Evidence for adrenergic control of transcellular calcium distribution in liver

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Free Ca^{2+} concentration and ^{45}Ca flux were measured in the perfusate and bile of the perfused rat liver. With a perfusate Ca^{2+} concentration of 1 mM, the bile concentration was 0.35 mM. The ratio of ^{45}Ca in bile to that in blood increased from 0.3 to 0.6 over 90 min of perfusion. Both verapamil and adrenaline (via α -adrenergic receptors) increased the ^{45}Ca bile/perfusate ratio to 0.8. Adrenaline infusion increased the bile Ca^{2+} concentration to 0.8 mM. This decreased to 0.35 mM after the infusion was stopped.

Increases in cytosolic Ca^{2+} concentration mediate the metabolic effects of a variety of hormonal and non-hormonal substances in the liver (Murphy *et al.*, 1980; Williamson *et al.*, 1981; Charest *et al.*, 1983; Reinhart *et al.*, 1983). The sources of mobilizable Ca^{2+} have been suggested to be extracellular and/or intracellular (Althaus-Salzmänn *et al.*, 1980; Poggioli *et al.*, 1980; Blackmore *et al.*, 1982; DeWitt & Putney, 1983; Kneer & Lardy, 1983; Burgess *et al.*, 1984; Dawson & Irvine, 1984; Mauger *et al.*, 1984; Shears & Kirk, 1984). The Ca^{2+} -channel antagonist verapamil transiently inhibits ^{45}Ca flux from the perfused liver, although without having any apparent effect on the total ^{45}Ca content of the liver (Kimura *et al.*, 1981). This suggests that a decrease in influx must have occurred, but that the methods used were not sensitive enough to detect it. In contrast, verapamil has no apparent effect on Ca^{2+} fluxes in isolated hepatocyte suspensions (Blackmore *et al.*, 1979). The role of bile in hormone-stimulated Ca^{2+} redistribution has been overlooked, perhaps as it is assumed that the bile Ca^{2+} concentration passively reflects blood Ca^{2+} concentrations (Wheeler, 1968) and that hormone-mediated responses are independent of any functional polarity of the hepatocyte. We report here that, under basal conditions, the bile Ca^{2+} concentration in perfused rat liver preparations is maintained at a significantly lower value than that in the perfusate. In contrast, exposure to α -adrenergic agonists or Ca^{2+} -channel blockers transiently increases the bile Ca^{2+} concentration and the bile/perfusate ^{45}Ca ratio. The

results suggest that a mechanism for Ca^{2+} reabsorption exists in the liver and that this mechanism is sensitive to both α -adrenergic agonists and Ca^{2+} -channel blockers. We postulate that a transcellular Ca^{2+} current may exist in the liver and that this current is maintained through a canalicular Ca^{2+} channel and a sinusoidal Ca^{2+} pump.

Experimental

Livers from fed female albino rats (225–275 g body wt.) were perfused essentially by the method of Hems *et al.* (1966), with cannulation of the portal vein, the vena cava via the right atria, and the bile duct. A synthetic perfusate was used containing, in mmol/litre, Na^+ 143.0, K^+ 5.9, Cl^- 124.9, Ca^{2+} 1.0, Mg^{2+} 1.2, PO_4^{2-} 1.2, HCO_3^- 25.0, glucose 5.0, lactate 1.3, pyruvate 0.1, taurocholate 0.01 [reflecting the physiological concentration (Boyer & Klatskin, 1970; Preisig *et al.*, 1962)], and 1% (w/v) bovine serum albumin (Sigma Fraction V, defatted and dialysed). The perfusate was constantly gassed with O_2/CO_2 (19:1). The flow rate was maintained at 1–1.5 ml/min per g of liver. No significant changes in our results were seen when flow rates of 2.5–5.0 ml/min per g were used, or when a volume-constant rather than pressure-constant perfusion apparatus was used. After 30 min of non-recirculation, 150 ml of perfusate was recirculated for the remainder of the perfusion. After 5 min of recirculation $^{45}\text{CaCl}_2$ (0.033 $\mu\text{Ci}/\mu\text{mol}$ of Ca^{2+} per ml of perfusate) and $^3\text{H}_2\text{O}$ (0.1 $\mu\text{Ci}/\text{ml}$ of perfusate) were added to the reservoir. Taurocholate (10 nmol/ml of perfusate) was added every 20 min after starting the recirculation to maintain a constant and detectable bile-flow rate. Bile samples were collected over succes-

