Relationships between the Exchange of Calcium and Phosphate in Isolated Fat-Cells

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1. The uptake and the washout of $^{45}\text{Ca}^{2+}$ and $^{32}\text{P}_i$ is described in free fat-cells and whole epididymal fat-pads from fed rats. 2. In isolated fat-cells, the uptake of $^{45}\text{Ca}^{2+}$ proceeds with an initial rapid phase of about 1 min duration, followed by a slower subsequent accumulation. In contrast with the rapid phase, the slow phase is inhibited by 2,4-dinitrophenol, warfarin, oligomycin and verapamil, shows saturation, and presumably represents transport across the plasma membrane. 3. The washout of $^{45}\text{Ca}^{2+}$ from preloaded cells consists of a rapid (1 min) initial phase and a slow phase which is non-monoexponential, suggesting that the radioactive isotope is released from several cellular pools. 4. When P_i is omitted from the incubation medium, the slow phase of ^{45}Ca uptake is almost abolished, and the washout of ^{45}Ca from preloaded fat-cells is markedly accelerated. At elevated extracellular concentrations of P_i (2.4–6.2 mM), the uptake of ^{45}Ca is stimulated by 2–10-fold, and the release of the radioactive isotope from preloaded cells is inhibited. In whole epididymal fat-pads, variations in the extracellular concentration of P_i have no detectable effect on the uptake or the washout of ^{45}Ca . 5. In isolated fat-cells, the accumulation of $^{32}\text{P}_i$ is inhibited by 2,4-dinitrophenol or the omission of glucose from the incubation medium. In a Ca^{2+} -depleted buffer, the uptake of $^{32}\text{P}_i$ is diminished, and hyperosmolarity, which stimulates ^{45}Ca uptake, also accelerates the accumulation of $^{32}\text{P}_i$. 6. It is concluded that in free fat-cells, the uptake and release of Ca^{2+} and P_i take place by closely interrelated processes, which are dependent on mitochondrial energy production.

A wide variety of metabolic processes are markedly influenced by Ca^{2+} and this ion has been proposed to play an essential role in the hormonal control of cell function (Bygrave, 1967; Rasmussen, 1970, 1972). Studies with isolated kidney cells indicate that the exchange of Ca^{2+} and P_i are intimately linked (Borle, 1972). P_i may thus influence organic metabolism both in itself and via an effect on cellular Ca^{2+} distribution (Mansour, 1963; Kurokawa & Rasmussen, 1973).

In rat epididymal fat-pads and in isolated fat-cells, insulin and lipolytic hormones have been shown to affect both the distribution of Ca^{2+} and the metabolism of P_i (Hepp *et al.*, 1968; Akgün & Rudman, 1969; Clausen 1969*a,b*, 1970; Alm *et al.*, 1970; Martin *et al.*, 1973; Clausen *et al.*, 1974). However, since little is known about the basic characteristics of the exchange of Ca^{2+} and P_i in fat-cells, the interpretation of this type of information is difficult.

The present investigation was undertaken with the purpose of identifying mechanisms and cellular pools involved in the accumulation and release of

Ca^{2+} and P_i in fat-cells. A recently developed technique for rapid separation of isolated fat-cells from incubation medium (Gliemann *et al.*, 1972) enabled us to characterize the time-course of ^{45}Ca and ^{32}P uptake. Both of these tracers were found to be accumulated in fat-cells by an energy-requiring process. Whereas the uptake of ^{45}Ca in whole epididymal fat-pads was not affected by changes in the extracellular concentration of P_i , this ion markedly influenced the uptake and release of ^{45}Ca in isolated fat-cells. This together with the observation that the uptake of ^{32}P is influenced by the availability of Ca^{2+} in an analogous fashion, suggests that in free fat-cells the mitochondrial pool of Ca^{2+} and P_i is more readily available for exchange with the extracellular phase.

