Cytosolic Free Ca^{2+} Level in Isolated Hepatocytes: The Effect of Insulin

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Abstract. Cytosolic free Ca^{2+} level was estimated in rat hepatocytes using the method described by Murphy et al. (1980). For control hepatocytes, a value of 0.20 ± 0.06 μmol/l was obtained. Insulin increased cytosolic free Ca^{2+} level to 0.63 ± 0.24 μmol/l. No net fluxes of Ca^{2+} across the plasma membrane were observed during incubation of hepatocytes with insulin. Mitochondria were shown to be the main Ca^{2+} buffering system. FCCP released 77—88 % of releasable calcium from the cell. Dibucaine increased cytosolic free Ca^{2+} level to 1.16 μmol/l.

Key words: Hepatocytes — Cytosolic free Ca^{2+} — Insulin

Introduction

Although direct evidence for Ca^{2+} as a second messenger of hormone action is still missing, there is no doubt that free Ca^{2+} plays a substantial role in the regulation of cell metabolism. Problems preventing a better understanding of the involvement of Ca^{2+} in hormone action arise from the fact that it is extremely difficult to measure cytosolic free Ca^{2+} level directly. Methods using labelled ^{45}Ca are not fully suitable since calcium is in the cell in a dynamic state, being very rapidly released, bound and sequestered by many intracellular systems.

Rather controversial data exist about the involvement of extracellular calcium in activation of receptors with Ca^{2+} being the second, or at least the third, messenger. Some authors have assumed that after activation of α_1-receptors Ca^{2+} gates in plasma membrane open, extracellular Ca^{2+} enters the cell, and the cytosolic Ca^{2+} concentration rises (Michell 1975, 1979). This hypothesis has been based on the fact that, in some cases, extracellular calcium is necessary for hormone action: however, from many other data it seems that extracellular calcium is not an obligatory requirement for the supposed increase in cytosolic free Ca^{2+} after α_1-receptor stimulation (for a review, see Williamson et al. 1981). A group of authors have reported an increased Ca^{2+} efflux from the liver after α_1-receptor activation (Chen et al. 1978; Althaus-Salzmann et al. 1980). Murphy et al. (1980) observed no net movement of Ca^{2+} across the plasma membrane, and Foden and

Randle (1978) have reported an inhibition of Ca^{2+} efflux from hepatocytes after the addition of phenylephrine.

Insulin is one of the hormones, the mechanism of action of which does not involve cyclic AMP, and some of the metabolic effects of insulin can be mediated by Ca^{2+}, e.g. activation of pyruvate dehydrogenase. The principal problem, whether Ca^{2+} can be the second messenger of insulin or whether it is involved in the action of insulin, cannot be solved on the basis of data presently available without any knowledge on mechanism of calcium homeostasis in the cell.

In this paper, the method of Murphy et al. (1980) was used to study the mechanism of calcium homeostasis in isolated hepatocytes; in addition, the effect of insulin was studied.

Materials and Methods

Hepatocytes were prepared from male Wistar rats, which had been fed between 4 a.m. and 7 a.m. The animals were killed at 10.30 a.m. Hepatocytes were isolated according to the procedure of Seglen (1976) with minor modification (Beynen et al. 1979). Isolated hepatocytes were washed twice with Ca^{2+}- and Mg^{2+}-free modified Hanks' medium containing (in mmol/l): 137 NaCl; 5.4 KCl; 0.44 KH_2PO_4; 25 $NaHCO_3$; 0.33 Na_2HPO_3; and 20 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (Hepes), pH 7.4 equilibrated with 95 % O_2 and 5 % CO_2. For some experiments (as indicated in Results), liver cells were loaded with calcium by incubation in Hanks' medium, containing 10 mmol/l $CaCl_2$ for 15 minutes, followed by washing in Ca^{2+}- and Mg^{2+}-free medium. Prior to the experiments, hepatocytes were incubated for 1 hour at room temperature.

Free Ca^{2+} content was measured spectrophotometrically with arsenazo III using the wavelength pair 675—685 (Aminco).

Cytosolic free Ca^{2+} concentration was estimated according to Murphy et al. (1980), using approximately 15 μg of digitonin per mg of protein. Protein was determined by the method of Lowry et al. (1951).

