The measurement of Ca^{2+} inflow across the liver cell plasma membrane by using quin2 and studies of the roles of Na^+ and extracellular Ca^{2+} in the mechanism of Ca^{2+} inflow

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1. Rates of Ca^{2+} inflow across the hepatocyte plasma membrane in the presence of vasopressin were estimated by using quin2. 2. Plots of the rate of Ca^{2+} inflow as a function of the intracellular quin2 concentration reached a plateau at about 1.7 mM intracellular quin2. Ca^{2+} inflow was inhibited by 60 % in the presence of 400 μM -verapamil. 3. A plot of the rate of Ca^{2+} inflow as a function of the concentration of extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$) was biphasic. The second (slower) phase showed no sign of saturation at values of $[\text{Ca}^{2+}]_o$ up to 5 mM. It is concluded that, in the presence of vasopressin, Ca^{2+} flows into the liver cell by two different processes, one of which is not readily saturated by Ca^{2+} . 4. The effect of the replacement of extracellular NaCl by choline or tetramethylammonium chloride on cellular Ca^{2+} movement was found to depend on the presence or absence of intracellular quin2. 5. In cells loaded with quin2 and incubated in the presence of choline or tetramethylammonium chloride, a small decrease in the basal intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was observed, and the increase in $[\text{Ca}^{2+}]_i$ caused by the addition of vasopressin was considerably diminished when compared with cells incubated in the presence of NaCl. In cells loaded with quin2, replacement of NaCl by choline chloride caused a decrease in Ca^{2+} inflow in the presence of vasopressin, as measured by using quin2 or $^{45}\text{Ca}^{2+}$ exchange, whereas no change in Ca^{2+} inflow was observed in the absence of vasopressin. 6. In cells not loaded with quin2, replacement of NaCl by choline chloride did not alter Ca^{2+} inflow either in the presence or in the absence of vasopressin. 7. It is concluded that (i) Ca^{2+} inflow through the basal and receptor-activated Ca^{2+} inflow systems does not involve the inward movement of Ca^{2+} in exchange for Na^+ or the induction of Ca^{2+} inflow by intracellular Na^+ , and (ii) the presence of both intracellular quin2 and extracellular choline or tetramethylammonium chloride (in place of NaCl) inhibits Ca^{2+} inflow through the receptor-activated Ca^{2+} inflow system but not through the basal Ca^{2+} inflow system, and inhibits the release of Ca^{2+} from intracellular stores.

INTRODUCTION

An increase in the concentration of free Ca^{2+} in the cytoplasmic space ($[\text{Ca}^{2+}]_i$) of the liver cell acts as an intracellular messenger in the actions of a number of agonists, including vasopressin, α_1 -adrenergic agonists and angiotensin II (reviewed by Altin & Bygrave, 1988). The increase in $[\text{Ca}^{2+}]_i$ induced by agonists is due to both the release of Ca^{2+} from intracellular stores (Shears & Kirk, 1984) and enhanced inflow of Ca^{2+} across the plasma membrane (Barritt *et al.*, 1981; Mauger *et al.*, 1984; Reinhart *et al.*, 1984). Previous studies have provided some evidence for the presence of two systems which facilitate the movement of Ca^{2+} from the extracellular space to the cytoplasmic space across the liver cell plasma membrane (Barritt *et al.*, 1981; Parker *et al.*, 1983; Hughes *et al.*, 1986b). These are a basal system which is responsible for Ca^{2+} inflow in the absence of agonists, and a receptor-activated Ca^{2+} inflow system (Hughes *et al.*, 1986b). Little is known of the mechanisms by which Ca^{2+} moves across the plasma membrane by these two pathways. It has been proposed that Ca^{2+} inflow involves a channel or pore (Parker *et al.*, 1983;

Hughes *et al.*, 1986b) or a saturable exchange carrier (Mauger *et al.*, 1984; Mauger & Claret, 1988). Two other possibilities are a Ca^{2+} - Na^+ exchange system, which has been identified in liver plasma-membrane vesicles (Kraus-Friedmann *et al.*, 1982; Famulski & Carafoli, 1982) and has been proposed as a pathway of Ca^{2+} inflow in other cell types (Barry & Smith, 1982; Blaustein & Nelson, 1982; Snowdowne & Borle, 1985), and Ca^{2+} inflow stimulated by intracellular Na^+ (Na^+_i) (Schanne & Moore, 1986).

It has proved difficult to obtain a satisfactory method for the measurement of Ca^{2+} inflow to hepatocytes. The methods used so far have employed measurement of $^{45}\text{Ca}^{2+}$ uptake (Assimakopoulos-Jeannot *et al.*, 1977; Keppens *et al.*, 1977; Mauger *et al.*, 1984; Reinhart *et al.*, 1984), analysis of $^{45}\text{Ca}^{2+}$ exchange under steady-state conditions (Barritt *et al.*, 1981), measurement of $^{40}\text{Ca}^{2+}$ uptake by atomic-absorption spectroscopy (Blackmore *et al.*, 1984) and measurement of the rate of activation of glycogen phosphorylase (Binet *et al.*, 1985; Joseph *et al.*, 1985; Hughes *et al.*, 1986a). Many of these procedures provide only indirect estimates of the rate of plasma-membrane Ca^{2+} inflow. In the case of measurement of

Abbreviations used: TMA, tetramethylammonium; $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$, concentrations of intracellular free Ca^{2+} and Na^+ respectively; $[\text{Ca}^{2+}]_o$ and $[\text{Na}^+]_o$, concentrations of extracellular Ca^{2+} and Na^+ respectively; quin2-AM, the acetoxymethyl ester of quin2; DTPA, diethylenetriaminepenta-acetic acid.

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the rate uptake of $^{40}\text{Ca}^{2+}$ or $^{45}\text{Ca}^{2+}$, it has not been clearly established whether the movement of Ca^{2+} across the plasma membrane or an intracellular membrane is the rate-limiting step in the uptake of $^{40}\text{Ca}^{2+}$ or $^{45}\text{Ca}^{2+}$.