Experimental

Materials

Chemicals. Apart from the xylene and Triton X-114 used for the scintillation mixture, all chemicals were of analytical grade. Bovine serum albumin (fraction 5), collagenase (type 1), and soya-bean trypsin inhibitor (type 1s) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. ^{45}Ca (0.2 Ci/g) and $\text{NaH}_2^{32}\text{PO}_4$ (10 Ci/g) were obtained from the Danish Atomic Energy Commission Isotope Lab-

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oratory, Risø, Denmark. [U-¹⁴C]Sucrose (0.6Ci/mmol), 3-O-[¹⁴C]methylglucose (58mCi/mmol) and D-[1-³H]glucose (7.6Ci/mmol) were products of The Radiochemical Centre, Amersham, Bucks., U.K. [³H]Methoxyinulin (116mCi/g) and L-[1-¹⁴C]glucose (50mCi/mmol) were obtained from New England Nuclear Corp., Frankfurt, W. Germany.

Animals. Male Wistar rats (130–150g) bred in the laboratory and given free access to food and water were used in all experiments.

Methods

Preparation and isolation of free fat-cells. Isolated fat-cells were prepared from the epididymal fat-pads of fed Wistar rats (130–150g), as described by Rodbell (1964) with minor modifications (Gliemann, 1967). The standard incubation medium was Krebs–Ringer bicarbonate buffer containing 1.27mM-Ca²⁺, 1.2mM-P_i, 1% bovine serum albumin and 1mM-D-glucose (Cohen, 1951). Buffer containing albumin was dialysed overnight at 4°C against 10vol. of buffer without albumin before use. The cells (10–20 µl/ml of suspension) were incubated in 30ml plastic beakers immersed in a water bath at 37°C and stirred with a Teflon-coated magnet. Up to six vessels could be stirred with a single motor, ensuring identical rates of stirring in parallel incubations. The vessels were gassed with humidified CO₂+O₂ (5:95) throughout the incubations.

Sampling of fat-cells and counting of radioactivity. Cells were separated from the medium essentially as described by Gliemann *et al.* (1972). With an Eppendorf pipette, 0.2ml portions were transferred into 0.4ml soft polyethylene centrifuge tubes containing approx. 40µl (two drops) of dinonyl phthalate (density 0.98). The tubes were immediately centrifuged for 45s at 6000g in a Beckman Microfuge (catalogue no. 314 326). After centrifugation, the tube was cut through the oil layer with a scalpel, and the top section with the cell cake transferred to a counting vial containing 10ml of scintillation fluid (Anderson & McClure, 1973). The cell cake was dispersed by leaving the vials to stand for 2h, followed by agitation on a vortex mixer. Radioactivity was measured by using a Nuclear–Chicago Mark II liquid-scintillation spectrometer.

Determination of calcium content and glycerol release. Whole epididymal fat-pads, or fat-cells (30–60mg) separated by centrifugation through dinonyl phthalate, were ashed in a Beckman material oxidizer at 900°C, and after dissolution in 0.1M-HCl, the calcium content was determined by the method of von Hattingberg *et al.* (1966) by using a Zeiss PMQ II spectrofluorimeter. The recovery of calcium added to the fat-cake varied from 84 to 96%. Glycerol release was determined as described by Korn (1955).

Determination of cell volume and water spaces. A well-stirred suspension of cells was aspirated into a 1mm diameter haematocrit tube, and after centrifugation for 5min at 2000g, the ratio of cells to medium was measured. In experiments where it was difficult to ensure an even distribution of cells among parallel incubation vessels the cells were prepared in the presence of D-[1-³H]glucose so as to label the triacylglycerols. A known volume of cells was counted for radioactivity, and during subsequent procedures the ³H radioactivity of the cells was taken as a measure of cell volume.

Extracellular space was determined by using either [U-¹⁴C]sucrose, L-[1-¹⁴C]glucose, or more frequently [³H]methoxyinulin to allow the simultaneous measurement of the amount of ⁴⁵Ca or ³²P taken up by the cells. In agreement with earlier observations (Gliemann *et al.*, 1972) there was no significant difference between the results obtained by using these different markers for the determination of the volume of the extracellular phase trapped between cells during their separation. Extracellular spaces were found to vary between 0.7 and 2.1 µl/100µl of cells. In 18 experiments with different batches of cells the mean ± S.E.M. was 1.17 ± 0.08. In individual experiments the coefficient of variation did not exceed 10%. The space available to 3-O-[¹⁴C]methylglucose was taken as a measure of the total water space and the intracellular water space calculated as the difference between the total and the extracellular water spaces. Values for the intracellular space varied between 1.4 and 2.5 µl/100µl of cells, the average being 1.83.

