Hormone-induced increase in free cytosolic calcium and glycogen phosphorylase activation in rat hepatocytes incubated in normal and low-calcium media

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The action of α_1 -adrenergic agonists (noradrenaline in the presence of propranolol), vasopressin and angiotensin on the intracellular free Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, was determined by using the fluorescent dye quin2 in isolated rat liver cells. In the presence of external Ca^{2+} (1.8 mM), 1 μM -noradrenaline induced an increase in $[\text{Ca}^{2+}]_i$ up to about 800 nM without apparent delay, whereas 10 nM-vasopressin and 1 nM-angiotensin increased $[\text{Ca}^{2+}]_i$ to values higher than 1500 nM with a lag period of about 6 s. The successive addition of the hormones and of their specific antagonists indicated that the actions of the three Ca^{2+} -mobilizing hormones occurred without apparent desensitization (over 6 min) and via independent receptors. The relative contributions of internal and external Ca^{2+} pools to the cell response were determined by studying the hormone-mediated $[\text{Ca}^{2+}]_i$ increase and glycogen phosphorylase activation in low- Ca^{2+} media (22 μM). In this medium: (1) $[\text{Ca}^{2+}]_i$ was lowered and the hormones initiated a transient instead of a sustained increase in $[\text{Ca}^{2+}]_i$; subsequent addition (2 min) of a second hormone promoted a lesser increase in $[\text{Ca}^{2+}]_i$; in contrast, the subsequent addition (2 min) of Ca^{2+} (1.8 mM) caused $[\text{Ca}^{2+}]_i$ to increase to a value close to that initiated by the hormone in control conditions, the amplitude of the latter response being dependent on the concentration of Ca^{2+} added to the medium; (2) returning to normal Ca^{2+} (1.8 mM) restored the resting $[\text{Ca}^{2+}]_i$ and allowed the hormone added 2 min later to promote a large increase in $[\text{Ca}^{2+}]_i$ whose final amplitude was also dependent on the concentration of Ca^{2+} added beforehand. Similar results were found when the same protocol was applied to the glycogen phosphorylase activation. It is concluded that Ca^{2+} influx is required for a maximal and sustained response and to reload the hormone-sensitive stores.

The α_1 -adrenergic agonists (noradrenaline in the presence of β -blockers), vasopressin and angiotensin act on liver cell metabolism via a mobilization of Ca^{2+} (Exton, 1981; Claret & Binet, 1984). A fast and sustained increase in $[\text{Ca}^{2+}]_i$, as monitored by indirect determination or by the fluorescent indicator quin2 (Tsien, 1981), has been described within the seconds after the addition of these hormones to rat hepatocytes in suspension (Murphy *et al.*, 1980; Charest *et al.*, 1983; Burgess *et al.*, 1983; Berthon *et al.*, 1984). The same resting value, about 160 nM,

was found by the different authors. In contrast, we observed that noradrenaline via α_1 -adrenergic receptors enhanced $[\text{Ca}^{2+}]_i$ to about 800 nM, and the peptide hormones vasopressin and angiotensin increases it to more than 1.5 μM (Berthon *et al.*, 1984), values significantly higher than those (about 400 nM) found by Charest *et al.* (1983) and Thomas *et al.* (1984).

Both internal Ca^{2+} stores and external Ca^{2+} have been involved in the cell responses (glycogenolysis, gluconeogenesis, respiration etc.) (for reviews see Exton, 1983; Claret & Binet, 1984). Since a net Ca^{2+} efflux was demonstrated within the first seconds after the hormone addition, several papers had suggested that the early fraction of the

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; quin2/AM, quin2 tetra-acetoxymethyl ester.

mobilized Ca^{2+} predominantly comes from intracellular stores (Chen *et al.*, 1978; Reinhart *et al.*, 1982; Blackmore *et al.*, 1982, 1983; Joseph & Williamson, 1983). The external Ca^{2+} was thought to be required both to prolong cell responses (>1 min) and to replenish the intracellular pools mobilized during the hormone activation (Exton, 1983; Reinhart *et al.*, 1984b). However, an activation of Ca^{2+} influx by the hormones had been proposed (Keppens *et al.*, 1977; Assimacopoulos-Jeannet *et al.*, 1977; Barritt *et al.*, 1981), this activation occurring within 1 min after hormone addition (Mauger *et al.*, 1984; Poggioli *et al.*, 1985).

In the present paper we have re-examined the relative contributions of internal and external Ca^{2+} in the action of the three Ca^{2+} -mobilizing hormones in isolated rat liver cells. This has been performed by determining the dependency on external Ca^{2+} of two cell responses that play a major role in the hormonal action: the cytosolic free Ca^{2+} , as measured by the fluorescent indicator quin2, and the target enzyme glycogen phosphorylase *a*. We show that external Ca^{2+} is involved within the first seconds of the hormone action and that it is required for sustained responses and to replenish internal stores depleted by the hormones.

