Cytosolic free Ca^{2+} in isolated rat hepatocytes as measured by quin2

Effects of noradrenaline and vasopressin

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Cytosolic $[Ca^{2+}]$ has been measured by using the Ca^{2+}-sensitive indicator quin2 in rat liver cells. Optimal loading and hydrolysis have been obtained by equilibrating the cells with 50 μM quin2 acetoxymethyl ester for 150 s. The increase in $[Ca^{2+}]_i$ initiated by noradrenaline and vasopressin was reduced but not abolished by removing external Ca^{2+}.

Hepatocyte *Quin2* *Cytosolic Ca^{2+}* *Ca^{2+} influx* *α-Adrenergic agonist* *Vasopressin*

1. INTRODUCTION

α-Adrenergic agonists and peptide hormones such as (Arg^8)-vasopressin and (Ile^5)-angiotensin control glucose metabolism and ion movements in mammalian liver by mobilizing Ca^{2+} from internal stores [1–4]. A recent study, using the fluorescent intracellular Ca^{2+}-indicator quin2 [5,6], showed that the hormones increase within 5–10 s cytosolic Ca^{2+} [7], confirming that cytosolic Ca^{2+} could be the intracellular messenger of Ca^{2+}-mobilizing hormones in this tissue [3,4,8].

Here, we have defined the experimental conditions under which the fluorescent indicator can be used the experimental conditions under which the fluorescent indicator can be used to monitor cytosolic $[Ca^{2+}]$ in isolated rat liver cells. Optimal cell loading and hydrolysis have been determined from the shift of fluorescence signals of the ester quin2/AM to quin2 and from the uptake of [^{3}H]quin2/AM. The origin of Ca^{2+} involved in the $[Ca^{2+}]_i$ rise has been analysed by examining the dependence of the response on external Ca^{2+} and by measuring the unidirectional influx of Ca^{2+} initiated by noradrenaline (via α_1-receptors) and vasopressin.

Abbreviation: quin2/AM, quin2 acetoxymethyl tetraester

2. METHODS

2.1. *Preparation of dispersed rat liver cells*

Parenchymal cells were isolated from the livers of fed female rats using the collagenase method as in [9]. The cells were resuspended in Eagle's solution containing (mM): NaCl, 116; KCl, 5.4; $CaCl_2$, 1.8; $MgSO_4$, 0.8; sodium phosphate, 0.96; $NaHCO_3$, 25 and (mg/ml): glutamine, 292; glucose, 1000; vitamins, 8.1; amino acids, 805. The medium was supplemented with 2 g/100 ml bovine serum albumin and was equilibrated at 37°C. The pH was maintained by passing CO_2/O_2 (1:19) over the surface.

2.2. *Loading and hydrolysis of quin2/AM by the hepatocytes*

Unless specified, rat hepatocytes (4 mg cell dry wt/ml) were incubated with the esterified indicator (50 μM) for 150 s in Eagle's medium. The final

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concentration of the solvent dimethylsulfoxide from the quin2/AM stock was 0.1% (v/v). The cells were then centrifuged at 50 × *g* for 60 s, washed twice and resuspended (1 mg dry wt/ml) in Eagle's medium containing no vitamins, amino acids or albumin. The cell suspension was then transferred to the oxygenated spectrofluorimeter cuvette (under CO_2/O_2, 1:19) and gently agitated with a magnetic barrel at 37°C. Under these conditions, it was estimated that quin2 loading was 1.55 nmol/mg dry wt, corresponding to a cytosolic concentration of 650 μM (see section 3). This is about 13 times the concentration of the ester present in the loading solution (50 μM). The maximal loading efficiency representing the percentage of ester molecules that became trapped and hydrolysed was typically 20% for a cell density of 4 mg cell dry wt/ml.

