Biochem. J. (1982) **208**, 619–630
Printed in Great Britain

Calcium ion fluxes induced by the action of α-adrenergic agonists in perfused rat liver

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(*Received 25 May 1982/Accepted 17 August 1982*)

Phenylephrine (2.0 μM) induces an α_1-receptor-mediated net efflux of Ca^{2+} from livers of fed rats perfused with medium containing physiological concentrations (1.3 mM) of Ca^{2+}. The onset of efflux (7.1 ± 0.5 s; $n = 16$) immediately precedes a stimulation of mitochondrial respiration and glycogenolysis. Maximal rates of efflux are observed between 35 s and 45 s after α-agonist administration; thereafter the rate decreases, to be no longer detectable after 3 min. Within seconds of terminating phenylephrine infusion, a net transient uptake of Ca^{2+} by the liver is observed. Similar effects were observed with vasopressin (1 m-unit/ml) and angiotensin (6 nM). Reducing the perfusate [Ca^{2+}] from 1.3 mM to 10 μM had little effect on α-agonist-induced Ca^{2+} efflux, but abolished the subsequent Ca^{2+} re-uptake, and hence led to a net loss of 80–120 nmol of Ca^{2+}/g of liver from the tissue. The administration at 5 min intervals of short pulses (90 s) of phenylephrine under these conditions resulted in diminishing amounts of Ca^{2+} efflux being detected, and these could be correlated with decreased rates of α-agonist-induced mitochondrial respiration and glucose output. An examination of the Ca^{2+} pool mobilized by α-adrenergic agonists revealed that a loss of Ca^{2+} from mitochondria and from a fraction enriched in microsomes accounts for all the Ca^{2+} efflux detected. It is proposed that the α-adrenergic agonists, vasopressin and angiotensin mobilize Ca^{2+} from the same readily depleted intracellular pool consisting predominantly of mitochondria and the endoplasmic reticulum, and that the hormone-induced enhanced rate of mitochondrial respiration and glycogenolysis is directly dependent on this mobilization.

The stimulation of hepatic glycogenolysis by α-adrenergic agonists, vasopressin and angiotensin appears to be mediated by a cyclic AMP-independent mechanism, possibly involving the direct activation of phosphorylase *b* kinase through an elevation of the cytosolic free Ca^{2+} concentration (Sherline *et al.*, 1972; Tolbert *et al.*, 1973; Hutson *et al.*, 1976; Cherrington *et al.*, 1976; Birnbaum & Fain, 1977; Chan & Exton, 1977; Van de Werve *et al.*, 1977; Blair *et al.*, 1979). The pool of Ca^{2+} mobilized during this activation, however, remains a controversial issue. Some investigators have proposed that α-adrenergic agonists can induce the inflow of Ca^{2+} from the external medium into the cell (Assimacopoulos-Jeannet *et al.*, 1977; Foden & Randle, 1978; Poggioli *et al.*, 1980; Barritt *et al.*, 1981*a*), whereas others have suggested that the pool is derived from stores located at, or close to, the plasma membrane (Althaus-Salzmann *et al.*, 1980). Also, a number of reports indicate that a major portion of the Ca^{2+} may be released from mitochondria or other intracellular organelles (Chen *et al.*, 1978; Blackmore *et al.*, 1979; Babcock *et al.*, 1979; Murphy *et al.*, 1980; Barritt *et al.*, 1981*a*,*b*; Berthon *et al.*, 1981).

Several features of the experimental conditions employed in the different studies cited may account for the seemingly conflicting conclusions. First, recent work has established that the effects of α-adrenergic agonists on hepatic metabolism are very rapid, and in many instances transient (Scholz & Schwabe, 1980; Reinhart *et al.*, 1981, 1982*a*). Hence studies involving, for example, the preincubation of isolated liver cells with adrenaline for up to 60 min (Foden & Randle, 1978) may give misleading information.

A second point is that isolated cells or perfused livers have been exposed in most studies to low Ca^{2+} concentrations (10–50 μM) in order to facilitate detection of Ca^{2+} flux exchanges. In those studies,

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Ca^{2+} release from hepatocytes or perfused livers has been measured by using atomic-absorption spectroscopy, chlortetracycline fluorescence or Ca^{2+}-sensitive electrodes (Chen *et al.*, 1978; Blackmore *et al.*, 1979*a*; Babcock *et al.*, 1979; Althaus-Salzmann *et al.*, 1980). Since 10μM-Ca^{2+} represents only about 1% of unbound extracellular Ca^{2+}, the prolonged use of such media considerably depletes the Ca^{2+} content of the whole liver, or of subsequently prepared liver fractions (Blackmore *et al.*, 1979*a*,*b*), as well as altering the cellular response to α-adrenergic agonists (Chan & Exton, 1977; Van de Werve *et al.*, 1977; Blair *et al.*, 1979; Assimacoupoulos-Jeannet *et al.*, 1977). A third point is that studies attempting to correlate changes in cellular Ca^{2+} fluxes with the Ca^{2+} content of subsequently prepared liver organelles have utilized extended fractionation procedures that allow a possible Ca^{2+} redistribution between organelles during their isolation (Blackmore *et al.*, 1979*a*,*b*; Babcock *et al.*, 1979; Althaus-Salzmann *et al.*, 1980; Murphy *et al.*, 1980; Poggioli *et al.*, 1980; Barritt *et al.*, 1981*a*,*b*; Berthon *et al.*, 1981).

In an attempt to overcome these problems, we have used a Ca^{2+}-specific electrode to continuously measure rapid cellular Ca^{2+} flux changes in response to α-adrenergic agonists and other hormones, in livers perfused with media containing 1.3mM-added Ca^{2+}. In addition, we have correlated the extent and direction of cellular Ca^{2+} fluxes with the Ca^{2+} content of liver organelles prepared by a recently-developed rapid fractionation procedure (Reinhart *et al.*, 1982*b*). The rapid hormone-induced changes in liver O_2 consumption and glucose output were also determined, and correlated with changes in Ca^{2+} flux and Ca^{2+} content.

We conclude that the earliest measurable effect of α-adrenergic agonists on perfused liver is to stimulate the rate of Ca^{2+} efflux from cells. The source of this mobilized Ca^{2+} appears to be intracellular organelles, predominantly mitochondria and the endoplasmic reticulum. Both α-adrenergic agonist-induced glycogenolysis and mitochondrial respiration (Reinhart *et al.*, 1982*a*) are dependent on the mobilization of Ca^{2+} from this pool.