Materials: Digitonin (Sigma Chemical Co.) was recrystalised three times from hot ethanol. Arsenazo III was purified according to Kendrick (1976). In our experiments 40 μmol/l of Arsenazo III was used. Ionophore A 23 187 was a gift of Eli Lilly Co., and a final concentration of 10 μmol/l was used. Uncoupler FCCP (carbonyl cyanide p. fluoroxyphenylhydrazone) was used in a concentration of 0.8 μmol/l.

Dibucaine from ICN (K + K Rare Chemicals) was used in a final concentration of 0.15 mmol/l. Insulin was a gift of Eli Lilly Co. (85 nmol/l).

Results

Determination of cytosolic free Ca^{2+} level and calcium homeostasis in rat hepatocytes

According to Murphy et al. (1980), free Ca^{2+} penetrates into digitonin treated hepatocytes from external medium untill an equilibrium between the extracellular free Ca^{2+} concentration is reached (Fig. 1*A*). The final equilibrium serves as a rough estimation of cytosolic free Ca^{2+}. Exact values were obtained from a series of experiments with different initial extracellular concentrations of free Ca^{2+}. The extent of Ca^{2+} changes after the addition of digitonin (15 μg per mg of protein) was plotted as a function of the initial free Ca^{2+} concentration (Fig. 2).

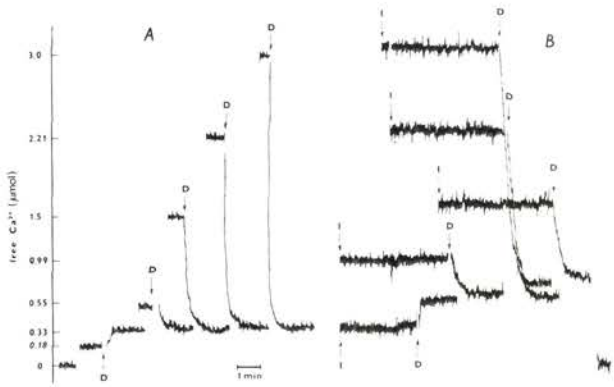

Fig. 1. Effect of digitonin (D) in a concentration of 15 μg per mg of protein on calcium uptake by hepatocytes. Cells (10 mg of protein) were incubated in 2.4 ml of Ca^{2+}- Mg^{2+}-free modified Hanks' medium, pH 7.4 at 37 °C, in the presence of 100 μl arsenazo III (in final concentration of 40 μmol/l). *A* — control samples, *B* — response after 5 minutes of incubation of cells with insulin (85 nmol/l).

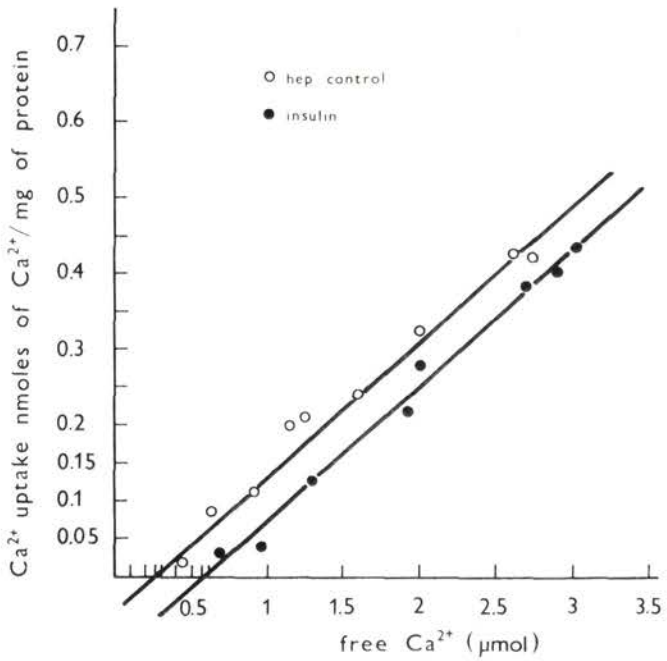

Fig. 2. An example of an exact estimation of the cytosolic free Ca^{2+} level from one experimental series. The best fit lines through the experimental points and the intercept with the abscissa were calculated by linear regression analysis. I — cells incubated with insulin for 5 minutes.

