α -Adrenergic stimulation of respiration in isolated rat hepatocytes

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1. The α -adrenergic agonists noradrenaline (in the presence of β -blocker) and phenylephrine cause a transient stimulation of the respiration in isolated rat hepatocytes. After a lag period of 12 s, this activation first attains its maximal value (+24%) for about 1 min and then falls to a sustained value (+15%). The effect is blocked by the α -antagonists phenoxybenzamine and phentolamine. It is dose-dependent, with an half-maximal stimulation by 16 nM-noradrenaline, which is similar to that found for other cell responses to the hormone. 2. Vasopressin and ATP, which in common with α -agonists are believed to increase intracellular $[Ca^{2+}]$, induce similar activation in the respiration rate. 3. The α -adrenergic-mediated respiration depends on extracellular Ca^{2+} . The activation is decreased or abolished when extracellular $[Ca^{2+}]$ is decreased by adding EGTA, or when the Ca^{2+} antagonists Mn^{2+} and La^{3+} are present in the incubation medium. 4. It is suggested that the activation of the mitochondrial respiration rate results from the increase in cytosolic Ca^{2+} concentration, presumably via Ca^{2+} influx or Ca^{2+} release from the plasma membrane or endoplasmic reticulum.

Noradrenaline, via α -adrenergic receptors, alters the glucose metabolism and the movements of ions in mammalian livers by a Ca^{2+} -dependent mechanism (Keppens *et al.*, 1977; for reviews, see Jenkinson *et al.*, 1978; Williamson *et al.*, 1981; Exton, 1981). It is thought that the hormone increases cytosolic Ca^{2+} concentration by mobilizing Ca^{2+} from internal stores. In addition to these effects, it has been shown that α -adrenergic stimulation may induce stable changes in the properties of mitochondria isolated from rat livers pretreated with the agonist (Taylor *et al.*, 1980). This includes increased State-3 respiration and Ca^{2+} -uptake rate, and prolonged retention time for accumulated Ca^{2+} . The stimulation of α -receptors also increases the respiration rate of perfused livers (Jakob & Diem, 1975; Reinhart *et al.*, 1980, 1982) and of isolated cells (Dehay *et al.*, 1981). Reinhart *et al.* (1982) showed that this oxygen uptake was largely due to mitochondrial respiration, and related it to glycogenolysis and glucose release initiated by α -agonists.

In the present work, we have studied the effects on the respiration rate in isolated rat liver cells of noradrenaline and other agents (vasopressin and ATP), which, in common with α -agonists, are believed to increase cytosolic $[Ca^{2+}]$ by mobilizing internal stores. The results show that the hormones promote a delayed biphasic activation of the cell

respiration, which is dependent on extracellular Ca^{2+} .

Experimental

Cell isolation

Liver cells were prepared as previously described (Poggioli *et al.*, 1980). At the end of the isolation procedure, the cells were suspended in Eagle's medium containing (mM): NaCl, 116; KCl, 5.4; $CaCl_2$, 1.8; $MgSO_4$, 0.81; NaH_2PO_4 , 0.96; $NaHCO_3$, 25; as well as (mg/l): glutamine, 292; amino acids, 805; vitamins, 8.1; glucose, 2000 (for detailed composition see Eagle, 1959). This medium was supplemented with 2% bovine serum albumin and incubated for 30 min at 37°C. Cells were centrifuged and resuspended in the same medium containing 5 μ M-propranolol to obtain a cell concentration of 10–12 mg of cell protein/ml. Cell viability as checked by exclusion of 0.4% Trypan Blue was higher than 90%.

Hepatocyte respiration

Suspended cells (1.5 ml), maintained under O_2/CO_2 (19:1), were put into the measuring cell of a Gilson oxygraph. The cell suspension was immediately isolated from the atmosphere, and stirred magnetically. O_2 uptake was measured with a Clark electrode. All further additions were made with a

15 μ l micro-syringe. Routinely, hormone or agent was added in the cell chamber 3 min after cells. The respiration rate was graphically measured at 3 min (i.e. immediately before the hormone addition), 3.5 and 6 min.

Materials

Collagenase was from Boehringer: (–)-noradrenaline, vasopressin, phenylephrine, propranolol and albumin (fraction V) were from Sigma. Phenoxybenzamine was a gift from Smith, Kline and French. All other materials were of analytical grade.