The uptake of quin2/AM and hydrolysis to the Ca^{2+}-indicator quin2 was followed from the shift of fluorescence emission spectra of the ester peaking at 440 nm to the hydrolysed marker quin2 peaking at 492 nm (10 nm slit width). Excitation wavelength was 342 nm (4 nm slit width). At the end of each experiment, the fluorescence signal was calibrated by equilibrating the internal quin2 with known concentrations of Ca^{2+}. The cell membrane was made permeable to quin2 and Ca^{2+} with 4 μM digitonin (see fig.2b). The addition of the agent to the medium immediately increased fluorescence (F_{max}) due to quin2 saturation by Ca^{2+}. Minimal fluorescence (F_{min}) was obtained by setting $[Ca^{2+}]$ to 2 nM by adding EGTA to the suspension of lysed cells, then F_{max} was recovered by adding back Ca^{2+} at maximal concentration as shown in fig.2b. The Ca^{2+} concentration corresponding to fluorescence F emitted by intracellular quin2 was calculated from the equation [6]:

$$[Ca^{2+}]_i = K_d\,(F-F_{min})/(F_{max}-F) \qquad (1)$$

where K_d is the apparent dissociation constant of the Ca^{2+}-quin2 complex.

2.3. *Influx, efflux and accumulation of [^{3}H]quin2/AM*

The rate of quin2/AM uptake by the hepatocytes was determined by incubating the cells (4 mg dry wt/ml) with 1 μCi of [^{3}H]quin2/AM in a medium containing 50 μM ester. As a function of time, 100-μl cell samples were diluted in 5 ml of a 'washing solution' (NaCl, 150 mM; EGTA, 2 mM; pH 7.4) and then filtered through Whatman GF/C filters at 0°C. The filters were then rinsed 3 times with 5 ml of the same solution. The efflux of hydrolysed indicator [^{3}H]quin2 was determined from cells preloaded for 150 s with 50 μM of the ^{3}H-labelled ester. The cells were washed and centrifuged twice with Eagle's solution to eliminate the non-incorporated indicator. The pellets were then resuspended in Eagle's solution. The radioactivity remaining in the cells was determined by using the same separating method as for the uptake experiments.

2.4. *Non-toxicity of quin2 in rat liver cells*

Quin2 did not alter cell viability as checked by their ability to exclude Trypan blue (4%). By this test the viability was 95% in control and in quin2-loaded cells. Further evidence of non-toxicity of the indicator was that: (i) the cells remained responsive to the hormones (fig.2); (ii) Ca^{2+} influx was sensitive to noradrenaline and vasopressin (table 1); (iii) $[Ca^{2+}]$ was independent of quin2 loading.

2.5. *Unidirectional $^{45}Ca^{2+}$ influx*

Cells (4 mg dry wt/ml) were incubated in Eagle's medium containing $^{45}Ca^{2+}$ (1 μCi/ml) and the hormones. Samples were removed at 15, 45, 75 and 105 s, diluted with 5 ml of 'washing solution' and then filtered and washed as indicated above. When used, antagonists were added 6 min beforehand.

2.6. *Total internal cell Ca^{2+}*

The total internal cell Ca^{2+} was determined by centrifuging 3 times 1 ml of cell samples (4 mg/ml) at 50 × *g* for 90 s with 5 ml of the 'washing solution'. All the steps were performed at 1°C. The pellets were dispersed in 2 ml of 0.1 M HNO_3 for 24 h and then total $[Ca^{2+}]$ was measured by atomic absorption spectrophotometry.

2.7. *Measurement of radioactivity*

All isotopes were counted in an Intertechnique scintillation spectrometer after addition of an appropriate scintillation fluid to GF/C Whatman filters.

2.8. *Chemicals*

Collagenase was from Boehringer, quin2/AM was from Lancaster Synthesis (Morecambe), [^{3}H]quin2/AM was from Amersham. All the chemicals and drugs were of analytical reagent grade.

