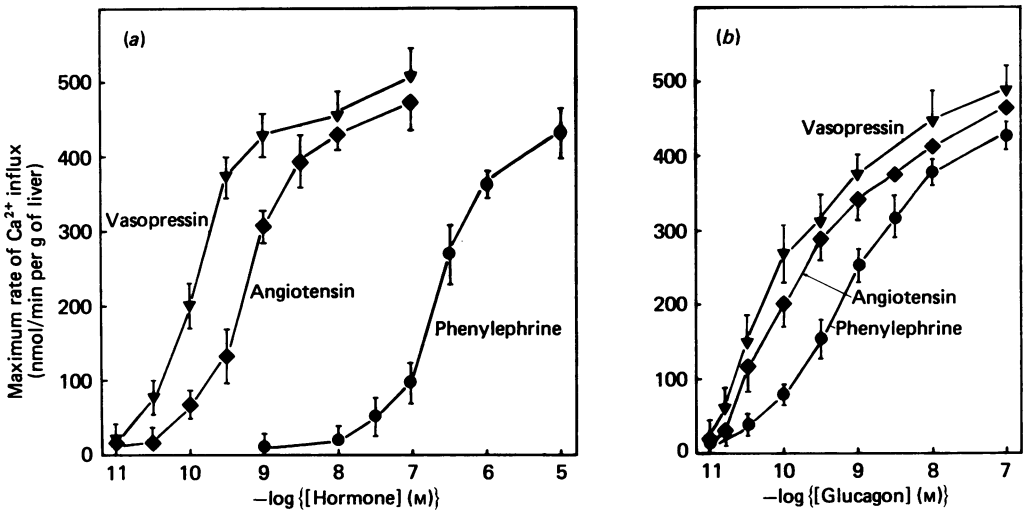

Fig. 2. Dose–response: effect of varying the concentration of Ca^{2+}-mobilizing hormone (*a*) or glucagon (*b*) on the maximum rate of Ca^{2+} influx

Liver perfusions were conducted as described in the legend to Fig. 1. At 10 min of perfusion, vasopressin, angiotensin or phenylephrine was infused for 2 min. This was done to avoid the spontaneous influx of Ca^{2+} that is normally observed with a prior administration of these hormones (Altin & Bygrave, 1985). At 25 min of perfusion, glucagon was infused at the concentration indicated. At 29 min of perfusion (4 min later), vasopressin, angiotensin or phenylephrine was also infused. The perfusate Ca^{2+} concentration was continuously monitored with a Ca^{2+}-selective electrode as previously described. Each point denotes the maximum rate of Ca^{2+} uptake that is induced at the different concentrations of either Ca^{2+}-mobilizing hormone (*a*) or glucagon (*b*). For all the points in (*a*), a maximal dose of glucagon (10 nM) was used; conversely, for all points in (*b*) maximal doses of vasopressin and angiotensin (10 nM) and phenylephrine (2 μM) were used. In experiments where phenylephrine was used, the specific α_2-adrenergic receptor antagonist yohimbine (5 μM) was co-infused with glucagon for 4 min before the infusion of phenylephrine to inhibit α_2-receptor binding. Each point represents the mean ± S.E.M. of three to six independent experiments conducted at each particular concentration of hormone.

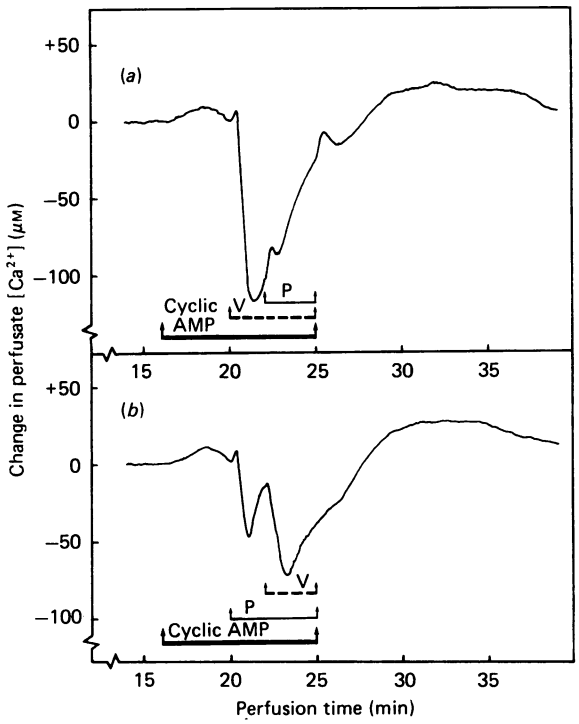

Fig. 3. Effect of the successive infusion of two different Ca^{2+}-mobilizing hormones on the Ca^{2+}-uptake response potentiated by cyclic AMP