The aims of the present experiments were firstly to develop an alternative method for measurement of Ca^{2+} inflow through the receptor-activated Ca^{2+} -inflow system in the liver cell plasma membrane, secondly to determine whether Ca^{2+} inflow through this pathway is readily saturated by extracellular Ca^{2+} (Ca^{2+}_o), and thirdly to determine whether Ca^{2+} inflow through the basal and receptor-activated Ca^{2+} -inflow systems involves the exchange of Ca^{2+}_o for intracellular Na^+ or a process dependent on the presence of a normal $[\text{Na}^+]_i$. The method developed for the measurement of Ca^{2+} inflow through the receptor-activated Ca^{2+} -inflow system is based on the use of the intracellular Ca^{2+} chelator quin2 (Tsien, 1980; Joseph *et al.*, 1985; Kao & Schneider, 1986; Hughes *et al.*, 1987b). The results indicate that Ca^{2+} inflow through the receptor-activated Ca^{2+} inflow system can be measured by using quin2. With this and other methods for the measurement of Ca^{2+} inflow, it has been shown that Ca^{2+} inflow through the receptor-activated system is not readily saturated by Ca^{2+}_o , does not involve the exchange of Ca^{2+}_o for Na^+_i , and is not stimulated by Na^+_i .

EXPERIMENTAL

Isolation of hepatocytes

Hepatocytes were isolated from fed male Hooded Wistar rats by collagenase digestion by the method of Berry & Friend (1969) as described previously (Barritt *et al.*, 1981). The incubation medium contained 117 mM-NaCl, 4.7 mM-KCl, 1.2 mM- KH_2PO_4 , 1.2 mM- MgSO_4 , 24 mM- NaHCO_3 , 20 mM-Tes/KOH (Na^+ medium), other additions as indicated, and hepatocytes [30 mg wet wt. (2×10^6 cells)/ml]. Na^+ -replaced media contained 117 mM-choline chloride and 24 mM-choline bicarbonate (choline medium) or 117 mM-tetramethylammonium (TMA) chloride and 24 mM-choline bicarbonate (TMA medium) in place of 117 mM-NaCl and 24 mM- NaHCO_3 . Vasopressin was dissolved in Na^+ medium.

Measurement of $[\text{Ca}^{2+}]_i$ in cells loaded with quin2

Hepatocytes were loaded with quin2 by the method of Tsien *et al.* (1982), as described previously (Hughes *et al.*, 1986a). A stock solution of 25 mM of the acetoxymethyl ester of quin2 (quin2-AM) in dimethyl sulphoxide was used. Cells were incubated with quin2-AM for 15 min in a shaking water bath (90 oscillations/min) at 37 °C in Na^+ medium which also contained 0.5 mM- Ca^{2+} , 5 mM-glucose and 1.5% (w/v) gelatin. A loading concentration of 100 μM -quin2-AM was routinely used, except in the experiments where the rate of Ca^{2+} inflow was measured as a function of the cytoplasmic quin2 concentration ([quin2]). In these experiments, the loading concentrations of quin2 were varied between 50 and 120 μM . The final concentration of dimethyl sulphoxide during the loading period was between 0.2 and 0.5% (v/v).

After incubation with quin2-AM, the cells were centrifuged at room temperature at 500 *g* for 1 min and resuspended in 5 ml of Na^+ , choline or TMA medium, which also contained 5 mM-glucose and 0.5% (w/v) gelatin. Except where indicated otherwise, the medium contained no added Ca^{2+} . The cells were incubated for

10 min at 37 °C before being centrifuged again and resuspended at 20–25 mg wet wt./ml in the same medium as used for the first resuspension. The cells were then incubated for a further 5 min at 37 °C before being placed in the cuvette for fluorescence measurements. The cells were gassed with O_2/CO_2 (19:1) throughout the loading procedure and while in the cuvette. Unloaded cells were treated in the same way as loaded cells, except that dimethyl sulphoxide (final concn. 0.4%) was added in place of quin2-AM.

The viability of cells after the loading of quin2 was assessed by using Trypan Blue (Barritt *et al.*, 1981). Cells loaded with quin2 and maintained for 10 min in Na^+ medium had a viability of $92 \pm 1\%$, compared with $90 \pm 2\%$ for unloaded cells (mean $\pm$ range, $n = 2$). Cells loaded with quin2 and placed in choline medium had a viability of $92 \pm 4\%$ compared with $88 \pm 2\%$ for unloaded cells.

The fluorescence (F) of quin2-loaded cells was measured at 37 °C by using excitation and emission wavelengths of 340 nm and 490 nm respectively. $[\text{Ca}^{2+}]_i$ was calculated by using the equation of Tsien *et al.* (1982) and a value of 115 nm for the dissociation constant for the binding of Ca^{2+} to quin2 (Tsien *et al.*, 1982). Values for F_{max} and F_{min} of cells loaded with quin2 were obtained by using 0.1% (w/v) Triton X-100 and 1 mM- Mn^{2+} respectively, as described by Rink & Pozzan (1985). The values of F , F_{max} and F_{min} were corrected for autofluorescence by subtracting the fluorescence of unloaded cells which had received the same additions in the cuvette as the loaded cells. A value for the component of fluorescence due to extracellular quin2 was obtained immediately before the determination of F_{max} by the addition of 50 μM - Mn^{2+} to quench the extracellular quin2, followed by 100 μM -diethylenetriaminepenta-acetic acid (DTPA) to chelate the Mn^{2+} and allow F_{max} to be determined subsequently (Rink & Pozzan, 1985). The fluorescence of extracellular quin2 was found to account for 20% of the total quin2 fluorescence.

