Biochem. J. (1984) **220**, 35–42
Printed in Great Britain

The contribution of both extracellular and intracellular calcium to the action of α-adrenergic agonists in perfused rat liver

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(*Received 5 August 1983/Accepted 31 January 1984*)

The role of both intracellular and extracellular Ca^{2+} pools in the expression of α-adrenergic-agonist-mediated responses was examined in perfused rat liver. Responses studied included glycogenolysis, respiration, lactate and pyruvate formation, ketone-body production, changes in the cytoplasmic and mitochondrial redox ratio and cellular K^+ fluxes. Each of these was shown to be dependent on the mobilization of intracellular Ca^{2+} and can be grouped into one of two response types. Transient responses (ion fluxes and the redox ratios) are obligatorily dependent on the mobilization of intracellular Ca^{2+} and occur irrespective of the extracellular Ca^{2+} concentration. Sustained responses, on the other hand, initially require intracellular Ca^{2+} and, subsequently, extracellular Ca^{2+}. The data indicate that α-adrenergic agonists mobilize extracellular Ca^{2+} as well as intracellular Ca^{2+} and that both pools are required for the full expression of hormone-induced responses in rat liver.

A redistribution of cellular Ca^{2+} is involved in the action of α-adrenergic agonists in liver (reviewed by Exton, 1981; Williamson *et al.*, 1981; Taylor *et al.*, 1983*b*). The earliest detectable movement of Ca^{2+} induced by α-adrenergic agonists is the mobilization of an intracellular pool of the ion (Blackmore *et al.*, 1982; Reinhart *et al.*, 1982*b*). Although some responses to α-adrenergic agonists are dependent on the mobilization of the intracellular Ca^{2+} stores (Reinhart *et al.*, 1982*b*), there is also some evidence for an additional role of extracellular Ca^{2+} in α-adrenergic effects. Firstly, extracellular Ca^{2+} is in rapid equilibrium with the intracellular hormone-sensitive pool of Ca^{2+}; for example, after cessation of hormone administration to the perfused rat liver, extracellular Ca^{2+} rapidly repletes the intracellular hormone-sensitive pool (Reinhart *et al.*, 1982*b*). Secondly, some of the prolonged α-agonist-induced responses become transient in the absence of extracellular Ca^{2+}; and thirdly, a kinetic analysis of $^{45}Ca^{2+}$ fluxes in isolated hepatocytes has indicated that α-adrenergic agonists stimulate the flux of Ca^{2+} from the extracellular to the cytoplasmic compartment (Barritt *et al.*, 1981).

In the present study we set out to examine the relative contribution of both intracellular and extracellular Ca^{2+} pools in the expression of a number of α-adrenergic-agonist-induced responses in the perfused rat liver. We conclude that both extracellular and intracellular Ca^{2+} pools are important in the full expression of the α-agonist-induced effects, and that the regulation of Ca^{2+} fluxes across the plasma membrane constitutes an important event in the action of these agonists.

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Experimental

Animals and perfusions

Male Wistar-strain albino rats weighing between 230 and 270g and having free access to food were used for all experiments. Rats were anaesthetized with sodium pentobarbitone (50mg/kg body wt.), and the livers perfused with Krebs–Henseleit (1932) bicarbonate medium equilibrated with O_2/CO_2 (19:1) as described previously (Reinhart *et al.*, 1982*a*). All livers were pre-perfused for 15min with medium containing 1.3mM added $CaCl_2$ at 35°C and at a flow rate of 3.5ml/min per g of wet liver. At 15min the free Ca^{2+} concentration in the perfusate was altered either by decreasing the concentration of added $CaCl_2$ or by adding low concentrations of EGTA to the perfusate. All experiments were performed between 08:00h and 12:00h to minimize diurnal fluctuations of basal metabolism.

