Rapid Ca^{2+} influx induced by the action of dibutylhydroquinone and glucagon in the perfused rat liver

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Glucagon induces a slight Ca^{2+} efflux when administered to the perfused rat liver. However, the hormone promotes rapid and significant Ca^{2+} influx after the prior administration of 2,5-di(*t*-butyl)-1,4-hydroquinone (BHQ), an agent that promotes Ca^{2+} release from the endoplasmic reticulum (ER). The concentrations of glucagon that promote Ca^{2+} influx are similar to those that promote glycogenolysis and gluconeogenesis in isolated hepatocytes. The permeable analogue of cAMP, but not that of cGMP, is able to duplicate the Ca^{2+} -mobilizing effects of glucagon. The influx of Ca^{2+} into liver is blocked by Ni^{2+} . Administration of sodium azide, an inhibitor of mitochondrial electron transport, also blocks the BHQ plus glucagon-induced Ca^{2+} influx and this

is reversed when azide administration is terminated. The actions of azide are evident within 60 s after administration or withdrawal, and also occur when either oligomycin or fructose is co-administered; this provides evidence for an effect of azide independent of cellular ATP depletion. Measurement of total calcium in mitochondria that were isolated rapidly from perfused livers after the combined administration of glucagon and BHQ confirmed that large quantities of extracellular Ca^{2+} had entered these organelles. These experiments provide evidence that in the perfused rat liver the artificial emptying of the ER Ca^{2+} pool allows glucagon to promote rapid and sustained Ca^{2+} influx that seems to terminate in mitochondria.

INTRODUCTION

Cellular Ca^{2+} influx across the plasma membrane is crucial for many cellular physiological and biochemical events, yet the molecular mechanism(s) involved in Ca^{2+} entry in many cell types is particularly unclear, with numerous factors implicated (reviewed in [1,2]). Crucial to current hypotheses of Ca^{2+} entry is the role of the endoplasmic reticulum (ER) and particularly the extent to which the Ca^{2+} pools therein are 'empty' or 'full' (see [1,2]).

Until now, little attention seems to have been paid to the possible role of mitochondria in agonist-induced Ca^{2+} inflow. Evidence from several laboratories has existed for some years, however, that signalling cross-talk induced by the co-administration of glucagon (generating cAMP) and Ca^{2+} -mobilizing agonists (generating $\text{Ins}(1,4,5)\text{P}_3$) promotes Ca^{2+} movement into the mitochondria of whole liver [3,4] or liver cells [5,6] (reviewed in [7]). The mechanisms involved in this cross-talk are complex and slowly becoming apparent (see, for example, [8]).

In the present study we investigated further an aspect of the mechanism of this signalling cross-talk. We used the perfused rat liver by employing a non-invasive Ca^{2+} -sensitive electrode [9]; this system enables quantitative and direct measurements to be made of Ca^{2+} influx and/or efflux across the plasma membrane in response to the action of a range of Ca^{2+} -mobilizing agents [10]. Use was made also of the observation [11] that 2,5-di(*t*-butyl)-1,4-hydroquinone (BHQ) in the presence of glucagon and vasopressin increased Mn^{2+} influx in hepatocytes. We tested the effects of Ca^{2+} depletion in the endoplasmic reticulum (ER) on glucagon action independent of the generation of $\text{Ins}(1,4,5)\text{P}_3$ by a Ca^{2+} -mobilizing agent such as vasopressin. The experiments show that depletion of ER Ca^{2+} by non-physiological agents

permits glucagon to promote a rapid and sustained Ca^{2+} influx that seemingly terminates in mitochondria.