Table 1. Cytosolic free Ca^{2+} concentration in rat hepatocytes (μmol/l)
n = number of various initial extracellular free Ca^{2+} concentrations used in one series of determinations.

n	Zero point	Correlation coeficient	Mean ± SEM
5	0.23	0.998	
4	0.16	0.998	
4	0.10	0.994	0.20 ± 0.06
9	0.27	0.945	
9	0.15	0.899	
7	0.25	0.919	
5	0.27	0.990	

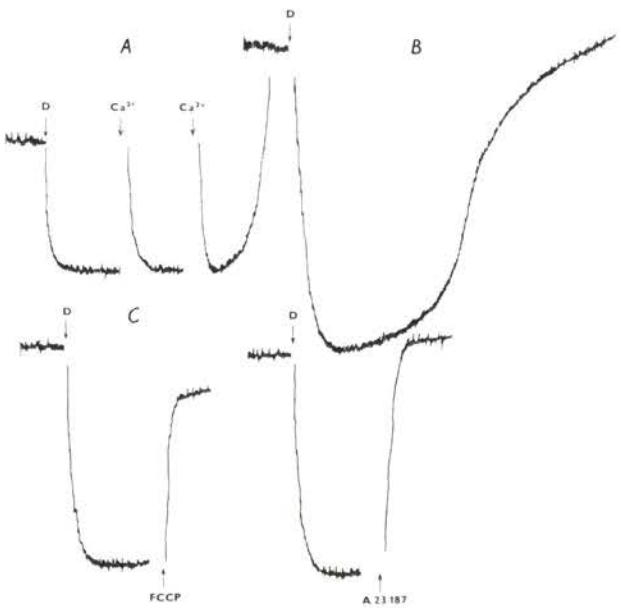

Fig. 3. Calcium homeostasis in hepatocytes treated with digitonin (D). Hepatocytes were incubated in Ca^{2+}- Mg-free Hanks' medium, pH 7.4. Thirty nmoles of free Ca^{2+} were added to the suspension of leaky cells and it was taken up by Ca^{2+} buffering systems to maintain a constant cytosolic free Ca^{2+} concentration. If these systems were overloaded, Ca^{2+} was extruded from the cells. *A* — control hepatocytes, *B* — hepatocytes preloaded with calcium by incubation in Krebs-Ringer with 10 mmol/l of $CaCl_2$, pH 7.4, at 37 °C, for 15 minutes, then washed and incubated in Ca^{2+}-free Hanks' medium. *C* — release of Ca^{2+} which entered the cell after digitonin action by 0.8 μmol/l of FCCP or by ionophore A 23 187 (10 μmol/l).

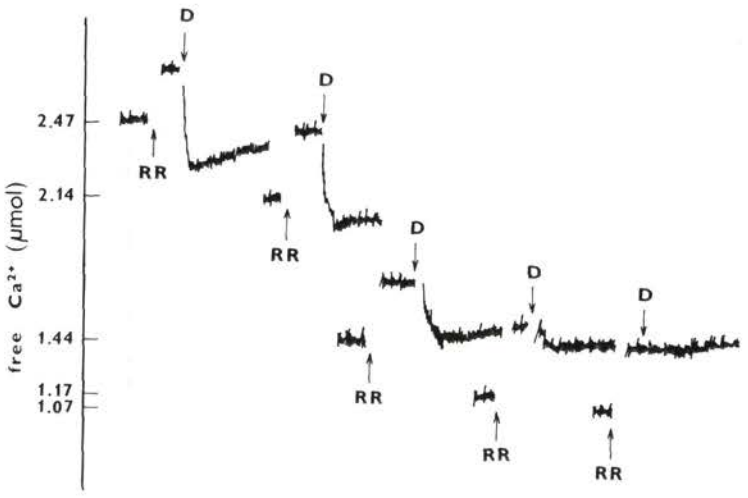

Fig. 4. The effect of 15 μmol/l of ruthenium red (RR) on Ca^{2+} uptake from extracellular medium after the action of digitonin (D). The addition of ruthenium red is followed by an artifact caused by RR (interference with arsenazo III).