It was found that the fluorescence of a given amount of quin2 free acid was lower when the quin2 was dissolved in choline or TMA medium than in Na^+ medium. The degree of suppression of fluorescence by choline or TMA was determined by using the following protocol. Cells were treated with dimethyl sulphoxide as described above for unloaded cells and washed and resuspended in Na^+ , choline or TMA medium which contained 1.3 mM- CaCl_2 . To 2.5 ml of cells suspended in the appropriate medium in the spectrophotometer cuvette were added vasopressin (13 nM), MnCl_2 (50 μM), DTPA (100 μM) and Triton X-100 (0.1%). This procedure was followed in order to determine the effects of choline and TMA on the fluorescence of quin2 under conditions identical with those employed for the measurement of $[\text{Ca}^{2+}]_i$. The successive addition of four equal amounts of quin2 free acid to Na^+ medium (final concentrations 10, 20, 30 and 40 μM) caused increases in fluorescence (340 nm excitation and 490 nm emission) which were 119 ± 4 , 119 ± 3 , 118 ± 3 and 118 ± 2 ($n = 8$)% of the corresponding increases observed for the successive addition of four equal amounts of quin2 free acid to choline medium. In a similar experiment conducted with TMA medium, the values obtained for the Na^+ medium were 125 ± 6 , 120 ± 3 , 119 ± 3 and 118 ± 2 ($n = 7$)% of those for the TMA medium. Consequently, the observed values of F_{max} obtained in choline medium were corrected by

multiplying by a factor of 1.18, and the observed values of F_{max} , obtained in TMA medium were corrected by multiplying by a factor of 1.19. The corrected values of F_{max} , were used in the calculation of $[\text{Ca}^{2+}]_i$.

Measurement of Ca^{2+} inflow by using quin2-loaded cells

Conditions for the measurement of rates of Ca^{2+} inflow to the cytoplasmic space were chosen in order to maximize the proportion of Ca^{2+} that is bound to quin2, and to minimize the outflow of Ca^{2+} from the cytoplasmic space to intracellular stores and the extracellular medium. This was achieved by using high concentrations of intracellular quin2 (cf. Kao & Schneider, 1986) and by incubating the cells in the presence of vasopressin. It has been shown that vasopressin releases Ca^{2+} from the endoplasmic reticulum and inhibits the uptake of Ca^{2+} by this organelle (Burgess *et al.*, 1984; Joseph *et al.*, 1984; Charest *et al.*, 1985; Hughes *et al.*, 1986a; Putney, 1986).

Hepatocytes loaded with quin2 were incubated in the absence of added Ca^{2+} . Vasopressin (13 nM) and CaCl_2 (1.3 mM) were then added as shown in Fig. 1. After addition of CaCl_2 , there was a short lag period before fluorescence increased (Fig. 1). It has previously been shown that the increase in fluorescence that follows the addition of extracellular Ca^{2+} represents the increase in $[\text{Ca-quin2}]$ which results from the movement of Ca^{2+} from the extracellular medium to the cytoplasmic space (Joseph *et al.*, 1985; Hughes *et al.*, 1986a).

Rates of Ca^{2+} inflow were calculated as follows. A straight line was drawn to represent the maximum slope of the plot of fluorescence as a function of time, after the addition of CaCl_2 , as shown in Fig. 1. Two time points on this line, at t and $(t+1)$ min, were selected and the value of $[\text{Ca-quin2}]$, in nmol/mg wet wt. of cells, at each time point was calculated by using the following equation:

$$[\text{Ca-quin2}] = a[\text{quin2}]/(a+1)$$

where a represents $(F - F_{\text{min}})/(F_{\text{max}} - F)$, F is the fluorescence at a given time, and $[\text{quin2}]$ is the intracellular concentration of quin2 in nmol/per mg wet wt. of cells. The fluorescence of unloaded cells was subtracted, and a correction was made for fluorescence caused by extracellular quin2. The expression for $[\text{Ca-quin2}]$ is a re-arrangement of the equations described by Tsien *et al.* (1982). For cells incubated in choline or TMA medium, the value of F_{max} was corrected as described above. The change in $[\text{Ca-quin2}]$ as a function of time, expressed as nmol/min per mg wet wt. of cells, was obtained by subtracting the value of $[\text{Ca-quin2}]$ at t min from that at $(t+1)$ min and was taken as a measure of Ca^{2+} inflow.

In order to measure the intracellular concentration of quin2, the cells were lysed with 0.1 % Triton X-100, and the amount of quin2 released was determined from a standard curve constructed by adding quin2 free acid to a suspension of lysed unloaded hepatocytes in the same medium. Concentrations of quin2 expressed as nmol/mg wet wt. of cells were converted into units of mM by using the relationship that the volume of the cytoplasmic space of 1 mg wet wt. of cells is 0.5 μl (Krebs *et al.*, 1974).

Measurement of $^{45}\text{Ca}^{2+}$ exchange and uptake, glycogen phosphorylase activity and Na^+

Rates of $^{45}\text{Ca}^{2+}$ exchange were measured as follows.

Freshly isolated hepatocytes were twice centrifuged and resuspended at room temperature in Na^+ medium or choline medium which contained 1.3 mM- CaCl_2 , 0.5 % gelatin and 5 mM-glucose. Rates of $^{45}\text{Ca}^{2+}$ exchange after the addition of tracer amounts of $^{45}\text{Ca}^{2+}$ to cells incubated in the appropriate medium in the presence of 1.3 mM- Ca^{2+} , were measured as described previously (Barritt *et al.*, 1981). For the measurement of $^{45}\text{Ca}^{2+}$ uptake, cells were twice centrifuged and resuspended at room temperature in Na^+ medium or choline medium which contained 0.5 % gelatin and 5 mM-glucose but no added Ca^{2+} . Rates of $^{45}\text{Ca}^{2+}$ uptake after addition of 1.3 mM- $^{45}\text{Ca}^{2+}$ to cells incubated in the absence of Ca^{2+} were measured as described previously (Hughes *et al.*, 1987a).

Glycogen phosphorylase *a* activity was measured by using [^{14}C]glucose 1-phosphate (Hutson *et al.*, 1976). One unit of enzyme activity is defined as 1 μmol of [^{14}C]glucose 1-phosphate incorporated into glycogen/min per g wet wt. of cells (Hutson *et al.*, 1976). For the measurement of glycogen phosphorylase, 'mock-loaded' cells were put through the procedure for loading with quin2-AM, except that an equal volume of dimethyl sulphoxide replaced quin2-AM. The cells were resuspended in Na^+ or choline medium which contained 1.3 mM- Ca^{2+} before incubation at 37 °C. Cells that were not 'mock-loaded' were treated as follows. Freshly isolated hepatocytes were washed at room temperature and resuspended twice in Na^+ or choline medium which contained 1.3 mM- Ca^{2+} before incubation at 37 °C. Both groups of cells were incubated for 15 min before addition of 13 nM-vasopressin ($t = 15$ min).