Perfusate calcium determinations

For some experiments the perfusate Ca^{2+} concentration was continuously monitored exactly as described previously (Reinhart *et al.*, 1982*b*). A Radiometer F2112 Ca^{2+}-selective electrode was placed into a small flow-through chamber, close to the liver, and coupled to a Radiometer GK 2401 C combination electrode via an agarose/KCl salt bridge. Signal amplification was achieved by connecting both electrodes to an Orion model 901 microprocessor ion analyser set to the grounded-solution mode. Output was channelled through a bucking-voltage device (Madeira, 1975) to a Spectra-Physics SP4100 computing integrator which was programmed (Autolab BASIC) to display changes in the perfusate Ca^{2+} concentration (peak height) and the total amounts of Ca^{2+} taken up or extruded by the liver (integration mode). Further details are described in Reinhart *et al.*, (1982*b*, 1983). As the logarithm of the electrode response becomes non-linear at Ca^{2+} concentrations below 1 μM, atomic-absorption spectroscopy was used to determine total Ca^{2+} concentration changes in experiments where EGTA was used to lower the Ca^{2+} concentration below 1 μM. For this procedure, samples of perfusate (4ml) were assayed in 0.1% KCl (5ml total volume) by using a NO_2/acetylene flame (medical-grade gases). Standard $CaCl_2$ solutions were prepared in 80% (v/v) Krebs–Henseleit medium plus 0.1% KCl.

The K^+ concentration in the outflow medium was measured by using an Orion potassium electrode (model 931900) coupled to a potassium-sensing module 93-19-01 and using reference electrode-filling solution 90-00-19. The electrode was calibrated by using KCl standard solutions, and coupling of signal output was the same as for Ca^{2+} measurements.

Free-calcium-concentration calculations

Free Ca^{2+} concentrations were calculated by using an algorithm of the program (COMICS) developed by Perrin & Sayce (1967). The logarithm of the total formation constants (Sillen & Martell, 1971) for the following complexes were considered (pH7.4): $PO_4^{3-}+H^+$ (11.8), $EGTA^{4-}+Ca^{2+}$ (11.0), $CO_3^{2-}+H^+$ (10.25), $EGTA^{4-}+H^+$ (9.54), $HEGTA^{3-}+H^+$ (8.93), $HPO_4^{2-}+H^+$ (7.15), $HEGTA^{3-}+Ca^{2+}$ (5.3), $EGTA^{4-}+Mg^{2+}$ (5.2), $HEGTA^{3-}+Mg^{2+}$ (3.4), $HCO_3^-+Mg^{2+}$ (3.4), $CO_3^{2-}+Ca^{2+}$ (3.2), $HPO_4^{2-}+Ca^{2+}$ (2.77), $H_2EGTA^{2-}+H^+$ (2.73), $HPO_4^{2-}+Mg^{2+}$ (2.5), $H_2PO_4^-+Ca^{2+}$ (1.5).

Perfusate glucose and oxygen determinations

Glucose release by the liver was determined by the glucose oxidase/peroxidase method as previously described (Reinhart *et al.*, 1982*b*). Perfusion-circuit lag times, from the point of hormone infusion to the sampling point, were routinely determined by using [^{3}H]inulin. All data shown are corrected for this lag time. The oxygen consumption by the liver was calculated from the difference between influent and effluent oxygen concentrations measured with a Clark-type oxygen electrode modified for a flow-through mode of operation as described previously (Reinhart *et al.*, 1982*a*). The linearity of the electrode response was established by using the colorimetric oxygen assay of Hamlin & Lambert (1971). Perfusion-circuit lag times were determined by using sodium dithionite, and data shown are corrected for this lag time.

Other analytical procedures

For some experiments, 1.5ml perfusate samples were centrifuged to remove contaminating red blood cells, and the supernatants mixed with 0.2ml of 2M-$HClO_4$ containing 5mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]. After being stood on ice for 10min, samples were neutralized with 0.2ml of KOH and the precipitate removed by centrifugation. Portions of the supernatant were taken to assay pyruvate, lactate (Gutman & Wahlefeld, 1974), β-hydroxybutyrate and acetoacetate (Williamson & Mellanby, 1974).

Results

Previously we have shown that responses to α-adrenergic agonists, such as a stimulation in the rates of glycogenolysis, respiration (Reinhart *et al.*, 1982*b*) and gluconeogenesis (Taylor *et al.*, 1983*a*), are obligatorily dependent on the mobilization of an intracellular pool of Ca^{2+}. To assess the extent to which such a mobilization forms part of a more general mechanism whereby α-adrenergic agonists induce intracellular events, we have examined whether or not a number of other α-agonist-induced metabolic changes are similarly dependent initially on intracellular Ca^{2+}. In these experiments we have employed a technique whereby the ability to alter rapidly both the perfusate Ca^{2+} concentration and the duration of α-adrenergic-agonist administration, allows the manipulation of a small, intracellular hormone-sensitive pool of Ca^{2+} (Reinhart *et al.*, 1982*b*). Experiments were carried out therefore under conditions either facilitating the depletion of the hormone-sensitive Ca^{2+} pool (4 μM perfusate Ca^{2+}) or under conditions allowing the continued repletion of the pool (1.3mM perfusate Ca^{2+}; Reinhart *et al.*, 1982*b*).