Preliminary accounts of some of these results have been published (Binet *et al.*, 1984).

Methods

Preparation of hepatocytes

Rat liver cells were isolated by the conventional collagenase method from fed Wistar rats as described previously (Burgess *et al.*, 1981; Berthon *et al.*, 1984). Female rats were routinely used, but a few experiments were performed on male rats (see the Results section). After isolation, the cells (4 mg dry wt./ml) were equilibrated in Eagle's medium, containing 116 mM-NaCl, 5.4 mM-KCl, 1.8 mM- CaCl_2 , 0.81 mM- MgCl_2 , 0.92 mM- NaH_2PO_4 , 25 mM- NaHCO_3 , 1000 mg of glucose/ml, and supplemented with 2% serum albumin, vitamins and amino acids (see Burgess *et al.*, 1981), and maintained under continuous gassing with O_2/CO_2 (19:1) at 37°C. The media also contained 5 μM -propranolol to block the β -adrenergic receptors.

Quin2 loading

The cells were loaded as described by Berthon *et al.* (1984). Routinely, 2 mg dry wt. of hepatocytes was incubated with 500 μl of 50 μM -quin2/AM for 150 s at 37°C under continuous gassing. The cells were then washed twice in Eagle's medium containing no vitamins, amino acids or albumin, and centrifuged at 50g for 1 min. Finally the cells were resuspended in the same medium at about 1 mg dry wt./ml and transferred into the cuvette of

a Jobin-Yvon JY3D spectrofluorimeter, at 37°C and under continuous magnetic agitation and gassing. Fluorescence (excitation at 342 nm and emission at 492 nm) was recorded on a potentiometric recorder. At the end of each experiment, the $[\text{Ca}^{2+}]_i$ was calibrated by successive additions of 4 μM -digitonin to equilibrate external Ca^{2+} and internal quin2 and to saturate the quin2 fluorescence, and of 24 mM-EGTA to obtain the minimal fluorescence. Each determination in quin2-loaded cells was followed by a parallel control experiment carried out under the same conditions in non-quin2-loaded cells. The slight variations of the cell autofluorescence (owing to modifications of the redox state of the nicotinamide nucleotides) were taken into account in calculating fluorescence in terms of Ca^{2+} concentration. In addition, the fluorescence emission was also corrected for the small component (4–6%) generated from quin2 in the external medium or trapped in dead cells.

In experiments carried out in 22 μM external Ca^{2+} , the hepatocytes were first loaded with quin2 in the presence of 1.8 mM- Ca^{2+} as usual, then washed first in a 1.8 mM- Ca^{2+} medium and then in a 22 μM - Ca^{2+} medium. The final pellet was resuspended in this 22 μM - Ca^{2+} medium for 6 min, including the second washing period.

Phosphorylase determination

Glycogen phosphorylase activity was determined by the method of Burgess *et al.* (1983). Cell samples (50 μl) were taken and added to 150 μl of ice-cold medium containing 0.5% glycogen, 100 mM-NaF, 20 mM-EDTA and 168 mM-glycylglycine (pH 7.4). Samples were immediately frozen and stored in liquid N_2 until assayed for phosphorylase *a* activity.

Materials

Collagenase was from Boehringer, quin2/AM from Lancaster Synthesis (Morecambe, Lancs., U.K.), and [^{14}C]glucose 1-phosphate from NEN. All other chemicals or drugs were of analytical-reagent grade.

Results

Hormonal action in normal- Ca^{2+} medium

Quin2 loading. In a previous paper (Berthon *et al.*, 1984) we had shown that optimal loading and hydrolysis of quin2/AM were obtained by incubating the hepatocytes with 50 μM -quin2/AM for 2.5 min at 37°C. This was shown both from the shift of the fluorescence emission spectra (measured by the ratio of the emission at 492 nm to that at 440 nm) and from the incorporation of [^3H]quin2/AM. Under these conditions the amount of quin2 was about 1.5 nmol/mg dry wt.

During this incubation period, the total cellular Ca²⁺ increased by about 20% (results not shown). This net uptake of cellular Ca²⁺ most probably results from equilibration of the internal chelator by Ca²⁺ via net Ca²⁺ uptake (Berthon *et al.*, 1984).

