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Concomitant stimulation by vasopressin of biliary and perfusate calcium fluxes in the perfused rat liver

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Changes in perfusate Ca^{2+} (measured with a Ca^{2+}-selective electrode) and changes in bile calcium (measured by atomic absorption spectroscopy) were continuously and simultaneously monitored after infusion of (*a*) vasopressin, (*b*) glucagon and (*c*) both vasopressin and glucagon together to the perfused rat liver. Also monitored were perfusate glucose and oxygen concentrations and bile flow. Vasopressin induces a sharp, transient, pulse of increased bile flow and increased bile calcium within 1 min of infusion, concomitant with rapid changes in perfusate Ca^{2+} fluxes, glucose output and oxygen uptake. This is immediately followed by a decrease in both bile flow and bile calcium for as long as the hormone is administered. Changes induced by glucagon are a relatively slow onset of perfusate Ca^{2+} efflux and oxygen uptake, but rapid glucose output, and a small but significant and transient decrease in bile flow and bile calcium which, despite the continued infusion of the hormone, spontaneously and rapidly returns to normality. However, the greatest responses are observed after co-administration of both hormones. Coincident with the augmented perfusate Ca^{2+} fluxes (influx) seen in earlier work, there occurs within 1 min of vasopressin infusion a sharp increase in bile secretion and bile calcium greater in magnitude than that produced by vasopressin alone. Immediately thereafter bile secretion and bile calcium decline below basal values and remain there for as long as the hormones are administered. Glucagon and vasopressin therefore each have opposing effects on bile flow and bile calcium. However, the action of vasopressin is enhanced by the prior administration of glucagon. The data thus reveal features about the actions of glucagon and Ca^{2+}-mobilizing hormones on bile flow and bile calcium not previously recorded and provide a novel framework around which the whole issue of hepato-biliary Ca^{2+} homoeostasis can be assessed in normal and diseased liver.

INTRODUCTION

Changes in the cytoplasmic free Ca^{2+} concentration play a vital role in the metabolic network of most cell types. These changes result from movements of the ion between the cell and its external environment and between the cytoplasm and intracellular organelles (reviewed by Carafoli, 1987; Dawson, 1990; Kraus-Friedmann, 1990). Among the various stimuli that induce changes in the cytoplasmic Ca^{2+} concentration are a number of Ca^{2+}-mobilizing hormones, whose mechanism(s) of action are under investigation (for a review see Exton, 1988).

Studies in liver of the mechanism of basal and hormone-stimulated Ca^{2+} fluxes appear to have taken account mainly of movements of the ion across the (blood) sinusoidal plasma membrane. However, work with intact rats by Yamaguchi and colleagues has shown that, after a single oral dose of $CaCl_2$, there occurs a significant time-dependent increase in calcium in the bile (Yamaguchi & Sugii, 1980) that is attenuated in thyroparathyroid rats but is further elevated by calcitonin administration (Yamaguchi & Yamamoto, 1981) as well as by several other agonists (Yamaguchi & Imase, 1988). In consonance with this, Hill *et al.* (1985), using perfused rat liver, showed that adrenaline, acting through α-receptors, induces verapamil-sensitive Ca^{2+} movements across the bile-canalicular plasma membrane. Together these studies provide evidence that Ca^{2+} fluxes across the sinusoidal and bile-canalicular plasma membranes are each under hormonal control, leading to transcellular movements of the ion (Hill *et al.*, 1985). Since then others have shown that Ca^{2+}-mobilizing hormones are involved also in the regulation of the permeability of tight junctions in the perfused rat liver (Lowe *et al.*, 1988; Llopis *et al.*, 1991).

In previous studies from our laboratory, we have made extensive use of a perfused rat liver system incorporating a Ca^{2+}-selective electrode in the outflow medium (Reinhart *et al.*, 1982*a*) to examine the Ca^{2+} fluxes associated with the action of Ca^{2+}-mobilizing hormones (reviewed in Reinhart *et al.*, 1984; Altin & Bygrave, 1988*a*,*b*); this has enabled continuous and sensitive monitoring of changes in the perfusate free Ca^{2+} concentration that arise specifically from those fluxes that occur across the sinusoidal plasma membrane. The present paper is the first report of a study that we have undertaken with this perfused rat liver system in which, besides continuously monitoring changes in the perfusate Ca^{2+} concentration (Altin & Bygrave, 1986), we also have monitored the accompanying changes in bile calcium after administration of vasopressin in the absence and presence of glucagon. By comparing these changes with those in bile flow and with the associated intracellular Ca^{2+}-sensitive metabolic responses of glycogenolysis and respiration (Denton & McCormack, 1990), we have been able to gain a greater understanding not only of hepato-biliary Ca^{2+} homoeostasis but also of the role of Ca^{2+} and Ca^{2+}-mobilizing hormones in the mechanism of bile secretion. Further work is needed on the alterations to hepato-biliary Ca^{2+} homoeostasis that occur in pathological conditions such as cholestasis.