The total Ca content of hepatocytes was measured as follows. Freshly isolated hepatocytes were incubated for 10 min at 37 °C in Na^+ medium which contained 1.3 mM- CaCl_2 and 1 % gelatin in a shaking water bath (90 oscillations/min). The cells were then twice centrifuged and resuspended at room temperature in either Na^+ or choline medium in the presence of 1.3 mM- CaCl_2 before incubation at 37 °C in a shaking water bath. Total Ca was determined by using atomic-absorption spectroscopy after extraction of the cells with HClO_4 as described previously (Barritt *et al.*, 1981; Parker & Barritt, 1981).

For the measurements of Na^+ , freshly isolated hepatocytes were twice centrifuged and resuspended at room temperature in either Na^+ or choline medium containing 1.3 mM- CaCl_2 , before incubation at 37 °C. After 12 min, samples (0.9 ml) of the incubation mixture were removed, the cells were pelleted by centrifugation, the supernatant was removed, and the pellet was resuspended in 0.5 ml of water. The cells were lysed by freezing and thawing three times in liquid N_2 , the cell debris was removed by centrifugation, and the amount of Na present in the supernatant was measured by flame photometry. Correction for the presence of Na trapped in the extracellular fluid of the cell pellet was made by using [^{14}C]inulin and $^3\text{H}_2\text{O}$. Concentrations of Na expressed as nmol/mg wet wt. were converted into units of mM by using values obtained for the [^{14}C]inulin and $^3\text{H}_2\text{O}$ spaces.

Materials

Quin2-AM, [^{14}C]inulin and $^3\text{H}_2\text{O}$ were purchased from Amersham (Australia), Surrey Hills, N.S.W, Australia; quin2 free acid from Lancaster Synthesis, Morecambe, Lancs., U.K.; quin2-AM, quin2 free acid, choline chloride, TMA hydroxide pentahydrate and choline bicarbonate were purchased from Sigma Chemical Co.,

St. Louis, MO, U.S.A. The sources of other chemicals used have been described previously (Barritt *et al.*, 1981; Parker & Barritt, 1981; Hughes *et al.*, 1986a). TMA chloride was prepared by neutralizing TMA hydroxide pentahydrate with HCl.

RESULTS

Effects of vasopressin, verapamil and intracellular quin2 on the rate of Ca^{2+} inflow in cells loaded with quin2

Representative traces of changes in fluorescence which follow the addition of 1.3 mM-Ca^{2+} to cells loaded with quin2 and incubated in the presence and absence of vasopressin are shown in Figs. 1(a) and 1(b) respectively. The rates of Ca^{2+} inflow observed after addition of 1.3 mM-Ca^{2+} in the presence or absence of vasopressin were 0.19 ± 0.01 and $0.14 \pm 0.01 \text{ nmol/min per mg wet wt.}$ respectively (mean $\pm$ S.E.M. for nine determinations using six separate cell preparations; $P < 0.05$, Student's *t* test for unpaired samples). The values of $[\text{Ca}^{2+}]_i$ (mean $\pm$ S.E.M.) were 222 ± 33 , 428 ± 72 , 187 ± 23 and $451 \pm 61 \text{ nM}$ immediately before the addition of vasopressin, at the peak after the addition of vasopressin, immediately before the addition of 1.3 mM-CaCl_2 and at the peak after the addition of CaCl_2 respectively (trace a), and 208 ± 18 and 354 ± 31 before and after the addition of CaCl_2 respectively (trace b).

Verapamil ($400 \mu\text{M}$) inhibited by 60 % the rate of increase in $[\text{Ca-quin2}]$ observed after the addition of 1.3 mM-Ca^{2+} to cells incubated in the presence of vasopressin (Fig. 2). Previous studies have shown that verapamil does not displace vasopressin from its receptor (Hughes *et al.*, 1986b). The effect of increasing the intracellular $[\text{quin2}]$ on the rate of Ca^{2+} inflow is shown in Fig. 3. A plateau is reached at a $[\text{quin2}]$ concentration above about 1.7 mM . The rate of Ca^{2+} inflow observed at

Fig. 1. Changes in fluorescence after addition of Ca^{2+} to cells loaded with quin2 and incubated in the presence (a) or absence (b) of vasopressin

The loading of hepatocytes with quin2 and the measurement of fluorescence were carried out as described in the Experimental section. Vasopressin (13 nM) and CaCl_2 (1.3 mM) were added at the times indicated. The straight line represents the maximum rate of increase in fluorescence after addition of CaCl_2 . The slope of this line was used to calculate the rate of Ca^{2+} inflow, as described in the Experimental section. A representative trace is shown for each condition.

Fig. 2. Inhibition by verapamil of the increase in $[\text{Ca-quin2}]$ which follows the addition of 1.3 mM-Ca^{2+} to cells loaded with quin2

The loading of hepatocytes with quin2, measurement of fluorescence and calculation of the change in $[\text{Ca-quin2}]$ in the presence (●) and absence (○) of verapamil were carried out as described in the Experimental section and in Fig. 1. Approx. 1 min after the addition of vasopressin (cf. Fig. 1), verapamil ($400 \mu\text{M}$) or dimethyl sulphoxide (final concn. 0.9%) was added. CaCl_2 (1.3 mM) was added 4 min after the verapamil (0 s on the graph). The abscissa represents the time elapsed after addition of CaCl_2 . Each point is the mean $\pm$ S.E.M. for nine determinations obtained from three separate cell preparations.

the plateau was $0.22 \text{ nmol/min per mg wet wt.}$ In subsequent experiments an intracellular concentration of quin2 of about 1.5 mM was used for the measurement of Ca^{2+} inflow.