Experimental

Animals and perfusions

Male Wistar-strain albino rats, weighing between 200 and 250g and having free access to food, were used for all experiments. Rats were anaesthetized with sodium pentobarbitone (50mg/kg body wt.), and the livers were perfused with Krebs–Henseleit bicarbonate medium (Krebs & Henseleit, 1932) equilibrated with O_2/CO_2 (19:1) and containing either 1.30mM-added $CaCl_2$ or no added $CaCl_2$ (Reinhart *et al.*, 1982*a*). To reduce the time for which livers were exposed to a low Ca^{2+} concentration, $CaCl_2$ was administered by infusion syringe, the $CaCl_2$ infusion being terminated only 5min before a low-Ca^{2+} medium was required. At 3–4min after the termination of Ca^{2+} infusion, the perfusate Ca^{2+} concentration had decreased from 1300μM to approx. 10μM, as determined both by atomic-absorption spectroscopy and Ca^{2+}-electrode measurements. All experiments were carried out between 08:00h and 12:00h to minimize diurnal fluctuations in the amount of basal glucose output.

Perfusate Ca^{2+} and O_2 determinations

The perfusate Ca^{2+} concentration was continuously monitored with a Radiometer F2112 Ca^{2+}-selective electrode, 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 ionanalyzer, set to the grounded-solution mode. The output of the ionanalyzer was modified by Orion Research, Sydney, Australia, to yield a 2mV output signal per mV displayed. Data was recorded by coupling the microprocessor ionanalyzer to a Spectra-Physics SP4100 computing integrator through a bucking-voltage device similar to that described by Madeira (1975). Shielded cables were used for all connections. The computing integrator was programmed to display both changes in perfusate Ca^{2+} concentration (peak height) and the total amounts of Ca^{2+} taken up or released by the liver (integration mode).

The Ca^{2+}-sensitive electrode was placed in a small flow-through reaction vessel mounted above a magnetic stirring unit, as close as practicable to the liver. Both the Ca^{2+} electrode, and the reference combination electrode vessels were kept at 20°C in a Faraday cage. For each experiment the Ca^{2+} electrode response was calibrated during the initial 15min of perfusion, by infusing known amounts of $CaCl_2$, at a constant flow-rate, between the liver and the electrode. In this way changes in mV output could be related to increases or decreases in the total Ca^{2+} concentration. Electrode membranes were discarded if the voltage change deviated by more than 15% from theoretical (Nernstian) values.

Preliminary experiments indicated that under some conditions the Ca^{2+}-sensitive electrode responds to changes in the concentration of perfusate O_2. This was traced to the existence of earth loops, and could be overcome by electrically isolating the perfusion circuit from any earth leaks. The only earth connection is from the solution in which the reference combination electrode is submerged, to the microprocessor ionanalyzer. Thereafter, altering the O_2 concentration in the perfusion medium between 50μM and 800μM did not alter the Ca^{2+} electrode signal.

A second initial observation was that the basal Ca^{2+} electrode potential is dependent on the flow rate of medium through the liver. As the vasoconstrictive action of some of the hormones and agonists used in the present study tend to transiently reduce the flow rate through the liver (P. H. Reinhart, W. M. Taylor & F. L. Bygrave, unpublished work), the perfusion circuit was changed from constant pressure to constant flow-rate.

For some experiments the performance of the Ca^{2+} electrode was independently checked by using atomic-absorption spectroscopy (see Fig. 6). Perfusate samples were extracted with 2 M-$HClO_4$ and analysed in 0.1% (w/v) KCl using an N_2O/acetylene flame (medical-grade gases).

The O_2 consumption by the liver was calculated from the difference between influent and effluent O_2 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 electrode was calibrated before every experiment, and after each experiment the lag time, from the point of hormone infusion to either the Ca^{2+} or oxygen electrode, was determined by using 10mM-$CaCl_2$ and 50mM-$Na_2S_2O_4$ respectively. Lag times varied as a function of flow rate and liver weight, but were usually from 5 to 8s for Ca^{2+}, and 8 to 12s for O_2. Within any one experiment, lag times could be reproducibly measured to within 0.1s. All rates of O_2 and Ca^{2+} concentration changes are expressed as μM. To express data as μmol/g wet wt. of liver per min, simply multiply by the constant 0.0035 [since the flow rate for all perfusions was constant at 3.5ml/g of liver per min, μM = μmol/liver wt. (g) per 1000/3.5 liver wt. (g)/(min)].

Glucose-output determinations

Effluent perfusate was assayed for glucose by using the glucose oxidase/peroxidase method as previously described (Reinhart *et al.*, 1982*a*).

Liver fractionation and Ca^{2+} content assay

In some experiments the liver was fractionated by a rapid procedure, recently developed in this laboratory (Reinhart *et al.*, 1982*b*). Briefly, this involved excising the median lobe and homogenizing in a medium consisting of 210mM-mannitol, 60mM-sucrose, 10mM-KCl, 10mM-sodium succinate, 1mM-ADP, 0.25mM-dithiothreitol, 2μM-EGTA, 5μM-Ruthenium Red, 1mM-nupercaine and 10mM-Hepes/KOH (pH7.4). A portion of the resulting homogenate (2ml) was layered on to a discontinuous density gradient of iso-osmotic Percoll, and centrifuged for 30s at 39600 $g_{av.}$ in a Sorvall RC-5B refrigerated centrifuge with the SS 34 rotor. In this way nine liver fractions were obtained within a total preparation time of 6min. In the present study only five of the nine fractions (fractions 1, 2, 4, 6 and 8) were further assayed, since more than 90% of the total protein and Ca^{2+} could be recovered in these five fractions (see Fig. 1 in Reinhart *et al.*, 1982*b*). The recovery of marker enzyme activities for mitochondria, endoplasmic reticulum, plasma membrane, peroxisomes and lysosomes in the nine fractions has previously been described (Reinhart *et al.*, 1982*a*). Ruthenium Red and nupercaine had no significant effects on the distribution of marker enzyme activities (results not shown). Immediately after isolation, fractions 1, 2, 4, 6 and 8 were extracted with ice-cold 2M-$HClO_4$ for 30min. Portions of the supernatant were analysed for Ca^{2+} using the atomic-absorption spectroscopy procedure described above.

Chemicals and materials

Phenylephrine, glucagon, [Arg^8]vasopressin, [Val^5]-angiotensin, Ruthenium Red and the glucose assay kit (510-A) were obtained from Sigma. Percoll and density-marker beads were from Pharmacia Fine Chemicals AG, Uppsala, Sweden. Nupercaine (cinchocaine hydrochloride) was supplied by Astra Chemicals, Sydney, N.S.W., Australia, Ca^{2+}-electrode membranes (F2002) and filling solution S 43316 were obtained from Radiometer, Copenhagen, Denmark. Prazosin and yohimbine were gifts from Dr. N. H. Hunt, John Curtin School of Medical Research, Australian National University. Other chemicals used were of analytical grade.