Lactate-plus-pyruvate formation and the cytoplasmic redox ratio

Consistent with previous observations (Althaus-Salzmann *et al.*, 1980; Scholz & Schwabe, 1980),

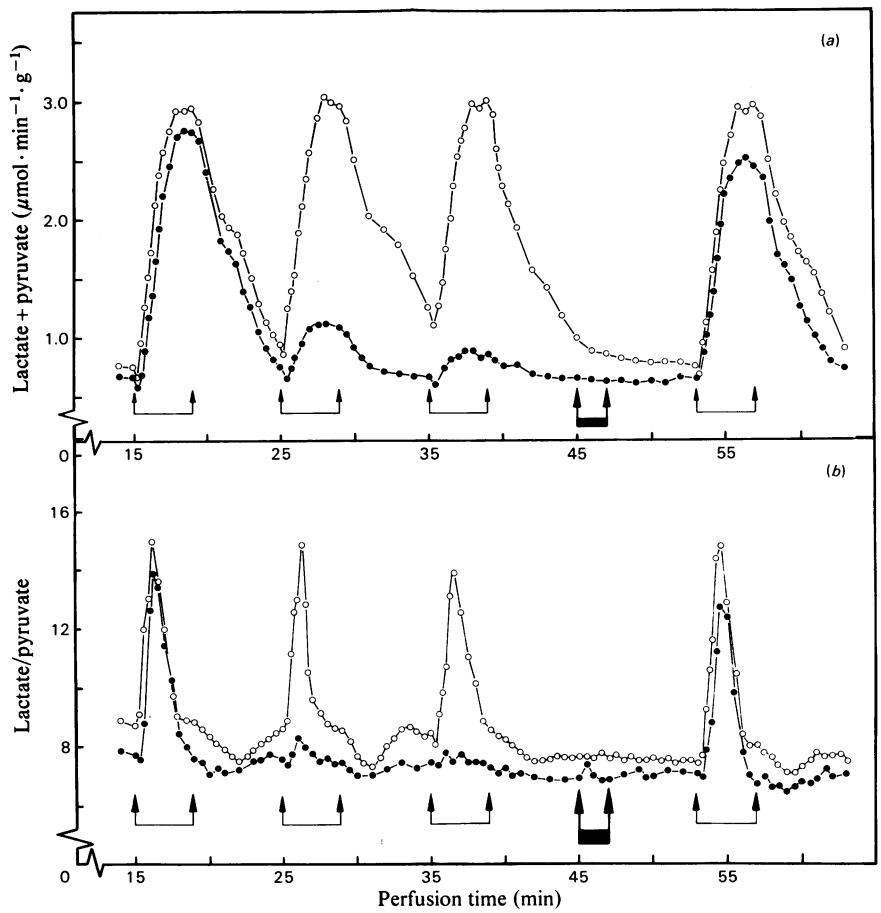

Fig. 1. *Effect of brief repeated infusions of phenylephrine on liver lactate-plus-pyruvate formation and on the cytoplasmic redox ratio*

Livers of fed rats were perfused as described in the Experimental section. The perfusion medium contained 1.3mM-Ca^{2+} for the first 10min. At 10min the perfusate Ca^{2+} concentration was decreased to 4μM for some livers (●), whereas for others it remained at 1.3mM (○). At 15, 25 and 35min, livers were infused with phenylephrine (2μM) for 4min (faint arrows). At 45min, livers perfused with medium containing 4μM-Ca^{2+} were infused with 1.3mM-$CaCl_2$ for 2min (bold arrows). At 53min, livers were again infused with phenylephrine for 4min. Perfusate lactate and pyruvate, were determined as described in the Experimental section. Results shown are the means from between three and six independent experiments. For the sake of clarity, S.E.M. bars have been omitted.

the data in Fig. 1(*a*) indicate that phenylephrine rapidly stimulates the rate of lactate-plus-pyruvate formation. By 5–6min after the termination of hormone infusion, the rates of output return to basal values. The present data in addition show that, at an extracellular Ca^{2+} concentration of 1.3mM, repeated pulses of phenylephrine administration induce repeated stimulations (by almost 3-fold) of lactate-plus-pyruvate output. When the experiment is carried out at an extracellular Ca^{2+} concentration of 4μM, only the first of the three pulses of phenylephrine induces significant production of lactate plus pyruvate. A single 2min administration of 1.3mM-$CaCl_2$ is sufficient to restore the response almost totally.