Effects of the extracellular Ca^{2+} concentration on the rate of Ca^{2+} inflow

A plot of the rate of Ca^{2+} inflow, measured with quin2-loaded hepatocytes incubated in the presence of 13 nM -vasopressin, as a function of $[\text{Ca}^{2+}]_o$ is shown in Fig. 4. The curve is biphasic, showing a greater increase in the rate of Ca^{2+} inflow with increasing $[\text{Ca}^{2+}]_o$ at lower $[\text{Ca}^{2+}]_o$. As the $[\text{Ca}^{2+}]_o$ was increased, the observed rate of Ca^{2+} inflow in the second phase showed no sign of saturation at 5 mM-Ca^{2+} , the highest Ca^{2+} concentration tested. The increases in the rate of Ca^{2+} inflow observed with increasing $[\text{Ca}^{2+}]_o$ were associated with an increase in the steady-state value of $[\text{Ca}^{2+}]_i$ reached after the addition of Ca^{2+} (Fig. 4, inset).

Fig. 3. Effect of increasing concentrations of quin2 on the measured rate of Ca^{2+} inflow

The loading of hepatocytes with quin2 by using different concentrations of quin2-AM and measurement of rates of Ca^{2+} inflow were carried out as described in the Experimental section and in the legend of Fig. 1. Each point represents the mean $\pm$ S.E.M. (obtained from five separate loadings with quin2) for the [quin2] (abscissa) and the mean $\pm$ S.E.M. for rate of Ca^{2+} inflow (ordinate). The curve was drawn from a fit of the data points by eye.

Effects of replacement of extracellular NaCl with choline chloride or TMA chloride on the rate of Ca^{2+} inflow and $[\text{Ca}^{2+}]_i$

When hepatocytes loaded with quin2 were resuspended in choline medium (in the absence of added Ca^{2+}) instead of Na^+ medium, a small decrease in basal $[\text{Ca}^{2+}]_i$ was observed (Table 1). The increases in $[\text{Ca}^{2+}]_i$ observed after addition of vasopressin and 1.3 mM- CaCl_2 to cells incubated in the presence of choline were considerably less than the increases observed after addition of these agents to cells incubated in the presence of Na^+ (Table 1). Results similar to those obtained with choline medium were observed for cells suspended in the presence of

Fig. 4. Effect of increasing concentrations of Ca^{2+}_o on the rate of Ca^{2+} inflow to cells loaded with quin2

The loading of hepatocytes with quin2, the measurement of fluorescence and calculation of the rates of Ca^{2+} inflow were carried out as described in the Experimental section and in the legend of Fig. 1. The concentration of added Ca^{2+}_o was varied between 0.25 and 5.0 mM. The inset shows the maximum value of $[\text{Ca}^{2+}]_i$ reached after the addition of Ca^{2+}_o , plotted as a function of $[\text{Ca}^{2+}]_o$. Each point is the mean $\pm$ S.E.M. for two to six determinations each made with a separate cell preparation.

TMA (Table 1). The rate of Ca^{2+} inflow measured after addition of 1.3 mM- Ca^{2+} to cells incubated in the presence of vasopressin was decreased from 0.20 ± 0.01 (12 determinations using seven separate cell preparations) nmol/min per mg wet wt. for cells suspended in Na^+ medium to 0.11 ± 0.01 (five determinations using three separate cell preparations) and 0.12 ± 0.01 (six determinations using four separate cell preparations)

Table 1. Effect of replacement of extracellular NaCl with choline or TMA chloride on $[\text{Ca}^{2+}]_i$ in hepatocytes incubated in the absence of Ca^{2+}_o

The loading of hepatocytes with quin2, the replacement of NaCl with choline or TMA chloride at the first resuspension, and the calculation of $[\text{Ca}^{2+}]_i$, were conducted as described in the Experimental section. No Ca^{2+} was added to the media used to wash and resuspend the cells after they had been loaded with quin2. Additions were made as shown in Fig. 1. Values of $[\text{Ca}^{2+}]_i$ were calculated at the following time points: immediately before the addition of vasopressin (13 nM), at the peak after addition of vasopressin, immediately before addition of CaCl_2 (1.3 mM), and at the peak after addition of CaCl_2 . The values are means $\pm$ S.E.M. for the numbers of determinations indicated, at least three separate cell preparations being used for each condition. The value of P (Student's t test for unpaired samples) for a comparison of cells in choline or TMA medium with cells in Na^+ was $*P < 0.05$.

Medium	$[\text{Ca}^{2+}]_i$ (nM)			
	Before vasopressin	After vasopressin	Before CaCl_2	After CaCl_2
Sodium	174 ± 13 (12)	365 ± 39 (12)	174 ± 12 (12)	504 ± 59 (12)
Choline	103 ± 6 (5)*	125 ± 4 (5)*	102 ± 5 (5)*	155 ± 9 (5)*
TMA	150 ± 25 (6)	193 ± 41 (6)*	132 ± 14 (6)*	200 ± 19 (6)*

Fig. 5. Effect of replacement of NaCl with choline chloride or TMA chloride on $[Ca^{2+}]_i$ in cells loaded with quin2 and incubated in the presence of Ca^{2+} .

The loading of hepatocytes with quin2, the replacement of NaCl with choline chloride or TMA chloride at the first resuspension, the measurement of fluorescence and the calculation of $[Ca^{2+}]_i$ were carried out as described in the Experimental section. Ca^{2+} (0.5 mM) was present in all media during loading with quin2 and washing of the cells. After the final suspension, $CaCl_2$ (0.8 mM) was added to raise the value of $[Ca^{2+}]_o$ to 1.3 mM. Vasopressin (13 nM) was added, as indicated by the arrow, to cells incubated in Na^+ (○), choline (△) or TMA (●) medium. The values shown are the means $\pm$ S.E.M. of the results for nine (Na^+) or eight (TMA) determinations obtained from five separate cell preparations, or five determinations obtained from three separate cell preparations (choline).

($P < 0.05$, Student's t test for unpaired samples) nmol/min per mg wet wt. for cells suspended in choline and TMA media respectively.

The results of experiments in which hepatocytes were loaded with quin2 and incubated in the presence of 1.3 mM- Ca^{2+} in Na^+ , choline or TMA medium are shown in Fig. 5. The value of $[Ca^{2+}]_i$ under basal conditions (before addition of vasopressin) is lower in cells suspended in choline or TMA medium. Addition of vasopressin to cells suspended in choline or TMA medium caused only a very small increase in $[Ca^{2+}]_i$ (Fig. 5).