The dissociation constant of the complex Ca²⁺-quin2 has been estimated as 115 nM in a saline medium (Tsien *et al.*, 1982). A similar value was found by calibrating the fluorescence obtained in digitonin-permeabilized quin2-loaded cells equilibrated at various Ca²⁺ concentrations. Free external Ca²⁺ was fixed with Ca²⁺-EGTA buffer. Fig. 1 shows that an excellent fit of the fluorescence signal was found for a 1:1 Ca²⁺/quin2 binding. The K_d calculated under these conditions was 110 nM. This shows that the cells fully regenerate the indicator without modification of the apparent affinity of quin2 for Ca²⁺.

Action of the hormones on [Ca²⁺]_i. The increase in [Ca²⁺]_i mediated by the Ca²⁺-mobilizing hormones is now well documented in isolated rat liver cells (Charest *et al.*, 1983; Berthon *et al.*, 1984; Thomas *et al.*, 1984). The maximal response was observed within 6 s for 1 μ M-noradrenaline and within 15–20 s for 10 nM-vasopressin (Figs. 2a and 2b) or 1 nM-angiotensin (results not shown). There was no apparent delay in the [Ca²⁺]_i increase induced by noradrenaline, whereas a short lag period of about 6 s was observed for the activation

by vasopressin (see Fig. 4). Discrepancies about the value attained by [Ca²⁺]_i appeared in the literature. In our hands the resting [Ca²⁺]_i was about 160 nM, and was increased to values close to 800 nM with noradrenaline and to above 1500 nM with vasopressin and angiotensin, whereas markedly lower maximal values (400 nM) were reported by others (Charest *et al.*, 1983; Thomas *et al.*, 1984). Several reasons may explain this discrepancy. First, the other authors incubated the hepatocytes with higher quin2/AM concentrations, and/or for longer periods (15 or 20 min), which should lead to a quin2 load substantially higher than that used here (see Berthon *et al.*, 1984). This necessarily enhances the buffering capacity of the indicator, which in turn may alter the maximal amplitude of the response to the hormone. Second, the external Ca²⁺ concentration was lower (0.5–0.6 mM) than that used in the present work (1.8 mM). This decreases the contribution of external Ca²⁺ influx to the rise in [Ca²⁺]_i. In addition, the extracellular pH was not buffered with CO₂/bicarbonate. Figs. 2c and 2d show that under these conditions (absence of O₂/CO₂ gassing above the measuring cell or replacement of bicarbonate by Hepes buffer) the stimulated [Ca²⁺]_i concentration was markedly lower than in the CO₂/bicarbonate-buffered medium (326 nM for noradrenaline and 400 nM for vasopressin). This presumably results from some modification in the regulation of internal and/or external pH and may suggest that the [Ca²⁺]_i metabolism is not independent of the pH control.

Absence of sex difference in the hormonal responses. Studer & Borle (1982, 1983) reported a sex difference in the cellular metabolism of Ca²⁺ and the α_1 -adrenergic activation of glycogen phosphorylase. The determination of the resting and hormone-stimulated [Ca²⁺]_i in male and female rat hepatocytes did not lead us to observe any difference in the [Ca²⁺]_i variations induced by noradrenaline, vasopressin and angiotensin, in the time course or the amplitude of the [Ca²⁺]_i increase or for the dose-response curves for the hormones (A. Binet & B. Berthon, unpublished work). This apparent lack of discrepancy was also observed for the inhibition of the hormonal responses in low-Ca²⁺ medium (see below) or for the cell responses to β -adrenergic agents and glucagon (results not shown).

Successive additions of the hormones. In the presence of normal extracellular Ca²⁺ (1.8 mM), the stimulated [Ca²⁺]_i was maintained as long as the hormone was present in the incubation medium of hepatocytes (Figs. 2a and 2b), indicating that no apparent desensitization occurred for at least 6 min. The dose-response curves for the hormones tested were in the same range as those reported for

Fig. 1. Determination of dissociation constant for the Ca²⁺-quin2 complex in digitonin-treated cells

The rat liver hepatocytes were loaded with quin2/AM as described in the Methods section. Free external Ca²⁺ concentrations were applied by using controlled EGTA and Ca²⁺ concentrations. Fluorescence was then measured in 4 μ M-digitonin-treated cells, and F_{max} (100) and F_{min} (0) were determined by adding excess concentrations of Ca²⁺ and EGTA respectively.

Fig. 2. Effects of noradrenaline and vasopressin on $[Ca^{2+}]_i$ of rat hepatocytes incubated with 1.8mM external Ca^{2+} . The cells were loaded with quin2 and incubated as indicated in the Methods section. Quin2 fluorescence was measured (342–492nm) in the spectrofluorimeter cuvette with constant agitation and gassing. Fluorescence was calibrated in terms of $[Ca^{2+}]_i$ after the addition of 4 μ M-digitonin (F_{max}) and 24mM-EGTA (F_{min}). In (a) and (b), the hepatocytes were incubated in Eagle bicarbonate medium gassed with O_2/CO_2 . In (c) and (d), they were suspended and incubated in a 20mM-Hepes medium (without bicarbonate but gassed with O_2). Noradrenaline (1 μ M; NOR), vasopressin (10nM; VP) or digitonin: (4 μ M; D) were added as indicated.