The best fit lines through the experimental points and the intercept with the abscissa were calculated by linear regression analysis. Table 1 shows Results of a series of these experiments, with a cytosolic free Ca^{2+} concentration of 0.20 ± 0.06 μmol/l. If extracellular free Ca^{2+} concentration was increased again by the addition of 30 nmoles of free Ca^{2+}, this latter calcium penetrated into the cells and the equilibrium concentration corresponding to the original cytosolic free Ca^{2+} concentration was reached again. Uptake of additional free Ca^{2+} was dependent on saturation of Ca^{2+} binding systems in the cell. If hepatocytes incubated in Ca^{2+}-free Hanks' medium were used, it was possible to repeat the addition of 30 nmoles of free Ca^{2+} after the first calcium penetration 1—3 times until Ca^{2+} was extruded from the cells into medium (Fig. 3*A*), and the system became overloaded by Ca^{2+}. In hepatocytes preloaded with Ca^{2+} (see Methods), extrusion of Ca^{2+} was observed immediately after the first response to digitonin (Fig. 3*B*).

Calcium taken up by intracellular systems could be fully released by the addition of 10 μmol/l of ionophore A 23 187. FCCP (0.8 μmol/l) released 77—88 % only (Fig. 3*C*).

Incubation of cells in the presence of 15 μmol/l of ruthenium red gave a rather different response to digitonin (Fig. 4). Ca^{2+} uptake by the cells was substantially lowered, and the equilibrium concentration has never reached the concentration, corresponding to cytosolic free Ca^{2+} level in control hepatocytes. The equilibrium concentration differed with various initial free Ca^{2+} concentration. This non-mitochondrial free Ca^{2+} uptake was not active below 0.82 ± 0.32 μmol/l free Ca^{2+} cytosolic concentration (determined in 6 series of experiments).

Table 2. The effect of insulin (85 μmol/l) on the cytosolic free Ca^{2+} concentration in rat hepatocytes after 5 min of incubation in Ca^{2+} -, Mg^{2+} -free Hanks' medium. The same hepatocytes, incubated in the absence of insulin were used as control.

n	Zero point	Correlation coeficient	% of control	Mean ± SEM
5	0.30	0.995	187	
4	0.53	0.999	230	
5	1.02	0.995	196	
8	0.56	0.992	209	0.63 ± 0.24
7	0.80	0.970	320	
5	0.58	0.990	216	

The effect of insulin

Incubation of hepatocytes with 85 nmol/l of insulin for 1—10 minutes was not followed by any changes in net Ca^{2+} fluxes across the plasma membrane (Fig. 1*B*). Table 2 shows results of a series of experiments, estimating cytosolic free Ca^{2+} concentration after 5 minutes of incubation in the presence of insulin. The cytosolic free Ca^{2+} levels were increased to 0.63 ± 0.24 μmol/l.

An increase in the cytosolic free Ca^{2+} concentration (to the similar extent) was observed after 1 ; 2 ; 5 ; and 10 minutes (data not shown) of incubation with insulin. Incubation of cells with insulin limited the capacity of uptake of additional Ca^{2+} ; it diminished the number of additions, and in some cases, Ca^{2+} entering the cell after digitonin was extruded as soon as equilibrium had been reached (Fig. 5, cf. Fig. 3*A*).

Variations of the cytosolic free Ca^{2+} level

The above results show that the level of cytosolic free Ca^{2+} in hepatocytes is buffered very rapidly, and changes evoked by penetration of extracellular Ca^{2+} into leaky cells are transient. Only when mitochondrial Ca^{2+} uptake is blocked with ruthenium red, Ca^{2+} level is not well controled. We have therefore checked other possibilities to increase the cytosolic free Ca^{2+} level to mimick the action of insulin. Ionophore A 23 187 and a local anaesthetic dibucaine were used. Fig. 6 shows that 0.15 nmol/l of dibucaine increased the cytosolic free Ca^{2+} concentration to 1.16 μmol/l. The effect of 10 μmol/l of A 23 187 was quite different (Fig. 7). The addition of the ionophore to hepatocytes was immediately followed by Ca^{2+} release into extracellular medium. The amount of Ca^{2+} released was the same at different external Ca^{2+} concentrations, and represented about 0.5 nmoles of total calcium per mg of protein. After the addition of digitonin to hepatocytes treated with the ionophore, a small uptake of Ca^{2+} was observed, dependent on extracellular Ca^{2+}

