The involvement of calcium in the stimulation of respiration in isolated rat hepatocytes by adrenergic agonists and glucagon

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The effects of adrenergic agonists and glucagon on respiration and cytosolic free Ca^{2+}, measured with quin2, in female rat hepatocytes have been compared. In the presence of lactate, all three agonists caused a permanent stimulation of respiration. However, unlike phenylephrine, glucagon induced only a transient increase in cytosolic free Ca^{2+}, and isoprenaline caused no detectable change. Mechanisms other than Ca^{2+} for respiratory stimulation by cAMP-linked agonists in liver are indicated.

Respiration *Glucagon* *cytosolic Ca^{2+}* *Phenylephrine* *Isoprenaline* *Hepatocyte*

1. INTRODUCTION

Adrenaline, glucagon and other glycogenolytic hormones stimulate respiration in perfused liver [1,2] and isolated hepatocytes [3,4], but the regulatory mechanisms have not been resolved. These hormones increase the proportion of pyruvate dehydrogenase in the active, non-phosphorylated form [5,6] and, indirect evidence suggests, activate α-oxoglutarate dehydrogenase [7,9]. However, there is no evidence for stimulatory increases in CoA/acetyl-CoA, ADP/ATP or NAD/NADH and indeed the latter ratio decreases [5,10,11]. These enzymes together with NAD-linked isocitrate dehydrogenase are markedly activated by Ca^{2+} in a number of mammalian tissues including liver [12,13]. Noradrenaline, glucagon and the α-adrenergic agonist phenylephrine all raise cytosolic free Ca^{2+} in liver cells [14,15] and, in the absence of any hormonally imposed constraint on mitochondrial Ca^{2+} transport, would be predicted to raise mitochondrial Ca^{2+} as well. Evidence for a role of intramitochondrial Ca^{2+} in the activation of these enzymes in liver in response to adrenaline and glucagon has been reported recently [16].

The question of whether glucagon and Ca^{2+}-mobilizing hormones both affect mitochondrial oxidative metabolism via Ca^{2+} is fundamental since, if this is the case, the role of Ca^{2+} as an intracellular messenger in liver would be extended to the action of cAMP-linked agonists. This study examines this issue and concludes that whereas respiration correlates with cytosolic free Ca^{2+} with phenylephrine as agonist, no such correlation is evident with either glucagon or the β-adrenergic agonist, isoprenaline.

2. MATERIALS AND METHODS

2.1. *Preparation of dispersed liver cells loaded with quin2*

Hepatocytes were prepared from 200–300 g fed, female Sprague Dawley rats by the collagenase method as in [17] and suspended finally in Krebs-Henseleit medium containing (mM) NaCl (124), KCl (5.0), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $CaCl_2$

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(1.3), $NaHCO_3$ (26) and 2% (w/v) bovine serum albumin. Cell DNA was determined as in [23].

The cells were loaded with quin2 by incubation (approx. 3 mg cell dry wt/ml) with the esterified indicator (acetoxymethyl tetra ester, 100 μM in dimethyl sulphoxide) as described in [14]. After 15 min the cells were centrifuged at approx. 50 × *g* for 1 min, washed twice by resuspension in Krebs-Henseleit medium (above) and sedimentation. The cells were stored on ice.

2.2. *Hepatocyte incubations*

All incubations were carried out at 37°C in Krebs-Henseleit medium (above) containing 10 mM lactate unless otherwise stated. A preincubation period of 15 min was observed in all cases. Respiration was measured with Clarke-type O_2 electrodes. In each experiment two separate incubations with O_2 electrodes (polarized by a common circuit) were performed in parallel, agonist was added to one of these, and the outputs displayed on a dual pen recorder (respiration vs time). The voltage difference between the two high-potential inputs was measured on a second recorder to give change in respiration at any time caused by the agonist (Δ respiration vs time).

Changes in quin2 fluorescence were measured in parallel with respiration with a Perkin-Elmer 3000 spectrometer operating at 342 nm excitation and 492 nm emission. The suspension was stirred continuously. The maximal fluorescence signal (Ca^{2+} saturation of quin2) was obtained by dissolving the cells with 1% Triton X-100. The signal at 2 nM Ca^{2+} was obtained by further addition of 20 mM EGTA. The fluorescence signal was converted to free Ca^{2+} using an apparent dissociation constant of 110 nM [15].