Fig. 3. Effects of successive additions of the hormones and their specific antagonists on $[Ca^{2+}]_i$. Rat liver hepatocytes were loaded and incubated as indicated in the Methods section. Quin2 fluorescence (342–492nm) was recorded and further calibrated from the F_{max} and F_{min} determined in the presence of 4 μ M-digitonin and 24mM-EGTA respectively. Successive additions to the incubation medium were: vasopressin (VP; 1 nM); [1-(β -mercapto- $\beta\beta$ -cyclopentamethylenepropionic acid),2-(*O*-ethyltyrosine),4-valine,8-arginine]vasopressin (AT/VP; 0.4 μ M); noradrenaline (NOR; 1 μ M); phentolamine (PHE; 10 μ M); angiotensin (AII; 1 nM); digitonin (D; 4 μ M); EGTA (24mM).

other hormone-stimulated functions in rat hepatocytes (half-maximal with 150nM-noradrenaline and about 0.4nM-vasopressin).

The addition of the specific vasopressin antag-

onist rapidly decreased $[Ca^{2+}]_i$ from the hormone-stimulated value (>1500 nM with 10nM-vasopressin) to the basal value (Fig. 3). This reversal was antagonist-dose-dependent, and was achieved

within 1–3 min, probably depending on the relative affinities of each agonist and antagonist for their specific receptor. The subsequent addition of a second Ca²⁺-mobilizing hormone (1 μ M-noradrenaline) provoked a complete response, which was suppressed by adding a specific antagonist for α_1 -adrenergic receptor (10 μ M-phentolamine). The addition of a third agonist (1 nM-angiotensin) then triggered a maximal stimulated [Ca²⁺]_i. These results resemble previous reports showing that an increase in cytosolic free Ca²⁺ may be initiated by vasopressin after successive additions of the α_1 -adrenergic agonist phenylephrine and antagonist phentolamine (Charest *et al.*, 1983).

Hormonal actions in low-Ca²⁺ media

Involvement of Ca²⁺ influx and internal Ca²⁺ stores. It has been previously shown that removing external Ca²⁺ for 30s–2 min before the hormone slightly decreased the resting [Ca²⁺]_i and considerably decreased the hormone-induced increase in [Ca²⁺]_i (Berthon *et al.*, 1984). Fig. 4 compares the fast time-courses of the [Ca²⁺]_i variations recorded

after the addition of 10 nM-vasopressin in the presence of 1.8 mM or 72 nM external Ca²⁺. In low-Ca²⁺ media, a maximal [Ca²⁺]_i of about 300 nM was observed at 30s, which returned to the basal value at 90s. This decreased and transient increase in low-Ca²⁺ media indicates that both influx of extracellular Ca²⁺ and internal Ca²⁺ store(s) contribute to the cell response. Incubating the cells in EGTA for periods longer than 30s (up to 10 min) promoted a progressive decrease in the resting and hormone-stimulated [Ca²⁺]_i. This presumably resulted from the suppression of Ca²⁺ influx, which was no longer able to reload the internal Ca²⁺ stores. Similar results were observed with noradrenaline and angiotensin.

The quantitative contribution of the internal Ca²⁺ stores could not be directly estimated from the rises in [Ca²⁺]_i. This could alternatively be done by calculating the amount of Ca²⁺ bound to quin2 as described by Berthon *et al.* (1984). If one assumes 1.5 nmol of internal quin2/mg dry wt., an increase in [Ca²⁺]_i from 160 to more than 1000 nM corresponds to a mobilization of 500 pmol of Ca²⁺/mg dry wt. In the absence of external Ca²⁺, the hormone-mediated rise from 125 to 330 nM corresponds to decrease to 325 pmol/mg dry wt.

Reloading of internal Ca²⁺ pools. In order to avoid secondary effects of the massive addition of external EGTA, the following low-Ca²⁺-medium experiments were performed in the presence of controlled 22 μ M external Ca²⁺. The hepatocytes were first loaded with quin2/AM in normal 1.8 mM-Ca²⁺, then incubated in 22 μ M-Ca²⁺ for 6 min (including the washing period). As expected from the above results, decreased and transient increases in [Ca²⁺]_i were observed with both noradrenaline and vasopressin (Figs. 5a and 5c). However, the addition of 1.8 mM-Ca²⁺ 2 min after the hormone induced a rapid increase in [Ca²⁺]_i to a value similar to that triggered by noradrenaline (about 800 nM) and by vasopressin (1500 nM) in the presence of external Ca²⁺.