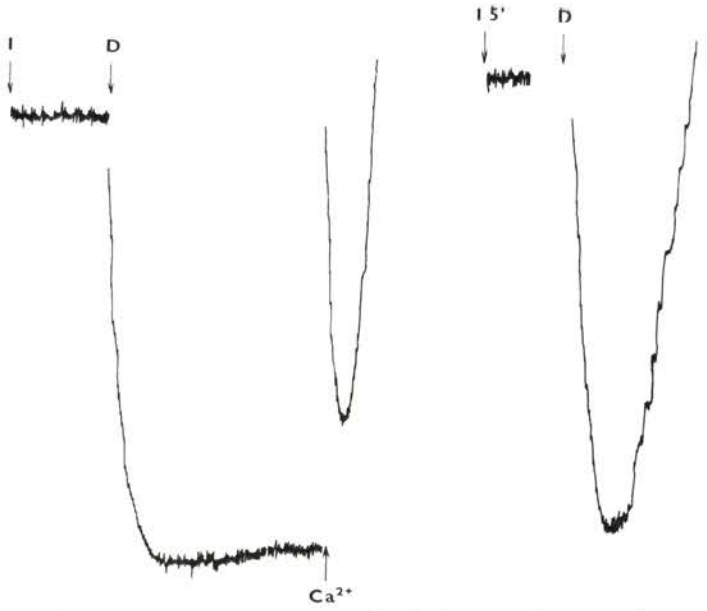

Fig. 5. Hepatocytes incubated in the presence of insulin have a reduced capacity to take up additional Ca^{2+} (cf. control response in Fig. 3*A*).

concentration, and bellow a concentration in external medium of about 1 μmol/l of Ca^{2+} no calcium uptake was observed.

Our results show that there exists a very efficient buffering system and the cell is fully able to increase its cytosolic free calcium level without an increased influx of calcium extracellular medium.

Discussion

Cytosolic Ca^{2+} level and calcium homeostasis in hepatocytes

The fact that the same equilibrium concentration of extracellular free Ca^{2+} is reached with different initial free Ca^{2+} concentration shows that Ca^{2+}, penetrating into the cell after the action of digitonin is removed by Ca^{2+} sequestering systems to maintain a constant cytosolic free Ca^{2+} concentration.

Our value of 0.20 ± 0.06 μmol/l for the cytosolic free Ca^{2+} level in hepatocytes is in good agreement with values reported by Murphy et al. (1980) (100—200 nmol/l), and by Becker et al. (1980), who measured the equilibrium Ca^{2+} concentration in a medium with digitonin-treated hepatocytes using electrodes (0.27 ± 0.04 μmol/l). In their experiments, this concentration of Ca^{2+} close to 0.2 μmol/l was also maintained by a suspension of isolated rat liver mitochondria and microsomes. Repeatly increased external Ca^{2+} concentration in a medium