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sive 2.5 or 5 min intervals in glass capillary tubes fitted over the end of the bile-duct cannula; the volume was recorded and the samples were diluted to 0.5 ml with distilled water. Samples of the perfusate (0.5 ml) were taken from the reservoir periodically. The perfusate and diluted bile samples were mixed with 3 ml of scintillation fluid [toluene/Triton X-100 (3:2, v/v), containing 4.67 g of 2,5-diphenyloxazole/litre] and assayed for radioactivity with a Phillips 4700 dual-channel liquid-scintillation counter. The data were corrected for carry-over and quenching and expressed as the ratio (c.p.m./ μ l of bile)/(c.p.m./ μ l of perfusate). To measure the Ca^{2+} concentration in the bile and perfusate, samples were collected from non-recirculated perfusions and assayed by atomic absorption spectrophotometry (Unicam SP191) or Arsenazo III absorbance ($A_{524} - A_{586}$; Aminco DW2 spectrophotometer in the dual-wavelength mode). For atomic absorption, the samples were diluted 1:100 (v/v) into 1.7% (w/v) trichloroacetic acid containing 0.1% KCl. For absorbance measurements, the samples were diluted 1:300 (v/v) into 0.2 mM-Hepes/NaOH, pH 7.4, containing 20 μ M-Arsenazo III. Absorbance of Arsenazo III was calibrated against 0–30 μ M- CaCl_2 prepared in perfusate without Ca^{2+} , diluted 1:300 (v/v). Both of these methods gave essentially identical results. In some experiments albumin was omitted from the perfusate, and essentially the same results were observed. We have also shown that the spectrum of Arsenazo III in the presence of Ca^{2+} is not affected by albumin when albumin and Ca^{2+} are present in the assay at the same concentration ratio as in the perfusate. Barnwell *et al.* (1983) have shown that bovine serum albumin present in perfusate at 1% (w/v) equilibrates to 0.05% (w/v) in bile. These combined results indicate that albumin could not have a significant effect on the biliary Ca^{2+} concentration, at least under the experimental conditions reported herein. All experiments were repeated at least twice, and representative results from one liver perfusion in each group are shown.

Results and discussion

^{45}Ca and $^3\text{H}_2\text{O}$ distribute within the perfusate and the bile of perfused rat livers in a biphasic manner (Fig. 1a). The fast phase is a complex of the perfusion-apparatus mixing time, the washout of the liver vasculature, cell water and biliary tree, and the rate of attainment of steady-state labelling of the perfusate and the bile. The half-time of this phase for both tracers is about 4.0 min. Whereas $^3\text{H}_2\text{O}$ equilibrates to a bile/perfusate ratio of 1.0 within 8.0 min, the second phase of the ^{45}Ca ratio increases slowly from 0.35 to 0.6 after 90 min of exposure to label. The kinetics of equilibration

Fig. 1. Effects of adrenergic agonists and antagonists on the bile/perfusate ratio of ^{45}Ca ($\blacktriangle$) and $^3\text{H}_2\text{O}$ ($\bullet$) (a) In the absence of adrenaline, (b) 1 μ M-adrenaline added at 32.5 min, (c) 1 μ M-adrenaline added 5 min before label addition, (d) 10 μ M-propranolol added at 30.5 min and 1 μ M-adrenaline added at 34.5 min, (e) 10 μ M-phentolamine added at 30.5 min and 1 μ M-adrenaline added at 34.5 min. Livers were perfused as described in the text.

do not indicate whether these substances take a route through and/or between the hepatocytes during bile formation. However, bile is iso-osmotic with blood (Wheeler, 1968), radiotracers of Na^+ , K^+ and plasma-membrane-impermeable substances equilibrate much more rapidly between blood and bile than between blood and intracellular pools (Graf & Peterlik, 1975), the kinetics of washout of preloaded ^{22}Na are similar in blood and bile (Graf & Peterlik, 1975), and cytological characteristics of the hepatocyte junctional com-

plex are similar to those seen in classical leaky epithelia (Boyer, 1982). Thus movement of substances between blood and bile can be summarized by Scheme 1.

Exposure of either perfused liver preparations or isolated hepatocyte suspensions to adrenaline is associated with a redistribution of Ca^{2+} within the hepatocyte. When $1\text{ }\mu\text{M}$ -adrenaline was added to the perfusate reservoir 32.5 min after label addition, there was a rapid increase in the ^{45}Ca bile/perfusate ratio to a plateau of 0.8 within 15 min (Fig. 1b). The same response was seen when verapamil (20 or $100\text{ }\mu\text{M}$ perfusate concentration) was added to the perfusate, suggesting the involvement of Ca^{2+} channels in the observed response to adrenaline (results not shown). If adrenaline is added 5.0 min before label addition, the ^{45}Ca ratio between bile and perfusate parallels that seen for $^3\text{H}_2\text{O}$, increasing to 0.8 within 7.5 min of label addition (Fig. 1c). The β -adrenergic antagonist propranolol added 3 min before the adrenaline did not significantly affect the response to the latter (Fig. 1d). However, when the α -adrenergic antagonist phentolamine was added to the system before adrenaline, there was an almost complete suppression of the response to adrenaline (Fig. 1e).