The data in Fig. 1(*b*) show that, as well as stimulating total lactate-plus-pyruvate output, phenylephrine also raises by almost 2-fold the lactate/pyruvate ratio, thought to reflect the cytosolic redox ratio (Williamson *et al.*, 1967). However, it is noteworthy that, in contrast with the sustained effect of the α-agonist on lactate and pyruvate output (Fig. 1) and respiration and glucose output (Reinhart *et al.*, 1982*b*), the effect on the cytosolic redox ratio is transient. Maximum changes are observed between 45 and 60s of agonist infusion; thereafter the lactate/pyruvate ratio returns to basal values even while phenylephrine is still being infused. Subsequent brief administrations of phenylephrine result in qualitatively similar responses

when the perfusate Ca^{2+} concentration is 1.3mM. When the perfusate Ca^{2+} concentration is decreased to 4 μM, only the first of the three pulses of phenylephrine induces a significant increase in the lactate/pyruvate ratio. Again a single 2min administration of 1.3mM-$CaCl_2$ is sufficient to restore the response.

β-Hydroxybutyrate-plus-acetoacetate formation and the mitochondrial redox ratio

Having observed that changes in the cytoplasmic redox ratio induced by repeated administration of α-agonists are dependent on extracellular Ca^{2+}, we carried out a similar set of experiments examining the mitochondrial redox ratio. Measurements of β-hydroxybutyrate and acetoacetate are considered to provide such an assessment (Williamson *et al.*, 1967).

The data in Fig. 2(*a*) show the effect of repeated administration of phenylephrine on ketone-body formation. With 1.3mM-Ca^{2+} in the perfusion medium, the concentration of these metabolites almost doubled following each pulse of phenylephrine. With 4 μM-Ca^{2+} in the perfusion medium, however, only the first of the three pulses of hormone induces any significant formation of these metabolites. As found in Fig. 1(*a*), a short exposure to 1.3mM-Ca^{2+} fully restores the phenylephrine-induced response.

Fig. 2(*b*) shows that the ratio β-hydroxybutyrate/acetoacetate doubles after each pulse of phenylephrine in the presence of 1.3mM-Ca^{2+}; like the lactate/pyruvate ratio (Fig. 1*b*), these also are transient, and are decreased to be approx. 40% above basal values by 4min of phenylephrine administration. If the duration of phenylephrine treatment is increased to 5min or longer, the redox ratios return to basal values (results not shown). These data are qualitatively similar to the effects of α-adrenergic agonists on mitochondrial redox ratios previously observed in livers of starved rats (Taylor *et al.*, 1983*a*). At 4 μM perfusate Ca^{2+}, only