3. RESULTS AND DISCUSSION

Under the standard conditions chosen here, i.e., 4 mg cell dry wt/ml and 50 μM quin2/AM, the shift of fluorescence signal from quin2/AM to quin2 was complete within 5 min. This was followed by a slight decrease in the emission suggesting a slow release of the trapped indicator. Reducing the temperature from 37°C to 22°C prevented the loss, but substantially reduced hydrolysis. The analysis of the ester uptake by the cells incubated with 50 μM [^{3}H]quin2/AM and the efflux of [^{3}H]quin2 from preloaded hepatocytes confirmed this observation. This is illustrated in fig.1. The cells rapidly accumulated [^{3}H]quin2/AM with a maximal uptake of 3.0 nmol/mg dry wt at 5–10 min. The first phase was followed by a slow decrease of the intracellular indicator. When the cells were first loaded with [^{3}H]quin2/AM for 150 s and then resuspended in unlabelled medium, a gradual decrease of quin2 content confirmed the small loss of the indicator. The time constant of release was 37 ± 4 min ($n = 3$). From these experiments, it was decided to load the cells (4 mg dry wt/ml) with 50 μM quin2/AM at 37°C for 150 s and then to transfer them to the spectrofluorimeter. At that time, intracellular quin2 amounted to 1.55 nmol/mg dry wt. This corresponds, on the basis of 2.4 μl of intracellular water/mg dry wt of cells, to an internal concentration of 650 μM.

Quin2 is a high affinity Ca^{2+} chelator that is expected to lower $[Ca^{2+}]_i$. It has been shown that cell Ca^{2+} homeostatis restores $[Ca^{2+}]_i$ by allowing net entry of extracellular Ca^{2+} [6]. In rat liver cells, the internal total Ca^{2+} content as measured by atomic absorption spectrophotometry was 4.62 ± 0.66 nmol/mg dry wt ($n = 8$). The addition of quin2/AM initiated a net increase in the total Ca^{2+} of 0.66 nmol/mg dry wt within 5–10 min. This

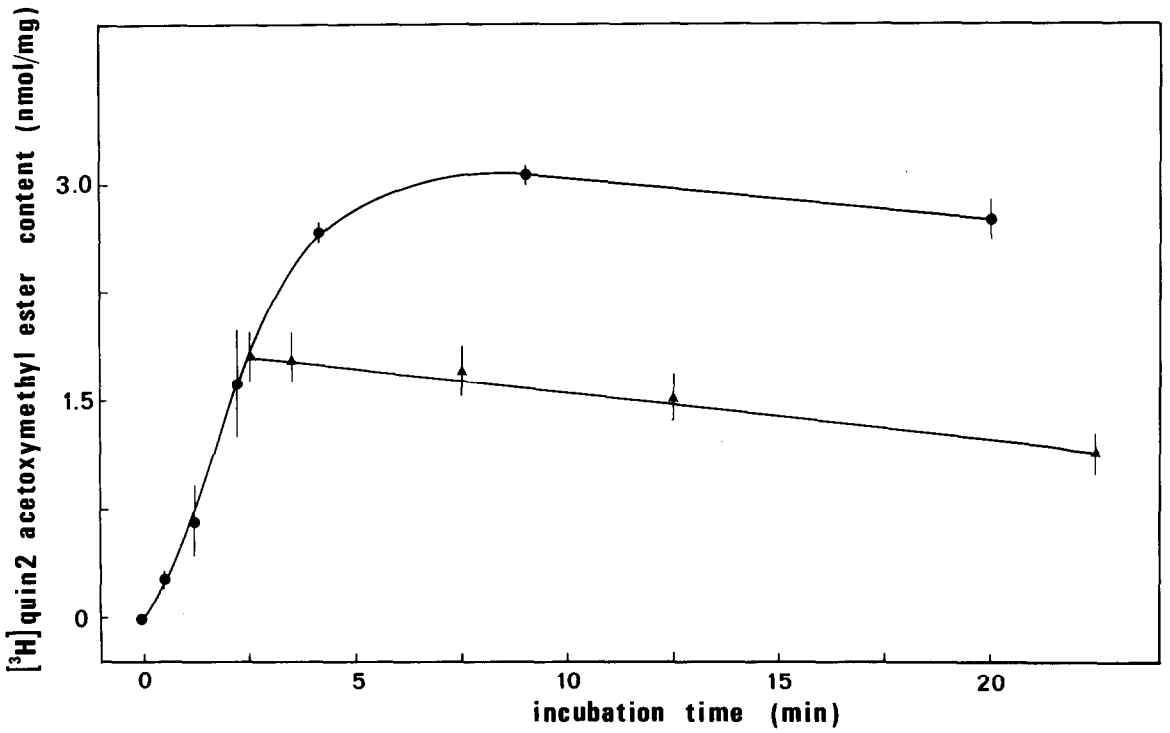

Fig. 1. Uptake (●) and efflux (▲) of [^{3}H]quin2 acetoxymethyl ester in isolated rat liver cells preloaded with the indicator for 150 s. For experimental details, see section 2. Results are means ± SE of 3 independent experiments.