Evaluation of separation method. The method for the separation of fat-cells has been criticized by Horn *et al.* (1973) on the grounds that the cells may be damaged by the high rate of centrifugation. We therefore decided to compare our method with that used by Horn *et al.* (1973). We found that centrifugation through silicone oil at 600g as described by Horn *et al.* (1973) resulted in an extracellular space twice as high as that found after centrifugation through dinonyl phthalate at 6000g. However, the intracellular water space and the ⁴⁵Ca space not available to L-[1-¹⁴C]glucose were shown to be identical with the two methods. Centrifugation at 6000g does not therefore appear to cause any measurable damage or squeezing of the cells.

The amount of ⁴⁵Ca present in the dinonyl phthalate was less than 0.1% of the amount taken up by the cells after 60min of incubation. In some experiments we assessed whether ⁴⁵Ca was taken up in the triacylglycerol globule. The cells were incubated for 120min with ⁴⁵Ca and after transfer to a glass tube the suspension was frozen for 15min. After thawing, the ice-cold suspension was shaken on a vortex mixer, thus completing disruption of the cells. The frozen lipids were transferred to

another vial, heated to 37°C, sucked into haematocrit tubes, and centrifuged for 3 min at 1000g. This led to the separation of the suspension into a clear oil phase (neutral lipids) and a water phase containing the same concentration of ^{45}Ca as the original incubation medium. The clear lipid phase contained less than 0.3% of the ^{45}Ca radioactivity present in the same volume of intact cells incubated for 120 min.

Experiments with whole epididymal fat-pads. Whole epididymal fat-pads were prepared from rats of the same strain and size and incubated in the same standard medium as the fat-cells. At the end of the incubation, the pads were lightly blotted on dry filter paper, weighed and homogenized in 2 ml of 5% (w/v) trichloroacetic acid. After centrifugation for 10 min at 1000g, 1 ml of the clear interphase was withdrawn for radioactivity counting in 10 ml of scintillation mixture. Control experiments showed that in this acid extract there was no preferential binding of ^{45}Ca to the lipids or to the protein sediment. Washout experiments were performed essentially as described by Clausen (1969a).

Results and Discussion

Fig. 1 shows the time-course of ^{45}Ca accumulation in whole epididymal fat-pads and in isolated fat-cells from fat-pads obtained from animals of the same size. All values have been corrected for the amount of ^{45}Ca present in the space available to extracellular markers. In isolated fat-cells there is a rapid initial phase lasting less than 2 min followed by a slow phase which approaches a plateau within 60 min. In the whole fat-pads the accumulation of ^{45}Ca reached values of about 50 ng-atoms/ml of cells. Owing to the diffusional delay in the intact tissue the initial part of the time-course of uptake in cells and fat-pads cannot be compared. It cannot be determined whether the rapid initial phase is a feature of the uptake of ^{45}Ca in whole fat-pads or not but it should be noted that the amount of ^{45}Ca taken up by isolated cells in the period of 120 min after the first minute of incubation is almost the same as that accumulated by intact fat-pads during the same period.

If all the ^{45}Ca taken up by the isolated fat-cells after 2 h was evenly distributed in the intracellular water the concentration would be 5 mM, or 3 mM if the amount taken up in the first minute is ignored. This indicates that ^{45}Ca is either precipitated or bound on the outside of the cells or accumulated inside the cell to give average concentrations in excess of the external medium (1.27 mM).

Table 1 shows the calcium content of fresh tissue measured fluorimetrically. The total amount of calcium in both freshly isolated fat-cells and in freshly excised whole fat-pads is considerably in excess of the amounts of ^{45}Ca accumulated, showing

that under these conditions only a fraction of the total calcium pool is available for exchange within 2 h.