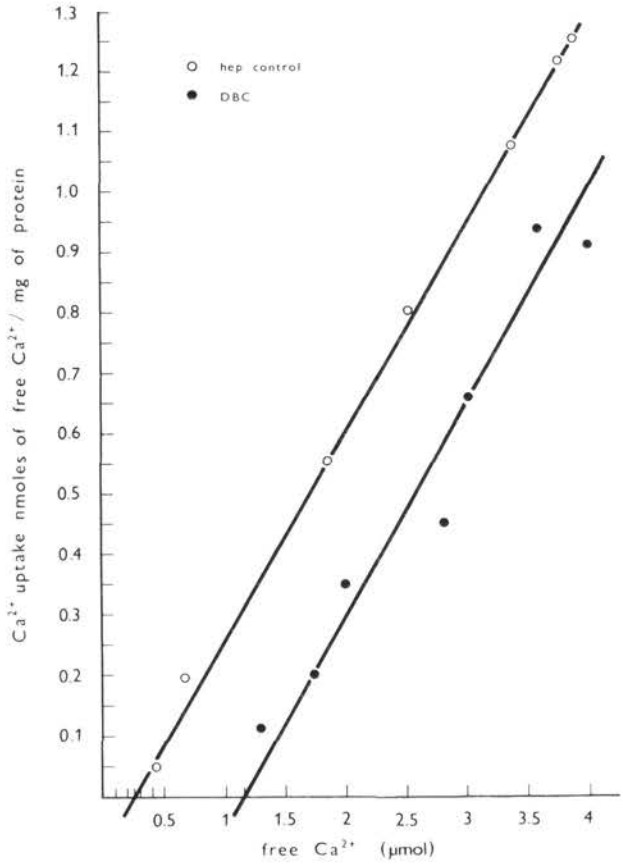

Fig. 6. Effect of 0.15 mmol/l of dibucaine on cytosolic free Ca^{2+} level after 5 minutes of incubation in Ca^{2+}- Mg^{2+}-free Hanks' medium, pH 7.4.

with leaky cells (Fig. 3*A*) showed that a very efficient buffering system exists in cells, which has a considerable capacity to bind free Ca^{2+}. Preloading of hepatocytes with calcium is probably unphysiological, and hepatocytes prepared by normal procedure are more convenient for the study of Ca^{2+} homeostasis in the cell.

Ruthenium red has been reported to be a specific inhibitor of mitochondrial Ca^{2+} uptake (Mc Donald et al. 1976; Bygrave 1978). From our results (Fig. 4) we can conclude that mitochondria are responsible for the major part of free Ca^{2+} uptake in hepatocytes (77—88 %) the plasma membrane of which was made permeable for calcium by the action of digitonin. The equilibrium concentration reached was higher in the presence of ruthenium red and it varied with variations in the initial free Ca^{2+} concentration in the medium.

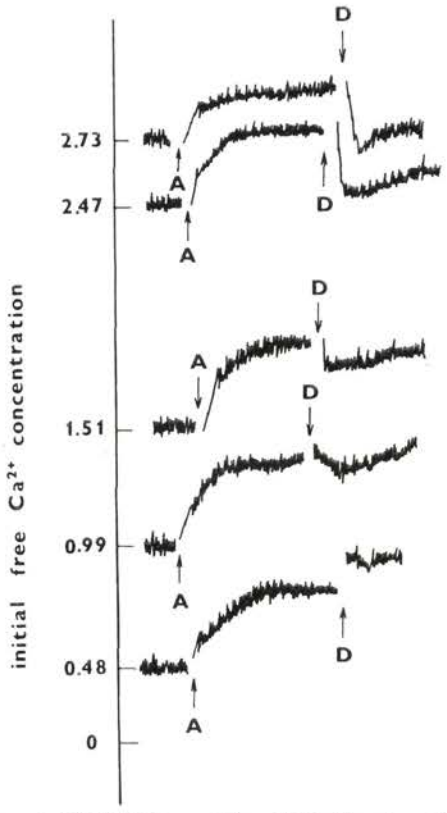

Fig. 7. Effect of ionophore A 23 187 (10 μmol/l) on Ca^{2+} efflux from hepatocytes, and responses after the addition of digitonin (D).

We conclude that nonmitochondrial Ca^{2+} uptake has not the capacity to buffer sufficiently cytosolic free Ca^{2+} level and different cytosolic free Ca^{2+} levels are obtained with different extracellular free Ca^{2+} concentrations in the presence of ruthenium red. In this respect, our results differ from conclusions of Murphy et al. (1980) who showed that Ca^{2+} zero point determination is independent of the activity of the mitochondrial Ca^{2+} uptake system. From our measurement it seems that nonmitochondrial Ca^{2+} system is inactive in the range of physiological Ca^{2+} level, since no Ca^{2+} was taken up below 0.82 ± 0.32 μmol/l Ca^{2+}. Becker et al. (1980) determined the activity of the microsomal Ca^{2+} uptake system more accurately in an in vitro system and concluded that, at a free Ca^{2+} concentration of 0.25 μmol/l or less, the net uptake of Ca^{2+} by liver endoplasmic reticulum is negligible. From our results it is clear that nonmitochondrial uptake of Ca^{2+} is inable to keep cytosolic free Ca^{2+} levels in the range between 0.1—0.2 μmol/l, and that mitochondria are main buffering system, most important for the cytosolic free Ca^{2+} levels.