Results

Activation of cell respiration by noradrenaline

Fig. 1(a) illustrates a typical effect of noradrenaline on the respiration of rat hepatocytes as monitored by a polarographic electrode placed in the cell chamber. The basal oxygen uptake by cells was 14.1 ± 0.7 nmol of O_2 /min per mg of protein ($n = 19$). Noradrenaline (trace a, 2 μ M, in the presence of 5 μ M-propranolol to block β -receptors) promoted, after a lag period of 12 ± 1 s ($n = 24$), a biphasic stimulation of the respiration. The respiration rate first increased to a maximal value for 63 ± 4 s ($n = 24$), then fell to a sustained but significantly elevated value.

Three parameters have been selected to quantify the effect of the hormone: the increase in the respiration rate 30 s (a) and 3 min (b) after noradrenaline addition and the extra O_2 consumption measured at 3 min (c). Expressed as a percentage of control value, the respiration rate was increased by $24.0 \pm 1.0\%$ ($n = 36$) and $15.2 \pm 1.3\%$

($n = 30$) at 30 s and 3 min respectively and the extra O_2 consumption was 5.3 ± 0.4 nmol of O_2 /mg of protein ($n = 30$). The dose-response curve of the response (a) (Fig. 2) shows that 50% of the maximal activation was obtained with 16 nM-noradrenaline. Similar curves were obtained for parameters (b) and (c) (results not shown).

Fig. 3 illustrates results of experiments performed in the presence or in the absence of α - and β -antagonists. The respiration initiated by 2 μ M-noradrenaline (parameter a) was little affected by the addition of 5 μ M-propranolol. In contrast, preincubation of the cells with the α -antagonist phenoxybenzamine (7 μ M) substantially decreased the cell response. Similar results were found when hepatocytes were pre-equilibrated with the α -blocker phentolamine (1 μ M). This strongly suggests that the observed effects were the consequence of the activation of α -receptors in this tissue. Further confirmation of this was obtained by using the relatively more specific α -agonist phenylephrine (1 μ M), which stimulates the cell respiration similarly. The activation of β -receptors by isoprenaline (1 μ M) also increased the respiration of isolated hepatocytes. However, the magnitude as well as the time-course of the effect differed from those initiated by the α -agonist addition: the increase was smaller (15%), with a longer lag period (30 s) (results not shown).

Effect of vasopressin and ATP

In common with α -agonists, vasopressin and ATP are thought to alter ion movements and/or glycogen phosphorylase in liver by mobilizing Ca^{2+} from

Fig. 1. Effect of noradrenaline, vasopressin and ATP on hepatocyte respiration

Isolated rat liver cells were prepared as described in the Experimental section, and equilibrated in Eagle's medium supplemented with 2% albumin and 5 μ M-propranolol, in the presence of O_2/CO_2 (19:1). Temperature was always maintained at 37°C. Cell suspension (1.5 ml) was put into the incubation cell of a Gilson oxygraph and the decrease in the O_2 concentration immediately recorded. After 3 min, the agonist was added (arrow). Numbers on the curves indicate the respiration rates in nmol of O_2 /min per mg of cell protein. Abbreviations: NOR, 2 μ M-noradrenaline; VP, 50 nM-vasopressin; ATP, 10 μ M-ATP. Broken line represents a control experiment with saline addition at 3 min.

Fig. 2. Dose-response curve of noradrenaline on respiration activation

Isolated hepatocytes were incubated as indicated in Fig. 1, and various concentrations of noradrenaline added at 3 min. The stimulation of the respiration 30 s after the hormone addition was measured for each curve and expressed as a percentage of the maximal stimulation obtained with the optimal dose of noradrenaline. Results are given as means $\pm$ S.E.M. for three to six experiments.

Fig. 3. Effects of α - and β -adrenergic antagonists on the noradrenaline-stimulated respiration

Isolated hepatocytes were incubated as in Fig. 1 (except that there was no propranolol in the equilibration medium), and noradrenaline (2 μ M) was added after 3 min. The effect measured was the percentage of the maximal stimulation, as in Fig. 2. The following antagonists were added at zero time: (a) 5 μ M-propranolol; (b) no addition; (c) 7 μ M-phenoxybenzamine; (d) 5 μ M-propranolol + 7 μ M-phenoxybenzamine. Results are expressed as means $\pm$ S.E.M. for four experiments.

internal stores (van de Werve *et al.*, 1977; Keppens *et al.*, 1977; Burgess *et al.*, 1979, 1981; Burnstock, 1981; see also Exton, 1981). Fig. 1 shows that vasopressin (50 nM) and ATP (10 μ M) also activated the respiration rate in rat hepatocytes, with magnitude and time-courses similar to that with noradrenaline.