3. RESULTS AND DISCUSSION

In assessing the involvement of Ca^{2+} in the regulation of hepatic oxidative metabolism by α-adrenergic and cAMP-linked agonists it was advantageous to use cells that display both α-adrenergic and β-adrenergic responses. Studer and Borle [19,20] concluded from studies of glycogen phosphorylase activation that female rats utilize both types of adrenergic receptor, unlike adult male rats in which α-adrenergic mechanisms predominate. Initial experiments therefore investigated the relative capacity of the α-adrenergic agonist phenylephrine and the β-adrenergic agonist isoprenaline to stimulate respiration in female rat hepatocytes.

In the presence of lactate (table 1), all three agonists tested stimulated respiration; the stimulation with isoprenaline was about 75% of that with phenylephrine. Similar effects of all three agonists were observed when lactate was replaced with pyruvate (not shown). The phenylephrine- and glucagon-induced responses were unaffected by the absence of substrate. However, omission of substrate abolished significant stimulation of respiration by isoprenaline. Unlike with the other agonists, therefore, β-adrenergic stimulation of respiration depends critically on the presence of substrate. It is conceivable that with isoprenaline as agonist, rate limitation by pyruvate dehydrogenase must be overcome by increased [pyruvate] in order for respiratory stimulation to

Table 1

The effects of adrenergic agonists and glucagon on respiration in female rat hepatocytes in the presence and absence of lactate

Substrate	Control value of respiration[a]	Stimulation (%) of respiration by		
		Phenylephrine	Isoprenaline	Glucagon
None	2.1 ± 0.1	28 ± 3 (3)[b]	5 ± 5 (6)	32 ± 5 (4)[c]
Lactate	2.5 ± 0.2	32 ± 4 (3)[b]	24 ± 3 (5)[d]	34 ± 4 (4)[c]

Respiration was measured 5 min after agonist. The following [agonist] were added: 10 μM phenylephrine, 1 μM isoprenaline, 10 nM glucagon. Cells not loaded with quin2 were used. Units: [a] ngatom O/min per μg DNA. Significance, *P*: [b] <0.02; [c] <0.01; [d] <0.002. Data are given as means ± SE (no. of independent experiments)

be expressed, and that isoprenaline may stimulate oxidation of pyruvate at a step subsequent to pyruvate dehydrogenase.

Fig.1 reports changes in quin2 fluorescence, calibrated for [Ca^{2+}], and respiration in the presence of lactate. Following the 15 min preincubation period, cytosolic free Ca^{2+} in the absence of agonist was about 70 nM (fig.1a), which is rather lower than that reported previously in resting cells (100–200 nM [15]). In this and all other preparations reported, resting cytosolic free Ca^{2+} was maintained constant for at least 10 min after the preincubation period. Previous measurements of phenylephrine- and glucagon-induced changes in cytosolic Ca^{2+} were terminated at 30 s [14]; the effect of isoprenaline has not been reported. In the present study, phenylephrine induced a rapid rise in cytosolic free Ca^{2+} that was partially maintained with time. Glucagon caused a smaller, more slowly developing increase in cytosolic free Ca^{2+} that was transient. In contrast, isoprenaline did not change cytosolic free Ca^{2+} detectably. Other experiments revealed similar effects of these agonists in the absence of added lactate. In sham experiments (not shown) with hepatocytes subjected to the same loading procedure with the solvent dimethyl sulphoxide, but without quin2 ester, none of the above agonists caused any detectable fluorescence change at the wavelengths used to measure the quin2 response. Thus the fluorescence changes (or absence of) with quin2-loaded cells may be correlated with changes in quin2 fluorescence.