Livers of fed rats were perfused with Krebs–Henseleit bicarbonate medium containing 1.3 mM-Ca^{2+}. The perfusate Ca^{2+} concentration was monitored continuously with a Ca^{2+}-selective electrode. At 16 min of perfusion, cyclic AMP (10 mM) was administered by infusion syringe, as indicated by the bold line. At 20 min and 22 min of perfusion, vasopressin (V; 10 nM) and phenylephrine (P; 10 μM) were also infused, as indicated by the broken and thin lines respectively. The resulting trace of the Ca^{2+} response is shown in (*a*). The trace in (*b*) shows the Ca^{2+} response for a similar experiment in which the order of infusion of vasopressin and angiotensin was reversed. Each trace is a representative of three independent experiments. Almost identical results were obtained when angiotensin (10 nM) was used instead of vasopressin.

required to reach the maximum rate of Ca^{2+} efflux after the removal of hormone stimulation, these results appear to suggest that the extrusion of Ca^{2+} continues for the period of time necessary to re-establish the initial steady-state concentrations of Ca^{2+} before the effects of the hormones. It is only after this time has elapsed that a second response, identical with the first, can be induced. A tendency towards this effect can be seen after the second period of hormonal challenge in Figs. 1(*a*), 1(*b*) and 1(*c*). Again, similar results were obtained when glucagon (10 nM) was administered instead of cyclic AMP. These results indicate that, even after the co-administration of maximal doses of these hormones, the liver has a capacity for regulating the Ca^{2+}-transport processes necessary to re-establish a basal level of physiological response and metabolic activity once the hormones are removed.

Dose–response of the synergistic stimulation of Ca^{2+} influx by glucagon and Ca^{2+}-mobilizing hormones

In order to gain evidence as to whether the potentiation by glucagon (and cyclic AMP) of the Ca^{2+}-uptake response induced by phenylephrine, vasopressin and angiotensin could be of physiological significance, dose–response measurements were carried out. The data in Fig. 2 show the maximum rate of Ca^{2+} uptake that is induced by phenylephrine, vasopressin and angiotensin as a function of the concentration of these hormones (Fig. 2*a*), or the concentration of glucagon (Fig. 2*b*). Each concentration point represents a separate experiment in which glucagon (at the specified concentration) was administered 4 min before the infusion of phenylephrine, vasopressin or angiotensin.

Two features can be seen from the dose–response curves in Fig. 2. Firstly, the potentiation of Ca^{2+} influx by glucagon occurs at physiological concentrations of the hormone (0.1–0.01 nM). Secondly, vasopressin and angiotensin appear to be more sensitive to low concentrations of glucagon (and presumably cyclic AMP) than is phenylephrine in stimulating Ca^{2+} uptake. Further confirmation of the second effect is shown in Fig. 3(*b*), which shows that, after pre-treatment with a submaximal concentration of cyclic AMP (2 μM), both vasopressin and angiotensin can elicit further uptake of Ca^{2+} by the liver after the administration of a maximal dose of phenylephrine (10 μM). However, phenylephrine is incapable of inducing further Ca^{2+} uptake if it is administered after the administration of either vasopressin or angiotensin (Fig. 3*a*).

The sink for the spontaneous influx of Ca^{2+} stimulated by vasopressin and angiotensin

Our previous finding that the administration of vasopressin and angiotensin to the perfused rat liver leads to a spontaneous net uptake of Ca^{2+} (Altin & Bygrave, 1985), and also that glucagon and cyclic AMP can potentiate this response (Morgan *et al.*, 1983*a*; the present work), raises the question of the intracellular 'sink' for this Ca^{2+}, especially since such large amounts of the ion appear to be mobilized. The data in Fig. 4 show the total calcium content of each of eight subcellular fractions obtained by Percoll-density-gradient subfractionation of the liver. Comparison of the calcium content of fractions obtained from control (Fig. 4*a*) and hormone-treated animals (Figs. 4*b* and 4*c*) indicates that the greatest change in calcium content that is induced by the hormones occurs in fraction 8. This fraction previously has been shown (Reinhart *et al.*, 1982*a*) to contain the highest proportion of the total cytochrome *c* oxidase activity, a marker enzyme for mitochondria. This result thus provides evidence that the Ca^{2+} influx stimulated by the hormones (see Fig. 1) leads to an accumulation of Ca^{2+} by the mitochondria, and is consistent with the findings by Morgan *et al.* (1983*a*), who conducted similar measurements on hormone-treated hepatocytes.