Fig. 2 shows that, when the free Ca^{2+} concentration in the perfusate is 1.0 mM , the bile concentration is 0.35 mM , or about $0.3\text{ nmol of Ca}^{2+}/\text{min per g}$. Further, exposure to adrenaline over a 10 min infusion interval resulted in a rapid and sustained increase in the bile Ca^{2+} concentration to about 0.8 mM , and a transient decrease in the bile-flow rate. This corresponds to a maximum increase in the appearance of Ca^{2+} in the bile of about $0.55\text{ nmol/min per g}$. These parameters returned to their basal values when the adrenaline infusion was stopped.

To identify more clearly a change in the Ca^{2+} flux between perfusate and bile as a result of exposure of the liver to agonists, the data in Figs. 1 and 2 were plotted as clearance [(bile/perfusate Ca^{2+} or ^{45}Ca) $\times$ bile-flow rate] as functions of bile-flow rate (Fig. 3). This plot facilitates the comparison of the three techniques used to measure bile Ca. A plot that is linear (i.e. bile/perfusate Ca is proportional to bile flow) indicates a constant bile/perfusate ratio. A slope of unity indicates passive transport of the substance in question, i.e.

no active or reabsorptive component. The slope of the plots for ^{45}Ca (Fig. 3a) and Ca^{2+} (Fig. 3c) under basal conditions (open symbols) is about 0.35, indicating, again, that the bile Ca^{2+} concentration is maintained at lower values than the perfusate Ca^{2+} concentration and that the bile/perfusate Ca ratio is constant. In contrast, both adrenaline (Figs. 3a and 3c, closed symbols) and verapamil (Fig. 3b) increased the bile Ca^{2+} concentration, as indicated by the increase of the slope of the clearance-versus-bile-flow curves, to about 0.8.

The results show that the ratio of the Ca^{2+} concentration in bile and perfusate is constant, whereas the bile/perfusate ratio of ^{45}Ca changes

Fig. 2. Effect of adrenaline on the free Ca^{2+} concentration in (●) and perfusate (▲)

These, and the bile flow rate (—) in the absence or presence of adrenaline ($10\text{ }\mu\text{M}$ perfusate concentration, in $40\text{ }\mu\text{M}$ -ascorbic acid), are shown. Hatched bar indicates infusion of ascorbic acid alone; solid bar indicates infusion of adrenaline. Livers were perfused and Ca^{2+} was analysed as described in the text. The data in this Figure were obtained from atomic-absorption measurements.

Fig. 3. *Effects of adrenaline or verapamil on the clearance of Ca^{2+} between perfusate and bile* Clearance [(bile Ca/perfusate Ca) $\times$ bile flow] of (a and b) ^{45}Ca and (c) Ca^{2+} was plotted as a function of bile flow rate. Data were plotted from several experiments similar to those shown in Figs. 1 and 2 performed (a and c) in the absence (open symbols) or in the presence (closed symbols) of $10\text{ }\mu\text{M}$ -adrenaline, or (b) in the presence of $20\text{ }\mu\text{M}$ - ($\blacktriangle$) or $100\text{ }\mu\text{M}$ - ($\triangle$) verapamil. In (c) data were obtained from atomic-absorption ($\bullet$, $\circ$) or Arsenazo-III-absorbance ($\blacktriangle$, $\triangle$) measurements on bile and perfusate samples. Curves were drawn by eye. The dashed line in (b) is transposed from the curve representing control experiments in Figs. 1 and 2.

slowly over the duration of the perfusion. This suggests the presence of a pool of Ca^{2+} which is not in equilibrium with perfusate or bile over the time course of the experiments. This is in agreement with previous reports which have indicated the existence of very slowly exchanging pools of ^{45}Ca (time constant 223min) in perfused rat liver (Claret-Berthon *et al.*, 1977) and in isolated hepatocyte suspensions (Barritt *et al.*, 1981). In the presence of adrenaline or verapamil, both the free Ca^{2+} and the ^{45}Ca bile/perfusate ratios increase to about 0.8. The proposed routes for water and electrolyte transport during bile formation are intercellular and/or transcellular (Graf & Peterlik, 1975; Boyer, 1982). Thus Ca^{2+} movement between the blood and the bile may be via (i) intercellular flux only, or (ii) intercellular flux plus removal from the bile to an additional pool. It seems unlikely that Ca^{2+} flux occurs only through (i), firstly as this would require the junctional complex to have a low conductance for Ca^{2+} [which is not so, as the fast phase has a similar half-time to water movement, and ouabain-induced hydrocholerisis does not affect the clearance of ^{45}Ca (or $^3\text{H}_2\text{O}$)