Effect of replacement of extracellular NaCl with choline chloride on $^{45}Ca^{2+}$ exchange and total Ca content

For cells not loaded with quin2, substitution of choline chloride for NaCl did not alter the initial rate of $^{45}Ca^{2+}$

exchange measured in the presence of 1.3 mM- Ca^{2+} , in either the absence or the presence of 13 nM-vasopressin (results not shown). The degree of stimulation by vasopressin of the initial rate of $^{45}Ca^{2+}$ exchange in choline medium was similar to that observed in Na^+ medium. The initial rate of $^{45}Ca^{2+}$ uptake observed after addition of 1.3 mM- $^{45}Ca^{2+}$ to cells previously incubated in the absence of added Ca^{2+} was not significantly different for cells incubated in choline medium compared with cells incubated in Na^+ medium (results not shown).

For cells loaded with quin2, replacement of NaCl with choline chloride did not alter the initial rate of $^{45}Ca^{2+}$ exchange measured in the absence of vasopressin (Fig. 6a). However, in choline medium no stimulation of the initial rate of $^{45}Ca^{2+}$ exchange by vasopressin was observed (Fig. 6a).

In order to determine whether, in the absence of vasopressin, replacement of NaCl with choline chloride alters the amount of exchangeable Ca^{2+} in intracellular organelles, $^{45}Ca^{2+}$ -exchange experiments were conducted over a longer period of time. For both quin2-loaded and unloaded cells, replacement of NaCl with choline chloride did not alter the amount of $^{45}Ca^{2+}$ exchanged at times up to 40 min when this was measured in presence of 1.3 mM- Ca^{2+} (Fig. 6b). Since one of the possibilities being tested was that the replacement of NaCl with choline chloride increases the rate of Ca^{2+} inflow, and hence the amount of cellular exchangeable Ca^{2+} , the effects of choline were compared with those of La^{3+} . This trivalent cation has previously been shown to increase Ca^{2+} uptake by hepatocytes (Krell *et al.*, 1979; Parker & Barritt, 1981). La^{3+} caused a large increase in $^{45}Ca^{2+}$ exchange (Fig. 6b, inset).

The amount of total Ca, measured by atomic absorption spectroscopy, in cells not loaded with quin2 and incubated at 37 °C in the presence of 1.3 mM- Ca^{2+} for 5 and 10 min in choline medium was found to be 0.92 ± 0.03 and 1.00 ± 0.03 nmol/mg wet wt. ($n = 6$) respectively. This was not significantly different from the values of 0.89 ± 0.03 and 0.99 ± 0.04 ($n = 6$) respectively, for cells in Na^+ medium. The total amount of Na in hepatocytes not loaded with quin2 and incubated at 37 °C in the presence of 1.3 mM- Ca^{2+} for 12 min in either Na^+ or choline medium was 12.3 ± 2.9 mM and 1.48 ± 0.05 mM (four determinations using two separate cell preparations) respectively.

Effect of replacement of extracellular NaCl with choline chloride or TMA chloride on glycogen phosphorylase activity

The effect of the replacement of NaCl with choline chloride on the activation by vasopressin of glycogen phosphorylase was investigated by using (a) cells subjected to the incubation conditions employed to load cells with quin2, except that quin2 was omitted from the incubation media (mock-loaded cells), and (b) untreated cells. [It was not possible to use cells loaded with quin2, since it has previously been shown (Charest *et al.*, 1983) that quin2 itself activates glycogen phosphorylase.] In mock-loaded cells incubated in the presence of 1.3 mM- Ca^{2+} , the basal activity of glycogen phosphorylase was greater in cells incubated in choline medium than in cells incubated in Na^+ medium (Fig. 7). The maximum values of glycogen phosphorylase activity induced by vasopressin in choline and Na^+ media were similar (Fig. 7). Moreover, there was no difference in the shape of the

Fig. 6. Effect of replacement of extracellular NaCl with choline chloride on the rate of $^{45}\text{Ca}^{2+}$ exchange measured at 1.3 mM-Ca^{2+} , at short (a) and long (b) periods of time

(a) Hepatocytes were loaded with quin2 and transferred to Na^+ medium ($\circ$, $\bullet$), or choline medium ($\triangle$, $\blacktriangle$) which contained 1.3 mM-Ca^{2+} , as described in the Experimental section. After 10 min incubation at 37°C , vasopressin (13 nM) ($\bullet$, $\blacktriangle$) or carrier ($\circ$, $\triangle$) was added. After a further 15 min tracer amounts of $^{45}\text{Ca}^{2+}$ were added. The amount of $^{45}\text{Ca}^{2+}$ associated with the cells was measured as described in the Experimental section at the times indicated after addition of $^{45}\text{Ca}^{2+}$ tracer. Each point represents the mean $\pm$ S.E.M. of six or four (no-vasopressin) determinations obtained from two separate cell preparations. (b) Hepatocytes were loaded with quin2 ($\bullet$, $\blacktriangle$) or received an equal volume of dimethyl sulphoxide in place of quin2-AM ($\circ$, $\triangle$), then transferred to Na^+ medium ($\circ$, $\bullet$) or choline medium ($\triangle$, $\blacktriangle$) which each contained 1.3 mM-Ca^{2+} , as described in the Experimental section. The cell were incubated at 37°C . After 15 min tracer amounts of $^{45}\text{Ca}^{2+}$ were added, and the amounts of $^{45}\text{Ca}^{2+}$ associated with the cells were determined at the times indicated. The values shown are the means $\pm$ S.E.M. of the results for four determinations using two separate cell preparations. The inset shows the amounts of $^{45}\text{Ca}^{2+}$ exchanged at 10 and 40 min in cells incubated in Na^+ medium ($\circ$), choline medium ($\triangle$), and Na^+ medium which contained $60 \mu\text{M-La}^{3+}$ added 15 min before $^{45}\text{Ca}^{2+}$ ($\bullet$). Freshly isolated hepatocytes were twice centrifuged and resuspended at room temperature in Na^+ or choline medium before incubation at 37°C . Each point represents the mean $\pm$ S.E.M. of eight determinations obtained from four separate cell preparations.

curves of enzyme activity as a function of time at longer time periods (Fig. 7).