EXPERIMENTAL

Male Wistar-strain albino rats, initially weighing approx. 280 g and having free access to food, were used in all experiments. Rats were anaesthetized with sodium pentobarbitone (50 mg/kg

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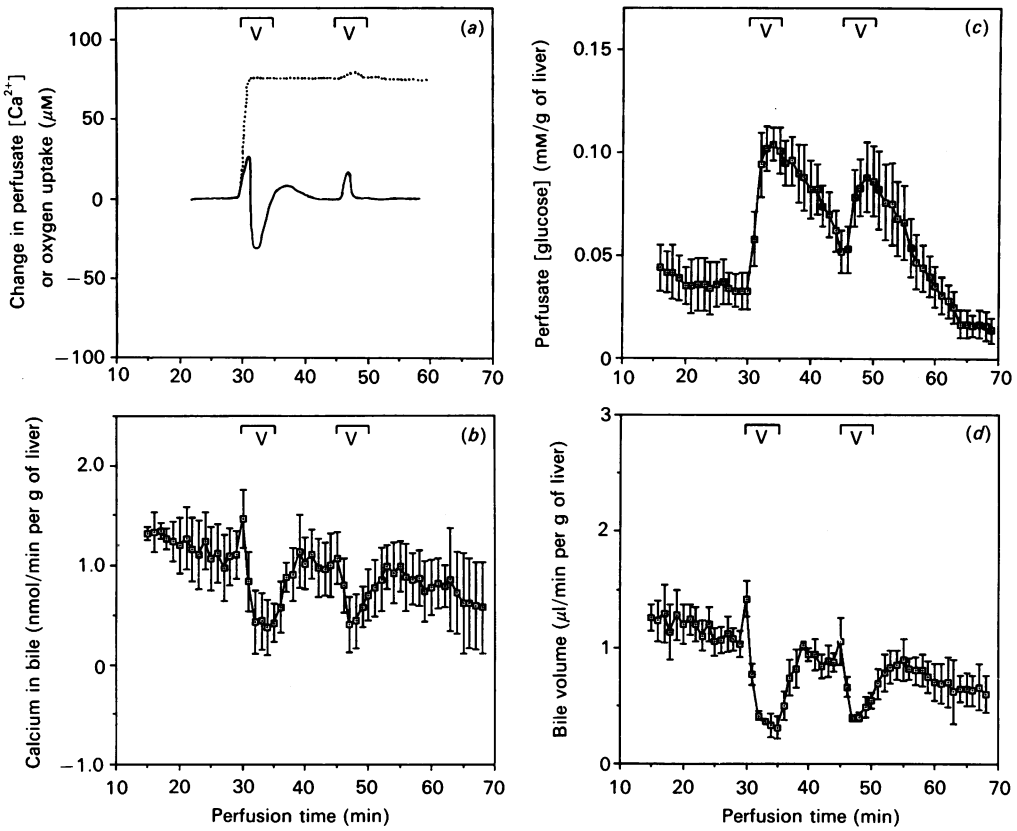

Fig. 1. Effect of vasopressin on perfusate Ca^{2+}, glucose output and oxygen uptake and on bile calcium and bile flow in the perfused rat liver

Livers were perfused with Krebs–Henseleit bicarbonate medium containing 1.3 mM-Ca^{2+}. After a pre-perfusion period of 30 min, two pulses of vasopressin (10 nM) were each infused for 5 min as indicated by 'V'. The Ca^{2+}-selective-electrode trace (——) and oxygen-electrode trace (·····) are shown in (*a*); the concentration of calcium in the bile determined by atomic absorption spectroscopy is shown in (*b*); perfusate glucose output is shown in (*c*); and bile flow is shown in (*d*). For further details see the Experimental section. Each trace is a representative of at least four experiments performed independently. The data in (*b*), (*c*) and (*d*) are means ± S.E.M. of four independent experiments.