Expression of data

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

Results

Effect of α-adrenergic agonists and other glycogenolytic hormones on liver Ca^{2+} fluxes

As the experimental approaches, previously used to examine the effects of α-agonists on liver Ca^{2+} fluxes, have serious shortcomings, we have investigated these flux changes in more detail. In the present study using a Ca^{2+}-sensitive electrode system, as described in the Experimental section, we show that phenylephrine induces a rapid and transient net efflux of Ca^{2+} from livers of fed rats perfused with medium containing physiological concentrations (1.3mM) of Ca^{2+}. Data in Fig. 1 indicate the time of onset of phenylephrine-induced Ca^{2+} efflux is very rapid, occurring at just 7.1 ± 0.5s ($n = 16$) after α-agonist administration. Ca^{2+} efflux occurs just before detection of phenylephrine-induced increases in O_2 consumption and glucose output, which, in this series of experiments, was

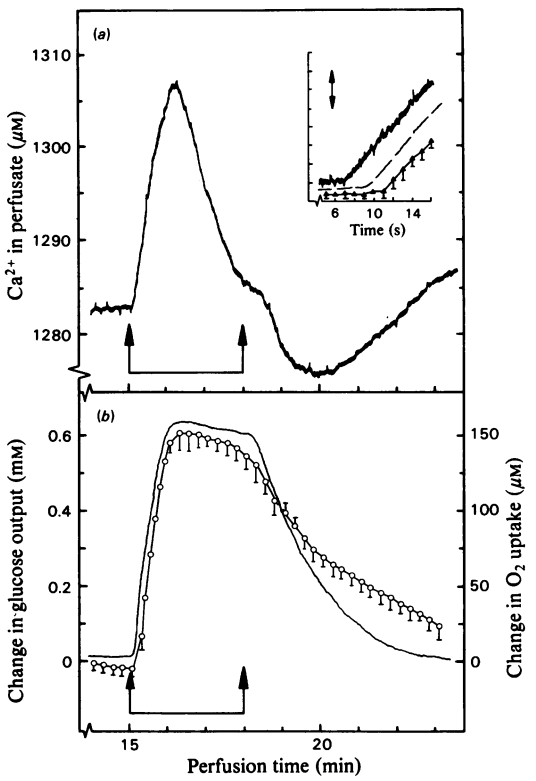


Fig. 1. *Effect of phenylephrine on effluent Ca^{2+} concentration (a), O_2 uptake and glucose release (b)*
Livers of fed rats were perfused initially for 15 min with Krebs–Henseleit buffer equilibrated with Carbogen (O_2/CO_2, 19:1) and containing 1.3 mM added $CaCl_2$ as described in the Experimental section. Livers were then infused with phenylephrine (2.0 μM, final concentration) for 3 min. (*a*) The effluent Ca^{2+} concentration was measured using a Ca^{2+}-selective electrode coupled to a microprocessor/ionalyzer and a computing integrator as described in the text. (*b*) O_2 uptake (continuous trace) was estimated from the difference between influent and effluent O_2 concentrations. Glucose (○) was assayed at 1 s intervals after removing contaminating erythrocytes by centrifugation. The inset in (*a*) shows the initial corrected time course (i.e. lag times subtracted) of phenylephrine-induced Ca^{2+} efflux (continuous trace), O_2 uptake (broken trace) and glucose output (▲). The interval shown represents 4 μM-Ca^{2+}, 20 μM-O_2 or 0.1 mM-glucose. Ca^{2+} and O_2 traces shown are from one typical experiment of 16 performed. Glucose output data are means ± S.E.M. for nine independent experiments.

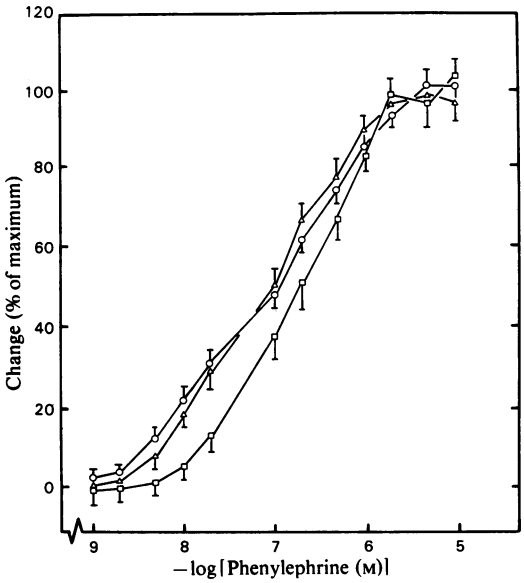


Fig. 2. *Dose–response data for the effect of phenylephrine on Ca^{2+} efflux, O_2 uptake and glucose output*
Livers of fed rats were perfused as described in the legend to Fig. 1. At 15 min of perfusion, phenylephrine, at the concentrations indicated, was infused and the maximal changes in Ca^{2+} efflux (○), O_2 uptake (△) and glucose output (□) were determined as described in the legend to Fig. 1. Maximal changes (100%) for Ca^{2+} efflux, O_2 uptake and glucose output were 21.5 ± 0.4 μM ($n = 8$), 164 ± 4.1 μM ($n = 12$), and 0.615 ± 0.04 mM ($n = 7$) respectively. Data are means ± S.E.M. for between four and 12 independent experiments.

shown to occur at 9.6 ± 0.6 s ($n = 16$) and 11.5 ± 0.4 s ($n = 12$) respectively, after α-agonist infusion (Fig. 1; Reinhart *et al.*, 1982*a*). No detectable influx of Ca^{2+} was seen before the Ca^{2+} efflux. The rate of Ca^{2+} efflux increased, to reach a maximum of 20–25 μM at 35–45 s of phenylephrine treatment, thereafter declining until being no longer detectable after 2–3 min. In contrast, phenylephrine-induced increases in O_2 consumption and glucose output were slower in reaching maximal values, requiring 50–60 s after α-agonist infusion, and were maintained near these rates for at least 3 min. Within 10–15 s of terminating the infusion of phenylephrine, a compensatory net uptake of Ca^{2+} by the liver is observed. The rate of this Ca^{2+} influx was slower but more prolonged (up to 5 min) than the preceding efflux rate and hence the total net amounts of Ca^{2+} involved in the efflux and influx phases (80–120 nmol/g wet wt. of liver) were approximately similar. Phenylephrine-stimulated rates of respiration and glucose output both declined toward basal values during this period of Ca^{2+} influx.

Fig. 2 shows the dose–response curves for the effect of phenylephrine on Ca^{2+} efflux, glucose output and O_2 uptake. The Ca^{2+} efflux and O_2 uptake dose–response curves are very similar,

showing significant stimulation at 8 nM-phenylephrine and half-maximal and maximal responses at 0.1 and 2 μM-phenylephrine respectively. Glucose output is slightly less sensitive to phenylephrine, with significant stimulation observed at 20 nM-phenylephrine and half-maximal and maximal responses evident at agonist concentrations of 0.2 and 1 μM respectively.