Effect of external Ca^{2+} on the α -adrenergically mediated respiration

The following experiments were done to test the dependence of the respiration initiated by noradrenaline on external Ca^{2+} . Cells were first incubated in a complete medium containing 1.8 mM- Ca^{2+} . EGTA (1.9 mM) was added 2–3 min before the hormone. This period was short enough to avoid a possible Ca^{2+} redistribution in hepatocytes (Claret-Berthon *et al.*, 1977) (EGTA had no effect by itself on the cell respiration). The free Ca^{2+} concentration, as estimated from the association constant for Ca^{2+} –EGTA complex proposed by Bartfai (1979), was decreased to about 4 μ M. Table 1 illustrates the results. The addition of EGTA caused a substantial inhibition of the respiration induced by a maximal dose of noradrenaline (2 μ M). The magnitude of the inhibitory effect ranged from 62 to 79% of the response observed in the absence of EGTA, depending on the selected parameter. It is noteworthy that the inhibitory effect of the Ca^{2+} chelator was maintained even if it was added 10 s before nor-

adrenaline. Shorter periods of preincubation with EGTA made the chelator unable to impair the response to the hormone. This confirms that the stimulation of respiration was dependent on external Ca^{2+} . A dose-response curve for the inhibitory effect of EGTA is shown in Fig. 4 [parameter (a)]. The respiration rate was already substantially inhibited in the sub-millimolar range of estimated Ca^{2+} concentration. Thus half-maximal inhibition was observed at about 0.05 mM-Ca^{2+} . The cell response to vasopressin ($0.1\text{ }\mu\text{M}$) was similarly inhibited by 1.9 mM-EGTA , in agreement with Balaban & Blum (1982). In contrast, the stimulatory effect of ATP ($10\text{ }\mu\text{M}$) was not affected by the same dose of the Ca^{2+} chelator (results not shown).

Table 1. *Effect of EGTA on noradrenaline stimulation of hepatocyte respiration*

Hepatocytes were incubated as described in the Experimental section: 1.9 mM-EGTA was added at zero time, and $2\text{ }\mu\text{M-noradrenaline}$ at 2 min. The results are expressed as the percentage inhibition of the noradrenaline activation measured in the absence of EGTA (means $\pm$ S.E.M. for seven experiments).

Effect measured	Inhibition by 1.9 mM-EGTA (%)
(a) Respiration activation 30 s after hormone addition	62 ± 5
(b) Respiration activation 3 min after hormone addition	89 ± 7
(c) Extra O_2 consumption in 3 min	79 ± 7

Our next aim was to exclude the possibility that EGTA by itself was able to inhibit the cell response by directly interfering with the mechanism of

Fig. 4. *Effect of EGTA on the noradrenaline-stimulated respiration*

Isolated hepatocytes were incubated as in Fig. 1 and the stimulation of the respiration by noradrenaline was expressed as in Fig. 2. Various concentrations of EGTA were added at zero time, and $2\text{ }\mu\text{M-noradrenaline}$ was added at 3 min. The stimulation of respiratory rate at 3.5 min was measured. Results are expressed as means $\pm$ S.E.M. for four to six experiments.

Fig. 5. *Effect of La^{3+} and Mn^{2+} on the noradrenaline-stimulated respiration*

Isolated hepatocytes were incubated as in Fig. 1, and the stimulation of respiration by noradrenaline was expressed as in Fig. 2. Various concentrations of La^{3+} (a) and Mn^{2+} (b) were added at zero time, and $2\text{ }\mu\text{M-noradrenaline}$ was added at 3 min. The stimulation of respiratory rate at 3.5 min is plotted. Results are expressed as means $\pm$ S.E.M. for three to six experiments. Ca^{2+} in the medium: ----, 1.8 mM ; —, 0.5 mM .