body wt.). Considerable care was taken to ensure the animal did not suffer pain in the course of isolating the liver, which was perfused with Krebs–Henseleit (1932) bicarbonate buffer equilibrated with O_2/CO_2 (19:1) and containing 1.3 mM-$CaCl_2$. Perfusions were conducted in a non-recirculating mode, and the perfusate was delivered at a constant volume of 3.5 ml/min per g wet wt. of liver by means of an LKB 2115 peristaltic pump. For each experiment the liver was pre-perfused for 15 min before the infusion of any hormone, each of which was administered by a pump-driven infusion syringe. Additionally, a fine plastic cannula (internal diam. 0.35 mm, external diam. 1.05 mm) was inserted into the bile duct to enable collection of samples after administration of agents to the inflow cannula in the portal vein.

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; this is described in detail elsewhere (Reinhart *et al.* 1982*a*). 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).

Other measurements

Oxygen consumption and glucose release by the liver were determined as previously described (Reinhart *et al.*, 1982*a*; Altin & Bygrave, 1985). Total atomic calcium in the bile samples was measured with a Varian AA20 atomic absorption spectrophotometer. Samples were extracted with 1 M-$HClO_4$ (final concn.), and, after centrifugation in an Eppendorf Microfuge, the resulting supernatant was analysed for calcium in the presence of $SrCl_2$ (0.2% final concn.) and KCl (0.1% final concn). Bile flow was measured by weighing the bile fluid collected for each 1 min, assuming that 1 μl is equivalent to 1 mg wet wt. Calculations carried out to determine the relative lag time in each of the perfusate and bile-flow cannulae showed that these were within 5–10 s of each other.

Chemicals and materials

Hormones were obtained from Sigma Chemical Co., St Louis, MO, U.S.A. These agents were dissolved in Krebs–Henseleit buffer before infusion into the liver. Ca^{2+}-selective electrode membranes (F2112) and filling solutions (S43316) were obtained from Radiometer, Copenhagen, Denmark. Other chemicals used were of analytical-reagent 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.