The pattern of ^{45}Ca accumulation is qualitatively similar to that described by Borle in kidney cells (Borle, 1970). He suggested that the initial rapid phase of uptake represents binding to prosthetic groups on the cell membrane, possibly the sialo-proteins that have been found to cover the outer surfaces of a variety of mammalian cell types (Rambourg & Leblond, 1967; Borle, 1972). The slow phase of uptake was said to be representative of

Fig. 1. Time-course of ^{45}Ca accumulation in isolated fat-cells and whole epididymal fat-pads

Isolated fat-cells were incubated in Krebs-Ringer bicarbonate buffer containing 1% dialysed bovine serum albumin, 1 mM-D-glucose and $[^3\text{H}]$ methoxyinulin (1 $\mu\text{Ci/ml}$). The cells were kept in suspension by stirring with a Teflon-coated magnet, and the buffer was gassed continuously with $\text{O}_2 + \text{CO}_2$ (95:5). At zero time ^{45}Ca (0.5 $\mu\text{Ci/ml}$) was added, and samples of 200 μl were withdrawn at the intervals indicated. The cells were separated from the medium by centrifugation through oil as described in the text, and the amount of ^{45}Ca taken up in the space not available to $[^3\text{H}]$ methoxyinulin was expressed as ng-atoms/ml of cells $\pm$ S.E.M. Each point represents the mean of four observations. Whole epididymal fat-pads were incubated in the same buffer and the amount of ^{45}Ca taken up was determined by counting a trichloroacetic acid extract of the tissue. The space available to $[\text{U-}^{14}\text{C}]$ sucrose was determined in separate experiments and used for the calculation of net cellular uptake of ^{45}Ca . To allow comparison with the isolated fat-cells this was expressed as ng-atoms/ml of cells $\pm$ S.E.M. This was calculated from the wet weight of the fat-pads on the basis that 1 ml of isolated fat-cells weighs 0.9375 g. The weight of the fat-pads was corrected for the extracellular water which they contained, which was determined from the $[\text{U-}^{14}\text{C}]$ sucrose space. Each point represents the mean $\pm$ S.E.M. of four observations. ○, Isolated fat-cells; ●, isolated fat-cells + verapamil (10 μM); △, epididymal fat-pads.

transport across the plasma membrane and accumulation within the cell.

In the present study several attempts have been made to differentiate the two major phases of ^{45}Ca accumulation and to define their properties. Verapamil [5-[N-(3,4-dimethoxyphenethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile hydrochloride], which has been shown to inhibit the transport of Ca^{2+} in the heart and uterus (Fleckenstein & Grün, 1969), caused no detectable change in the rapid initial phase but clearly decreased the subsequent rise, suggesting that this represents transport across the plasma membrane (Fig. 1). Figs. 2(a) and 2(b) show the results of experiments in which the amount of ^{45}Ca taken up during the first minute and the following 14 min respectively were measured in the concentration range 0.16–1.91 mM- Ca^{2+} . The initial phase of uptake (Fig. 2a) shows a linear relationship with the concentration of Ca^{2+} consistent with a low-affinity binding. The subsequent slow phase (Fig. 2b) shows saturation at the higher concentrations of Ca^{2+} , which suggests the involvement of a carrier mechanism. At the concentration of Ca^{2+} used in the standard medium (1.27 mM) the uptake of ^{45}Ca was 47.5 ng-atoms/min per ml of cells during the rapid initial phase and 1.44 ng-atoms/min per ml of cells during the subsequent slow phase. When expressed on the basis of the area of cell surface, determined on osmium-fixed cells from rats of the same size (Gliemann & Vinten, 1974), these values correspond to 0.79 and 0.024 pg-atom/cm² respectively. The value for the slow phase is comparable with the rates of Ca^{2+}

influx determined for a wide variety of tissues (Table 2).