Table 1

Effect of noradrenaline (1 μM, in the presence of 5 μM propranolol) and vasopressin (1 nM) on the $^{45}Ca^{2+}$ influx in isolated quin2-loaded rat liver cells

Controls	Noradrenaline	Vasopressin
	pmol · mg dry wt^{-1} · min^{-1}	
871 ± 180	1790 ± 180[a]	1753 ± 155[a]

Means ± SE of 3 separate experiments
[a]Significantly different from paired controls ($p < 0.05$)

amount represents about half-saturation of intracellular quin2 (1.55 nmol/mg dry wt) in agreement with the fact that the dissociation constant of Ca^{2+}-quin2 complex is close to $[Ca^{2+}]_i$ (see below). It is noteworthy that these rates of quin2/AM hydrolysis and Ca^{2+} loading are very high in hepatocytes compared with those found in other cells [6,9,10]. This probably results from both the high efficiency of cytosolic esterases and high Ca^{2+} fluxes in this tissue (see table 1). In this regard, low calcium fluxes may constitute a limitation of the quin2 method in that the chelator may partially deplete internal stores as long as it has not been loaded via Ca^{2+} influx. This may explain recent

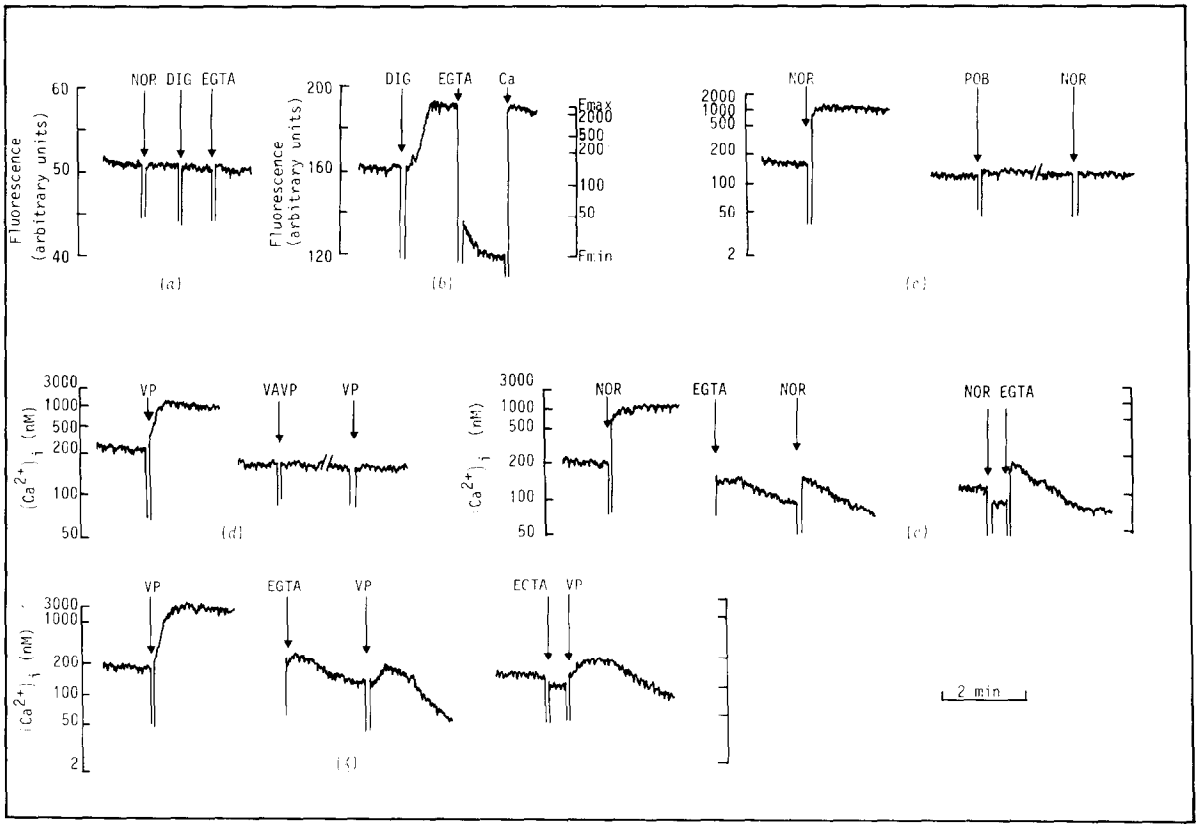

Fig. 2. Autofluorescence of the cells (a), calibration of quin2-loaded cells (b) and effects of Ca^{2+}-mobilizing hormones on $[Ca^{2+}]_i$ (c–f). (a) Note that non-loaded cells gave no fluorescence response to EGTA (24 mM), noradrenaline (NOR, 1 μM in the presence of 5 μM propranolol to block β-receptors) and digitonin (DIG, 4 μM). (b) The fluorescence signal was calibrated in terms of $[Ca^{2+}]_i$ (nM) as described in section 2 (DIG, 4 μM; EGTA, 24 mM; Ca^{2+}, 12 mM). (c,d) Effect of noradrenaline (NOR, 1 μM) and vasopresssin (VP, 1 nM) and antagonists on $[Ca^{2+}]_i$ (nM). When used, the antagonists phenoxybenzamine (POB, 50 μM (c), and (1-(β-mercapto-β-β-cyclopentamethylenepropionic acid), 2-(*o*-ethyltyrosine), 4-valine, 8-arginine vasopressin (VAVP, 20 nM) (d) were added 6 min before the hormones. (e,f) Effect of EGTA (8 mM) added 2 min and 30 s before noradrenaline (NOR, 1 μM) (e), and vasopressin (VP, 1 nM) (f).

