Insulin inhibition of α -adrenergic actions in liver

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The effects of insulin on α -agonist (phenylephrine)- and [Arg⁸]vasopressin-induced Ca^{2+} and glucose release and mitochondrial Ca^{2+} fluxes in isolated perfused rat livers were examined. Insulin (6 nM) inhibited the ability of phenylephrine (1 and 0.5 μM) to elicit Ca^{2+} and glucose release, whereas it was without effect on vasopressin (10 and 2.5 nM) actions. Correspondingly, insulin inhibited the action of phenylephrine to induce a stable increase in mitochondrial Ca^{2+} uptake, but it did not affect the alteration caused by vasopressin. Phenylephrine and vasopressin caused transient increases in hepatocyte respiration. Insulin inhibited the effect of phenylephrine on this parameter, but not that of vasopressin. Insulin added alone did not alter any of the above parameters. It is concluded from these data that insulin does not alter cellular Ca^{2+} fluxes and respiration themselves, but selectively inhibits α -adrenergic stimulation of these processes. It is proposed that insulin acts either to inhibit binding of α -agonists to their specific plasma-membrane receptors or to alter generation and/or degradation of the putative α -adrenergic 'second messenger'. If this latter possibility is the case, then the α -adrenergic 'second messenger' must be different from the 'second messenger' of vasopressin.

α -Adrenergic agonists, vasopressin and angiotensin II promote Ca^{2+} release from rat liver mitochondria and perhaps microsomal fractions (Blackmore *et al.*, 1979b,c; Babcock *et al.*, 1979; Taylor *et al.*, 1980; Murphy *et al.*, 1980). This release is postulated to increase the Ca^{2+} concentration in the cytosol (Murphy *et al.*, 1980), resulting in stimulation of phosphorylase *b* kinase and extrusion of Ca^{2+} into the extracellular medium by a plasma-membrane Ca^{2+} -sensitive Mg^{2+} -dependent ATPase (Assimakopoulos-Jeannot *et al.*, 1977; Blackmore *et al.*, 1978, 1979b). Phosphorylase *b* kinase is sensitive to micromolar concentrations of Ca^{2+} (Khoo & Steinberg, 1975; Shimazu & Amakawa, 1975; Vandenhede *et al.*, 1977) and catalyses the activation of phosphorylase, leading to glycogenolysis.

Previous studies from our laboratory have demonstrated that insulin inhibits α -adrenergic activation of hepatic phosphorylase and Ca^{2+} output (Blackmore *et al.*, 1979a). It was proposed that insulin acted by inhibiting the mobilization of intracellular store(s) of Ca^{2+} induced by α -adrenergic agents. The present

work supports this hypothesis by showing that insulin inhibits the release of Ca^{2+} from mitochondrial and post-mitochondrial fractions induced by phenylephrine treatment of isolated perfused rat liver.

Perfusion of rat livers with α -adrenergic agents has been shown to stimulate respiration (Jakob & Diem, 1975; Sugano *et al.*, 1980), and to cause a stable increase in the rate of Ca^{2+} uptake by subsequently isolated mitochondria (Taylor *et al.*, 1980). The ability of insulin to inhibit these changes was therefore tested. In addition, the possible modulation by insulin of the effects of vasopressin on hepatic respiration, Ca^{2+} efflux, glucose release and mitochondrial Ca^{2+} content and uptake was examined. None of the actions of this agent was found to be modified by insulin, suggesting a selective inhibitory action of insulin on α -adrenergic agents.

Experimental

Rat liver perfusion and isolation of hepatocytes

Livers from 220–250 g fed Sprague–Dawley rats were perfused with non-recirculating medium as previously described (Blackmore *et al.*, 1978, 1979b). Isolated rat liver parenchymal cells were prepared as described by Hutson *et al.* (1976).