EXPERIMENTAL

Liver perfusion

Male Wistar-strain albino rats, weighing from 250 to 350 g and having free access to food, were anaesthetized with sodium pentobarbitone (50 mg/kg body wt). Livers were perfused with Krebs–Henseleit [12] bicarbonate buffer equilibrated with O_2/CO_2 (19:1) and containing 1.3 mM CaCl_2 ; the final oxygen concentration was approx. 1 mM. Perfusions were conducted in a non-recirculating mode and the perfusate was delivered at a constant volume of 35 ml/min by means of a Gilson Minipuls 3 peristaltic pump. Livers were first pre-perfused for at least 20 min before the infusion of any hormone, each of which was administered by a pump-driven infusion syringe.

Ca^{2+} measurements

The perfusate Ca^{2+} concentration was monitored continuously with a Radiometer F2112 Ca^{2+} -selective electrode in a flow-through chamber placed on the outflow side of the liver; this is described in detail elsewhere [9,10]. The electrode was coupled to a Radiometer K801 reference electrode via an agarose/KCl salt bridge, and the combined signals were fed via an Orion Ionalyzer through a MacLab 2e recorder into a Macintosh computer. The Ca^{2+} data were based on a sample rate of 20 samples per second. Data were then analysed, calculated and graphed with Igor (Wavemetrics, Lake Oswego, OR, U.S.A.) software. Ca^{2+} concentration changes are expressed as $\mu\text{mol}/\text{min}$ per g of liver. An

average value of $0.1 \mu\text{mol}/\text{min}$ per g of liver corresponds to a change in perfusate Ca^{2+} of $40 \mu\text{M}$. Each trace was representative of at least three observations from different independent experiments. An upwards deflection in each trace reflects Ca^{2+} efflux and a downwards deflection Ca^{2+} influx. In all experiments the oxygen concentration in the outflow was also measured to ascertain that the inhibitors of mitochondrial function used in this study were having their expected effects on oxygen uptake.

Liver fractionation

Fractionation of the liver immediately after specific periods of perfusion was performed by the procedure of Reinhart et al. [13]; Ruthenium Red but not nupercaine was added to the homogenizing medium. Total calcium in liver fractions was measured by atomic absorption spectroscopy exactly as described in [4].

Reagents

Glucagon was from Eli Lilly (Australia) (Sydney, Australia). Ca^{2+} -selective electrode membranes (F2112) and filling solutions (S43316) were obtained from Radiometer (Copenhagen, Denmark). All other chemicals used were of analytical reagent grade.

RESULTS AND DISCUSSION

The upper trace of Figure 1(a) shows that 10 nM glucagon induces little Ca^{2+} efflux soon after its administration to the perfused rat liver; consistent with considerable earlier work (reviewed in [7]), no net influx of the ion occurs. Administration of BHQ, which empties the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} store in the ER and elevates the cytoplasmic Ca^{2+} concentration [14], induced slight Ca^{2+} efflux (Figure 1a, lower trace). BHQ always induced Ca^{2+} efflux; however, the magnitude of the response varied from experiment to experiment (see also the data presented in the figures below). After BHQ administration a substantial and prolonged influx of Ca^{2+} occurred when an identical concentration of glucagon (10 nM) was then administered (Figure 1a, lower trace). We calculate that over the course of 10 min, for example, at least 900 nmol of Ca^{2+} per g of liver is taken up in this way. As the concentration of glucagon administered after BHQ addition was varied, so was the rate and extent of Ca^{2+} inflow. Half-maximal and maximal concentrations of glucagon required were of the order of 0.5 and 1.0 nM respectively, with a tripling of Ca^{2+} inflow between 0.05 and 1.0 nM glucagon (Figure 1b). This response of Ca^{2+} inflow to glucagon concentration is virtually identical with that seen with the activation by this hormone of glycogenolysis and gluconeogenesis in hepatocytes [15].

Figure 2(a) shows the concentrations of BHQ needed to promote maximally the actions of glucagon to induce Ca^{2+} influx. A BHQ concentration of $25 \mu\text{M}$, which produced near-maximal responses (Figure 2b), was consistently used in this work; it is similar to the maximal effects induced in hepatocytes (see, for example, [14]).