The effect of a range of hormones, agonists or analogues on the maximal rate of net Ca^{2+} efflux is shown in Fig. 3. Vasopressin (3 m-units/ml) and angiotensin (10 nM), hormones thought to stimulate hepatic glycogenolysis in a cyclic AMP-independent manner (Hems & Whitton, 1973; Kirk & Hems, 1974; Keppens & De Wulf, 1976; Keppens *et al.*, 1977; Fain, 1978), and adrenaline (0.2 μM) all induce a rapid efflux of Ca^{2+} similar to that described for phenylephrine. For each of these agents the Ca^{2+} efflux response was transient, maximal effects of 19–26 μM being observed at between 35 and 50 s of treatment, and basal Ca^{2+} concentrations being re-established by 3 min (results not shown). In contrast, the administration of glucagon or of cyclic AMP analogous at concentrations producing maximal effects on glycogenolysis (Reinhart *et al.*, 1982*a*) had comparatively small effects only.

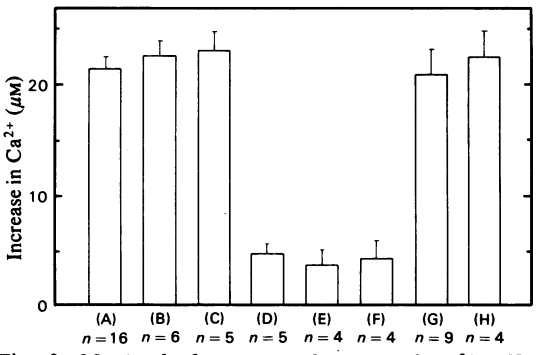


Fig. 3. *Maximal changes in the rate of Ca^{2+} efflux induced by hormones or agonists from livers perfused with Krebs–Henseleit medium containing 1.3 mM added $CaCl_2$*

Livers of fed rats were perfused as described in the legend to Fig. 1. At 15 min of perfusion hormones or agonists were infused at these final concentrations: phenylephrine (A), 2 μM; adrenaline (B), 0.2 μM; noradrenaline (C), 0.2 μM; glucagon (D), 10 nM; dibutyryl cyclic AMP (E), 0.1 mM; 8-bromo-cyclic AMP (F), 0.1 mM; vasopressin (G), 3 munits/ml; angiotensin (H), 10 nM. Maximal rates of Ca^{2+} efflux were determined as described in the legend to Fig. 1. Data are means ± S.E.M. for the numbers of independent experiments indicated.

The effect of α_1- and α_2-adrenergic binding inhibitors on phenylephrine-stimulated Ca^{2+} efflux

Livers cells have been shown to contain both α_1- and α_2-adrenergic receptors (Hoffman *et al.*, 1980), and the activation of either receptor class may lead to metabolic responses (Wikberg, 1979; Hoffman *et al.*, 1980; Fain & Garcia-Sainz, 1980; Tolbert *et al.*, 1980; Jard *et al.*, 1981). Stimulation of both glycogenolysis, and of mitochondrial respiration by phenylephrine, adrenaline or noradrenaline has previously been shown (Reinhart *et al.*, 1982*a*) to be mediated via the α_1-receptor system in studies detailing the relative sensitivity of these responses to specific α_1- or α_2-receptor antagonists. Phenylephrine-induced Ca^{2+} efflux is also apparently an α_1-receptor-mediated event, since the addition of the specific α_1-antagonist prazosin at either 20 nM or 0.2 μM almost completely abolished the response (Fig. 4). In contrast, the administration of 0.2 μM-yohimbine (an α_2-binding antagonist) had essentially no effect on either phenylephrine-induced (Fig. 4) or adrenaline-induced (results not shown) Ca^{2+} efflux.

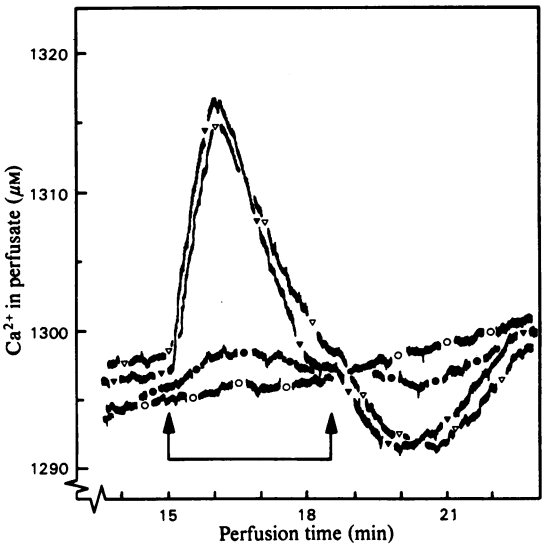


Fig. 4. *Effect of α_1- and α_2-adrenergic antagonists on phenylephrine-induced Ca^{2+} efflux*

Perfusion details are as outlined in the legend to Fig. 1. At 15 min of perfusion prazosin at 20 nM (●) or 0.2 μM (○) final concentration, or yohimbine at 20 nM (▼) or 0.2 μM (▽) were infused for 5 min before phenylephrine (2.0 μM) administration. Changes in the rates of Ca^{2+} efflux were determined as described in the legend to Fig. 1. Data for each agent are typical experiments for between four and six independent experiments performed.

The effect of perfusate Ca^{2+} concentration on α-adrenergic agonist or hormone-stimulated Ca^{2+} efflux, respiration and glucose output

The effects of phenylephrine (2 μM) on Ca^{2+} efflux, O_2 consumption and glucose output in rat livers perfused with either 1.3 mM- or approx. 10 μM-Ca^{2+}

are shown in Figs. 5(*a*) and 5(*b*) and Figs. 5(*c*) and 5(*d*) respectively. At each concentration the initial response to the first pulse of phenylephrine administration is very similar, with times of onset and maximal responses of Ca^{2+} efflux and enhanced O_2 consumption and glucose output being essentially as detailed in Fig. 1. Differences in Ca^{2+} efflux became evident, however, approx. 1 min after α-agonist administration. In perfusions performed with media containing approx. 10 μM-Ca^{2+}, the efflux of the ion was more prolonged and did not return to basal levels until 4–5 min after phenylephrine was first administered. In addition, no compensatory uptake of Ca^{2+} after efflux was observed in this medium (Fig. 5*c*). The absence of any observed Ca^{2+} uptake is not due to the possibility that the concentration of Ca^{2+} in the perfusate is below the detection limit of the Ca^{2+} electrode, since, at the experimental conditions employed, the relationship between pCa and mV remains linear up to approx. 0.1 μM (results not shown).

Further marked differences in response to phenylephrine were also observed when up to five consecutive short infusions of the agonist were performed at the two Ca^{2+} concentrations described. Whereas the stimulation of Ca^{2+} efflux, O_2 consumption and glucose output was essentially the same as described in Fig. 1 during each of five successive administrations of phenylephrine to livers perfused with 1.3 mM-Ca^{2+}, in livers perfused with 10 μM-Ca^{2+} the responses were all increasingly reduced with successive phenylephrine infusions. Under these latter conditions increases in the rate of Ca^{2+} efflux, O_2 consumption and glucose output were all inhibited to approximately the same degree, being virtually abolished after the third phenylephrine pulse. This inhibition could be overcome by exposing the liver to 1.3 mM-Ca^{2+} for 90 s before another pulse of phenylephrine infusion (Figs. 5*c* and 5*d*).