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Isolation of mitochondria

Mitochondria were isolated from separate lobes of perfused livers 2 min before and 6 min after hormone infusion (Blackmore *et al.*, 1979b). Subcellular fractionation was performed by differential centrifugation as described by Blackmore *et al.* (1979b). As described previously, subcellular fractions 1, 2 and 3 contained essentially all of the cytochrome *c* oxidase activity of the homogenate and were relatively free of 5'-nucleotidase and glucose 6-phosphatase, whereas the postmitochondrial fractions 4 and 5 had the greatest activity of glucose 6-phosphatase and contained 5'-nucleotidase, suggesting the presence of microsomal and plasma-membrane fragments (Blackmore *et al.*, 1979b). In experiments in which the initial rate of $^{45}\text{Ca}^{2+}$ uptake by mitochondria was measured, mitochondria from subcellular fractions 1 and 2 were pooled. These mitochondria had a respiratory control index [ADP-stimulated (State 3) respiration rate/ADP-depleted (State 4) respiration rate] of approx. 4.5, measured in a medium (final vol. 3 ml) containing 100 mM-sucrose, 50 mM-KCl, 15 mM-tris (hydroxymethyl)aminoethane, 10 mM- KH_2PO_4 , 2 mM- MgCl_2 , 1 mM-EDTA and 12.5 mM-sodium succinate, pH 7.4, at 25°C with 1 mg of mitochondrial protein. For each determination three additions, each of 200 μM -ADP, were made.

Measurement of hepatocyte respiration

To measure hepatocyte respiration rates, approx. 50 mg wet wt. of hepatocytes were incubated in a final volume of 5 ml in a medium of Krebs-Henseleit bicarbonate buffer (Krebs & Henseleit, 1932) containing 1.5% (w/v) gelatin, pH 7.4. This was equilibrated with O_2/CO_2 (19:1) at 37°C. Measurements of O_2 partial pressure were made with a YSI model 53 Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) connected to a Datamark Servocorder SR 6253 (Datamark Corp., Costa Mesa, CA, U.S.A.). Hormones and agents under test were added to the incubations with a glass micro-syringe.

Other methods

Methods used for the measurement of glucose, Ca^{2+} and protein were as described previously (Blackmore *et al.*, 1979b). The initial rate of mitochondrial $^{45}\text{Ca}^{2+}$ uptake was measured at 0°C as described by Taylor *et al.* (1980).

Chemicals

The sources of hormones and adrenergic blockers were as described previously (Blackmore *et al.*, 1979a,b). $^{45}\text{Ca}^{2+}$ was obtained from New England Nuclear (Boston, MA, U.S.A.).

Fig. 1. Effect of insulin on phenylephrine-induced release of Ca^{2+} from perfused liver

Livers were perfused as described in the Experimental section, and 3.5 ml samples of perfusate were collected every 30 s. Erythrocytes were removed by centrifugation and samples of the supernatant were taken for measurement of Ca^{2+} . In panels (a) and (b), insulin (6 nM) was present at zero time and phenylephrine was infused at 6 min. In panel (c) insulin infusion was started at 6 min. Δ , No insulin; $\blacktriangle$, insulin present. The numbers of experiments for each condition are four (a and b) or five (c). In all Figures, bars represent $\pm$ S.E.M.

Results

Insulin inhibition of phenylephrine actions on hepatic glucose and Ca^{2+} release

The results in Fig. 1 confirm previous findings (Blackmore *et al.*, 1979a) that pretreatment of the perfused rat liver with insulin (6 nM) inhibits Ca^{2+} release produced by continuous infusion of phenylephrine at $0.5 \mu\text{M}$ (a half-maximally effective concentration) and $1 \mu\text{M}$ (a maximally effective dose).