It is pertinent to point out that, although the calcium content of mitochondria from hormone-treated liver (approx. 50 nmol/mg of protein) is higher than the 16 nmol/mg of protein obtained by Morgan *et al.* (1983*a*), we believe that this is still an underestimate. This is because at such high calcium contents it can be expected that there will be calcium loss from the

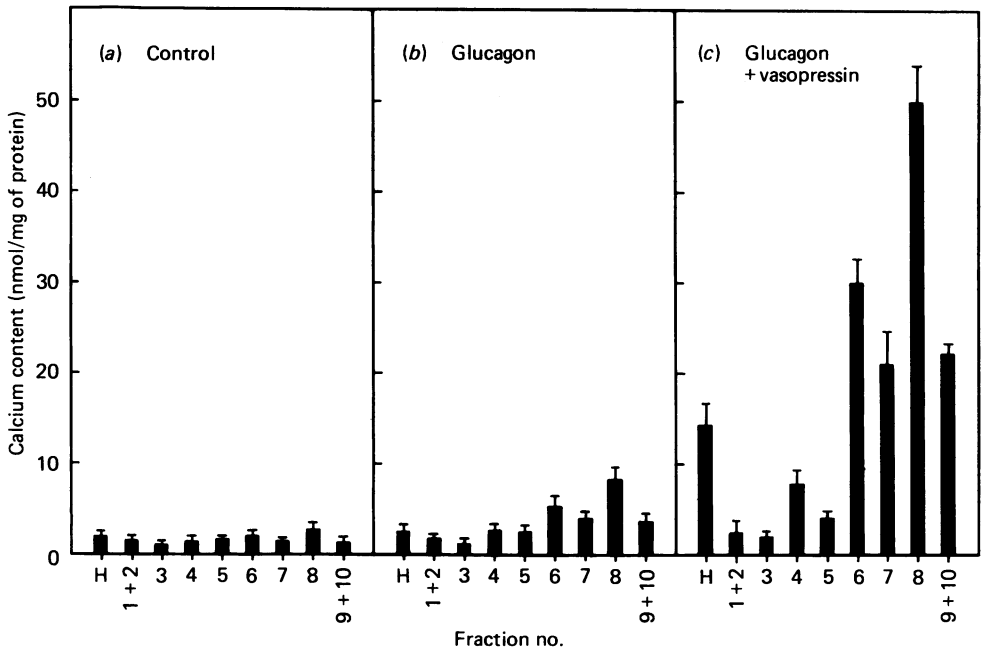

Fig. 4. Calcium content of subcellular fractions prepared from perfused rat liver after the administration of glucagon and vasopressin

Livers were perfused with Krebs–Henseleit medium, the 1.3 mM-Ca^{2+} being infused separately into the portal cannula by infusion syringe. At 15 min and 19 min of perfusion respectively, glucagon (10 nM) and vasopressin (10 nM) were also infused. At 39 min of perfusion the infusion of both hormones and 1.3 mM-Ca^{2+} was terminated and immediately replaced with 50 μM-EGTA (see Reinhart *et al.*, 1984*a*). After exactly 2 min the liver was excised and rapidly homogenized and then fractionated on a single-step Percoll gradient as indicated in the Experimental section. Of the ten fractions obtained (see Reinhart *et al.*, 1982*a*), fractions 1 and 2, and fractions 9 and 10, were each combined for convenience. All fractions were then assayed for calcium content by atomic-absorption spectroscopy; the results are shown in (*c*). The column marked 'H' represents the calcium content of the homogenate before fractionation. The results for the calcium contents of the liver fractions obtained from the corresponding control and glucagon-treated liver are shown in (*a*) and (*b*) respectively. The calcium content of fractions obtained from vasopressin-treated liver was not significantly different from that of control liver (results not shown). It should be noted that, although the amount of calcium/mg of protein in fraction 7, and fractions 9 and 10 combined, is relatively large (probably because of contamination with mitochondria), the total amount of calcium in these fractions is small, owing to their low protein content (see Reinhart *et al.*, 1982*a*). The results are means ± S.E.M. for three independent experiments.