results reported in pig lymphocytes [10] showing that quin2 alter certain physiological functions depending on Ca^{2+}.

Fig.2a shows that the cell autofluorescence presumably generating from intracellular NAD(P)H [7] was not affected by the hormones, EGTA or digitonin. Loading the hepatocytes with the Ca^{2+} indicator increased this basal emission intensity 3 times (fig.2b). This fluorescence signal was calibrated by equilibrating quin2 with maximal and minimal concentrations of Ca^{2+} in cells lysed by digitonin (see section 2). The same procedure was used to determine the dissociation constant K_d of the quin2-Ca^{2+} complex in digitonin-treated cells. Free Ca^{2+} concentrations were applied with EGTA-Ca^{2+} buffers. An excellent fit of the fluorescence signal was obtained with 1:1 dye/Ca^{2+} binding, with an effective dissociation constant of 110 ± 2 nM ($n = 12$). Identical K_d values were obtained with quin2 in water that has not been esterified and deesterified. This shows that the cell had fully regenerated the indicator with the correct affinity for Ca^{2+}.

Fig.2c,d confirm results in [7] showing that the α-agonists (noradrenaline, here) and vasopressin rapidly increased cytosolic Ca^{2+} from a basal level of about 100–200 nM. Similar results were found with angiotensin (10 nM, not shown). Half-maximal effects, as estimated from the dose-response relation for each hormone, were observed at 0.5 nM vasopressin and 0.2 μM noradrenaline. The specific antagonists phenoxybenzamine and (1-(β-mercapto-β,β-cyclopentamethylpropionic acid),2-(*o*-ethyltyrosine),4-valine,8-arginine) vasopressin, which by themselves had no effect on $[Ca^{2+}]_i$ totally blocked the cell response.