Contrary to the effects observed with isolated liver cells (Blackmore *et al.*, 1979a), it was necessary to pretreat the intact liver with insulin in order to observe maximal inhibition, since simultaneous infusion of phenylephrine with insulin produced only a small antagonism (results not shown). The reason for this difference in the perfused liver is not known. Infusion of insulin alone at 6 nM from 0 min did not produce a significant change in perfusate Ca^{2+} content (6 min values in Figs. 1a and 1b). Neither

Fig. 2. Effect of insulin on phenylephrine-induced release of glucose from livers perfused with non-recirculating medium. For other details see legend to Fig. 1. Δ , Phenylephrine alone; $\blacktriangle$, phenylephrine + insulin; $\circ$, insulin alone.

Fig. 3. Effect of insulin, phenylephrine and phenylephrine plus insulin on Ca^{2+} content in mitochondrial and postmitochondrial fractions

Subcellular fractions were isolated as described in the Experimental section from separate liver lobes sampled at 4 min and 12 min in the perfusion experiments shown in Fig. 1. $\square$, Insulin present in perfusion; $\square$, no insulin. Significance of differences, determined by Student's *t* test for paired samples, was: * $P < 0.05$; †not significant ($P > 0.1$).

did infusion of insulin commencing at 6 min significantly alter perfusate Ca^{2+} content (Fig. 1c). To confirm that the inhibition by insulin of Ca^{2+} release produced by phenylephrine was associated with a decreased glycogenolytic response (Blackmore *et al.*, 1979a), glucose release was also measured. The data in Figs. 2(a) and 2(b) show that insulin blocked the release of glucose produced by both 0.5 μM - and 1 μM -phenylephrine. Insulin alone produced a small inhibition of glucose release (Fig. 2a).

Insulin inhibition of phenylephrine-induced changes in hepatic mitochondria

The results in Fig. 3 show the effect of insulin alone and in the presence of phenylephrine on mitochondrial and postmitochondrial (microsomal) Ca^{2+} content (Blackmore *et al.*, 1979b). Insulin infused by itself from 6 min did not produce a significant change in the Ca^{2+} content of any of the subcellular fractions examined (Fig. 3a). Nor did insulin infusion from 0 min alter the Ca^{2+} content of subcellular fractions (Table 1). These observations are consistent with the lack of effect of insulin on the Ca^{2+} content of the perfusate (Fig. 1). Insulin,

however, inhibited the release of mitochondrial Ca^{2+} (fractions 1 and 2) and postmitochondrial Ca^{2+} (fractions 4 and 5) produced by phenylephrine infusion (Figs. 3b and 3c). These changes correlate with the inhibition of Ca^{2+} release from the liver shown in Figs. 1(a) and 1(b).

Table 1. Effect of insulin on Ca^{2+} content of mitochondrial and postmitochondrial fractions

Livers were perfused with non-recirculating medium. Liver lobes were removed at 4 min after treatment with either insulin (6 nM) or saline (control) and fractionated as described in the Experimental section (Blackmore *et al.*, 1979b). Results are means $\pm$ S.E.M. for the numbers of perfusions in parentheses.

Fraction no.	Ca^{2+} (nmol/mg of protein)	
	Saline (31)	Insulin (18)
1	2.31 $\pm$ 0.18	2.04 $\pm$ 0.22
2	1.94 $\pm$ 0.13	1.91 $\pm$ 0.18
3	3.11 $\pm$ 0.22	3.15 $\pm$ 0.31
4	4.71 $\pm$ 0.23	5.11 $\pm$ 0.53
5	6.79 $\pm$ 0.66	5.44 $\pm$ 0.42

Fig. 4. Effect of (a) phenylephrine, (b) phenylephrine plus insulin, (c) vasopressin and (d) vasopressin plus insulin on the ability of mitochondria to take up $^{45}\text{Ca}^{2+}$ in vitro

Perfusions were carried out as described in the legend to Fig. 1, except that Ca^{2+} (2.4 mM) was present in the perfusion medium (this gave more reproducible data). Mitochondria (combined fractions 1 and 2, Fig. 3) were isolated from separate liver lobes sampled at 4 min (control) and 12 min in the perfusion experiments. The data are the means $\pm$ S.E.M. for three or four experiments. Significant differences, as determined by Student's *t* test for paired samples, were: **P* < 0.05, ***P* < 0.025, ****P* < 0.01, †*P* < 0.005. (a), (b): ●, +phenylephrine; ○, control. (c), (d): ●, +vasopressin; ○, control.