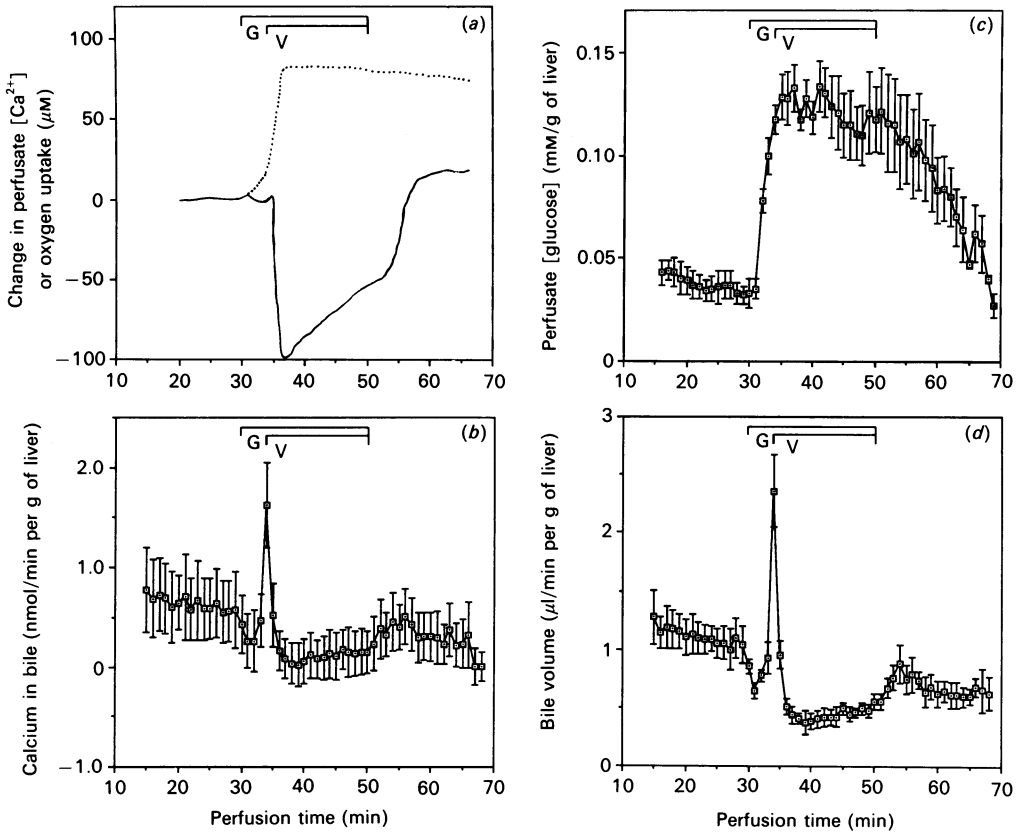

Fig. 2. Effect of the synergistic action of glucagon and vasopressin on perfusate Ca^{2+}, glucose output and oxygen uptake and on bile calcium and bile flow in the perfused rat liver

Livers were perfused with Krebs–Henseleit bicarbonate medium containing 1.3 mM-Ca^{2+}. After a pre-perfusion period of 30 min, glucagon (10 nM), was infused for the duration indicated by 'G'. Then 4 min later vasopressin (10 nM) was infused for the duration indicated by 'V'. The Ca^{2+}-selective-electrode trace (——) and oxygen-electrode trace (·····) are shown in (*a*); the concentration of calcium in the bile determined by atomic absorption spectroscopy is shown in (*b*); perfusate glucose output is shown in (*c*); and bile flow is shown in (*d*). For further details see the Experimental section. Each trace is a representative of at least four experiments performed independently. The data in (*b*), (*c*) and (*d*) are means ± S.E.M. of four independent experiments.

RESULTS

Effect of vasopressin on perfusate Ca^{2+}, glucose output and oxygen uptake and on biliary flow and calcium content

Although previous experiments from our laboratory have recorded the changes in perfusate Ca^{2+} concentration after administration of vasopressin to the perfused rat liver (reviewed in Altin & Bygrave, 1988*a*), we never have measured any concomitant biliary responses.

Fig. 1(*a*) shows the changes in perfusate Ca^{2+} and oxygen concentration after the administration of two 5 min pulses of vasopressin to the perfused rat liver. The responses are typical of those reported previously (e.g. Altin & Bygrave, 1985); immediately after the first pulse of the hormone, Ca^{2+} efflux occurs, followed by a spontaneous influx of the ion, and then a second, more gradual, efflux. A second pulse induces only a net efflux of the ion. A sustained increase in oxygen uptake occurs in response to vasopressin action, thought to result from Ca^{2+} mobilization and subsequent stimulation of mitochondrial citrate cycle activity (Denton & McCormack, 1990). Fig. 1(*c*) shows the well-documented hormone-induced increase in glucose output. Noteworthy here is the rapid increase in glucose output after the administration of each of the two pulses and the rapid decline in output in each instance once the vasopressin is removed.

By analysing samples of bile fluid taken at 1 min intervals, we were able to determine the concomitant changes in bile volume and bile calcium occurring at the same time as the changes mentioned above in the perfusate after the action of vasopressin (Figs. 1*b* and 1*d*). It is evident that immediately after vasopressin administration, and concomitant with the rapid elevation of perfusate Ca^{2+}, there occurs almost immediately, given the sampling times adopted, a highly reproducible sharp stimulation of bile flow (Fig. 1*d*) and of calcium in the bile (Fig. 1*b*). This is followed in the next 1 min by an apparent sharp diminution of biliary flow and calcium flux but each response lasts only as long as the hormone is being administered. Removal of the hormone allows biliary flow and biliary calcium to return to basal levels; a second pulse of vasopressin rapidly induces an attenuation in bile flow and bile calcium. Whether or not the decrease in bile secretion and bile calcium is attributable to the vasoconstrictive action of the hormone was not determined. The basal bile calcium concentration was 0.69 ± 0.26 mM ($n = 6$). During the first of the two transients shown in Fig. 1(*b*), the biliary calcium concentration increased by approx. 0.5 mM to just over 1 mM; the second transient of Fig. 1(*b*) did not result in any appreciable change in the biliary calcium concentration (results not shown). This compares favourably with the value of 0.35 mM obtained by Hill *et al.* (1985), who used a perfusate Ca^{2+} concentration of 1 mM in their experiments.