mitochondria during the isolation procedure itself, especially since the isolation medium contained 20 μM-EGTA. On the basis of the amount of Ca^{2+} uptake shown in Table 1, and assuming 60 mg of mitochondrial protein/g wet wt. of liver and that most of the Ca^{2+} taken up is accumulated by mitochondria, our results suggest that the calcium content of mitochondria from hormone-treated liver could be as high as 60–70 nmol/mg of protein. Since the calcium content of mitochondria from control liver appears to be around 1–2 nmol/mg of protein (Reinhart *et al.*, 1984*a*; Somlyo *et al.*, 1985), we conclude that in the perfused liver the synergistic action of glucagon (of cyclic AMP) with either vasopressin or angiotensin can lead to an increase in the calcium content of mitochondria to a value representing some 20–50 times that of control mitochondria.

In order to demonstrate the involvement of mitochondria in sinking the hormone-induced Ca^{2+} uptake *in situ*, we have examined the effect of respiratory-chain inhibitors on the Ca^{2+}-uptake response. The trace in Fig. 5(*a*) shows that the infusion of NaN_3 (5 mM) is followed by a net release of approx. 200 nmol of Ca^{2+}/g of liver, presumably from the mitochondria. This Ca^{2+} is then taken up when the NaN_3 is removed. The co-administration of NaN_3 (5 mM) with glucagon does not significantly affect the NaN_3-induced Ca^{2+} release (Fig. 5*b*). However, subsequent administration of either vasopressin or angiotensin, with or without glucagon pre-treatment, results in a complete abolition of the net uptake of Ca^{2+} that is normally associated with the administration of these hormones (cf. Figs. 1 and 5). As judged by manometric pressure measurements, there were no significant vasodilatory effects of the infusion of NaN_3. Similar results, but with slightly less effect, were obtained with antimycin A (5 μM); however, the use of NaN_3 in these experiments was more convenient, as it is water-soluble and its effects seemed to be rapidly reversed once its infusion was terminated. Although a possible inhibitory effect of these inhibitors on Ca^{2+} transport across the plasma membrane, or secondary effects owing to the possible lowering of cellular ATP concentrations, cannot be eliminated, these results are nevertheless consistent with the findings of Morgan *et al.* (1983*a*) using the mitochondrial inhibitors oligomycin, carbonyl cyanide *m*-chlorophenylhydrazone and antimycin A in isolated hepatocytes, and also with the fractionation studies detailed above, which implicate the mitochondria as the sink for the observed influx of Ca^{2+}.

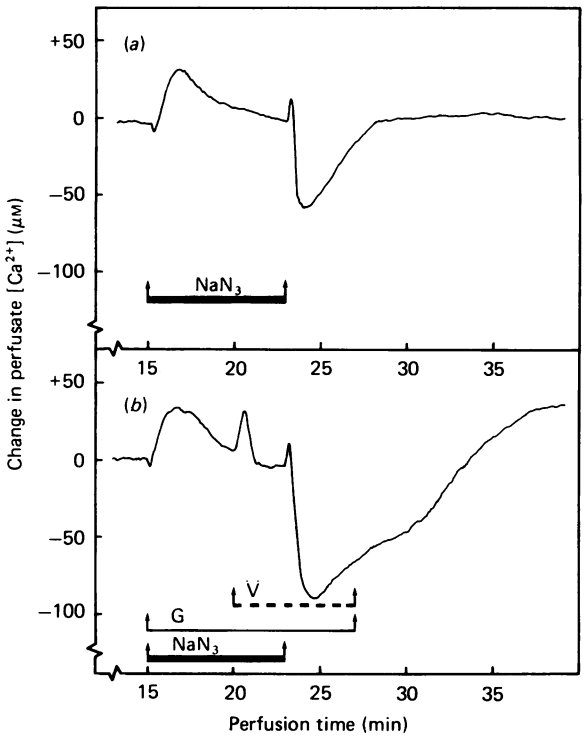

Fig. 5. Effect of NaN_3 on the Ca^{2+}-uptake response induced by the co-administration of glucagon and a Ca^{2+}-mobilizing hormone

Perfusions were conducted as described in the legend to Fig. 1. At 15 min of perfusion, NaN_3 (5 mM) was infused for the times indicated by the bold lines. The resulting trace of the Ca^{2+} response, obtained with the Ca^{2+}-selective electrode, is shown in (*a*). The trace in (*b*) shows the Ca^{2+} response obtained when NaN_3 was co-administered with maximal concentrations of glucagon (10 nM; thin line; G), followed by vasopressin (10 nM; broken line; V) 5 min later. Each trace is a representative of three independent experiments.