The effect of successive short infusions of different hormones or agonists on Ca^{2+} efflux in livers perfused with approx. 10 μM-Ca^{2+} is shown in Fig. 6. Clearly, prior infusion of phenylephrine diminishes not only the responses to the later re-administration of the agonist (Fig. 5) but also to vasopressin or angiotensin as well. Similarly the responses to phenylephrine are diminished after prior vasopressin (Fig. 6) or angiotensin (results not shown) infusion.

In contrast, the glycogenolytic action of glucagon was still near-maximal after prior infusion of phenylephrine and vasopressin. Furthermore the responses to phenylephrine, vasopressin or angiotensin were not altered appreciably by prior infusion of glucagon. [Values for glucose output were partially masked by the prolonged glycogenolytic effect of glucagon (Reinhart *et al.*, 1982*a*) in these latter experiments; results not shown.]

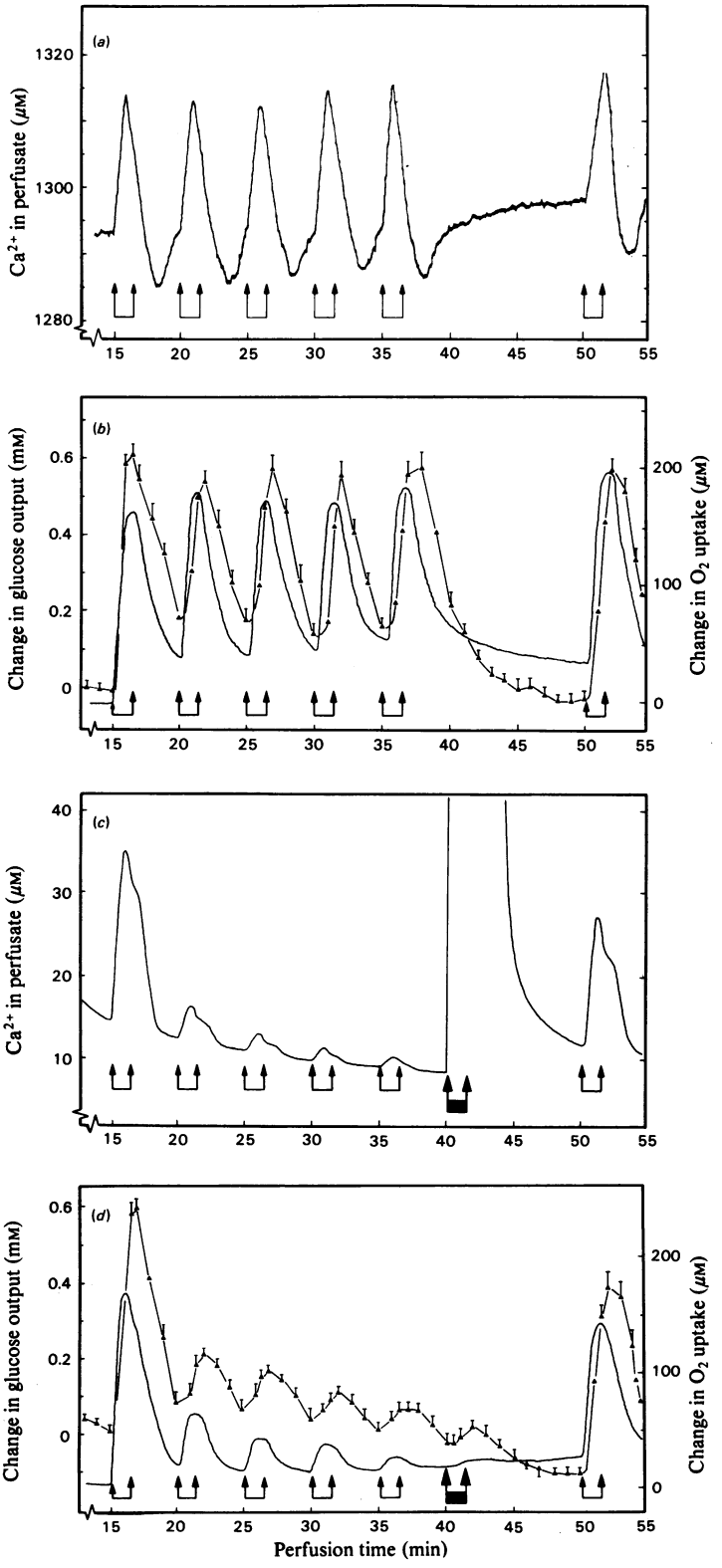
The effect of phenylephrine on the Ca^{2+} content of liver fractions

The data in Figs. 1–6 indicate that phenylephrine induces a rapid and transient release of Ca^{2+} from a cellular pool in the perfused liver. We attempted to locate the intracellular pool(s) by using a rapid liver fractionation technique (Reinhart *et al.*, 1982*b*). During these studies perfusions were carried out in media containing 10 μM-Ca^{2+}, since under these conditions the administration of phenylephrine results in a net loss of Ca^{2+} from cells, as efflux is not followed by uptake (Fig. 5). 'Low-Ca^{2+}' perfusions also have the effect of decreasing the total basal Ca^{2+} content of all subcellular fractions (Blackmore *et al.*, 1979*a*). However, it is not known whether this difference represents a depletion of Ca^{2+} in 10 μM-Ca^{2+}, or a sequestration of Ca^{2+} in 1.3 mM-Ca^{2+}-containing medium.

Fig. 7 shows the effect of phenylephrine on the Ca^{2+} content of five fractions isolated from liver treated for between 10 and 180 s with the α-agonist. The largest loss of Ca^{2+} induced by phenylephrine occurs in fraction 8, which we have previously shown to consist of relatively uncontaminated mitochondria (Reinhart *et al.*, 1982*b*). Significant

Fig. 5. *A comparison of the effects of short repeated pulses of phenylephrine on Ca^{2+} efflux, O_2 uptake and glucose output measured in 'high-Ca^{2+}' and 'low-Ca^{2+}' perfusion medium*

For 'high-Ca^{2+}' experiments (*a* and *b*) livers were perfused with media containing 1.3 mM added Ca^{2+} for 15 min, at which time phenylephrine (2.0 μM) was infused for 90 s; 3.5 min later another 90 s phenylephrine pulse was administered and this process was repeated three more times. At 50 min of perfusion, a sixth 90 s pulse of phenylephrine was administered. For 'low-Ca^{2+}' experiments (*c* and *d*), the $CaCl_2$ in the Krebs–Henseleit buffer was replaced with NaCl. During the first 10 min of perfusion 1.3 mM-$CaCl_2$ (final concentration) was administered by infusion syringe. At 10 min of perfusion time $CaCl_2$ infusion was terminated and at 15 min perfusion time, 90 s pulses of phenylephrine (2.0 μM) were administered every 5 min. At 40 min of perfusion, a 90 s pulse of $CaCl_2$ (1.3 mM final concentration) was administered (arrows) and 5 min later a final pulse of phenylephrine (2.0 μM). (*b*) and (*d*) show changes in O_2 uptake (continuous trace lines) and glucose output (▲). Ca^{2+} and O_2 traces are from representative experiments of between three and five performed independently. Glucose data are expressed as means ± S.E.M. for three to six independent experiments.