activation. This was examined by determining the effect of agents such as La^{3+} and Mn^{2+} on the cell respiration initiated by noradrenaline. La^{3+} , like other rare earths, is an inhibitor of the attachment of Ca^{2+} to most of its binding sites (Mela, 1968; Weiss, 1974), and Mn^{2+} is a competitive inhibitor of the Ca^{2+} influx into cells (Deth & Lynch, 1981). Figs. 5(a) and 5(b) illustrate the results. La^{3+} inhibited the respiration activation (parameter a) provoked by $2\mu\text{M}$ -noradrenaline. However, in contrast with the effect of EGTA, which blocked 60–80% of the control cell response (Table 1), La^{3+} completely abolished the response to noradrenaline, with a half-maximal inhibition value of $500\mu\text{M}$. Mn^{2+} partially (40%) inhibited the respiration activation at a concentration of 10mM . Decreasing the Ca^{2+} concentration to 0.5mM in the incubation medium drastically enhanced the efficiency of Mn^{2+} , probably because of the competitive nature of the interaction of this ion with Ca^{2+} -binding sites. The half-maximal inhibition of the effect in this low- Ca^{2+} medium was 5.5mM .

Discussion

The basal O_2 consumption measured in isolated liver cells is about 14nmol/min per mg of protein, which is not different from that found in slices or in isolated liver perfused with saline solution (Claret, 1978), a possible indication that the isolation procedure for cells does not influence the basal oxidative metabolism of the hepatocytes. As previously reported in perfused liver (Reinhart *et al.*, 1982), this respiration may be ascribed to mitochondria, since no O_2 uptake was detected when cells were incubated in the presence of the mitochondrial electron-transfer inhibitor antimycin A ($1\mu\text{M}$; results not shown).

Noradrenaline in the presence of the β -blocker propranolol stimulates cell respiration. The effect is dose-dependent, is inhibited by the α -antagonists phenoxybenzamine and phentolamine, and may be mimicked by the more selective α -adrenergic agonist phenylephrine. These results suggest that in rat liver the effect of noradrenaline on the cell respiration is mediated through its effect on α -receptors (Jakob & Diem, 1975; Taylor *et al.*, 1980; Dehaye *et al.*, 1981). It is noteworthy that vasopressin (Dehaye *et al.*, 1981; Balaban & Blum, 1982; the present work) and externally applied ATP (which, in common with α -agonists, are thought to mediate their effects by increasing cytosolic $[\text{Ca}^{2+}]$) induced similar activation of the respiration of isolated hepatocytes. The half-maximal activation by noradrenaline was obtained at 16nM . This value is close to but slightly lower than those reported for the stimulation of glycogen phosphorylase (approx. 50nM ; El-Refai *et al.*, 1979), for Ca^{2+} release

(approx. 70nM ; El-Refai *et al.*, 1979) or for the stimulation of the Na^+-K^+ pump (approx. 100nM ; Capiod *et al.*, 1982) by the hormone in this tissue.

Our finding that EGTA, La^{3+} and Mn^{2+} may partially or totally inhibit the cell response to noradrenaline and vasopressin suggests that the respiration activation is dependent on extracellular Ca^{2+} . Balaban & Blum (1982) have also shown that the effect of vasopressin on respiration in rat hepatocytes is blocked in the presence of EGTA. However, there is little to indicate the mechanism of action of this ion. The simplest hypothesis is that the response is mediated by an influx of Ca^{2+} . Noradrenaline, via α -receptors (and presumably ATP and vasopressin, which are active in this regard), could trigger the opening of Ca^{2+} channels and the increase in cytosolic Ca^{2+} concentration, before releasing Ca^{2+} from internal stores. Alternatively, it may be proposed that the binding of extracellular Ca^{2+} to 'specific sites' located on the plasma membrane is necessary for the hormone to exert its action. Other workers have reported that various cell responses to the α -agonists are dependent on the presence of extracellular Ca^{2+} . Short-term incubations in low- Ca^{2+} media, which are believed to block Ca^{2+} influx and not to modify internal stores, have been shown to inhibit protein phosphorylation (Garrison *et al.*, 1979), fatty acid synthesis inactivation (Ly & Kim, 1981) and gluconeogenesis inactivation (Yip & Lardy, 1981). Althaus-Salzmann *et al.* (1980) also indicated that, if an uptake of Ca^{2+} did not seem to be required, the absence of extracellular Ca^{2+} affects the response to phenylephrine. In contrast, Blackmore *et al.* (1978, 1982) and Murphy *et al.* (1980) have shown that decreasing extracellular Ca^{2+} to very low concentrations had no influence on the capacity of α -agonists and vasopressin to stimulate the cell Ca^{2+} efflux mediated by the membrane Ca^{2+} pump and the activation of Ca^{2+} -dependent phosphorylase in isolated rat hepatocytes. We thus consider that there is not one but several sequences of events which lead to different responses of the cells to the α -adrenergic stimulation. The site of action of Ca^{2+} could depend on the cell response studied. Thus it may well be that certain responses are dependent on extracellular Ca^{2+} , whereas others are not impaired by its absence from the medium.