DISCUSSION

The present work demonstrates that glucagon, and its intracellular messenger cyclic AMP, can exert quite profound effects on the Ca^{2+}-flux changes induced by Ca^{2+}-mobilizing hormones in the intact liver perfused with 1.3 mM-Ca^{2+}. Although some evidence for the potentiation of the effects of Ca^{2+}-mobilizing hormones by glucagon and cyclic AMP has previously been reported (Friedmann & Park, 1968; Morgan *et al.*, 1983*a*), hitherto it appears that no detailed studies of the combined effects of these hormones on long-term Ca^{2+}-flux changes have been conducted. The perfused rat liver is an ideal system for such studies, as it allows for an integrated approach to many facets of Ca^{2+} mobilization and hormone action in near-physiological conditions. The data obtained with the Ca^{2+}-selective electrode (Fig. 1) show the magnitude and the temporal relationship of the Ca^{2+} fluxes that occur after the combined administration of cyclic AMP (or the cyclic-AMP-producing hormone, glucagon) and the Ca^{2+}-mobilizing hormones phenylephrine, vasopressin and angiotensin. Several important features of the combined effect of these hormones on Ca^{2+} fluxes that were not previously reported are revealed.

The first observation relates to the magnitudes of the maximum net amount, and maximum net rate, of Ca^{2+} uptake that occurs after the administration of both cyclic-AMP-dependent and Ca^{2+}-mobilizing hormones. These Ca^{2+} changes are over an order of magnitude greater than the Ca^{2+}-flux changes induced by phenylephrine, vasopressin and angiotensin alone, approx. 140 nmol/g of liver (see Reinhart *et al.*, 1982*b*; Altin & Bygrave, 1985). The results clearly demonstrate the importance of 'communication' between the cyclic AMP-dependent and Ca^{2+}-dependent pathways under physiological conditions where it is conceivable that both cyclic AMP-dependent and Ca^{2+}-mobilizing hormones can be present simultaneously.

The possible relevance of these effects *in vivo* is evident from the dose–response curves in Fig. 2, which show that the effects are apparent even at physiological concentrations of glucagon and Ca^{2+}-mobilizing hormones (Buhler *et al.*, 1978). More importantly, the dose–response curves, together with the finding that maximal concentrations of phenylephrine, vasopressin and angiotensin display different abilities to induce Ca^{2+} influx when co-administered with a relatively low concentration of cyclic AMP (10 μM) (Fig. 5), appear to suggest that the difference in the ability of these hormones to induce Ca^{2+} influx when administered alone (Altin & Bygrave, 1985) may be due to a greater sensitivity to endogenous cyclic AMP of the specific events triggered by vasopressin and angiotensin. It is envisaged that such events may involve the phosphorylation of the Ca^{2+} transporter or Ca^{2+} channel across the plasma membrane by some cyclic AMP-dependent protein kinase(s) (Vargas *et al.*, 1982; Reuter, 1983; Bean *et al.*, 1984; Mauger *et al.*, 1984).

Differences in the ability of phenylephrine, vasopressin and angiotensin to stimulate Ca^{2+} influx (see Figs. 1 and 5) are less evident when glucagon (10 nM) is administered instead of cyclic AMP in the range 10 μM–0.2 mM (results not shown). However, since the intracellular cyclic AMP concentrations generated by glucagon (10 nM) through activation of adenylate cyclase are above the range, it can be presumed that, under these conditions, the binding of phenylephrine to α_1-adrenergic receptors is further enhanced (Morgan *et al.*, 1984). It is therefore possible that the observed differences may be associated with a greater number of 'active' receptors for vasopressin and angiotensin on the liver cell plasma membrane, and consequently, the ability for these hormones to induce a greater stimulation of phosphoinositide breakdown, a larger accumulation of inositol 1,4,5-trisphosphate (Lynch *et al.*, 1985), or a higher increase in the cytosolic Ca^{2+} concentration (Charest *et al.*, 1985; Binet *et al.*, 1985), by comparison with phenylephrine. A direct involvement of phosphoinositide breakdown, or any metabolite thereof, in the stimulation of Ca^{2+} influx has yet to be demonstrated, however. Alternatively, the differences may be more directly linked to individual differences in the nature of the hormone–receptor interaction that is involved between phenylephrine, vasopressin and angiotensin and their specific receptors, perhaps involving specific hormone-operated Ca^{2+} channels (Berridge, 1982). This area requires further investigation.