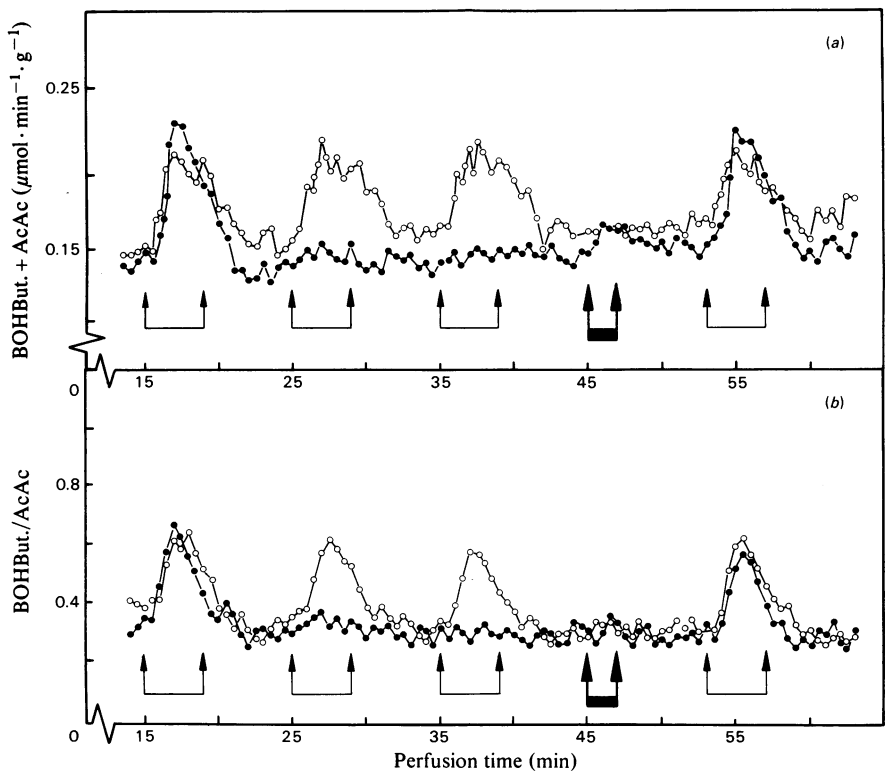

Fig. 2. *Effect of brief repeated infusions of phenylephrine on the formation of β-hydroxybutyrate (BOHBut.) and acetoacetate (AcAc) and the mitochondrial redox ratio in perfused rat liver*

The experiments were carried out exactly as described for Fig. 1, except that perfusate β-hydroxybutyrate and acetoacetate were determined as described in the Experimental section. The perfusate Ca^{2+} concentration was either 1.3mM (○) or 4 μM (●). Faint and bold arrows show the infusion of phenylephrine (2 μM) or Ca^{2+} (1.3mM) respectively. Results shown are the means for between three and six independent experiments. For the sake of clarity, S.E.M. bars have been omitted.

the first pulse of hormone induces a response, and this is restored after a short exposure to 1.3mM-Ca^{2+}.

The data in Figs. 1 and 2 thus provide evidence that the mobilization of intracellular Ca^{2+} forms part of a general mechanism whereby α-adrenergic agonists induce hepatic responses, and that some of these responses are transient, whereas others are prolonged.

K^+ fluxes

Evidence exists that administration of phenylephrine induces rapid changes in K^+ fluxes in rat liver. (Jacob & Diem, 1975; Burgess *et al.*, 1979; Blackmore *et al.*, 1979; Althaus-Saltzman *et al.*, 1980; Burgess *et al.*, 1981; Capiod *et al.*, 1982). Consistent with these reports, the data in Fig. 3 show that, at 4μM-Ca^{2+}, phenylephrine induces significant K^+ flux changes consisting of two components: K^+ influx followed by a more prolonged phase of K^+ efflux from the cell. Repeated administrations of phenylephrine under these conditions result in the inhibition of predominantly the K^+-influx phase, with little effect on the secondary K^+-uptake phase. A single 2min administration of 1.3mM-$CaCl_2$ fully restores the magnitude of the initial K^+-influx phase. With 1.3mM-Ca^{2+} in the perfusate, a different pattern of K^+ fluxes can be seen. Although the initial K^+-influx phase is very similar, the magnitude of K^+ efflux is relatively small during the first phenylephrine administration and increases during each successive phenylephrine treatment. The significance of these differences in K^+ fluxes was not further examined in this series of experiments.

Ca^{2+} fluxes, glycogenolysis and respiration

Further experiments were designed to define the role of extracellular Ca^{2+} in the action of α-agonists. The data in Fig. 4 show how the magnitude of the Ca^{2+} gradient across the plasma membrane influences the expression of phenylephrine-induced Ca^{2+} fluxes, glucose output and respiration. When the Ca^{2+} gradient is high (i.e. 1.3mM-Ca^{2+} is perfused through the liver), phenylephrine stimulates respiration (Fig. 4*c*) and glucose output (Fig. 4*b*) for the duration of α-agonist infusion. The effect on Ca^{2+} fluxes (Fig.