The ability of glucagon to induce Ca^{2+} inflow after BHQ administration was duplicated when dibutyryl cAMP was added in place of the hormone (Figures 3a and 3b); however, dibutyryl cGMP was ineffective (Figure 3c).

Ca^{2+} inflow induced by the combined actions of BHQ and glucagon was blocked when 0.5 mM Ni^{2+} was pre-administered (results not shown). Previous work from this laboratory [10] had shown that Ni^{2+} inhibits Ca^{2+} inflow in the perfused rat liver, consistent with its effects on hepatocytes [16]. This indicates that

Figure 1 BHQ treatment of liver permits glucagon-induced Ca^{2+} influx in the perfused rat liver

Details of the perfusion and chemicals employed are described in the Experimental section. In (a) the concentration of glucagon (G) was 10 nM and that of BHQ (B) was $25 \mu\text{M}$; in (b) the concentration of glucagon was varied as indicated, in experiments that were otherwise identical with that performed in the lower trace of (a). Areas above the curve observed after glucagon administration (a, lower trace) were calculated to give the Ca^{2+} uptake data (means $\pm$ S.E.M. for at least four independent observations from different experiments) shown in (b).

the Ca^{2+} is entering the liver cells through the Ni^{2+} -sensitive, receptor-operated Ca^{2+} influx pathway [17]. Because BHQ is continuously administered over the course of the experiments, the ER Ca^{2+} store remains depleted.

With the knowledge that BHQ inhibits bile flow (T. L. Applegate, A. Karjalainen and F. L. Bygrave, unpublished work) and thus presumably any major exit of calcium from the liver by this route [18], we then examined the question as to where else the accumulated Ca^{2+} might terminate in the liver. Incidental observations that oxygen uptake always increased when plasma membrane Ca^{2+} uptake was enhanced suggested to us that mitochondria were a possible site of accumulation (see also [19]). The following experiments were designed to address this point.

Entry of Ca^{2+} is totally abolished when sodium azide is pre-administered (Figure 4). This water-soluble agent inhibits mitochondrial electron transport [20] and thereby collapses the membrane potential obligatory for mitochondrial electrophoretic Ca^{2+} uptake (see, for example, [21]). The collective experiments in Figure 4 provide further information relevant to the route(s) of Ca^{2+} inflow after the actions of BHQ plus glucagon. First, as

Figure 2 Optimal concentrations of BHQ to permit glucagon-induced Ca^{2+} inflow

The experimental set-up was identical with that described in Figure 1 except that the glucagon (G) concentration was 10 nM and concentrations of BHQ (B) of 25, 15 and 5 mM as shown were used. Each trace shown in (a) is representative of at least four independent observations from different experiments. The Ca^{2+} uptake data shown in (b) (means $\pm$ S.E.M. for at least four independent observations from different experiments) were obtained by calculating the initial maximal rate of Ca^{2+} inflow at 4 min after the administration of glucagon.

long as azide is administered with or without BHQ, glucagon cannot induce Ca^{2+} inflow (Figure 4a). Secondly, administration of azide to liver that is already accumulating Ca^{2+} (Figure 4b) inhibits Ca^{2+} inflow within approx. 60 s; this inhibition is reversed extremely rapidly after the termination of azide infusion (Figures 4b and 4c). Thirdly, repeated additions and terminations of azide are able to inhibit and promote Ca^{2+} inflow reversibly (results not shown). The data in the figure also make the point that Ca^{2+} inflow induced by glucagon is specific to ER Ca^{2+} depletion; depletion of mitochondrial Ca^{2+} is ineffective.