The slow phase of ^{45}Ca uptake was suppressed by 2,4-dinitrophenol, warfarin and oligomycin, which either uncouple oxidative phosphorylation or interfere with the accumulation of Ca^{2+} in the mitochondria (Lehninger, 1970). Omission of glucose from the incubation medium led to a significant

Table 1. *Calcium content of epididymal fat-pads and isolated fat-cells*

The calcium content was determined as described in the Experimental section. The results are corrected for the amount of calcium present in the extracellular space. In the fat-pads this was calculated by multiplying the space available to [U- ^{14}C]sucrose (0.18 ml/g wet wt.) by the plasma concentration of calcium (1.7 mM; Akgün & Rudman, 1969). In the fat-cells, the extra- and intracellular water spaces were determined by using respectively L-[1- ^{14}C]glucose and 3-O-[^{14}C]methylglucose. The results are expressed as ng-atoms/ml of cells (or per μl of intracellular water) $\pm$ S.E.M. with the number of observations in parentheses.

Preparation	Expt no.	Calcium content	
		ng-atoms/ml of cells	ng-atoms/ml of intracellular water
Whole epididymal fat-pads	I	1125 $\pm$ 140 (3)	
	II	1165 $\pm$ 290 (4)	
Isolated fat-cells	III	600 $\pm$ 78 (4)	28.6 $\pm$ 3.7 (4)
	IV	353 $\pm$ 51 (4)	18.6 $\pm$ 2.7 (4)

Fig. 2. *Rapid and slow phases of ^{45}Ca accumulation at various Ca^{2+} concentrations*

The experimental conditions were as described in the legend to Fig. 1. Isolated fat-cells were suspended in Krebs-Ringer bicarbonate buffer containing various concentrations of Ca^{2+} , ^{45}Ca was added at zero time and samples were withdrawn 1 min and 15 min later. (a) Amounts of ^{45}Ca taken up in the first 1 min; (b) amount taken up per min during the following 14 min. Each point represents the mean of three observations with bars denoting S.E.M.

Table 2. Comparison between the rates of calcium influx in various tissues and cells

Tissue	Extracellular concentration of calcium (mM)	Calcium influx (pmol/s per cm ² of cell surface)
Frog sartorius muscle (Bianchi & Shanes, 1959)	1.00	0.094 ± 0.006
Guinea-pig atria (Winegrad & Shanes, 1962)	1.25	0.016 ± 0.003
Frog heart ventricles (Niedergerke, 1963)	1.00	0.009
HeLa cells (Borle, 1969)	1.30	0.055
Kidney cells (Borle, 1970)	1.30	0.048 ± 0.006
Isolated fat-cells (present study)	1.27	0.024 ± 0.003

decrease in the total amount of ⁴⁵Ca accumulated during a 60 min period (Fig. 3). These observations support the hypothesis that the slow phase represents uptake of Ca²⁺ into the cell and that this uptake is related to energy production. None of these conditions had any effect on the amount of ⁴⁵Ca taken up in the first minute of incubation, however.

In isolated cell preparations the magnitude of ⁴⁵Ca accumulation during the initial rapid phase showed considerable variation between preparations. Since the collagenase used to disrupt the fat-pads is relatively impure and is known to contain tryptic activity it seemed possible that cleavage of peptide bonds in plasma membrane proteins might lead to an increase in the number of sites available to bind Ca²⁺. It was found that treatment with trypsin and to a lesser extent prolonged exposure to collagenase did lead to a significant rise in the capacity of the cells to accumulate ⁴⁵Ca during the first minute of exposure. This lends further support to the view that the initial rapid phase of uptake represents the interaction of Ca²⁺ with sites on the outside of the plasma membrane.