The effect of insulin

Data about hormonal effect on Ca^{2+} fluxes across plasma membrane are confusing (see Bygrave 1978; Kirk et al. 1980; Williamson et al. 1981); however, the hypothesis trying to explain the action of hormones which mobilize Ca^{2+} in the cell, has assumed a change in plasma membrane permeability for Ca^{2+} (Michell 1979). As seen from Fig. 1, hepatocytes incubated in Ca^{2+}-Mg^{2+}-free Hanks' medium have no detectable net fluxes of Ca^{2+} both in the absence and presence of insulin. An incubation of hepatocytes in Ca^{2+} free Hanks' medium for 0.5—1 hour before an experiment is necessary. Similar observations (no net fluxes of Ca^{2+} in hepatocytes incubated in the absence of hormone) were presented by Chen et al. (1978); however, these authors measured net release of Ca^{2+} from isolated hepatocytes after the action of norepinephrine, vasopressin, glucagon and ionophore A 23 187, using Ca^{2+} electrode or efflux of ^{45}Ca.

Release of Ca^{2+} from hepatocytes after the action of α_1-adrenergic agonists was described by Blackmore et al. (1978). Our results have showed that insulin increases the cytosolic free Ca^{2+} level without any effect on plasma membrane permeability for calcium. The lower capacity of leaky cells to take up Ca^{2+} from extracellular medium after incubation with insulin affects, by some way, the capacity of Ca^{2+} binding systems, most probably that of mitochondria. The capacity of non mitochondrial uptake was not effected by incubation of hepatocytes with insulin.

Variations of the cytosolic free Ca^{2+} level

Local anaesthetics and ionophore A 23 187 are often used to vary free Ca^{2+} levels in the cytosol. The capacity of local anaesthetic to interfere with Ca^{2+} binding sites in membranes has been well documented (Siddle and Halles 1974). The effect of ionophore A 23 187 depends on the conditions (for a review, see Halles et al. 1977). Murphy et al. 1980 reported a 4-fold increase in the cytosolic free Ca^{2+} concentration in the presence of 4 μmol/l of A 23 187. Our estimation of the amount of Ca^{2+} released from hepatocytes (about 1.5 μmoles of calcium per mg of protein) is identical with the value obtained by Babcock et al. (1979). These authors have used digitonin-treated hepatocytes to characterize the hormone responsive pool of Ca^{2+}, and believed that Ca^{2+} released from intact hepatocytes after the action of 10 μmol/l of A 23 187 represents the calcium content of the mitochondria from hepatocytes. In our experiments, the amount of Ca^{2+} released from digitonin-treated hepatocytes was higher after A 23 187 then after FCCP (Fig. 3). Bellomo et al. (1982) distinguished two pools of intracellular calcium: mitochondrial released by FCCP, and nonmitochondrial released by A 23 187, the mitochondrial pool representing 60 % and the latter 40 % of calcium releasable

from hepatocytes. The rapid cell fractionation technique used by Murphy et al. (1980) has shown that the mitochondria in hepatocytes contain 70—80 % of the total intracellular calcium.

Dibucaine increases cytosolic free Ca^{2+} levels in the same way as insulin, independently on external Ca^{2+} concentration. The effect of insulin on the free Ca^{2+} level in the cell is quite different from that of ionophore A 23 187. After the addition of A 23 187, a net release of Ca^{2+} from the cells was observed, an attempt to estimate the cytosolic Ca^{2+} level showed that, in the presence of the ionophore, it is higher than in control hepatocytes, and that it is dependent on extracellular Ca^{2+}. Our observation of the effect of ionophore A 23 187 is in good agreement with findings of Segal and Ingbar (1982), who showed that, at very low concentrations of Ca^{2+}, the ionophore A 23 187 had no specific effect on ^{45}Ca uptake by rat thymocytes.

It seems that the method developed by Murphy et al. (1980) enables to study the mechanism of calcium homeostasis in isolated cells. One limitation exists here: media with very low calcium concentrations and Mg^{2+}-free solutions can only be used.

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