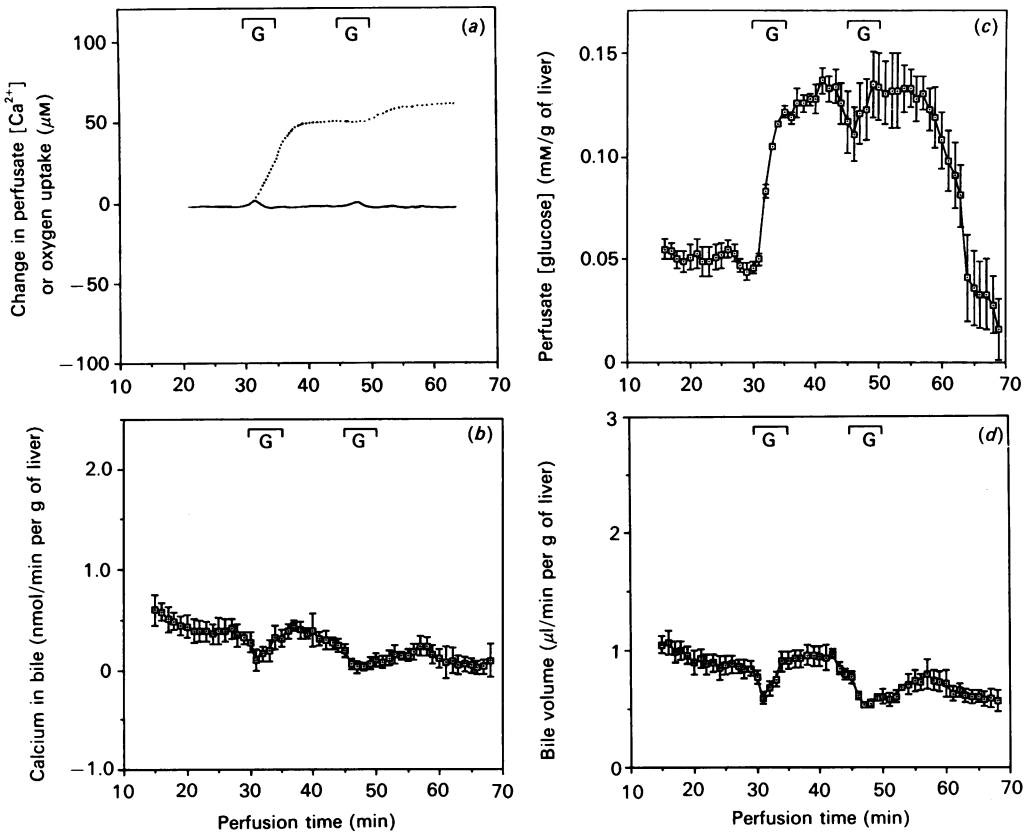

Fig. 3. Effect of glucagon on perfusate Ca^{2+}, glucose output and oxygen uptake and on bile calcium and bile flow in the perfused rat liver

Livers were perfused with Krebs–Henseleit bicarbonate medium containing 1.3 mM-Ca^{2+}. After a pre-perfusion period of 30 min, two pulses of glucagon (10 nM) were each infused for 5 min, as indicated by 'G'. The Ca^{2+}-selective-electrode trace (——) and oxygen-electrode trace (·····) are shown in (*a*); the concentration of calcium in the bile determined by atomic absorption spectroscopy is shown in (*b*); perfusate glucose output is shown in (*c*); and bile flow is shown in (*d*). For further details see the Experimental section. Each trace is a representative of at least four experiments performed independently. The data in (*b*), (*c*) and (*d*) are means ± S.E.M. of four independent experiments.

Effect of co-administration of glucagon and vasopressin on perfusate Ca^{2+}, glucose output and oxygen uptake and on biliary flow and calcium content

The experiments undertaken in Fig. 1 were repeated but under conditions that are known to lead to a more pronounced mobilization of Ca^{2+}, i.e. situations where glucagon is first administered to the liver, followed soon after by vasopressin infusion (Altin & Bygrave, 1986). Figs 2(*a*) and 2(*c*) show the changes occurring in perfusate Ca^{2+}, oxygen and glucose. As previously documented (Altin & Bygrave, 1986), glucagon administration induces a slight Ca^{2+} efflux, little oxygen uptake but a large glucose output. However, the most significant response to co-administration of vasopressin is the very small Ca^{2+} efflux, followed by the pronounced and prolonged Ca^{2+} influx. Oxygen uptake is enhanced, but little extra glucose output is observed.