The possibility that the initial phase was related to the precipitation of Ca₃(PO₄)₂, Ca(HCO₃)₂ or CaSO₄ on the surface of the cell was also considered. The accumulation of ⁴⁵Ca was measured in buffers from which each of these ions had been omitted, and where necessary the pH was adjusted by the addition of Tris (10 mM). Under these conditions there was no significant change in the initial phase of accumulation but the following slower phase was abolished (results not shown). This observation, together with the report by Borle (1972) that P_i stimulates the accumulation of ⁴⁵Ca by kidney cells, promoted a

Fig. 3. Effect of metabolic inhibitors and the omission of glucose on the accumulation of ⁴⁵Ca in free fat-cells

Experimental conditions were as described in the legend to Fig. 1. ●, Control; ○, glucose-free buffer; □, 2,4-dinitrophenol (0.1 mM); △, warfarin (10 μM); ▲, oligomycin (0.5 μg/ml). Each point represents the mean of three observations with bars denoting S.E.M.

more detailed examination of the role of P_i in the exchange of Ca²⁺ in fat-cells.

Fig. 4 shows that when P_i was omitted from the standard incubation medium no ⁴⁵Ca accumulation occurred after the first minute of incubation. Increasing the P_i concentration above the standard value (1.2 mM) markedly stimulated the accumulation of ⁴⁵Ca, 4.8 mM-P_i producing a more than twofold increase after 1 min and a more than sixfold increase after 30–60 min. 2,4-Dinitrophenol clearly suppressed the stimulatory effect of P_i (6.2 mM) (Table 3), indicating that this is not simply the outcome of precipitation of Ca₃(PO₄)₂ on the cell surface.

In whole epididymal fat-pads, changes in the extracellular concentration of P_i (from 0 to 4.8 mM) had no detectable effect on the uptake of ⁴⁵Ca as measured after 60 or 120 min of incubation (results not shown).

In muscle cells the uptake of Ca^{2+} was found to be stimulated by hyperosmolarity (Homsher *et al.*, 1974) or by replacing extracellular Na^+ by Li^+ (Niedergerke, 1963; van Breemen *et al.*, 1973). These effects presumably reflect a favouring of the net accumulation of Ca^{2+} in the cells owing to changes in the transport across the plasma membrane. Table 3 shows that both hyperosmolarity (induced by the addition of 200mM-mannitol) and Li^+ substitution produce a stimulation of the slow phase of ^{45}Ca accumulation in isolated fat-cells.

Washout experiments with ^{45}Ca

Fig. 5 shows the time-course of ^{45}Ca washout from preloaded fat-cells during incubation in unlabelled buffer. After an initial rapid phase, which presumably represents the release of radioactive isotope from the readily exchangeable pool detected in the uptake experiments, the washout proceeds at a steadily decreasing rate, suggesting that ^{45}Ca is retained in more than one cellular pool. 2,4-Dinitrophenol, which has been shown to accelerate release of ^{45}Ca from isolated mitochondria (Lehninger, 1970), whole epididymal fat-pads (Clausen, 1970) and other tissues (Carafoli *et al.*, 1969; Case & Clausen, 1973; Clausen *et al.*, 1975), also increased the rate of ^{45}Ca washout from the isolated fat-cells.

The most striking change in the washout of ^{45}Ca , however, was produced by altering the concentration of P_i . The omission of P_i from the incubation medium led to a marked acceleration of the release of ^{45}Ca , and after 120min the cells contained only one-third of the radioactivity retained after washout in the standard medium with 1.2mM- P_i . Conversely, increasing the concentration of P_i in the medium to 6.2mM led to a decrease in the rate of washout,

which was particularly marked in the later stages of the experiment. Thus in addition to stimulating ^{45}Ca uptake in fat-cells P_i also favours ^{45}Ca retention.

Fig. 4. Effect of P_i on ^{45}Ca accumulation in isolated fat-cells

Experimental conditions were as described in the legend to Fig. 1. Fat-cells were incubated in Krebs-Ringer bicarbonate buffer modified so as to contain the concentrations of P_i indicated. Each point represents the mean of three observations with bars denoting S.E.M. ■, no P_i ; □, 1.2mM- P_i ; ●, 2.4mM- P_i ; ○, 4.8mM- P_i .

Table 3. Effects of P_i , 2,4-dinitrophenol, Na^+ substitution and hyperosmolarity on the uptake of ^{45}Ca in isolated fat-cells

Experimental conditions were as described in the legend to Fig. 1. The amount of ^{45}Ca taken up in the space not available to [^3H]methoxyinulin during the slow phase of accumulation (1–15 min after the onset of exposure to the radioactive isotope) was determined and is expressed as ng-atoms/min per ml of cells $\pm$ S.E.M. with the numbers of observations indicated in parentheses.