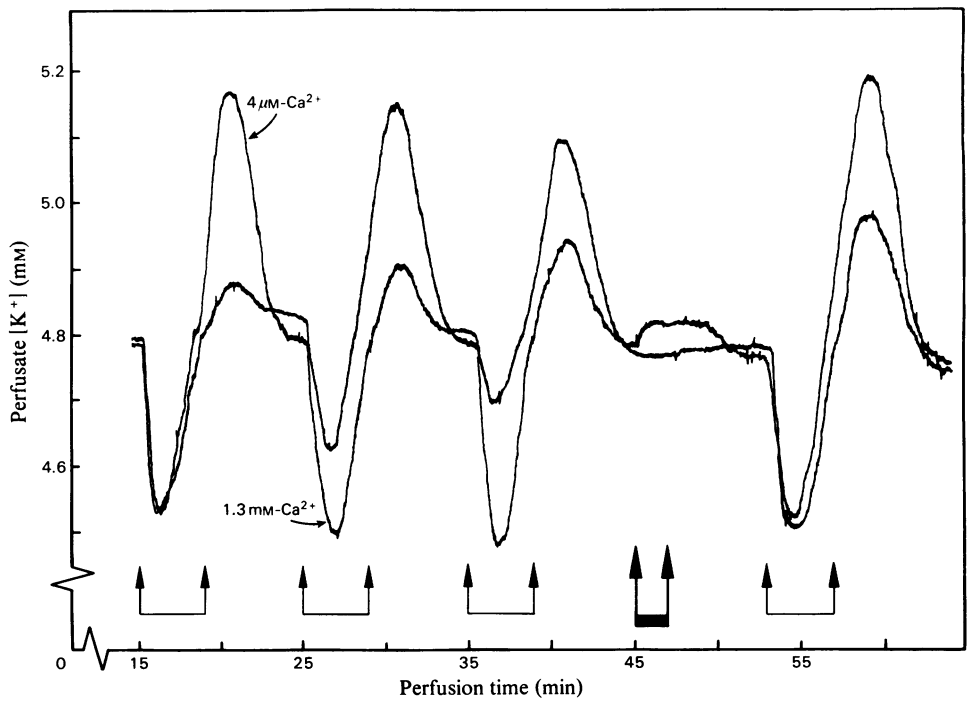

Fig. 3. *Effect of brief repeated infusions of phenylephrine on liver K^+ fluxes*
The experiments were carried out exactly as described for Fig. 1, except that the K^+ concentration in the effluent medium was continuously monitored with a potassium electrode as described in the Experimental section. The perfusate Ca^{2+} concentration was either 1.3mM or 4μM. Faint and bold arrows show the infusion of phenylephrine (2μM) or Ca^{2+} (1.3mM) respectively. Results shown are representative recordings from between three and five independent experiments performed.

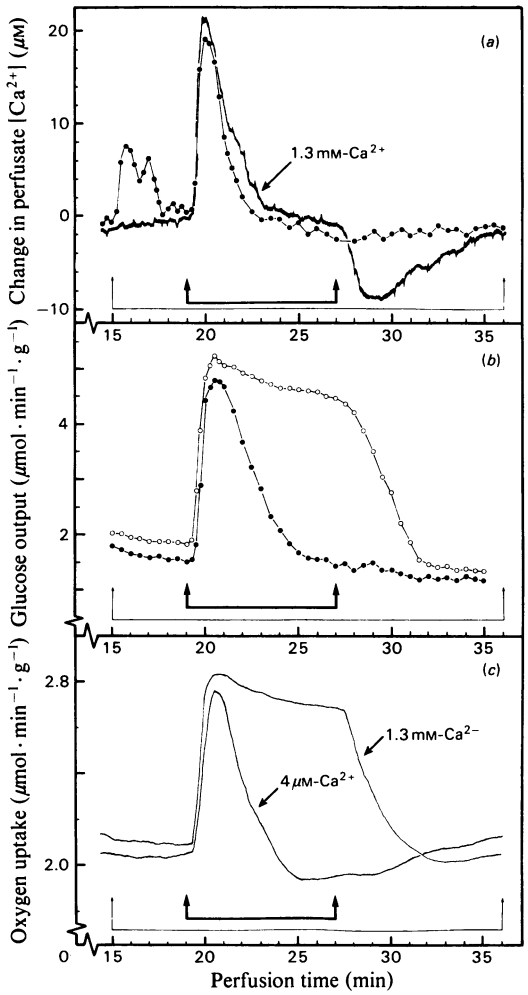

Fig. 4. *Effect of the extracellular Ca^{2+} concentration on phenylephrine-stimulated Ca^{2+} fluxes, glucose output and respiration in perfused rat liver*

Livers of fed rats were perfused as described in the Experimental section. For all livers the perfusion medium contained 1.3mM-Ca^{2+} for the first 12min. At 12min the perfusate Ca^{2+} concentration was either decreased to 4 μM (●) and at 15min reduced further to less than 10^{-7} M by the continuous infusion of a small excess (30 μM) of EGTA, or maintained at 1.3mM. At 19min all livers were infused with phenylephrine (2 μM) for a total of 8min. The perfusate Ca^{2+} concentration (*a*) was determined either by atomic-absorption spectroscopy (●) or Ca^{2+}-Selectrode. Changes in perfusate glucose and oxygen concentration were determined as described in the Experimental section. The continuous recordings (*a* and *c*) are from single recordings representative of between three and eight independent experiments performed. All other data are expressed as means, the S.E.M. bars having been omitted for the sake of clarity.