Results similar to those shown in Fig. 7 were obtained for untreated cells incubated in Na^+ medium, choline medium and TMA medium (results not shown). However, the basal values of phosphorylase activity in untreated cells were higher than those in mock-loaded cells (results not shown).

Fig. 7. Effect of replacement of extracellular Na^+ with choline on the activation by vasopressin of glycogen phosphorylase α

Hepatocytes were 'mock-loaded' with quin2, and incubated in Na^+ ($\circ$) or choline ($\bullet$) medium which contained 1.3 mM-CaCl_2 as described in the Experimental section. Samples were taken for determination of glycogen phosphorylase activity immediately before addition of vasopressin (indicated by the arrow) and at the times indicated after vasopressin addition. Each point represents the mean $\pm$ S.E.M. for three determinations each made with a separate cell preparation. The P value (Student's t test for unpaired samples) for comparison of cells in choline medium with cells in Na^+ medium was $*P < 0.05$.

DISCUSSION

Measurement of Ca^{2+} inflow by using quin2

In this study, rates of Ca^{2+} inflow across the hepatocyte plasma membrane have been estimated from the initial rate of change of fluorescence of the Ca–quin2 complex in cells loaded with quin2. This would be an accurate estimate of the rate of Ca^{2+} inflow if, during the period of measurement, there is no significant removal of Ca^{2+} from the cytoplasmic space. The main pathways of Ca^{2+} removal are inflow to intracellular organelles, outflow across the plasma membrane and binding to proteins, metabolites and other components of the cytoplasmic space (Altin & Bygrave, 1988). The experiments were conducted in the presence of vasopressin and a high concentration of intracellular quin2. These conditions are expected to minimize the removal of Ca^{2+} from the cytoplasmic space. Previous studies have shown that vasopressin, through the action of inositol 1,4,5-trisphosphate, releases Ca^{2+} from the endoplasmic reticulum (Burgess *et al.*, 1984; Joseph *et al.*, 1984; Charest *et al.*, 1985), and evidence that this action of vasopressin inhibits the accumulation of Ca^{2+} by the endoplasmic reticulum has been presented (Hughes *et al.*, 1986a; Putney, 1986).

The use of an intracellular quin2 concentration of 1.5 mM in the measurement of Ca^{2+} inflow should have ensured that the binding sites for Ca^{2+} provided by quin2 are in excess of the amount of Ca^{2+} which enters the cell. This should maximize the binding of Ca^{2+} to quin2 and minimize the removal of Ca^{2+} from the cytoplasmic space by intracellular processes. That this in fact occurred is indicated by the finding that plots of the rate of Ca^{2+} inflow as a function of the concentration of intracellular quin2 reached a plateau at about 1.5 mM–quin2. In addition to the presence of vasopressin and a high concentration of quin2, measurement of the rate of change of fluorescence at early time points after the addition of Ca^{2+} should have decreased the effect of any removal of Ca^{2+} from the cytoplasmic space by the pathways of Ca^{2+} disposal, since $[\text{Ca}^{2+}]_i$ will be near its minimum value during this period.

The value for the rate of Ca^{2+} inflow obtained for quin2-loaded cells incubated at 1.3 mM- Ca^{2+}_o in the presence of vasopressin is 0.2 nmol/min per mg wet wt. This is in good agreement with the value of 0.15 nmol/min per mg wet wt. for the rate of Ca^{2+} inflow measured in the presence of adrenaline from a compartmental analysis of steady-state kinetic data (Barritt *et al.*, 1981). Moreover, the degree of stimulation of Ca^{2+} inflow by vasopressin (35 %) is in good agreement with that observed by using the initial rate of $^{45}\text{Ca}^{2+}$ exchange under steady-state conditions as a measure of the rate of Ca^{2+} inflow (Hughes *et al.*, 1986a).

Effects of $[\text{Ca}^{2+}]_o$ on rate of Ca^{2+} inflow

The biphasic nature of plots of Ca^{2+} inflow as a function of $[\text{Ca}^{2+}]_o$ suggests that two different processes are responsible for Ca^{2+} inflow to hepatocytes incubated in the presence of vasopressin. The first process appears to be saturated at about 1 mM- Ca^{2+}_o , whereas the second is not saturated at values of Ca^{2+}_o up to 5 mM. It is considered unlikely that the observed increase in the rate of Ca^{2+} inflow with increasing values of $[\text{Ca}^{2+}]_o$ is a consequence of the increase in the steady-state value of $[\text{Ca}^{2+}]_i$ reached after the addition of Ca^{2+}_o , since the

initial rate of increase in the fluorescence of the Ca–quin2 complex rather than changes in fluorescence at longer times after the addition of Ca^{2+}_o has been used as the measure of Ca^{2+} inflow, and the value of $[\text{Ca}^{2+}]_i$ before the addition of $[\text{Ca}^{2+}]_o$ is the same under each condition. In previous studies conducted in this laboratory, in which Ca^{2+} inflow was measured either by the initial rate of $^{45}\text{Ca}^{2+}$ exchange under steady-state conditions (Parker *et al.*, 1983) or by the initial rate of $^{45}\text{Ca}^{2+}$ uptake to Ca^{2+} -deprived cells (Hughes *et al.*, 1987a) plots of the rate of Ca^{2+} uptake as a function of $[\text{Ca}^{2+}]_o$ also showed no saturation at values of $[\text{Ca}^{2+}]_o$ up to 5 and 10 mM respectively.

The present results differ from those of Mauger *et al.* (1984), obtained by using $^{45}\text{Ca}^{2+}$ uptake, and Joseph *et al.* (1985), obtained with hepatocytes loaded with quin2. In the studies by Mauger *et al.* (1984), plots of the estimated rate of Ca^{2+} inflow as a function of $[\text{Ca}^{2+}]_o$ in the presence of vasopressin approached a plateau at 5 mM- Ca^{2+}_o . In the studies by Joseph *et al.* (1985), plots of the estimated rate of Ca^{2+} inflow as a function of $[\text{Ca}^{2+}]_o$ obtained in the presence and absence of vasopressin reached a plateau at about 2 mM- Ca^{2+}_o . The differences between these results and those reported here may be due to the conditions employed in the present study, which should have minimized the removal of Ca^{2+} from the cytoplasmic space during the period in which the rate of Ca^{2+} inflow is being estimated.