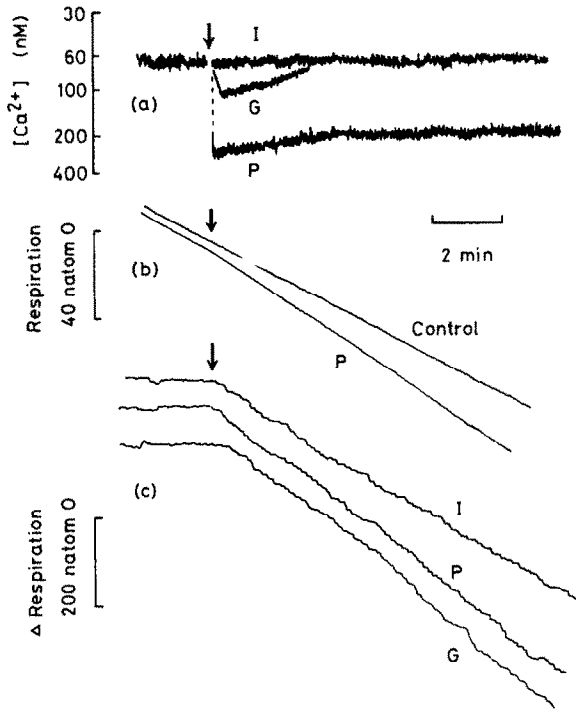

Fig.1. The effects of adrenergic agonists and glucagon on cytosolic Ca^{2+} and respiration in quin2 loaded hepatocytes. (a) Quin2 fluorescence traces calibrated for free [Ca^{2+}]. All the baselines before agonist addition were superimposable. The initial stage of phenylephrine-induced change (indicated by the dashed line) was not recorded since it occurred within the mixing time (5 s). (b) O_2 consumption vs time. (c) ΔO_2 consumption (test minus control) vs time. The following agonists were introduced at the arrows: phenylephrine (P, 10 μM), isoprenaline (I, 1 μM), glucagon (G, 10 nM). Control, no agonist.

Respiration was measured in parallel with the fluorescence measurements. In practice it was difficult to discern small changes in respiration with time in response to agonist from conventional traces (fig.1b), and therefore the difference in O_2 content between control and test incubations (test minus control) was determined as in fig.1c (section 2). In fig.1c a downward deflection of the trace corresponds to stimulation by agonist and the negative slope of the trace at any instant is proportional to the degree of respiratory stimulation at that instant. It is evident that all three agonists stimulated respiration irrespective of their quite different effects on cytosolic free Ca^{2+}.

Calibration of the maximal fluorescence change (2 nM–1.3 mM Ca^{2+}) with quin2 indicated a cellular quin2 content of 0.7–1.5 nmol/mg dry wt. With the assumption of 2.4 μl intracellular H_2O/mg dry wt of cells [14], this amount of quin2 corresponds to an internal [quin2] of 0.29–0.62 mM. Since this would introduce significant additional Ca^{2+} buffering to the cytosol, experiments examined whether the agonist-induced activation of respiration was changed by quin2 loading. Fig.2 compares agonist-induced activation of respiration in quin2-loaded and non-loaded cells of the same preparations. The responses were not affected significantly by quin2 loading. Unlike phenylephrine, glucagon-induced stimulation involved a pronounced lag phase of almost 30 s.

Fig.3 collates data from a number of

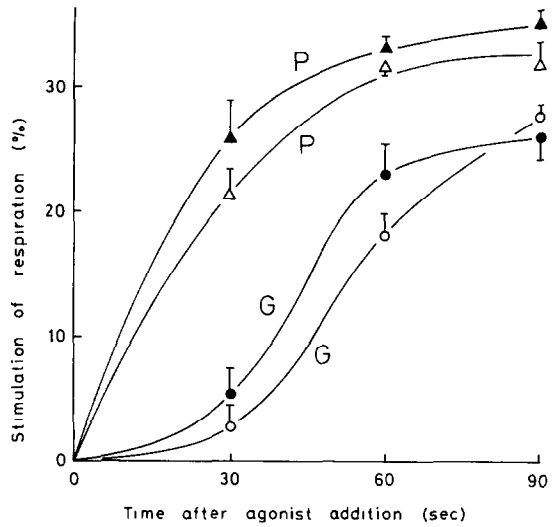

Fig.2. The effect of quin2 loading on the stimulation of respiration by phenylephrine and glucagon. The respiratory stimulations with phenylephrine (P, 10 μM) and glucagon (G, 10 nM) were calculated from traces of the type in fig.1c using hepatocytes from the same stock with quin2 loading (closed symbols) and without quin2 loading (open symbols). Data are given as means of either 3 (P) or 4 (G) determinations with separate hepatocyte preparations. Bars indicate SE.