Confirming the observations of Taylor *et al.* (1980), mitochondria isolated from livers perfused with 1 μ M-phenylephrine exhibited an increased initial rate of Ca^{2+} uptake (Fig. 4). A similar change was observed in mitochondria from livers treated with 5 nM-vasopressin (Fig. 4). Inclusion of insulin in the perfusion medium reversed the effect of phenylephrine on this parameter (Fig. 4), but did not alter control values (results not shown).

The addition of phenylephrine to a suspension of hepatocytes caused a rapid (within 15 s) stimulation of the rate of oxygen utilization by the cells of approx. 25% (Fig. 5). The stimulated rate of respiration was maintained for a period of about 2 min and then declined (Fig. 5). Addition of vasopressin or angiotensin II had a similar effect (Fig. 5).

The effect of phenylephrine (1 μ M) on hepatocyte respiration was unaffected by the inclusion of propranolol (10 μ M) in the incubation medium, but was abolished by the inclusion of phentolamine (10 μ M) (results not shown). The effect of phenylephrine on hepatocyte respiration was inhibited when insulin was present in the incubation (Table 2). The addition of insulin alone to the incubation had no effect (results not shown).

Lack of insulin inhibition of vasopressin actions

Previously, we (Blackmore *et al.*, 1979a) demonstrated a lack of inhibition by insulin of vasopressin- and angiotensin II-stimulated Ca^{2+} efflux and phosphorylase activation in isolated rat liver parenchymal cells. These results suggested that the mechanism of action of these agents on Ca^{2+}

mobilization was not identical with that of the α -adrenergic agonist phenylephrine. To confirm this lack of effect of insulin on vasopressin-stimulated Ca^{2+} efflux, we performed experiments in the perfused liver similar to those with phenylephrine. The results in Fig. 6 show that insulin also did not modify vasopressin-stimulated Ca^{2+} efflux from the perfused liver or glucose release (Figs. 6a and 6b). Similarly, insulin did not modify the ability of vasopressin to induce a loss of mitochondrial Ca^{2+} (Fig. 7). The increases in mitochondrial Ca^{2+} uptake

Table 2. *Effect of phenylephrine, phenylephrine plus insulin, vasopressin or vasopressin plus insulin on hepatocyte respiration*

Hepatocyte respiration was measured as described in the Experimental section and in the legend to Fig. 5. Insulin when present was added approx. 3 min before either phenylephrine or vasopressin. The data represent the maximally hormone-stimulated respiration rate as a percentage of the control rate. The data are means $\pm$ S.E.M. for the numbers of experiments shown in parentheses. Significant differences, as determined by Student's *t* test for paired samples, between the hormone-stimulated rate and the control rate for each experiment were: **P* < 0.01, ***P* < 0.005.

Hormone	Respiration (%)	
	Insulin absent	Insulin (6 nM)
Phenylephrine (1 μ M)	120 $\pm$ 1 (5)**	105 $\pm$ 3 (4)
Vasopressin (5 nM)	115 $\pm$ 4 (4)**	115 $\pm$ 3 (4)*

Fig. 5. *Effect of phenylephrine, vasopressin or angiotensin II on hepatocyte respiration*

Hepatocytes were incubated as described in the Experimental section. At the point indicated by the arrow the following additions were made: (a) saline (0.9% NaCl); (b) phenylephrine (10 μ M, Phe); (c) vasopressin (10 nM, Vaso); or (d) angiotensin II (10 nM, Angio). Broken lines indicate control (saline) rates.