Effect of replacement of NaCl by choline chloride or TMA chloride

The effects of replacement of NaCl by choline chloride were found to depend on whether or not the cells were loaded with quin2. In cells that were not loaded with quin2, replacement of NaCl by choline chloride did not alter the rate of Ca^{2+} inflow in the absence of vasopressin, as judged by measurement of $^{45}\text{Ca}^{2+}$ exchange and the initial rate of $^{45}\text{Ca}^{2+}$ uptake by cells previously incubated in the absence of Ca^{2+} , or in the presence of vasopressin, as judged by measurement of $^{45}\text{Ca}^{2+}$ exchange and comparison of the time courses for the activation by vasopressin of glycogen phosphorylase at longer times after addition of the hormone. Moreover, the ability of vasopressin to stimulate Ca^{2+} inflow was not impaired by replacement of NaCl by choline chloride.

In cells loaded with quin2 and incubated in the absence of vasopressin, replacement of NaCl with choline chloride did not alter Ca^{2+} inflow, as judged by the measurement of $^{45}\text{Ca}^{2+}$ exchange. However, in cells loaded with quin2 and incubated in the presence of vasopressin, replacement of NaCl with choline chloride caused a decrease in Ca^{2+} inflow, as measured by using quin2 or $^{45}\text{Ca}^{2+}$ exchange. These observations indicate that the combination of intracellular quin2 and extracellular choline chloride in place of NaCl inhibits Ca^{2+} inflow through the receptor-activated Ca^{2+} -inflow system, but not through the basal Ca^{2+} -inflow system. An inhibition by quin2 of Ca^{2+} inflow in squid axons has been reported by Allen & Baker (1985). However, in this case the effect of quin2 was an inhibition of Na^+ -dependent Ca^{2+} inflow through the Na^+ – Ca^{2+} exchange system, whereas, as discussed below, the present findings provide no evidence for Na^+ -dependent Ca^{2+} inflow to hepatocytes.

Cells loaded with quin2 and incubated in the presence of choline chloride or TMA chloride in place of NaCl show a marked diminution of the increase in $[\text{Ca}^{2+}]_i$

elicited by the addition of vasopressin in both the presence and absence of added Ca^{2+}_o . The results obtained in the absence of added Ca^{2+}_o indicate that, in addition to an effect on Ca^{2+} inflow, the substitution of choline chloride for NaCl in the presence of quin2 decreases the amount of Ca^{2+} released by vasopressin from intracellular stores. This effect may be due to a decreased efficiency of coupling between receptors and the release of Ca^{2+} from inositol trisphosphate-sensitive stores, to a decreased efficiency of action of inositol trisphosphate in inducing Ca^{2+} release from intracellular stores, or to a decreased binding of vasopressin to its receptors. It is considered unlikely that the decrease in Ca^{2+} released from intracellular stores by vasopressin in the presence of quin2 and choline is due to a decreased binding of vasopressin to its receptors or to a decrease in the amount of Ca^{2+} present in intracellular stores. When present alone, neither choline or quin2 inhibits the ability of vasopressin to release Ca^{2+} from intracellular stores, as judged by measurement of quin2 fluorescence (quin2) or glycogen phosphorylase (choline). Moreover, the results of $^{45}\text{Ca}^{2+}$ -exchange experiments conducted at times up to 40 min indicate that the substitution of choline for Na^+ does not alter the amount of Ca^{2+} in intracellular stores.

The present observation that the replacement of extracellular Na^+ by choline decreases the transient increase in $[\text{Ca}^{2+}]_i$ induced by the addition of vasopressin to quin2-loaded hepatocytes is similar to results obtained for the effect of thrombin on $[\text{Ca}^{2+}]_i$ in blood platelets loaded with quin2 and incubated in the presence of choline (Siffert *et al.*, 1987). However, other workers (Sage & Rink, 1986) did not observe this effect of choline on the action of thrombin.

The results indicate that replacement of Na^+_o by choline leads to an increase in basal $[\text{Ca}^{2+}]_i$ (as judged by an increase in the activity of glycogen phosphorylase) in the absence of quin2, but a decrease in basal $[\text{Ca}^{2+}]_i$ as measured from the fluorescence of quin2. The difference between these two sets of results may be due to the ability of quin2 to buffer $[\text{Ca}^{2+}]_i$ (Tsien *et al.*, 1982; Pozzan *et al.*, 1982) or to an effect of altered $[\text{Na}^+]_i$ on the activity of glycogen phosphorylase.

The results obtained under a variety of conditions provide no evidence for an increase in Ca^{2+} inflow when NaCl is replaced by choline chloride. In particular, no increase was observed in the contents of $^{45}\text{Ca}^{2+}$ or $^{40}\text{Ca}^{2+}$ in cells not loaded with quin2 and incubated for up to 40 min in the presence of 1.3 mM $^{45}\text{Ca}^{2+}$ or $^{40}\text{Ca}^{2+}$. If the mechanism of plasma-membrane Ca^{2+} inflow involved the exchange of Ca^{2+}_o for Na^+_i , it would be expected that the replacement of NaCl by choline chloride would increase the value of the $[\text{Na}^+]_i/[\text{Na}^+]_o$ gradient, which, in turn, would increase the rate of Ca^{2+} inflow (Barry & Smith, 1982; Blaustein & Nelson, 1982; Snowdowne & Borle, 1985), and the amount of Ca^{2+} present in intracellular stores. Since an increase in the rate of Ca^{2+} inflow was not observed under any condition when NaCl was replaced by choline chloride, it is concluded that the inflow of Ca^{2+} to the liver cell cytoplasmic space through the basal and receptor-activated Ca^{2+} -inflow systems does not involve the inward movement of Ca^{2+} in exchange for the outward movement of Na^+ . A similar observation has been made in other cell types, including Ca^{2+} inflow stimulated by muscarinic cholinergic receptors in pheochromocytoma (PC12) cells (Inoue & Kenimer, 1988). The present results also indicate that

Ca^{2+} inflow through both the basal and receptor-activated Ca^{2+} -inflow systems is not stimulated by Na^+_i , as suggested by the results of Schanne & Moore (1986) obtained with liver cell plasma membranes.

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