The concomitant changes in biliary secretion and calcium content are shown in Figs. 2(*b*) and 2(*d*). Immediately after the administration of glucagon, a small but discernible decline in both parameters occurs. However, the most striking finding was that, immediately after vasopressin co-administration, a sharp rise in calcium secretion (Fig. 2*b*) and in bile flow (Fig. 2*d*) occurs. As with Fig. 1(*b*), the biliary calcium concentration during the transient in Fig. 2(*b*) increased by approx 0.5 mM to just over 1 mM (results not shown). The stimulation of bile flow and of bile calcium by vasopressin (Figs. 2*b* and 2*d*) is more than doubled by prior infusion of glucagon (cf. Figs. 1*b* and 2*b* and Figs. 1*d* and 2*d*); this stimulatory action thus contrasts with the attenuating action of glucagon on the initial phase of vasopressin-induced Ca^{2+} efflux in the perfusate. In another experiment (results not shown), we measured bile calcium in this early phase every 20 s and found that the peak occurred, as far as we could judge, very close to 40 s after vasopressin administration. A second important action of the glucagon is to offset partially, after this spike, the vasopressin-induced decreases in bile secretion and biliary calcium (Figs. 1*b* and 1*d*). Thus it would appear that both glucagon and vasopressin are able to influence bile flow and biliary calcium content. During the period for which the co-administration of the two hormones continues, both bile secretion and bile calcium remain slightly attenuated, but these return to basal values once the hormones are removed. A further noteworthy feature of the data is that, when one extrapolates either the rate of bile flow or calcium content before and after administration of the hormones, there appears to be little change in rate. This implies that the hormones together induced transient changes in these two parameters, particularly at the onset of their action, when Ca^{2+} release also occurs from the endoplasmic reticulum (see, e.g., Dawson, 1990).

Effects of glucagon alone on perfusate Ca^{2+}, glucose output and oxygen uptake and on biliary flow and calcium content

Data in Fig. 3 show results from experiments carried out similar to those described in Fig. 1 but with glucagon administered in place of vasopressin. Fig. 3(*a*) shows that the

effect of the hormone on perfusate Ca^{2+} is to induce a slight efflux of the ion, a finding seen consistently in other similar experimental situations (see Altin & Bygrave, 1986). An increase in oxygen uptake occurs less rapidly, as revealed in earlier work (cf. Reinhart *et al.*, 1982*b*), but the change in glucose output is expectedly significant. Of note, however, is that glucose output is slower in onset than that induced by vasopressin (cf. Figs. 1*c* and 3*c*).

Changes in biliary calcium and biliary flow are shown in Figs. 3(*b*) and 3(*d*). No increase in either parameter is observed, but there are indications of a slight but significant decrease in both bile flow and calcium content; this is more clearly evident after the second pulse of the hormone. This is evident also in the data of Fig. 2(*b*) and is unlikely to be attributable to the possible vasoconstrictive effects observed with vasopressin (cf. Fig. 1*b*).

DISCUSSION

The experiments reported in this paper are, we believe, the first to record the changes in the perfusate calcium concentration of perfused rat liver that take place simultaneously across the bile-canalicular plasma membrane on the one hand and the sinusoidal plasma membrane on the other after the action of the Ca^{2+}-mobilizing agonist vasopressin. Although the studies by Hill *et al.* (1985) were most useful in providing evidence for adrenergic-induced transcellular Ca^{2+} transport in the liver, the technique employed by them for measuring changes in perfusate calcium (atomic absorption spectroscopy) was not sufficiently sensitive to detect significant changes therein. Their use of adrenaline, which induces both α- and β-adrenergic responses, and of 3 min sampling times also would not have revealed the added information gained in the present study. In our experiments, care was taken to sample the bile in the shortest possible time intervals that allowed a sufficient volume of sample for analysis of bile volume and atomic calcium; this was 1 min. An additional set of relevant measurements made in the present work was whole-tissue respiration and glycogenolysis, each of which are Ca^{2+}-sensitive metabolic events that provide information about changes taking place in intracellular (cytoplasmic) Ca^{2+} after the action of Ca^{2+}-mobilizing hormones. Thus our experimental approach was one in which we have been able to gain information about concurrent changes in Ca^{2+} fluxes occurring on two different surfaces of the hepatocyte as well as those occurring within the cell.

