Sources of calcium mobilized by glucagon in isolated rat hepatocytes

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Abstract. Effects of glucagon on cytoplasmic concentration of free calcium, [Ca\textsuperscript{2+}]\textsubscript{c}, were studied in aequorin-loaded hepatocytes. Addition of 5 nmol/l glucagon resulted in a prompt, but transient increase in aequorin bioluminescence. Glucagon, at 5 nmol/l, induced an increase in [Ca\textsuperscript{2+}]\textsubscript{c} even in medium containing 1 μmol/l calcium, although the response was considerably smaller than that observed in medium containing 1.0 mmol/l calcium. When hepatocytes incubated in the presence of 1 μmol/l extracellular calcium were first stimulated by phenylephrine and subsequently by either glucagon or angiotensin II, there was a response of [Ca\textsuperscript{2+}]\textsubscript{c} to glucagon, but not to angiotensin II. Dantrolene (50 μmol/l), which inhibits an increase in [Ca\textsuperscript{2+}]\textsubscript{c} induced by phenylephrine, did not inhibit the increase in [Ca\textsuperscript{2+}]\textsubscript{c} induced by glucagon. In contrast, dinitrophenol (50 μmol/l) abolished [Ca\textsuperscript{2+}]\textsubscript{c} response to glucagon without abolishing the increase in [Ca\textsuperscript{2+}]\textsubscript{c} induced by angiotensin II. These results suggest that glucagon mobilizes calcium from both intracellular and extracellular pools and that the intracellular calcium pool involved in glucagon action may be different from that mobilized by either phenylephrine or angiotensin II.

The glucagon action on cellular calcium has been well documented. When cytoplasmic free calcium concentration, [Ca\textsuperscript{2+}]\textsubscript{c}, is measured using the fluorescent indicator quin 2 (Charest et al. 1983; Sistare et al. 1985; Staddon & Hansford 1986), glucagon increases [Ca\textsuperscript{2+}]\textsubscript{c}. As to the source of calcium mobilized by glucagon, Claret and colleagues (Mauger et al. 1985; Mauger & Claret 1986; Poggioli et al. 1986; Combettes et al. 1986) demonstrated that glucagon mobilizes calcium from an intracellular pool and stimulates calcium influx. Altin & Bygrave (1987) also demonstrated that glucagon stimulates calcium uptake in the perfused rat liver.

Regarding the location of the intracellular calcium pool, Kraus-Friedmann (1986) reported that glucagon releases calcium from an FCCP-sensitive pool, which is considered to reside in mitochondria. Baddams et al. (1983) too demonstrated that glucagon acts on the liver to decrease mitochondrial calcium stores. Also, Chen et al. (1978) suggested that glucagon and vasoactive agents have a common intracellular calcium pool, which is located inside mitochondria. On the other hand, Combettes et al. (1986) and Mauger et al. (1986) reported that glucagon induces a release of calcium from the same internal store as do vasoactive agents. Thus, the source of the intracellular calcium pool affected by glucagon remains to be elucidated. The aim of the present study was to characterize the action of glucagon on [Ca\textsuperscript{2+}]\textsubscript{c} and to assess the intracellular source of calcium affected by glucagon. Our results suggest that the intracellular calcium pool mobilized by glucagon is different from that affected by vasoactive agents.
Material and Methods

Methods

Preparation of parenchymal liver cells. Parenchymal liver cells were prepared by the method of Berry & Friend (1969): Cells were suspended in modified Hanks' solution containing (in mmol/l) NaCl 137; KCl 3.5; KH2PO4 0.44; NaHCO3 4.2; Na2HPO4 0.33; CaCl2 1.0; HEPES/NaOH (pH 7.4) 20; equilibrated with 100% O2 gas.

Measurement of cytoplasmic free calcium concentration by aequorin. Changes in [Ca2+]c were monitored by measuring aequorin bioluminescence. Aequorin was loaded into hepatocytes by making plasma membrane reversibly permeable by the method of Morgan & Morgan (1982) as described previously (Mine et al. 1986, 1987). Aequorin-loaded cells were incubated in modified Hanks' solution. Aequorin signal was monitored using a Chronolog platelet aggregometer (Havertown, PA). Cell suspension containing 10^7 aequorin-loaded cells in 1 ml was filled into a cuvette and incubated at 37°C under constant stirring. [Ca2+]c under steady conditions was estimated as described in Snowdowne & Borle (1984) assuming an intracellular magnesium concentration of 1 mmol/l. [Ca2+]c in the non-steady condition was not calibrated, since no information is available at present as to the spatial distribution of calcium in the cell. Aequorin-loaded hepatocytes responded to glucagon normally in terms of glycogenolysis (Mine et al. 1986), and more than 99% aequorin-loaded cells excluded trypan blue. In some experiments, we loaded aequorin by a centrifugation method described by Borle et al. (1986) and results were essentially similar.