When 1.8 mM-Ca²⁺ was added to the cells preincubated for 6 min in 22 μ M-Ca²⁺ medium, [Ca²⁺]_i rose to about 300–400 nM, i.e. slightly higher than the initial resting [Ca²⁺]_i. This presumably resulted from some reorganization in the Ca²⁺ homeostasis disturbed by the depletion and repletion phases, but also from the uncertainty about the Ca²⁺-quin2 calibration caused by addition of Ca²⁺ during the course of the experiment (see the Methods section). However, it is noteworthy that the addition of the hormone 2 min later triggered a normal maximal response (Figs. 5b and 5d), indicating a complete restoration of the Ca²⁺ response at that time.

Similar experiments were carried out by adding increasing concentrations of Ca²⁺ before or after

Fig. 4. Time course of the [Ca²⁺]_i increase after vasopressin stimulation in the presence of 1.8 mM or 72 nM external Ca²⁺

The hepatocytes were prepared and incubated as described in the Methods section. At zero time, 10 nM-vasopressin was added with a syringe with the needle plunged into the medium to decrease the mixing time to less than 1 s (○); ●, 8 mM-EGTA was added 1 min before the hormone. [Ca²⁺]_i was calculated from the fluorescence of quin2 as indicated in the Methods section.

Fig. 5. Addition of the hormones and Ca^{2+} on hepatocytes equilibrated in $22\mu\text{M}$ external Ca^{2+} . The rat hepatocytes were loaded with quin2/AM as indicated in the Methods section. The last washing was made with a saline medium containing $22\mu\text{M}$ - Ca^{2+} and the cells were allowed to equilibrate for 6 min at 37°C in the cuvette of the spectrofluorimeter before the first addition. Quin2 fluorescence ($342\text{--}492\text{ nm}$) was further calibrated as usual. The additions performed were: (a) and (b) vasopressin (VP; 10 nM), Ca^{2+} (1.8 mM) and digitonin (D; $4\mu\text{M}$); (c) and (d) noradrenaline (NOR; $1\mu\text{M}$), Ca^{2+} (1.8 mM) and digitonin (D; $4\mu\text{M}$). The horizontal bar represents 1 min.

the hormones. In each case, the amplitude of the hormone-induced $[\text{Ca}^{2+}]_i$ increase was related to the external Ca^{2+} concentrations. Fig. 6 combines the results of several experiments of this kind in which the maximal $[\text{Ca}^{2+}]_i$ elicited by noradrenaline and vasopressin has been plotted as a function of the external $[\text{Ca}^{2+}]$ added 2 min beforehand. Dose-responses curves show that half-maximal activations were obtained with about $250\mu\text{M}$ external Ca^{2+} for noradrenaline and with higher than $800\mu\text{M}$ for vasopressin.

Glycogen phosphorylase activation. One of the most studied metabolic effects of Ca^{2+} -mobilizing hormones in liver is the activation of glycogen phosphorylase, responsible for the breakdown of glycogen mediated by activation of the phosphorylase kinase by Ca^{2+} . The phosphorylase *a* activity was measured under the same conditions as those used for the measurements of $[\text{Ca}^{2+}]_i$. However, in the quin2-loaded cells the hormone-mediated activation of phosphorylase was not easily measurable because the presence of the chelator alters the basal activity of the enzyme (Charest *et al.*, 1983; Thomas *et al.*, 1984; Poggioli *et al.*, 1985). As a consequence the next experiments were performed in the absence of quin2 loading.

In the presence of $22\mu\text{M}$ external Ca^{2+} , a fast activation of the phosphorylase by $1\mu\text{M}$ -noradrenaline was observed (Fig. 7a). The activation was

Fig. 6. Ca^{2+} -dependence of the hormone-induced increase. Rat hepatocytes were loaded with quin2/AM and equilibrated in $22\mu\text{M}$ - Ca^{2+} as indicated in Fig. 5. Various Ca^{2+} concentrations were first added, and 2 min later $1\mu\text{M}$ -noradrenaline (○) or 10 nM -vasopressin (●). Quin2 fluorescence was recorded and calibrated as usual in terms of $[\text{Ca}^{2+}]_i$. The maximal $[\text{Ca}^{2+}]_i$ was measured 30 s after the hormone addition. Results are means of two to five separate experiments.

transient and phosphorylase *a* activity decreased slowly within the 4 min after the hormone addition. At that time, addition of Ca^{2+} to the medium induced a second activation of the phosphorylase

Fig. 7. Glycogen phosphorylase activation by noradrenaline and Ca²⁺ in the presence of 22 μM external Ca²⁺