Confirmation that the effects of azide were attributable to the predicted actions on mitochondria was obtained by directly measuring the changes in total calcium in the subsequently

Figure 3 The actions of cAMP in inducing Ca^{2+} inflow are not seen with cGMP

The experimental set-up was identical with that described in Figure 1 except that 50 μM dibutyryl cAMP (dbcAMP; a and b) or 50 μM dibutyryl cGMP (dbcGMP; c) replaced glucagon. Each trace is representative of at least three independent observations from different experiments.

isolated organelles after the treatments indicated in Figure 4. Data in Table 1 show that the inflow of Ca^{2+} revealed by Ca^{2+} electrode measurements (Figure 4) is seen also when total calcium is measured in the whole homogenate and the mitochondrial fraction. In the latter case, the large calcium/ Ca^{2+} inflow induced by the combined actions of BHQ plus glucagon is prevented by azide. It is of additional interest that the amounts of calcium taken up by the mitochondria in the present conditions are similar to those taken up by the synergistic action (cross-talk) of glucagon plus vasopressin (see [4]).

In control experiments (results not shown) the following points were established: thapsigargin was able to replace BHQ in inducing Ca^{2+} efflux as observed in hepatocytes [22] and in promoting glucagon-induced Ca^{2+} inflow; rotenone was able to replace azide despite its actions being slower, perhaps owing to its poor solubility in the perfusate medium. Other experiments (results not shown) were aimed at investigating whether the depletion of cellular ATP by azide (and not its action on mitochondrial Ca^{2+} fluxes) was a factor contributing to the observed inhibition of Ca^{2+} inflow [23]. First, the inclusion of 5 mM glucose in the perfusate had no effect on the observed Ca^{2+} fluxes shown in Figure 4(b). Secondly, the inclusion of 5 mM fructose to maintain high ATP/ADP ratios [24] likewise did not alter the responses seen in Figure 4(b). Finally, the addition of oligomycin to block ATP generation did not mimic the effects of azide. However, azide could still prevent the inflow of Ca^{2+} when oligomycin was present.

We believe that these results reveal several new features about the mechanism of receptor-operated Ca^{2+} inflow in non-excitable cells. The results show the following: first, that glucagon, which

Figure 4 Glucagon-induced Ca^{2+} influx in the presence of BHQ is inhibited by sodium azide

The experimental set-up was identical with that described in Figure 1, with glucagon (G; 10 nM) and BHQ (B; 25 μM) used in varying sequence combinations with sodium azide (Az; 5 mM). Each trace, labelled (a), (b) and (c), is representative of at least three independent observations from different experiments.

Table 1 Calcium content of mitochondria isolated after the actions of BHQ, glucagon and sodium azide in the perfused rat liver

Experimental conditions and concentrations of agents administered were the same as described in the legend to Figure 4. In each instance the liver was homogenized at 45 min after the start of the perfusion. In Expt. 2 BHQ was added at 20 min and glucagon at 30 min. In Expt. 3 azide was added at 13 min, BHQ at 20 min and glucagon at 30 min. The homogenate was fractionated by the Percoll-density procedure of Reinhart et al. [13]. Portions of the homogenate and the mitochondrial fraction were then assayed for calcium content by atomic absorption spectroscopy. All data shown are the means $\pm$ S.E.M. for four independent observations from different experiments.

Experiment	Cell fraction	Calcium content	
		(nmol/mg of protein)	(nmol/g of liver)*
1. No additions	Homogenate	5.62 $\pm$ 0.39	566.3 $\pm$ 39.7
	Mitochondria	6.13 $\pm$ 0.26	
2. BHQ + glucagon	Homogenate	23.24 $\pm$ 1.91	2559.3 $\pm$ 312.4
	Mitochondria	30.75 $\pm$ 1.81	
3. Azide + BHQ + glucagon	Homogenate	7.90 $\pm$ 0.48	885.5 $\pm$ 60.7
	Mitochondria	10.22 $\pm$ 0.85	

* Data derived from the homogenate calcium content.