Another finding from the present work is that the order of administration of glucagon (or cyclic AMP) and

a Ca^{2+}-mobilizing hormone can significantly affect both the maximum net rate and the maximum net amount of Ca^{2+} influx that is induced. Although no dependence on the order of addition was observed in the work of Morgan *et al.* (1983*a*), our results appear to be consistent with the more recent work by Mauger *et al.* (1985), which suggests that the initial rate of $^{45}Ca^{2+}$ uptake by hepatocytes is less when vasopressin is added 1 min before glucagon than when vasopressin and glucagon are added at the same time. Since the dependence of Ca^{2+} influx in the order of hormone addition occurs even when exogenous cyclic AMP is used instead of glucagon (Fig. 1, the present work), it seems that the effect is not due to the inhibition of glucagon-induced cyclic AMP accumulation by vasopressin (see Morgan *et al.*, 1983*b*), but instead is probably related to the sequential phosphorylation steps that may be involved in the stimulation of Ca^{2+} transport across the plasma membrane.

The correlation of Ca^{2+}-flux changes with an increase in the calcium content of fractions enriched in mitochondria obtained by Percoll-density-gradient subfractionation of the liver after hormonal challenge strongly indicates that the Ca^{2+} taken up by the liver is accumulated by the mitochondria, a conclusion consistent with that of Morgan *et al.* (1983*a*). The data also are consistent with previous observations from our and other laboratories that the administration of glucagon *in vivo* to rats, or to the perfused rat liver, greatly enhances the ability of the subsequently isolated mitochondria to retain Ca^{2+} (Hughes & Barritt, 1978; Prpic *et al.*, 1978; Andia-Waltenbaugh *et al.*, 1978, 1981; Prpic & Bygrave, 1980; Taylor *et al.*, 1980).

Mauger *et al.* (1985), who studied the stimulation of $^{45}Ca^{2+}$ uptake induced by the combined addition of glucagon and Ca^{2+}-mobilizing hormones using isolated hepatocytes, concluded that plasma-membrane Ca^{2+} channels are the principal sites affected. Morgan *et al.* (1983*a*) have suggested that the mitochondrial membrane is the likely control point for the Ca^{2+} influx stimulated by glucagon and Ca^{2+}-mobilizing hormones, since the Ca^{2+} uptake can be abolished by inhibitors of mitochondrial respiration. Our work indicates that the prolongation of the uptake response is dependent on the continued presence of both glucagon (or cyclic AMP) and a Ca^{2+}-mobilizing hormone (results not shown). Since the hormones are known to interact with receptors at the plasma membrane which also contain the necessary transporters to permit the entry of Ca^{2+}, it appears to us that the plasma membrane would be the least energy-demanding control point to regulate the influx of Ca^{2+}. However, our data show that a prolonged stimulation with these hormones results in a transient decrease in the rate of Ca^{2+} influx, which decays even while the hormones are still being administered (Fig. 1). This may be an indication that, under these conditions, the calcium content of the mitochondria has reached saturation. We therefore argue that both the plasma membrane and the mitochondrial membrane are involved in regulating the Ca^{2+}-uptake response.

A final important aspect of the present work relates to the magnitude of Ca^{2+} taken up by the mitochondria in the intact liver. Stimulation with near-maximal concentrations of glucagon (or cyclic AMP) and a Ca^{2+}-mobilizing hormone results in a 20–60-fold increase in mitochondrial calcium content (to approx. 60 nmol/mg of mitochondrial protein; see Fig. 4). Whether this increase is relevant to the activation of mitochondrial-matrix enzymes (Denton & McCormack, 1985; McCormack, 1985*a*,*b*) remains to be established. This appears to be the first time that mitochondria have been shown to accumulate such large amounts of Ca^{2+} in a reversible manner in the intact liver, while at the same time maintaining (or recovering) apparent full integrity of mitochondrial and cellular function. These findings thus seem to be important in many pathological states involving calcification. Hence, as well as its usefulness in the study of the ways in which cells regulate mitochondrial Ca^{2+} fluxes *in vivo*, it appears that this could be a useful system with which to study the ability of mitochondria to tolerate Ca^{2+} reversibly in conditions of cellular toxicity that are associated with cellular necrosis or cell death.

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