The rat liver cells (2 mg dry wt./ml) were resuspended in Eagle's medium containing 22 μM-Ca²⁺ and equilibrated at 37°C with constant agitation and gassing for 6 min before the first addition. Samples were taken for determination of phosphorylase activity. In (a), 1 μM-noradrenaline (NOR) was added at zero time, and various concentrations of Ca²⁺ at 4 min: ◆, none; ▲, 200 μM; ■, 600 μM; ●, 1.8 mM. In (b), Ca²⁺ was added at zero time (same concentrations and symbols as above) and 1 μM-noradrenaline (NOR) 2 min later.

whose amplitude and duration were dependent on the Ca²⁺ concentration added. Similar results were reported for glucose output in perfused rat liver by Reinhart *et al.* (1984a).

As [Ca²⁺]_i increases, the hormone-mediated phosphorylase activation was dependent on the Ca²⁺ added 2 min beforehand (Fig. 7b). Increasing Ca²⁺ concentration from 22 μM to 1800 μM induced a more sustained activation of the phosphorylase. It is noteworthy that phosphorylase activation was not maximal at 600 μM-Ca²⁺, whereas the noradrenaline-mediated [Ca²⁺]_i increase was complete above 500 μM (Fig. 6).

Successive additions of the hormones on [Ca²⁺]_i and phosphorylase in low-external-Ca²⁺ medium. In contrast with the result in Fig. 3, showing that a second and then a third hormonal challenge are

Fig. 8. Effects of successive hormone or antagonist addition on the [Ca²⁺]_i in the presence of 22 μM external Ca²⁺

The rat liver cells were prepared, loaded and incubated as in Fig. 5. Quin2 fluorescence was measured and calibrated as usual, and values of [Ca²⁺]_i were recalculated for each point indicated on the Figure. Noradrenaline (1 μM; NOR) was added 6 min after the equilibration of the cells in 22 μM-Ca²⁺ (a and b) and vasopressin (10 nM; VP) 3 min later (a, b, c). In (b), 10 μM-phenoxybenzamine (POB) was added 30 s after the hormone.

efficient in initiating maximal responses in the presence of 1.8 mM external Ca²⁺, the experiments performed in low external Ca²⁺ (Fig. 8) showed that the hormone-sensitive pools could no longer be replenished and that a subsequent hormone addition was ineffective. In a 22 μM-external-Ca²⁺ medium the addition of 1 μM-noradrenaline induced a [Ca²⁺]_i increase that tended to return to its basal value. If 10 nM-vasopressin were then added 3 min later, a lesser increase in [Ca²⁺]_i was observed (Fig. 8a), whereas the same dose of vasopressin added at 3 min (without prior noradrenaline addition) initiated a normal Ca²⁺ increase (Fig. 8c). Addition of the α-adrenergic antagonist phenoxybenzamine 30 s after noradrenaline accelerated the reversal to the basal value, and the subsequent addition of vasopressin induced an intermediate response. This indicates that, when internal Ca²⁺ pools are not replenished by external Ca²⁺, a first hormonal stimulation mobilizes a large fraction of the available Ca²⁺, so that a subsequent challenge with another hormone promotes an altered response.

Fig. 9. Effects of successive additions of the hormones and antagonist on the phosphorylase activity in the presence of $22\text{ }\mu\text{M-Ca}^{2+}$

The rat liver cells were prepared as indicated in Fig. 8. Noradrenaline ($1\text{ }\mu\text{M}$; NOR) was added at 6 min in $22\text{ }\mu\text{M-Ca}^{2+}$ (○); $10\text{ }\mu\text{M}$ -phenoxybenzamine (POB) was added 30 s later (■), and 10 nM -vasopressin (VP) 4 min after noradrenaline in noradrenaline-treated cells (○), in noradrenaline + phenoxybenzamine-treated cells (■) and in control cells (●). Results of a typical experiment are shown.

Fig. 9 shows the results of a similar experiment on the phosphorylase activity. When the rat hepatocytes were incubated in $22\text{ }\mu\text{M-Ca}^{2+}$, phosphorylase was immediately and transiently activated by $1\text{ }\mu\text{M}$ -noradrenaline. The addition of 10 nM -vasopressin 4 min after noradrenaline slightly re-activated phosphorylase. If $10\text{ }\mu\text{M}$ -phenoxybenzamine was added 30 s after noradrenaline, phosphorylase rapidly returned to its basal activity, and the subsequent addition of vasopressin then promoted a fast and transient activation of the enzyme.