on its own is not a potent inducer of Ca^{2+} influx, becomes so after the (artificial) depletion of ER but not of mitochondrial Ca^{2+} . In these circumstances only very small amounts of $\text{Ins}(1,4,5)\text{P}_3$, if any, might be generated by glucagon ([25]; reviewed in [8]). Secondly, they show that this entry of Ca^{2+} is blocked not only

by Ni^{2+} , which prevents its passage through the purported plasma membrane Ca^{2+} channel, but also by sodium azide, which prevents the entry of calcium/ Ca^{2+} into the mitochondria (Table 1). The rapidity of both the onset (results not shown) and release of this inhibition when azide is added and removed respectively (Figure 4), together with the above-mentioned arguments, indicates that it is unlikely that the inhibition of Ca^{2+} influx is attributable to the depletion of intracellular stores of ATP [23]. Moreover the actions of vasopressin and glucagon will have been to rapidly stimulate glycogenolysis and thus the production of glucose as an energy source for any energy-dependent Ca^{2+} fluxes. Finally, the amount of Ca^{2+} entering the liver in the above-mentioned circumstances is considerable and by far in excess of that leaving the liver following BHQ action (see, for example, [26]).

Conclusion

Several of these findings could assist in elucidating the mechanism of agonist-induced Ca^{2+} inflow, at least in liver. Although the notion that ER store depletion is necessary for Ca^{2+} inflow is not new (reviewed in [2]), it is evident from the present work that such inflow occurs despite the inability of the Ca^{2+} to accumulate in the ER owing to the continual depletion of ER Ca^{2+} (see also [11]). This inflow seems to be dependent on the ability of mitochondria to take up this Ca^{2+} from the cytoplasm after its entry through the plasma membrane. Among recent reports supporting roles for mitochondria in cellular Ca^{2+} inflow are (1) that of Ali et al. [27] showing that thapsigargin promotes Ca^{2+} inflow in rat basophilic RBL-2H3 cells into mitochondria, (2) that of Hoek et al. [28] showing that mitochondrial calcium content is increased after the action of thapsigargin and glucagon in hepatocytes and (3) that of Lawrie et al. [29] showing a close relationship between Ca^{2+} inflow and mitochondrial Ca^{2+} inflow in endothelial cells. Also relevant to this issue is the paper of Budd and Nicholls [30], in which it is suggested that the physiological function of mitochondria in cerebellar granule cells is to prolong (cell membrane) Ca^{2+} inflow by preventing the local Ca^{2+} concentration from rising too far at the mouths of the voltage-activated Ca^{2+} channels.

Earlier investigations into glucagon plus vasopressin-induced Ca^{2+} fluxes in liver had advocated a role for mitochondria (reviewed in [31]) in controlling hepatic Ca^{2+} fluxes. Others have suggested that a major role of mitochondrial Ca^{2+} uptake is to control enzymes of the citric acid cycle and thereby perhaps oxidative phosphorylation (see, for example, [19]). Further recent evidence (see [32]; reviewed in [33,34]) shows that many properties of mitochondrial Ca^{2+} uptake are most conducive to this organelle's being crucial in agonist-induced cellular Ca^{2+} regulation. Our present results also indicate that once the (receptor-operated) signals that initiate the events involved in cellular Ca^{2+} inflow are in place, the availability of mitochondrial stores can determine the extent of plasma membrane Ca^{2+} inflow, at least in this artificial system.

The chemical nature of the signal(s) involved remains to be elucidated. At least one set of signals seems to be necessary to communicate links from the ER to the purported Ca^{2+} inflow channel (discussed in [35,36], for example). In this regard the action of BHQ in promoting glucagon-induced Ca^{2+} inflow bears similarities to mechanisms of signalling cross-talk induced by the synergistic action of Ca^{2+} -mobilizing agonists and glucagon (reviewed in [7,8]). The present findings are of further relevance to these mechanisms in that little if any of the second messenger $\text{Ins}(1,4,5)\text{P}_3$, derived from 'classical' Ca^{2+} -mobilizing agonists,

will have been generated from the actions of BHQ and glucagon. Investigations on this issue are continuing.

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