4*a*), however, occurs in two phases: firstly there is a transient efflux of Ca^{2+} and, only after removal of the agonist, a transient re-uptake of Ca^{2+}. When the Ca^{2+} gradient is largely removed by decreasing the perfusate Ca^{2+} concentration and adding a slight excess of EGTA (30 μM), a number of different effects is observed. Firstly, EGTA mobilizes a pool of Ca^{2+} consisting of between 50 and 60nmol/g of liver, within 3min of its administration. Presumably this pool predominantly represents Ca^{2+} bound to the outside of the plasma membrane (Claret-Berthon *et al.*, 1977). However, neither the loss of this pool nor the low extracellular Ca^{2+} concentration diminishes the rate or amount of Ca^{2+} mobilized by phenylephrine, indicating that EGTA-accessible plasma-membrane-bound Ca^{2+} does

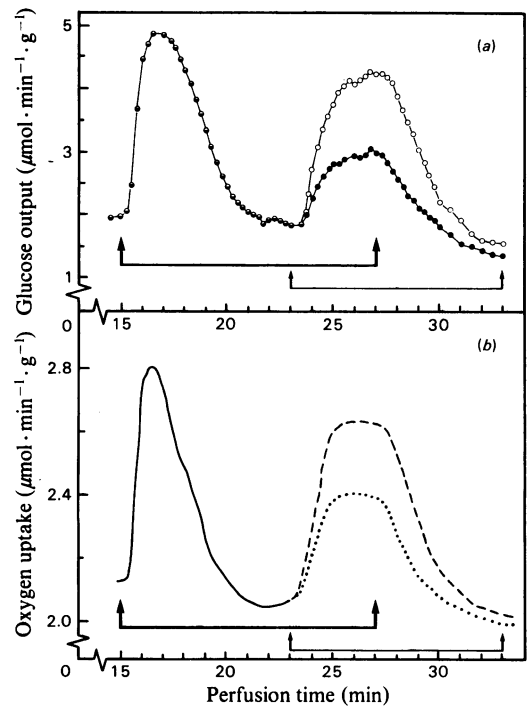

Fig. 5. *Effect of Ca^{2+} infusions on glucose output and respiration in phenylephrine-challenged perfused rat liver*

Livers of fed rats were perfused as described in the Experimental section. At 10min the perfusate Ca^{2+} concentration was decreased to less than 0.1 μM. At 15min all livers were infused with phenylephrine (2 μM) (——). At 23min the extracellular Ca^{2+} concentration was elevated to either 400 μM (····) or 1.3mM (----), and 4min later the infusion of phenylephrine terminated. The data in (*a*) are means from between three and five independent experiments, and the data in (*b*) represent recordings from between three and five independent experiments performed.

not form part of the cellular hormone-sensitive pool of Ca^{2+}. Secondly, no compensatory re-uptake of Ca^{2+} is observed after removal of the α-agonist. However, the major metabolic difference relates to the maintenance of the sustained glycogenolytic and respiratory responses. In the absence of extracellular Ca^{2+} these responses become transient, and although the maximal extent of stimulation is very similar to that observed at 1.3mM-Ca^{2+}, the responses gradually return to basal values within 5–6min.

The data in Fig. 5 further show the relationship between extracellular Ca^{2+} and phenylephrine on the sustained effects of the agonist. The transient glucose-output (*a*) or oxygen-uptake (*b*) responses observed in perfusions with 4μM-Ca^{2+} can be converted into prolonged responses by elevating the perfusate Ca^{2+} concentration to 1.3mM while the α-adrenergic agonist is still being administered. The extent of restoration is dependent on the extracellular Ca^{2+} concentration. At 1.3mM-Ca^{2+}, almost total restoration of the response is observed, whereas at 400μM-Ca^{2+} partial restoration is observed (Fig. 5), with little restoration apparent at 100μM-Ca^{2+} or less (results not shown). Both the time of onset, and the time taken to reach maximal rates of respiration or glucose output, is longer for the Ca^{2+}-induced restoration than for phenylephrine-induced rates, consistent with the interpretation that initial α-adrenergic-induced responses are due to the mobilization of intracellular Ca^{2+}. Hence this experimental approach allows the separation of phenylephrine-induced responses due to intracellular Ca^{2+} from those dependent on extracellular Ca^{2+}. Further aspects of this are discussed in the following paper (Reinhart *et al.*, 1984).

Discussion

The results presented here have revealed new insights about the redistribution of Ca^{2+} induced by α-adrenergic agonists and the role these play in the expression of α-agonist-induced effects.