(a)
1320
1300
1280
Ca^{2+} in perfusate (μM)
15 20 25 30 35 40 45 50 55
(b)
0.6
0.4
0.2
0
Change in glucose output (mM)
200
100
0
Change in O_2 uptake (μM)
15 20 25 30 35 40 45 50 55
(c)
40
30
20
10
Ca^{2+} in perfusate (μM)
15 20 25 30 35 40 45 50 55
(d)
0.6
0.4
0.2
0
Change in glucose output (mM)
200
100
0
Change in O_2 uptake (μM)
15 20 25 30 35 40 45 50 55
Perfusion time (min)

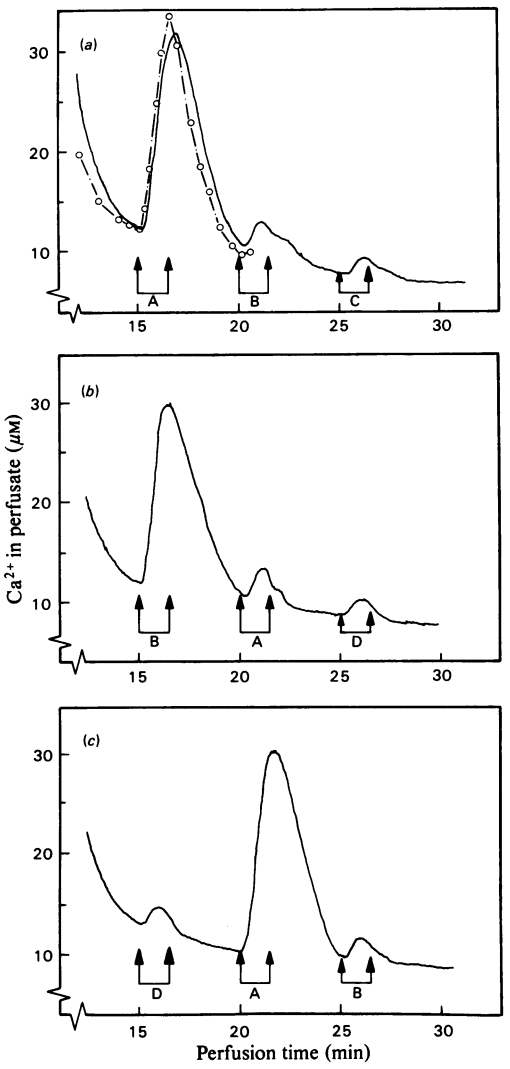


Fig. 6. *Effects of successive short infusions of different agonists or hormones on Ca^{2+} efflux*

Livers of fed rats were perfused with Krebs–Henseleit medium in which the $CaCl_2$ had been replaced with NaCl. During the first 10min of perfusion, $CaCl_2$ (1.3mM final concentration) was administered by perfusion syringe. At 15, 20 and 25min of perfusion, 90s pulses of different hormones or agonists (A, phenylephrine; B, vasopressin; C, angiotensin; D, glucagon) were administered at the concentrations described in the legend to Fig. 3. Ca^{2+} efflux was measured as described in the legend to Fig. 1 and the results shown are representative experiments for between three and five performed independently. Alternatively atomic-absorption spectroscopy was used to assay Ca^{2+} in the perfusate (O) as described in the Experimental section. Data are means from nine independent experiments. For the sake of clarity, standard errors of the mean have been omitted; however, $\pm 2.4\mu$M was never exceeded.

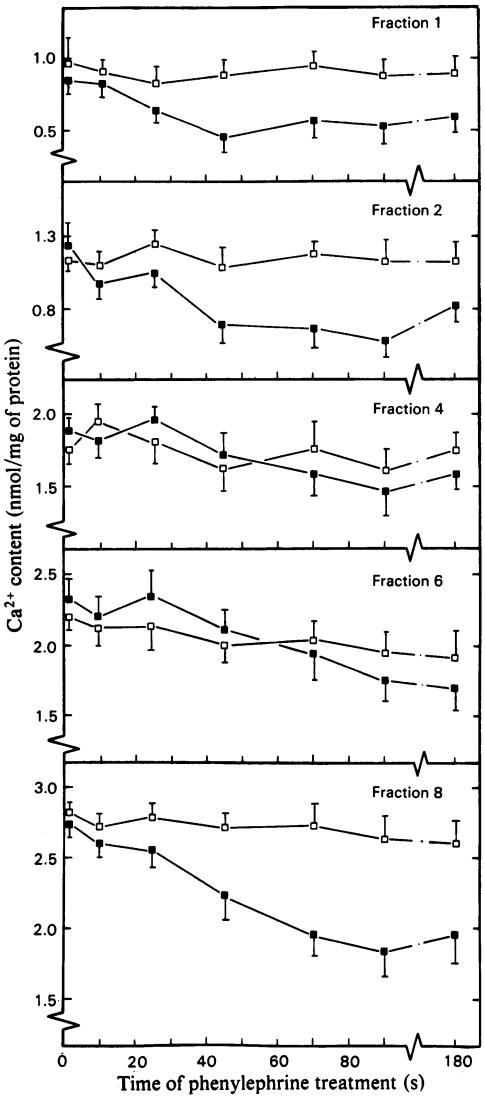


Fig. 7. *The effect of phenylephrine on the Ca^{2+} content of liver fractions*

Livers of fed rats were perfused as described in the legend to Fig. 6, with the infusion of $CaCl_2$ (1.3mM final concentration) being terminated at 10min of perfusion time. At 15min of perfusion phenylephrine (2.0μM; ■) or Krebs–Henseleit medium (□) was infused for the times indicated. At these times the median and left main lobes were excised and rapidly fractionated as described in the Experimental section. The Ca^{2+} content of the fractions shown was determined by atomic-absorption spectroscopy. Data are means ± S.E.M. for between three and nine independent experiments for each time point and condition.