Information about changes in extracellular Ca^{2+} in hepatocytes and in the perfused rat liver after the administration of Ca^{2+}-mobilizing hormones is extensive (see, e.g. Reinhart *et al.*, 1984; Altin & Bygrave, 1988*a*,*b*; Exton, 1988), but what now is evident from the work reported here and in the papers quoted in the Introduction is that such changes form only one set of several that take place across the plasma membrane. A major restriction in attempting to balance quantitatively the fluxes of calcium across each of the sinusoidal and bile-canalicular membrane surfaces, under the influence of glucagon and vasopressin action, was having to make measurements of biliary calcium by atomic absorption spectroscopy. This measures the total calcium, i.e. bound plus free, whereas that measured in the perfusate with the Ca^{2+}-selective electrode was free Ca^{2+}. Nevertheless we still are in a good position to report information previously not acquired by any group.

Our decision to take bile samples at short time intervals, although unfortunately increasing the standard error in some of the measurements of biliary calcium, proved to be particularly rewarding in that it has allowed detection of changes in bile secretion and bile calcium that otherwise would have been overlooked. Noteworthy in this respect was the detection of a sharp increase in each of these parameters well within 1 min after the administration of vasopressin to the liver. This vasopressin-induced increase was particularly evident after the 'priming' of the liver with glucagon (Figs. 2*b* and 2*d*). This event seemingly precedes the action of vasopressin on the early phase of appearance of horseradish peroxidase in the bile that occurs after passage through the tight junctions (Lowe *et al.*, 1988); this reached a peak at approx. 2 min, by contrast with the peak of bile flow and bile calcium, which occurred less than 1 min after vasopressin administration. We have assessed our biliary dead times of approx. 2.5 min to be similar to theirs. This raises the possibility that the mobilization of calcium seen in our study actually triggers the subsequent changes in permeability of the tight junctions. Kan & Coleman (1988) previously reported that the calcium ionophore A23187 increases tight-junction permeability in liver. It is noteworthy that the data of Lowe *et al.* (1988) reveal that cyclic AMP actually decreased permeability in tight junctions and that this action reached a peak some 12–15 min after its administration to the perfused rat liver.

Our work raises again (see, e.g., Mauger & Claret, 1988) the notion of glucagon being a modulator of cellular Ca^{2+} fluxes. This was manifest here by glucagon being able in effect to redirect the calcium fluxes induced by vasopressin. On its own, vasopressin induces rapid, significant, Ca^{2+} efflux into the perfusate originating from the endoplasmic reticulum as a result of $InsP_3$ formation (see, e.g., Dawson, 1990). Glucagon attenuates this efflux (cf. Figs. 1*a* and 2*a*) and concomitantly transiently redirects the flow of calcium to the bile. This in turn could be seen as a mechanism by which glucagon itself also can indirectly control bile secretion. Thus we envisage that, although calcium mobilization is basically driven by vasopressin action, glucagon modulates and directs this. Whether or not one site of action of this modulation is the attenuating action of glucagon on the CaATPase (Mallat *et al.*, 1987) remains to be determined. It is possible to imagine that the closing of the Ca^{2+}-efflux pathway at the sinusoidal plasma membrane occurs at the same time as the opening of the verapamil-sensitive Ca^{2+} channel at the bile-canalicular surface. This may relate too to the synergistic action of vasopressin and glucagon on massive and prolonged Ca^{2+} uptake by the liver (Fig. 2*a*), most of which, according to our previous experiments (Altin & Bygrave, 1986, 1988*a*), is associated with the mitochondria. An important determinant of Ca^{2+} homoeostasis, at least in liver, thus is not the mere action of Ca^{2+}-mobilizing hormones alone, but rather the fine balance between vasopressin and glucagon in respect of the timing of their action and their relative concentrations.

We cannot lose sight of the fact that glucagon itself appears to have quite rapid direct effects on bile secretion (Fig. 3*d*) apparently unrelated to its effects on gap-junction permeability (Lowe *et al.*, 1988). Indeed, our work reveals a direct correlation between glucagon-induced perfusate Ca^{2+} efflux on the one hand and glucagon-induced decreases in calcium secretion and bile flow on the other.

Since this paper was submitted for publication, we have noted that Krell *et al.* (1985) previously reported that adrenaline induces a rapid triphasic response in bile flow, similar to that observed in our study.

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