Firstly, by using a previously described technique to deplete gently the intracellular hormone-sensitive pool of Ca^{2+}, we have shown that the mobilization of this pool is obligatory for the expression of α-agonist-induced responses such as cellular Ca^{2+} efflux, mitochondrial respiration, glycogenolysis (Reinhart *et al.*, 1982*a*,*b*), cytoplasmic and mitochondrial-redox-ratio changes, glycolysis, ketogenesis, K^+ uptake (the present paper) and gluconeogenesis (Taylor *et al.*, 1983*a*). The mechanism whereby a redistribution of Ca^{2+} alters the rate of these responses has not been fully elucidated, but an elevation in the concentration of cytoplasmic Ca^{2+}, (Barritt *et al.*, 1981; Murphy *et al.*, 1980) probably plays a role.

Secondly, the hepatic responses to α-adrenergic agonists appear to be either 'transient' or 'sustained', depending on the temporal progression of the response during the first 3–5min of α-agonist administration in medium containing millimolar Ca^{2+} concentrations. The cytoplasmic and mitochondrial redox ratios, and Ca^{2+} and K^+ flux changes, appear to be transient. Changes in the rates of metabolite release, on the other hand, appear to be sustained. The mechanistic basis for this has not yet been established, but, because of the Ca^{2+}-dependence, it is tempting to speculate that transient responses are triggered by the initial change in cytoplasmic Ca^{2+} concentration, whereas sustained responses may require a continually elevated cytoplasmic Ca^{2+} concentration.

The third major observation made in the present study is that although virtually all hepatic responses to α-agonists are dependent on the redistribution of intracellular Ca^{2+}, this alone is not sufficient for the full expression of α-agonist-induced effects. The continued expression of sustained effects appears to be dependent on an extracellular pool of Ca^{2+}. Hence the redistribution of Ca^{2+} induced by α-adrenergic agonists appears to follow a strict temporal sequence wherein predominantly intracellular Ca^{2+} is mobilized during the first 1–2min of α-agonist action, and extracellular Ca^{2+} is utilized at longer times. Such a scheme is consistent with a steady-state kinetic analysis of α-agonist-induced $^{45}Ca^{2+}$ flux changes (Barritt *et al.*, 1981), and furthermore indicates that any long-term responses, i.e. responses activated after 3–4min of α-agonist administration, should be entirely dependent on extracellular Ca^{2+}.

A further point highlighted by these studies is the important role played by Ca^{2+}-transporting enzymes located in the plasma membrane of liver cells. Although these enzymes have not been clearly defined, both the Ca^{2+}-uptake and the Ca^{2+}-efflux components are regulated by α-adrenergic agonists, and this may have important consequences in maintaining both the total cellular Ca^{2+} content, and the free Ca^{2+} concentration, in one or more intracellular compartments. The Ca^{2+} efflux mechanism appears to be operating well below maximal rates, as judged by the reserve capacity for Ca^{2+} efflux observed after hormonal stimulation (Reinhart *et al.*, 1982*b*), consistent with observations made for the red-blood-cell Ca^{2+} pump (Lew & Ferreira, 1978; Schatzman & Burgin, 1978).

The regulation of Ca^{2+} uptake by liver is complex, since apparently this activity plays a role both during the administration and after the removal of α-adrenergic agonists (the following paper, Reinhart *et al.*, 1984). Fine regulation is

essential, since markedly elevated cytoplasmic Ca^{2+} concentrations may induce cell damage (Reinhart *et al.*, 1983).

We thus propose that an obligatory part of the mechanism whereby α-adrenergic agonists stimulate both transient and sustained responses in liver, is the redistribution of cellular Ca^{2+}. This redistribution appears to progress in two stages. Firstly, a bound pool of intracellular Ca^{2+} (Reinhart *et al.*, 1983) is mobilized and at least partially extruded from the cell, and secondly, the Ca^{2+}-uptake/efflux mechanism at the plasma membrane is altered in such a way as to allow the utilization of extracellular Ca^{2+} for the maintenance of some hormone-induced redistribution of intracellular Ca^{2+} (which may include an elevation in the concentration of cytoplasmic Ca^{2+}; Barritt *et al.*, 1981; Murphy *et al.*, 1981). Thus the signal(s) generated in response to α-adrenergic agonist/receptor binding appear to mobilize both intracellular and extracellular Ca^{2+}

This work was supported by a grant to F. L. B. from the National Health and Medical Research Council of Australia.

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