decreases in the Ca^{2+} content of this fraction can be measured after 25s of phenylephrine treatment with maximal effects observed after between 45 and 60s

of treatment. The maximal loss of Ca^{2+} from this pool is 0.82 ± 0.2 ($n = 6$) nmol/mg of protein, which corresponds to approx. 50 nmol/g of liver or approx. 50% of the Ca^{2+} detected by using the Ca^{2+} electrode. From Fig. 7 it is also evident that a small decrease in Ca^{2+} occurs in fractions 1 and 2 over a similar time course. The maximal amount of Ca^{2+} lost from these glucose 6-phosphatase-enriched fractions is 0.4 nmol/mg, but as the protein yield is higher, the total amount of Ca^{2+} lost from these two fractions is similar to that lost from fraction 8 (approx. 40 nmol/g of liver). No significant phenylephrine-induced decreases in Ca^{2+} content were observed in any of the other fractions (results for fractions 3, 5, 7 and 9 not shown), even though fraction 6 contains significant amounts of both cytochrome *c* oxidase and glucose 6-phosphatase activities. Presumably fraction 6 contains a hormone-insensitive subpopulation of these organelles (Prpić *et al.*, 1978; Taylor *et al.*, 1980; Reinhart & Bygrave, 1981) or non-intact organelles. Qualitatively similar data were obtained when perfusions were carried out in media containing 1.3 mM added $CaCl_2$, although quantitatively the changes were smaller and the experimental error was larger (Ca^{2+} content in fraction 8: control, 6.81 ± 0.53 mmol/mg of protein, $n = 3$; 3 min phenylephrine-treated, 6.07 ± 0.44 nmol/mg of protein, $n = 3$).

Discussion

The present study demonstrates that α-adrenergic agonists, vasopressin and angiotensin all induce a transient net efflux of Ca^{2+} within seconds after their administration to rat liver perfused with media containing physiological concentrations of Ca^{2+}. The onset of Ca^{2+} efflux immediately precedes the onset of hormone-induced increases in both respiration and glycogenolysis, a finding consistent with our previous report (Reinhart *et al.*, 1982*a*), showing that these hormones stimulate mitochondrial respiration just before glycogenolysis. Hence the mobilization of a cellular Ca^{2+} pool represents one of the earliest and most transient effects of α-adrenergic agonists on rat liver.

Previous attempts to determine the effects of α-agonists on cellular Ca^{2+} fluxes have yielded conflicting data and interpretations (Foden & Randle, 1978; Blackmore *et al.*, 1979*a*,*b*; Babcock *et al.*, 1979; Poggioli *et al.*, 1980; Althaus-Salzmann *et al.*, 1980; Chen *et al.*, 1978; Murphy *et al.*, 1980; Barritt *et al.*, 1981*a*,*b*; Berthon *et al.*, 1981). Hence a critical examination of some of the experimental details employed in previous studies is now warranted. Investigators using $^{45}CaCl_2$ to estimate net Ca^{2+} flux changes have suggested that noradrenaline or phenylephrine may stimulate cellular Ca^{2+} uptake (Assimacopoulos-Jeannet *et al.*, 1977; Foden & Randle, 1978), Ca^{2+} efflux (Barritt *et al.*, 1981*a*) or Ca^{2+} uptake followed by efflux (Poggioli *et al.*, 1980). These discrepancies may in part be due to unknown changes in pool sizes and specific radioactivities of $^{45}Ca^{2+}$, as well as the fact that in some studies $^{45}Ca^{2+}$ flux changes were not determined during the first 60 s of α-agonist administration, the time when we observe maximal changes in Ca^{2+} fluxes (Fig. 1). Furthermore, a rigorous analysis of the effects of adrenaline on $^{45}Ca^{2+}$ exchange curves in hepatocytes revealed both long-term (steady-state) and short-term (transient) effects of the hormone (Barritt *et al.*, 1981*a*), emphasizing the importance of correlating the temporal progression of Ca^{2+} flux changes with Ca^{2+}-regulated metabolic pathways such as glycogenolysis. Both the extent and the time-dependence of these responses were markedly altered by decreasing the extracellular Ca^{2+} concentration from 1.3 mM to 0.1 mM (Barritt *et al.*, 1981*a*), further highlighting the importance of the experimental conditions employed.

Previous studies using atomic-absorption spectroscopy (Blackmore *et al.*, 1979*b*), chlortetracycline fluorescence (Babcock *et al.*, 1979) or ion-sensitive electrodes (Althaus-Salzmann *et al.*, 1980; Chen *et al.*, 1978) to follow α-adrenergic agonist-induced Ca^{2+} flux changes have resorted to using media containing only very low concentrations of Ca^{2+} (10–50 μM). Flux changes observed under these conditions may not accurately reflect changes at more physiological Ca^{2+} concentrations. A dependence of α-adrenergic agonist-induced Ca^{2+} fluxes on the extracellular Ca^{2+} concentration was evident in the present study, in which we found that reducing the perfusate calcium concentration from 1.3 mM to 10 μM for only 1–2 min distinctly alters not only the Ca^{2+} efflux response, but also the respiratory and glycogenolytic responses, particularly at times of α-agonist administration longer than 60 s (see Fig. 5). The finding that the initial responses (<60 s) to α-agonists are not significantly altered by reducing the perfusate Ca^{2+} concentration indicates that extracellular Ca^{2+} appears not to play a role in the primary hormone signal. This conclusion was confirmed by experiments in which the extracellular Ca^{2+} concentration was further reduced from 10 μM to less than 0.1 μM by infusing 0.2 mM-EGTA for 3 min before phenylephrine treatment (results not shown). In these experiments the extent of Ca^{2+} efflux and glycogenolysis was only slightly less than that observed in 1.3 mM-Ca^{2+}-containing medium, a finding consistent with results presented by Blackmore *et al.* (1978), who showed that phenylephrine activates phosphorylase *a* and induces a decrease in the total Ca^{2+} content of hepatocytes incubated in the presence of stoichiometric concentrations of EGTA.

However, extracellular Ca^{2+} does appear to play

an important role in the longer term (>60s) effects of α-agonists, since both the respiratory and the glycogenolytic responses are more transient in 10 μM-Ca^{2+}-containing medium, and no re-influx of Ca^{2+} is observed under these conditions (Fig. 5). This last finding is of particular interest, since this hormone-induced irreversible loss of the Ca^{2+} allows the depletion of the α-agonist-sensitive pool of Ca^{2+} by three short consecutive pulses of hormone.

Assuming that the short-term perfusion of livers with media containing 10 μM-Ca^{2+} does not in itself decrease the size of the hormone-sensitive pool of Ca^{2+}, then by integrating the Ca^{2+} efflux peaks, we estimate that the total α-adrenergic agonist-mobilized pool contains approx. 140nmol of Ca^{2+}/g wet wt. of liver, a value that may be compared with 160–180 nmol/g wet wt. for the mitochondrial pool of exchangeable Ca^{2+} (Claret-Berthon *et al.*, 1977; Barritt, 1981). Our calculated pool size may be a slight overestimate, since we cannot rule out the possibility that a very slow Ca^{2+} uptake by cells is taking place between consecutive α-agonist pulses. This hormone-sensitive Ca^{2+} pool is apparently in rapid equilibrium with extracellular Ca^{2+}, since the re-administration of Ca^{2+} for 90s to livers whose hormone-sensitive Ca^{2+}-pool had been depleted by three consecutive phenylephrine pulses re-establishes the α-agonist-induced responses.