It has been claimed that the α-adrenergic agonists and vasopressin mobilize Ca^{2+} from internal stores in the rat liver because they promote a net loss of Ca^{2+} and they were able to activate Ca^{2+}-dependent glycogen phosphorylase in the absence of external Ca^{2+} [3,4]. This claim was examined: (i) by seeing whether an influx of Ca^{2+} may be initiated by the hormones in our cell preparation; (ii) by determining the dependence of $[Ca^{2+}]_i$ increase on external Ca^{2+}. Table 1 shows that noradrenaline and vasopressin increased the unidirectional Ca^{2+} influx in agreement with [1,12,13] and indicating that external Ca^{2+} is involved in the mobilization of cell Ca^{2+}. This conclusion is strongly supported by the experiments illustrated in fig.2e,f. The dependency of the hormone-mediated $[Ca^{2+}]_i$ rise on external Ca^{2+} was tested by utilizing the Ca^{2+} chelator EGTA to eliminate Ca^{2+} influx. An excess of EGTA was added to the medium 2 min or 30 s before the hormones, reducing the external estimated free Ca^{2+} to below 0.1 μM [14]. Recordings show that the magnitude of the effects of noradrenaline and vasopressin on $[Ca^{2+}]_i$ were substantially altered by abolishing Ca^{2+} influx. Also, the response was then transient instead of sustained.

Table 2 combines the results of several experiments of this kind and performed at different concentrations of external Ca^{2+}. From these, it was interesting to estimate the relative contribution of internal Ca^{2+} stores and Ca^{2+} influx to the cell response from the total amount of Ca^{2+} mobilized

Table 2

Effect of external $[Ca^{2+}]$ on the maximal $[Ca^{2+}]_i$ increase initiated by noradrenaline (1 μM in the presence of 5 μM propranolol) or by vasopressin (1 nM)

$[Ca^{2+}]_o$	Controls (nM)	Noradrenaline (nM)	Ca_m (pmol/mg)	Vasopressin (nM)	Ca_m (nmol/mg)
1.8 mM	171 ± 9 (9)	864 ± 98 (7)	400	1340 ± 300 (9)	460
0.5 mM	176 ± 6 (7)	426 ± 17 (4)	259	610 (2)	334
72 nM	101 ± 9 (5)	155 ± 12 (5)	152	182 ± 28 (10)	207

The hepatocytes were incubated with the indicated $[Ca^{2+}]_o$ for 2 min. When used (last line), EGTA (8 mM) was added 2 min before the hormones to the control medium containing 1.8 mM Ca^{2+}. In this case, estimated free $[Ca^{2+}]_o$ was calculated by using the EGTA-Ca^{2+} complex dissociation constant proposed in [14]. Ca_m represents the amount of total Ca mobilized by the hormones (see text)

by the hormones. This amount is the sum of free Ca^{2+} and Ca^{2+} bound to quin2 as a complex form. The latter may be calculated from the relation:

$$[Ca^{2+}\text{-quin2}] = [quin2_t] / (1 + K_{Ca}/[Ca^{2+}]_i) \quad (2)$$

where $[quin2_t]$ is the intracellular concentration of quin2 as measured in fig.1 and K_{Ca} is the dissociation constant estimated above. This has been done in table 2. In the presence of 1.8 mM external Ca^{2+}, noradrenaline and vasopressin mobilized similar amounts of Ca^{2+} (Ca_m). In the absence of external Ca^{2+}, about 40% of the response was maintained indicating equivalent contribution of internal Ca^{2+} stores and Ca^{2+} influx in increasing $[Ca^{2+}]_i$. The origin of the Ca^{2+} released from internal stores has not been yet established. Endoplasmic reticulum and plasma membranes [12,15,16] or mitochondria [8,17,18] have been proposed. However, a recent study [19] has suggested that both compartments are involved in the action of the hormones. Table 2 also shows that the contribution of Ca^{2+} influx to the increase in $[Ca^{2+}]_i$ is very dependent on external Ca^{2+} in the millimolar range concentrations. Reducing $[Ca^{2+}]_o$ from 1.8 mM to 0.5 mM substantially diminished the $[Ca^{2+}]_i$ increase generated by both hormones. This is in agreement with our finding that noradrenaline- and vasopressin-mediated Ca^{2+} influxes are not maximally activated at 0.5 mM (unpublished). The presence of these two components may explain controversies (review [3,4,20] about the dependence on external Ca^{2+}-mobilizing hormones in the liver.

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