Fig. 6. Effect of insulin on vasopressin-stimulated Ca^{2+} efflux (a) and glucose release (b) from perfused liver. Insulin (6 nM) was added at zero time to the perfusate. For other details, see legend to Fig. 1. Numbers in parentheses indicate numbers of perfusions for each treatment. ●, 10 nM-vasopressin (6); ○, 10 nM-vasopressin + insulin (4); ■, 2.5 nM-vasopressin (5); □, 2.5 nM-vasopressin + insulin (6).

Fig. 7. Effect of insulin, vasopressin and vasopressin plus insulin on Ca^{2+} content in mitochondrial and postmitochondrial fractions

Subcellular fractions were isolated from separate liver lobes sampled at 4 min and 12 min in the perfusion experiments shown in Fig. 4. ▨, Insulin present in perfusion; □, no insulin.

induced by perfusion of livers with vasopressin (Fig. 4) and in hepatocyte respiration induced by incubation with vasopressin (Table 2) were also not affected by insulin.

Discussion

The results presented here provide further support for the concept that the mobilization of

mitochondrial Ca^{2+} is a major factor in the mechanism of action of α -adrenergic agonists on the liver. Although this response was initially observed in livers or hepatocytes exposed to low- Ca^{2+} media, it also occurs in normal- Ca^{2+} media (Taylor *et al.*, 1980; P. F. Blackmore & J. H. Exton, unpublished work) and *in vivo* (Babcock *et al.*, 1979). A key observation in the present study is the demonstration that insulin inhibits α -adrenergic mobilization of hepatic mitochondrial and postmitochondrial Ca^{2+} . Insulin by itself was unable to produce any consistent effects on hepatic Ca^{2+} flux or mitochondrial Ca^{2+} content. However, it should be noted that the Ca^{2+} concentration of the perfused medium employed in the present experiments was decreased to $50\mu\text{M}$ in order to measure alterations in Ca^{2+} release from the liver. The possibility that insulin, by itself, causes alterations in cellular Ca^{2+} in livers perfused with normal concentrations of Ca^{2+} requires investigation.

The present findings are consistent with the proposal that insulin inhibits α -adrenergic-stimulated glycogenolysis by lessening the increase in the cytosolic Ca^{2+} concentration by preventing the release of Ca^{2+} from mitochondrial and postmitochondrial fractions. The diminution in the increase in cytosolic Ca^{2+} would act to blunt the activation of phosphorylase *b* kinase and hence decrease phosphorylase *a* activity, as has been observed previously (Blackmore *et al.*, 1979a). As discussed below, insulin is unlikely to act by stimulating the uptake of Ca^{2+} into subcellular fractions, since this would not account for the selectivity of its inhibitory action.

The observation that phenylephrine and vasopressin induce an increase in hepatocyte respiration confirms and extends findings made by others in the perfused liver (Jakob & Diem, 1975; Sugano *et al.*, 1980). In addition, the present studies demonstrate that insulin inhibits the effect induced by phenylephrine, but not that by vasopressin. The rapid onset and the transient nature of the stimulation of respiration induced by the two agents suggests that it is related to the changes responsible for the mobilization of mitochondrial Ca^{2+} in the intact liver. Taylor *et al.* (1980) have reported that mitochondria isolated from phenylephrine-treated livers exhibit increased State-3 respiration. This is also observed with mitochondria from livers perfused with vasopressin (B. P. Hughes, P. F. Blackmore & J. H. Exton, unpublished work).