Discussion

Quin2/AM loading conditions

In the present paper, we confirm our previous observation (Berthon *et al.*, 1984) that uptake and hydrolysis of quin2/AM is a rapid process in isolated rat liver cells. Optimal signal/noise ratios of the fluorescence emission were obtained by loading the cells for 2 min 30 s. This is shorter than the loading periods currently used in the liver or in other tissues: 15–20 min in rat hepatocytes (Charest *et al.*, 1983; Thomas *et al.*, 1984), 60 min with lymphocytes (Hesketh *et al.*, 1983) and neutrophils

(Pozzan *et al.*, 1983), 25 min with platelets (Rink *et al.*, 1982).

Hormone-dependent $[\text{Ca}^{2+}]_i$ increase

If the $[\text{Ca}^{2+}]_i$ resting value usually found in isolated rat hepatocytes is about 160 nM (Murphy *et al.*, 1980; Charest *et al.*, 1983; Berthon *et al.*, 1984), the maximal value attained during the hormone stimulation was higher in our hands than that reported by Charest *et al.* (1983) and Thomas *et al.* (1984). A possible reason for this discrepancy is that those authors incubated the hepatocytes with higher quin2/AM concentration and/or for longer periods (see above). In addition, differences in the experimental conditions of incubation should be mentioned. Particularly, it seems likely that the presence of $\text{CO}_2/\text{bicarbonate}$ was required for obtaining optimal $[\text{Ca}^{2+}]_i$ increases. Furthermore, low external Ca^{2+} concentrations were used by these authors (0.5 mM , Charest *et al.*, 1983; 0.65 mM , Thomas *et al.*, 1984), and the present paper shows that $[\text{Ca}^{2+}]_i$ increases are dependent on external Ca^{2+} (Figs. 5 and 6).

In normal (1.8 mM)- Ca^{2+} medium, the increase in $[\text{Ca}^{2+}]_i$ was sustained for at least 6 min. This indicates that there is no apparent desensitization of the receptors during this period. This was confirmed by the finding that the addition of a specific antagonist which displaces the hormone from its receptor caused $[\text{Ca}^{2+}]_i$ to return rapidly to its basal value. In other words, the agonist has to be bound to its receptor to maintain the high stimulated $[\text{Ca}^{2+}]_i$. Furthermore, the subsequent addition of a second Ca^{2+} -mobilizing hormone when $[\text{Ca}^{2+}]_i$ had returned to its basal value restored a maximal increase in $[\text{Ca}^{2+}]_i$. Incidentally, this is in agreement with the fact that the three Ca^{2+} -mobilizing hormones act via independent receptors (Keppens *et al.*, 1982; Jard, 1983).

Origin of the mobilized Ca^{2+}

The observation of a glycogen phosphorylase activation by Ca^{2+} -mobilizing hormones in the absence of external Ca^{2+} led different authors to suggest that the Ca^{2+} responsible for the activation of metabolic functions has an intracellular, and probably mitochondrial, origin (Babcock *et al.*, 1979; Studer & Borle, 1983; Blackmore *et al.*, 1983; Exton, 1983; Joseph & Williamson, 1983). On the other hand, a non-mitochondrial pool (endoplasmic reticulum or plasma membrane) was proposed by several groups (Poggioli *et al.*, 1980; Althaus-Salzmann *et al.*, 1980; Kimura *et al.*, 1982; Shears & Kirk, 1984a,b). The requirement for external Ca^{2+} was first suggested by Keppens *et al.* (1977) and Assimacopoulos-Jeannet *et al.* (1977), and more recently several other metabolic functions, including gluconeogenesis and specific protein

phosphorylation, have been shown to be altered when Ca²⁺ is removed from the medium (Weiss & Putney, 1978; Garrison *et al.*, 1979; Kneer & Lardy, 1983; Taylor *et al.*, 1983; Reinhart *et al.*, 1984a). Also, stimulation of mitochondrial respiration resulting from the Ca²⁺-dependent activation of intramitochondrial dehydrogenases (McCormack & Denton, 1981; Assimacopoulos-Jeannet *et al.*, 1983) is inhibited by removing Ca²⁺ 10 s before the hormones (Binet & Claret, 1983).

One of the purposes of the present paper was to determine the relative contribution of the internal Ca²⁺ stores and external Ca²⁺ in two key systems of hormonal action in the liver: the intracellular mediator [Ca²⁺]_i and the target enzyme responsible for the glycogen breakdown, phosphorylase *a*. The results of experiments performed under a variety of experimental conditions suggest that extracellular Ca²⁺ plays a part in this action.

Contribution of Ca²⁺ influx

The action of the Ca²⁺-mobilizing hormones on [Ca²⁺]_i was impaired in low-Ca²⁺ media, being transient instead of sustained. The amount of Ca²⁺ bound to quin2 6 s after the addition of noradrenaline was about 500 pmol/mg dry wt. in 1.8 mM-Ca²⁺ and 350 pmol/mg dry wt. in low-Ca²⁺ media. This indicates that internal pools are not sufficient to induce a maximal and sustained cell response, in keeping with the observation by Reinhart *et al.* (1984a) that both intra- and extra-cellular Ca²⁺ are required for the hormonal action in perfused rat liver.