Expt. no.	Additions and modifications of the incubation medium	^{45}Ca uptake (ng-atoms/min per ml of cells)	P
I	Krebs-Ringer bicarbonate buffer (1.2mM- P_i)	1.76 ± 0.32 (4)	<0.001
	Krebs-Ringer bicarbonate buffer (1.2mM- P_i), 2,4-dinitrophenol (0.1mM)	0.0 (4)	
	Krebs-Ringer bicarbonate buffer (6.2mM- P_i)	16.6 ± 1.6 (4)	<0.001
	Krebs-Ringer bicarbonate buffer (6.2mM- P_i), 2,4-dinitrophenol (0.2mM)	6.7 ± 0.8 (4)	
II	Krebs-Ringer bicarbonate buffer (1.2mM- P_i)	1.38 ± 0.35 (3)	<0.05
	Krebs-Ringer bicarbonate buffer (1.2mM- P_i). NaCl replaced by LiCl	2.46 ± 0.30 (3)	
III	Krebs-Ringer bicarbonate buffer (1.2mM- P_i)	1.58 ± 0.22 (4)	<0.01
	Krebs-Ringer bicarbonate buffer (1.2mM- P_i) with 200mM-mannitol	2.87 ± 0.17 (4)

Fig. 5. Effect of P_i and 2,4-dinitrophenol on the time-course of ^{45}Ca washout

Isolated fat-cells were prepared in the presence of D-[1- ^3H]-glucose to label the triacylglycerols and provide a measure of cell volume as described in the Experimental section. After washing in the usual manner cells were loaded with ^{45}Ca by incubation for 60 min in Krebs-Ringer bicarbonate buffer (200 μl of cells/ml of medium) containing 1 mM-D-glucose, 1.2 mM- P_i , 1.27 mM- Ca^{2+} and 2 μCi of ^{45}Ca /ml. At the end of this period a sample of cells was taken to determine the ^{45}Ca content as described in the Experimental section and legend to Fig. 1. The cells were then packed by centrifugation and the medium was aspirated off. The cells were resuspended in unlabelled buffer (30 μl of cells/ml of medium) containing no P_i , 1.2 mM- P_i , 1.2 mM- P_i + 0.1 mM-2,4-dinitrophenol or 6.2 mM- P_i as indicated. The subsequent incubation and sampling were as described in the legend to Fig. 1. Each point represents the mean of three observations with bars indicating S.E.M. ○, no P_i ; ●, 1.2 mM- P_i ; ■, 1.2 mM- P_i + 0.1 mM-2,4-dinitrophenol; □, 6.2 mM- P_i .

In whole epididymal fat-pads, however, it was not possible to detect any significant effect of P_i (0–4.8 mM) on the washout of ^{45}Ca (results not shown).

It has been suggested that in epididymal fat-pads, the uptake of ^{45}Ca is markedly dependent on the rate of lipolysis (Akgün & Rudman, 1969; Alm *et al.*, 1970), and extracellular concentrations of P_i higher than 20 mM were reported to stimulate lipolysis (Mosinger & Vaughan, 1967). It was therefore important to determine whether lipolysis was influenced by the concentrations of P_i used in the present experiments. In experiments where the ^{45}Ca uptake was suppressed by the omission of P_i or

Fig. 6. Effect of EGTA and 2,4-dinitrophenol on the accumulation of $^{32}\text{P}_i$ in isolated fat-cells

Experimental conditions were as described in the legend to Fig. 1. At zero time $^{32}\text{P}_i$ (1 $\mu\text{Ci}/\text{ml}$) was added, and samples were withdrawn at the intervals indicated. The amount of ^{32}P accumulated in the space not available to [^3H]methoxyinulin is expressed as nmol/ml of cells, with bars indicating S.E.M. Each point represents the mean of three observations. ○, Ca^{2+} (1.27 mM); ●, no Ca^{2+} + EGTA (0.5 mM); □, Ca^{2+} (1.27 mM), 2,4-dinitrophenol (0.1 mM); ■, no Ca^{2+} + EGTA (0.5 mM) + 2,4-dinitrophenol (0.1 mM).