Measurement of cyclic AMP. An aliquot of cell suspension (4 x 10^6 cells/ml) was incubated with glucagon for 5 min in the presence of 0.5 mmol/l 3-isobutyl-1-methyl-xanthine. When dinitrophenol was included, 50 µmol/l dinitrophenol was added 2 min before the addition of glucagon. The reaction was terminated by adding trichloroacetic acid. After the removal of trichloroacetic acid by washing with diethylether, cAMP was measured by radioimmunoassay after succinylation by using a radioimmunoassay kit (Yamasa, Tokyo, Japan).

Materials

Phenylephrine, angiotensin II, and dinitrophenol were obtained from Sigma Chemical Co (St. Louis, MO). Glucagon was obtained from Novo Industri A/S (Copenhagen, Denmark). Dantrolene was kindly donated from Yamanouchi Pharmaceutical Co, Ltd (Tokyo, Japan). Aequorin was purchased from Dr. J. R. Blinks of the Mayo Foundation (Rochester, MN).

Results

Role of calcium influx in [Ca2+]c response induced by glucagon in aequorin-loaded hepatocytes

As demonstrated in Fig. 1A, addition of 5 nmol/l glucagon resulted in a prompt increase in aequorin bioluminescence. The glucagon action on [Ca2+]c was only transient: [Ca2+]c returned to a value close to the resting level within 30 sec. To identify the source of calcium mobilized by glucagon, aequorin-loaded hepatocytes were incubated in modified Hanks' solution containing 1 µmol/l calcium. In medium containing this concentration of calcium, basal as well as stimulated calcium influx is negligible (Mauger et al. 1984). Furthermore, cellular calcium may not be depleted severely in this condition, since the concentration of extracellular calcium is higher than the resting [Ca2+]c. When these cells were stimulated by 5 nmol/l glucagon, [Ca2+]c increased immediately (Fig. 1B) even though the response was considerably smaller than that observed in cells incubated in modified Hanks' solution containing 1.0 mmol/l calcium. Thus, at least part of the calcium mobilized by glucagon is of intracellular origin.

Comparison of intracellular calcium pools affected by glucagon, phenylephrine and angiotensin II

To determine whether the intracellular pool of calcium mobilized by glucagon and by either an-
Effect of glucagon and angiotensin II on [Ca^{2+}]_c in cells pretreated with phenylephrine. Aequorin-loaded cells were first treated with 10 μmol/l phenylephrine (Ph) and then stimulated by either 10 nmol/l angiotensin II (AII) (A) or 5 nmol/l glucagon (Glu) (B). Concentration of extracellular calcium was 1 μmol/l. Traces presented are representative of at least six experiments with similar results.

Gliotensin II or phenylephrine were the same, sequential addition of agonists were performed in the presence of 1 μmol/l extracellular calcium. When hepatocytes were first stimulated by phenylephrine at a high concentration and subsequently stimulated by a high dose of angiotensin II, there was no response of [Ca^{2+}]_c to angiotensin II (Fig. 2A). By contrast, when the cells were first stimulated by phenylephrine and then by glucagon, a small but significant increase in [Ca^{2+}]_c was observed in response to glucagon (Fig. 2B), suggesting that glucagon mobilized calcium from a distinct intracellular pool.

In the next set of experiments, the effect of glucagon was studied in aequorin-loaded hepatocytes pretreated with dinitrophenol to determine whether glucagon mobilizes calcium from a dinitrophenol-sensitive pool. Although dinitrophenol may induce a rapid depletion of the cellular ATP, pretreatment with dinitrophenol did not change cAMP production in response to 5 nmol/l glucagon (5 nmol/l glucagon alone; 26.6 ± 1.9 pmol/
mg protein vs 5 nmol/l glucagon + 50 µmol/l dinitrophenol: 26.7 ± 0.8 µmol/mg protein, mean ± SEM, N = 4). In the presence of 1 µmol/l extracellular calcium, dinitrophenol induced a slow and transient increase in [Ca²⁺]c, suggesting that calcium was mobilized from an intracellular pool (Fig. 3A). In the presence of dinitrophenol, glucagon did not cause any increase in [Ca²⁺]c (Fig. 3A). In contrast, angiotensin II caused a small increase in [Ca²⁺]c in the presence of dinitrophenol, even though the magnitude of the response was much smaller than that in the absence of dinitrophenol (Fig. 3B).