from the perfusate to the bile (C. E. Hill, A. P. Dawson & J. S. Pryor, unpublished work)], and secondly this would require the junctional complex to be sensitive to substances which modulate blood/bile Ca^{2+} flux. The route described in (ii) would require the secondary pool(s) to be slowly exchanging, and the observed kinetics of the slow phase of ^{45}Ca distribution may be a measure of net flux between this pool and the bile. This pool would be sensitive to Ca^{2+} -perturbing agents so that exposure brings it into rapid equilibrium with the bile and/or blood Ca^{2+} pools. That verapamil, a Ca^{2+} -channel blocker, also stimulates this response suggests that the bile Ca^{2+} concentration is kept low through Ca^{2+} transport into the hepatocyte via a Ca^{2+} channel located in the canalicular membrane. The adrenaline response may also involve this proposed channel, although indirectly, possibly via inactivation of the putative Ca^{2+} channel through the increased cytosolic Ca^{2+} concentration.

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References

- Althaus-Salzmann, M., Carafoli, E. & Jakob, A. (1980) *Eur. J. Biochem.* **106**, 241–248
- Barnwell, S. G., Godfrey, P. P., Lowe, P. J. & Coleman, R. (1983) *Biochem. J.* **210**, 549–557
- Barritt, G. J., Parker, J. C. & Wadsworth, J. C. (1981) *J. Physiol. (London)* **312**, 29–55
- Blackmore, P. F., El-Refai, M. F. & Exton, J. H. (1979) *Mol. Pharmacol.* **15**, 598–606
- Blackmore, P. F., Hughes, B. P., Shuman, E. A. & Exton, J. H. (1982) *J. Biol. Chem.* **257**, 190–197
- Boyer, J. L. (1982) in *The Paracellular Pathway* (Bradley, S. E. & Purcell, E. F., eds.), pp. 209–221, Josiah Macy Foundation, New York
- Boyer, J. L. & Klatskin, G. (1970) *Gastroenterology* **59**, 853
- Burgess, G. M., Godfrey, P. P., McKinney, J. S., Berridge, M. J., Irvine, R. F. & Putney, J. W. (1984) *Nature (London)* **309**, 63–66
- Charest, R., Blackmore, P. F., Berthon, B. & Exton, J. H. (1983) *J. Biol. Chem.* **258**, 8769–8773
- Claret-Berthon, B., Claret, M. & Mazet, J. L. (1977) *J. Physiol. (London)* **272**, 529–552
- Dawson, A. P. & Irvine, R. F. (1984) *Biochem. Biophys. Res. Commun.* **120**, 858–864
- DeWitt, L. M. & Putney, J. W. (1983) *FEBS Lett.* **160**, 259–263
- Graf, J. & Peterlik, M. (1975) in *The Hepatobiliary System* (Taylor, W., ed.), pp. 43–60, Plenum Press, New York
- Hems, D. A., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966) *Biochem. J.* **101**, 284–292
- Kimura, S., Koide, Y., Tada, R., Abe, K. & Ogata, E. (1981) *Endocrinol. Jpn.* **28**, 69–78
- Kneer, N. M. & Lardy, H. A. (1983) *Arch. Biochem. Biophys.* **225**, 187–195
- Mauger, J.-P., Poggioli, J., Guesdon, F. & Claret, M. (1984) *Biochem. J.* **221**, 121–127
- Murphy, E., Coll, K., Rich, T. L. & Williamson, J. R. (1980) *J. Biol. Chem.* **255**, 6600–6608
- Poggioli, J., Berthon, B. & Claret, M. (1980) *FEBS Lett.* **115**, 243–246
- Preisig, R., Cooper, H. L. & Wheeler, H. O. (1962) *J. Clin. Invest.* **41**, 1152–1162
- Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. (1983) *Biochem. J.* **214**, 405–412
- Shears, S. B. & Kirk, C. J. (1984) *Biochem. J.* **220**, 417–421
- Wheeler, H. O. (1968) *Handbook Physiol. Sect. 6* **5**, 2409
- Williamson, J. R., Cooper, R. H. & Hoek, J. B. (1981) *Biochim. Biophys. Acta* **639**, 243–295