The contribution of the Ca²⁺ influx to the [Ca²⁺]_i increase occurs within the first seconds after the hormone addition. This is illustrated in the fluorescence records in Fig. 5. The hepatocytes whose hormone response was impaired in the presence of 22 μM external Ca²⁺ rapidly recovered their stimulated [Ca²⁺]_i after the subsequent addition of 1.8 mM-Ca²⁺. Since a stimulation of the Ca²⁺ influx has been demonstrated in hormone-treated cells (Keppens *et al.*, 1977; Assimacopoulos-Jeannet *et al.*, 1977; Barritt *et al.*, 1981; Mauger *et al.*, 1984; Poggioli *et al.*, 1985), it appears that, even in low-Ca²⁺ media, noradrenaline or vasopressin activate the mechanism underlying Ca²⁺ influx. No Ca²⁺ influx occurs in the absence of external Ca²⁺. It is then sufficient to add Ca²⁺ to the medium to increase [Ca²⁺]_i to a value close to that initiated by the hormones in control media. Furthermore, adding Ca²⁺ 2 min beforehand restores the cell hormone response (Figs. 5b and 5d). This indicates that the resting Ca²⁺ influx has reloaded the internal pools within this period. The dependence of the hormonal response on external Ca²⁺ concentration, as shown by Fig. 6, confirms the contribution of Ca²⁺ influx in the restoration of

the complete hormonal response. Half-maximal activation was found for 0.25 mM-Ca²⁺ with noradrenaline, in reasonable agreement with that reported for Ca²⁺ influx (0.17 mM; Mauger *et al.*, 1984). The apparent half-maximal activation by Ca²⁺ could not be similarly estimated for vasopressin because of quin2 saturation; it was probably higher than 0.8 mM (0.82 mM for Ca²⁺ influx).

In contrast with that observed in 1.8 mM-Ca²⁺, the effect of a second hormonal challenge in low-Ca²⁺ medium was substantially decreased. This reveals that the hormones induced the release of a large fraction of the internal mobilizable Ca²⁺, and that these depleted pools cannot be replenished unless Ca²⁺ is present in the external medium.

Glycogen phosphorylase activations

The dependence of the glycogen phosphorylase activation on the external Ca²⁺ leads to similar conclusions. However, the activation of the phosphorylase by the hormones in low-Ca²⁺ medium was as large as in high Ca²⁺ medium, but this effect was transient instead of sustained. It seems likely that if Ca²⁺ influx is abolished (for example in low-Ca²⁺ media, or by blocking the hormone action by its antagonist in control Ca²⁺ media) the resulting return of [Ca²⁺]_i to its basal value accounts for the slow reversal of the phosphorylase activity.

Addition of Ca²⁺ 4 min after a first hormonal response in 22 μM-Ca²⁺ promotes a further phosphorylase activation that is dependent on the concentration of Ca²⁺ added (Fig. 7a). It is noteworthy that, under these conditions, relatively high Ca²⁺ concentrations were required to obtain maximal phosphorylase activation (more than 500 μM-Ca²⁺ with noradrenaline). It appears then that the time courses of the successive addition and desensitization of phosphorylase activity mimic the hormone-mediated changes in [Ca²⁺]_i. However, more quantitative comparisons of the two cell responses could not be made because the experiments were not performed under the same conditions.

The present results clearly demonstrate that both internal and external Ca²⁺ are needed for optimal [Ca²⁺]_i increase and glycogen phosphorylase activation by α₁-adrenergic agonists and peptide hormones in isolated rat liver cells. Though no further information could be drawn about the origin of the internal Ca²⁺ mobilized, our results may be interpreted in the light of data recently published (Burgess *et al.*, 1984; Berridge, 1984; Dawson & Irvine, 1984). It seems likely that Ca²⁺ pool(s) permeabilized by the hormones is localized in the microsomal fraction. This Ca²⁺ might be mobilized by inositol 1,4,5-trisphosphate released from the plasma membrane after the activation of the hormone-dependent phospho-

lipase C. This effect could be responsible for the fast and transient increase in $[Ca^{2+}]_i$. Within the first seconds, the stimulation of Ca^{2+} influx (Mauger *et al.*, 1984) seems also to be required to complement the rise in $[Ca^{2+}]_i$. Ca^{2+} influx is also required to sustain the cell responses and to reload the depleted internal stores. These different actions can occur only in the presence of millimolar concentrations of external Ca^{2+} .

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