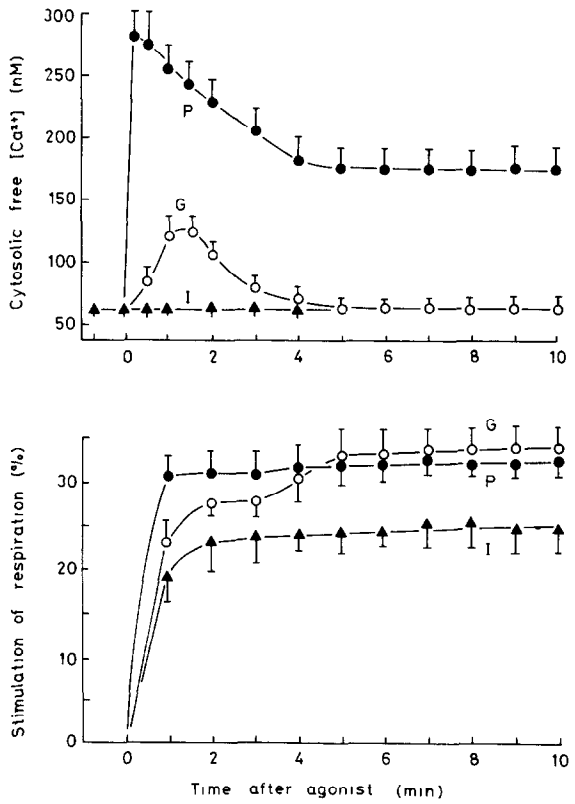

Fig.3. The increases in respiration and cytosolic free Ca^{2+} by adrenergic agonists and glucagon in quin2-loaded hepatocytes. Respiration and Ca^{2+} were determined in parallel in the same quin2-loaded cells. Results are given as means of determinations with the following number of cell preparations: P (10 μM phenylephrine), 5; G (10 nM glucagon), 5; I (1 μM isoprenaline), 3. Bars indicate SE.

quin2-loaded hepatocyte preparations. Phenylephrine induced an overall 4–5-fold increase in cytosolic free Ca^{2+} initially which decreased with time, but was then maintained at about 3-fold the resting level. The respiratory stimulation was quite constant at 30%. With glucagon as agonist the transient increase in cytosolic Ca^{2+} was not reflected in respiration; indeed in the period 3–5 min after glucagon administration, when cytosolic Ca^{2+} was restored to the resting level, there was a further small rise in respiration which was then maintained at the same rate as with phenylephrine. Moreover, the permanent stimulation of respiration by isoprenaline was not accompanied by any detectable increase in cytosolic Ca^{2+}.

Mitochondrial Ca^{2+} in liver is established by a transport cycle in which Ca^{2+} influx via the uniporter is balanced by Ca^{2+} efflux via the Na^+-Ca^{2+} carrier [21] and, possibly, an Na^+-independent Ca^{2+} transport system. Denton and McCormack [12,16] have proposed that hormone-induced increases in cytosolic free Ca^{2+} may be relayed by the cycle to the mitochondrial matrix with consequent activation of oxidative metabolism. The present data fully support this concept as applied to phenylephrine action on liver. The present study indicates, however, that the stimulation of respiration by isoprenaline cannot be attributed to relay of increased cytosolic free Ca^{2+} to the mitochondrial matrix, at least under the incubation conditions employed. The same conclusion applies to the later time course of glucagon action. Increased mitochondrial Ca^{2+} in the absence of increased cytosolic Ca^{2+} would require either uniporter activation or inhibition of the mitochondrial Ca^{2+} efflux systems. We have shown previously that administration of glucagon

or isoprenaline (but not phenylephrine) to perfused female rat livers stimulates Na^+-Ca^{2+} carrier activity 2–3-fold, at least in subsequently isolated mitochondria, and is without effect on the uniporter or the Na^+-independent system [22–24]. A decrease in hepatocyte mitochondrial $^{45}Ca^{2+}$ after glucagon addition has also been reported [25]. Taken together, these observations suggest that respiratory stimulation by cAMP-linked agonists can occur in liver without Ca^{2+} as mediator.

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