Noradrenaline triggers a biphasic and delayed stimulation of the respiration in isolated rat liver cells. The biphasic increase in the respiration rate may be related to the known capacity of mitochondria to transport and to accumulate Ca^{2+} . An increase in the extramitochondrial Ca^{2+} concentration promotes a Ca^{2+} uptake which is directly dependent on the inner-membrane potential and therefore stimulates the respiration rate (Bygrave, 1978; Carafoli, 1979; Nicholls & Crompton, 1980).

The resulting increase in the mitochondrial matrix Ca^{2+} concentration can in turn activate some Ca^{2+} -dependent dehydrogenases such as pyruvate dehydrogenase or oxoglutarate dehydrogenase (Denton & McCormack, 1980; Hansford, 1981; but see also Coll *et al.*, 1982). These two successive states of the Ca^{2+} -sensitive respiration could explain the presence of two phases in the O_2 -consumption increase initiated by the hormones. The first phase could reflect the increased Ca^{2+} transport mediated by the increase in cytosolic $[\text{Ca}^{2+}]$. The second could be ascribed to the increased mitochondrial free Ca^{2+} concentration. However, it should be mentioned that this interpretation is not in agreement with other results suggesting that the α -adrenergic stimulation decreases Ca^{2+} content in rat liver mitochondria (Blackmore *et al.*, 1979; Murphy *et al.*, 1980).

Noradrenaline stimulates the cell respiration with a delay of about 12 s, which has previously been observed in perfused liver and in isolated hepatocytes (Reinhart *et al.*, 1982; Dehay *et al.*, 1981). If we accept that this effect results from the increase in cytosolic $[\text{Ca}^{2+}]$, the presence of such a lag period suggests that the α -adrenergic-mediated respiration is not a primary event in the sequence of steps which lead to the cell response. This observation is based on the fact that several other responses caused by the cytosolic $[\text{Ca}^{2+}]$ increase (Murphy *et al.*, 1980), such as cell Ca^{2+} efflux and glycogen phosphorylase stimulation in rat hepatocytes (Blackmore *et al.*, 1982), K^+ efflux in guinea-pig liver cells (Burgess *et al.*, 1981), or the membrane hyperpolarization in guinea-pig liver slices (Haylett & Jenkinson, 1972) or cells (Green *et al.*, 1972), are substantially activated by that time.

The present data do not allow one to determine the source of the mobilized Ca^{2+} . However, two alternative possibilities may be proposed. (1) Ca^{2+} is first released from mitochondria that have been permeabilized by a putative second messenger (Blackmore *et al.*, 1979; Whiting & Barritt, 1982). In this case, this messenger or ionophore would have to mobilize all the Ca^{2+} responsible for the phosphorylase activation in a few seconds. It also would have to complete its action before the beginning of the Ca^{2+} reuptake (12 s after hormone addition) to avoid a 'futile' cycle of Ca^{2+} in these organelles. (2) Ca^{2+} is first released from other internal stores (plasma membrane or endoplasmic reticulum), as suggested by Althaus-Salzmann *et al.* (1980) and Berthon *et al.* (1981), and/or originates from extracellular Ca^{2+} . The resulting increase in cytosolic $[\text{Ca}^{2+}]$ may lead to an uptake of Ca^{2+} by mitochondria and to increased respiration. This hypothesis is in keeping with the finding that, between 10 and 60 s after the hormone addition, there is a transient increase in the mitochondrial Ca^{2+} content (Berthon *et al.*, 1981).

This issue remains unresolved, because determination of the time-course of net Ca^{2+} movements initiated by α -adrenergic agonists in mitochondria *in situ* and their dependence on extracellular Ca^{2+} concentration have given controversial results (Blackmore *et al.*, 1978; Chen *et al.*, 1978; Althaus-Salzmann *et al.*, 1980; Berthon *et al.*, 1981).

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