In contrast, consecutive phenylephrine pulses did not decrease the Ca^{2+} efflux response in livers perfused with 1.3 mM-Ca^{2+}, even when, in one series of experiments, 24 consecutive phenylephrine pulses were administered (results not shown). Hence it appears as though α-agonists rapidly mobilize an intracellular pool of Ca^{2+} that is expelled from the cell. Although the mechanism by which this Ca^{2+} is expelled is unclear, a recently characterized Ca^{2+}-dependent ATPase present in the plasma membrane, whose activity is stimulated by Ca^{2+} in the range 1–100nM (Lotersztajn *et al.*, 1981), may play a role. Presumably it is during the expulsion of Ca^{2+} from the cell that the cytosolic Ca^{2+} concentration is transiently increased, hence allowing the activation of phosphorylase. Subsequent to this mobilization, the hormone-sensitive pool is repleted by extracellular Ca^{2+}. The cytoplasmic Ca^{2+} concentration does not appear to be elevated during this period of Ca^{2+} uptake, since glucose release is decreasing rather than increasing.

The pools of Ca^{2+} sensitive to α-agonists, vasopressin and angiotensin are apparently identical, or at least in rapid equilibrium with each other, since depletion of these Ca^{2+} pools by multiple pulses of any one of these agents abolished the efflux of Ca^{2+} in responses to the subsequent addition of any other of these hormones. This depletion of the hormone-sensitive Ca^{2+} pool is unlikely to result from Ca^{2+}-dependent changes in hormone receptor binding, since all three different classes of hormone are equally affected, and we have found that adrenaline receptor binding in liver plasma-membrane preparations is unaffected by as much as 1 mM-EGTA in a medium containing no added Ca^{2+} (results not shown). Hence all these classes of hormones may have similar Ca^{2+}-dependent post-receptor mechanisms for stimulating hepatic glycogenolysis, and mitochondrial respiration.

Previous reports have suggested that glucagon and cyclic AMP analogues enhance liver glycogenolysis by a mechanism different to that of the agents described above. Our data reinforce this view, as we have consistently found that glucagon or 8-bromo-cyclic AMP elicits only a very small increase in Ca^{2+} efflux. In addition, under low-Ca^{2+} conditions, the prior stimulation of glycogenolysis by glucagon or 8-bromo-cyclic AMP does not deplete the Ca^{2+} pool(s) affected by α-agonists, vasopressin or angiotensin, as judged by their unimpaired ability to stimulate Ca^{2+} efflux. Likewise prior depletion of this Ca^{2+} pool by repeated administrations of α-adrenergic agonists or vasopressin does not appreciably diminish the glycogenolytic effect of subsequently administered glucagon or 8-bromo-cyclic AMP. Hence α-adrenergic agonists, and more importantly adrenaline, the physiologically relevant hormone, appear to stimulate liver glycogenolysis by a cyclic AMP-independent mechanism. This conclusion appears to be valid even after repeated pulses of hormone in medium containing 10 μM total Ca^{2+}, since under these conditions α-agonist-induced glycogenolysis was greatly diminished, although glucagon-induced glycogenolysis was unimpaired.

In only one previous study (Blackmore *et al.*, 1979*b*) has any attempt been made to examine the effect of α-agonists on the Ca^{2+} content of all subcellular fractions recovered, and to correlate these fractions with organellar marker-enzyme activities. Unfortunately the results of that study were not clear, since phenylephrine induced a loss of Ca^{2+} from all fractions examined. This may have been due to a redistribution of Ca^{2+} during the isolation of the fractions, as extended fractionation times were employed and the isolation medium was devoid of Ca^{2+} flux inhibitors such as Ruthenium Red and nupercaine, although containing very high (2mM) concentrations of EGTA for a perfusion medium containing only 50 μM total Ca^{2+}.

In the present study we have further utilized the observation (Fig. 4) that the administration of phenylephrine to livers perfused with 10 μM-Ca^{2+}-containing medium results in the irreversible depletion of the hormone-sensitive Ca^{2+} pool to show that this pool is located in mitochondria and to a lesser extent in the endoplasmic reticulum. Since the maximal loss of Ca^{2+} from the mitochondrial

fraction amounts to approx. 60nmol/g wet wt. of liver, and the Ca^{2+} lost from the endoplasmic-reticulum-enriched fraction is about 40nmol/g wet wt. of liver, together these pools approximately correlate with the amount of Ca^{2+} efflux detected using the Ca^{2+}-sensitive electrode (80–120nmol/g wet wt. of liver) after a single pulse of phenylephrine. The time course of Ca^{2+} decrease in these fractions also correlates well with the Ca^{2+} efflux detected using the Ca^{2+} electrode. After 25s of α-agonist treatment we can detect significant decreases in mitochondrial Ca^{2+} content and maximal effects are observed at between 60 and 90s of treatment. The slightly longer times required until Ca^{2+} efflux can no longer be detected using the Ca^{2+} electrode is probably the result of signal spreading due to the finite electrode-response time (particularly at low Ca^{2+} concentrations) and due to flow characteristics of the perfusion tubing. Hence we predict that the actual Ca^{2+} fluxes at the cellular membranes are even more rapid and transient than recorded. After this work was completed, Blackmore *et al.* (1982) confirmed the rapidity of α-adrenergic agonist-induced Ca^{2+} efflux by showing that the total $^{45}Ca^{2+}$ content of hepatocytes decreases after only 10s of agonist treatment.

In conclusion, we suggest that α-adrenergic agonists, vasopressin and angiotensin all rapidly mobilize a small intracellular pool of Ca^{2+}, consisting predominantly of mitochondria and endoplasmic reticulum. Most of this mobilized Ca^{2+} is extruded from the cell during the first minute of agonist administration, to be subsequently reaccumulated. Hence, although extracellular Ca^{2+} appears not to play a role in the initial response of α-agonists, it does play a role in replenishing the depleted pool. This leads us to postulate that within seconds of α_1-adrenergic receptors becoming occupied, some as yet undefined mechanism ensures the transfer of information from the plasma membrane to mitochondria and the endoplasmic reticulum, resulting sequentially in Ca^{2+} release from these organelles, efflux of the ion across the plasma membrane, an increase in respiration and a stimulation of glycogenolysis. The mechanism of α_1-adrenergic-activated Ca^{2+} release and the relationship of this response to mitochondrial respiration requires further investigation.

We are grateful to Miss Sue Howitt for capable assistance in some of the experiments, and to Dr. N. H. Hunt for a gift of α-adrenergic antagonists. 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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