Insulin was able to abolish the increase in the rate of mitochondrial Ca^{2+} uptake observed *in vitro* in mitochondria isolated from phenylephrine-treated livers, but did not antagonize that induced by vasopressin. As discussed elsewhere (Taylor *et al.*, 1980), the relationship of this change to those responsible for the mobilization of mitochondrial

Ca^{2+} observed in the intact liver is unclear. The change in mitochondrial Ca^{2+} uptake observed *in vitro* is clearly stable, since it persists through the isolation procedure (with 1 h duration) and for several hours thereafter. In contrast, the changes resulting in mitochondrial Ca^{2+} efflux *in vivo* are transient, e.g. release of Ca^{2+} is followed by re-uptake commencing within 2–3 min after infusion of α -agonists, vasopressin or angiotensin II (Blackmore *et al.*, 1979a; Dehaye *et al.*, 1980). The amount of Ca^{2+} taken up in the experiments measuring uptake *in vitro* (approx. 40nmol/mg of protein) is very much greater than that found in liver mitochondria *in vivo* (Blackmore *et al.*, 1979b; Babcock *et al.*, 1979). Their significance for the situation *in vivo* is therefore uncertain. In fact, it is considered by some investigators that the increased ability of mitochondria from hormone-treated livers to take up and retain Ca^{2+} probably reflects increased resistance to the uncoupling actions of high intramitochondrial Ca^{2+} concentration (Nicholls & Crompton, 1980). Whatever the mechanisms involved in the α -adrenergically induced change in mitochondrial Ca^{2+} uptake observed *in vitro*, it represents an additional example of an α -adrenergic response which is reversed by insulin.

Jarett and co-workers (McDonald *et al.*, 1976a,b, 1978) have reported insulin effects on the Ca^{2+} content of subcellular fractions in adipose tissue, and several groups have suggested that the effect of insulin to inhibit α -adrenergic inactivation of glycogen synthase in this tissue and in liver is due to a decrease in cytosolic Ca^{2+} (Blackmore *et al.*, 1979a; Lawrence & Larner, 1978; Massague & Guinovart, 1978). This is in contrast with the contention of two groups (Clausen & Martin, 1977; Hope-Gill *et al.*, 1976) that insulin acts on muscle and adipose tissue by increasing cytoplasmic Ca^{2+} . This conclusion was reached on the basis of studies of insulin effects on ^{45}Ca efflux from adipocytes (Hope-Gill *et al.*, 1976). However, as discussed in detail elsewhere (Blackmore *et al.*, 1978, 1979b; Lawrence & Larner, 1978), interpretation of such studies is seldom unequivocal.

Comparison of the effect of insulin on phenylephrine and vasopressin actions in the perfused liver gives some insight into the mode of action of insulin. Although vasopressin, angiotensin II and phenylephrine all produce a Ca^{2+} -dependent cyclic AMP-independent (Hems & Whitton, 1973; Kirk & Hems, 1974; Keppens & DeWulf, 1975, 1976) activation of hepatic phosphorylase and mobilization of mitochondrial Ca^{2+} , only the α -adrenergic mechanism is inhibited by insulin. This selectivity of action is apparent in earlier work (Whitton *et al.*, 1978; Ma *et al.*, 1978; Blackmore *et al.*, 1979a; Kirk & Hems, 1979; Strickland *et al.*, 1980) on glycogenolysis, gluconeogenesis, phosphorylase and glycogen syn-

thase. Thus insulin is unlikely to have a general effect to decrease mitochondrial Ca^{2+} release or to promote Ca^{2+} uptake by these organelles. In fact, it would seem that the site of insulin action is probably very close to the α -receptor. For example, insulin may act by decreasing binding of α -agonists to the receptor or by interfering with the generation of the putative second messenger, which is presumed to result from this interaction (Blackmore *et al.*, 1978, 1979b; El-Refai *et al.*, 1979; Dehaye *et al.*, 1980). Alternatively, it may be postulated that the α -adrenergic second messenger is not the same as that for vasopressin and antiotensin II. In this case, insulin may act by inactivating the α -adrenergic second messenger or inhibiting its action, and may not affect that (or those) of the other hormones. Clearly, discrimination between these two possibilities requires definition of the nature of the presumed intracellular second messenger(s) of the agents.

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