To further characterize the intracellular calcium pool affected by glucagon, we tested the effect of dantrolene on glucagon-induced increase in [Ca²⁺]c. In the presence of 1 µmol/l extracellular calcium, 50 µmol/l dantrolene inhibited the increase in [Ca²⁺]c induced by 10 µmol/l phenylephrine (Fig. 4B). However, as shown in Fig. 4B, 50 µmol/l dantrolene did not inhibit the increase in [Ca²⁺]c by 5 nmol/l glucagon in the presence of 1 µmol/l dantrolene did not inhibit the glucose output induced by 5 nmol/l glucagon (data not shown).

**Discussion**

In the present study, we have characterized the action of glucagon at a relatively high concentration on [Ca²⁺]c. As shown in Fig. 1, glucagon induced an increase in [Ca²⁺]c. Our present results agree with previous reports (Charest et al. 1983; Sistare et al. 1985; Combettes et al. 1986; Staddon & Hansford 1986). Studer et al. (1984) showed that glucagon does not increase aequorin bioluminescence in hepatocytes. The reason for this discrepancy is not clear at present, but it may be due to the difference in experimental conditions. We used an incubation system with constant stirring for the measurement of aequorin bioluminescence, whereas Studer and associates employed a flow-through perifusion system with hypoosmotic shock for aequorin loading. The present results are reproduced in hepatocytes loaded with aequorin by the method described by Borle et al. (1986).

As shown in Fig. 1B, the 5 nmol/l glucagon-mediated increase in [Ca²⁺]c is reduced but not abolished in medium containing 1 µmol/l calcium. In this condition, it is thought that the basal as
well as stimulated calcium influx is negligible (Mauger et al. 1984). Thus, in addition to stimulating calcium influx, 5 nmol/l glucagon mobilizes calcium from an intracellular pool(s). These results agree with the report of Combettes et al. (1986). Because aequorin bioluminescence induced by 5 nmol/l glucagon was considerably smaller in the presence of 1 mmol/l extracellular calcium, than in the presence of 1 mmol/l extracellular calcium, it is likely that calcium influx is also important in the action of glucagon on [Ca\(^{2+}\)]_c.

The intracellular pool of calcium mobilized by glucagon appears distinct from that mobilized by vasoactive agents, phenylephrine and angiotensin II for the following reasons. First, as shown in Fig. 2A, when aequorin-loaded hepatocytes incubated in 1 mmol/l calcium medium are first incubated with phenylephrine and subsequently with glucagon, glucagon induces an increase in [Ca\(^{2+}\)]_c. The effect of glucagon is in contrast to that of angiotensin II, which has no effect on [Ca\(^{2+}\)]_c when added following phenylephrine. These data suggest that glucagon can mobilize calcium from intracellular pools not depleted by phenylephrine. Second, an intracellular pool affected by glucagon is sensitive to dinitrophenol, an uncoupler of mitochondrial oxidative phosphorylation, whereas the intracellular pool affected by angiotensin II is less sensitive to dinitrophenol. Since dinitrophenol pretreatment did not change cAMP response to glucagon, it is unlikely that dinitrophenol inhibits glucagon action on [Ca\(^{2+}\)]_c by inhibiting cAMP production. When applied to isolated subcellular fractions, dinitrophenol releases Ca\(^{2+}\) specifically from mitochondria. Although the possibility cannot be excluded that dinitrophenol may have other effects than on mitochondrial oxidative phosphorylation in intact cells, including partial inhibition of the intracellular Ca\(^{2+}\) release by the IP\(_3\)-dependent mechanism, it seems possible that the intracellular pool affected by glucagon includes mitochondria. Third, as shown in Fig. 4, dantrolene does not inhibit the glucagon-induced increase in [Ca\(^{2+}\)]_c. In a previous study, we demonstrated that dantrolene (50 µmol/l) inhibits calcium release from an IP\(_3\)-sensitive pool since dantrolene inhibits phenylephrine-induced calcium mobilization without inhibiting IP\(_3\) production induced by phenylephrine (Mine et al. 1987). Although we did not measure IP\(_3\) production induced by glucagon, the present results suggest that glucagon mobilizes calcium from an intracellular pool which is different from the IP\(_3\)-sensitive pool affected by phenylephrine. Alternately, glucagon mobilizes calcium from a common pool by a dantrolene-insensitive mechanism. This possibility, however, seems unlikely in view of the above-mentioned two aspects of glucagon action.

Taken together, the present findings indicate that at a relatively high concentration, glucagon (5 nmol/l) mobilizes calcium from the intracellular calcium pool which may be different from that sensitive to phenylephrine and angiotensin II. In addition, a rise in [Ca\(^{2+}\)]_c in response to glucagon is at least in part dependent upon the influx of calcium from the extracellular milieu.

References