increased by high P_i (4.8 mM) the rate of lipolysis was unchanged (0.2 $\mu\text{mol}/\text{h}$ per ml of cells). The glycerol release induced by noradrenaline (1 $\mu\text{g}/\text{ml}$) was the same at all the concentrations of P_i tested (0, 1.2 and 4.8 mM), i.e. $4.1 \pm 0.14 \mu\text{mol}/\text{h}$ per ml of cells (mean $\pm$ S.E.M. of 12 observations). Thus the cells showed a normal lipolytic response and it is unlikely that the effect of P_i on the uptake and release of ^{45}Ca is related to changes in the rate of lipolysis.

Measurements of ^{32}P uptake

Fig. 6 shows the time-course of ^{32}P accumulation in fat-cells. After a rapid initial uptake which is not modified by 2,4-dinitrophenol or by the concentration of Ca^{2+} in the medium, the label is accumulated at an approximately linear rate for about 30 min. After 60 min of incubation, the concentration of ^{32}P in the water space not available to [^3H]methoxyinulin was 15 times that of the incubation medium. The

Table 4. *Effect of 2,4-dinitrophenol, glucose and hyperosmolarity on the accumulation of $^{32}\text{P}_i$ in fat-cells and fat-pads*

Isolated fat-cells or whole epididymal fat-pads were incubated as described in the legend to Fig. 1. The fat-cells were sampled 1 and 15 min after the onset of exposure to $^{32}\text{P}_i$ and the amount of ^{32}P taken up during this 14 min expressed as nmol/min per ml of cells. Epididymal fat-pads were incubated for 30 or 60 min in buffer containing both $^{32}\text{P}_i$ and $[\text{U-}^{14}\text{C}]\text{sucrose}$. The amount of ^{32}P taken up in the space not available to $[\text{U-}^{14}\text{C}]\text{sucrose}$ is expressed as nmol/min per ml of cells. The results are given as means $\pm$ S.E.M. with the numbers of observations in parentheses.

Preparation and incubation interval	Additions	^{32}P taken up (nmol/min per ml of cells)	P
Isolated fat-cells (1–15 min)	Control (glucose-free)	5.4 ± 0.6 (3)	
	1 mM-D-glucose	10.9 ± 0.5 (3)	<0.01
	1 mM-D-glucose + 2,4-dinitrophenol (0.2 mM)	2.6 ± 0.2 (3)	<0.01
	1 mM-D-glucose + 200 mM-mannitol	20.0 ± 1.5 (3)	<0.001
Fat-pads (0–60 min)	Control (glucose-free)	1.83 ± 0.2 (4)	
	2,4-dinitrophenol (0.5 mM)	0.70 ± 0.08 (4)	<0.005
	Mannitol (200 mM)	8.97 ± 1.12 (4)	<0.001
	Mannitol (200 mM) + 2,4-dinitrophenol (0.5 mM)	1.50 ± 0.17 (4)	

accumulation was clearly decreased by the omission of Ca^{2+} together with the addition of EGTA [ethanedioxybis(ethylamine)tetra-acetate] or the addition of 2,4-dinitrophenol (0.1 mM). The combination of these two modifications virtually abolished the late phase of ^{32}P uptake. This indicates that the accumulation of ^{32}P requires energy and is to some extent dependent on the availability of Ca^{2+} . The omission of glucose from the incubation medium led to some decrease in the ^{32}P uptake and when the accumulation of Ca^{2+} was stimulated by incubation in a hyperosmolar medium, considerably more ^{32}P was taken up (Table 4).

In whole epididymal fat-pads accumulation of ^{32}P was inhibited by 2,4-dinitrophenol (0.5 mM), and stimulated by hyperosmolarity induced by the addition of mannitol (200 mM). It should be noted that the rate of accumulation of ^{32}P in whole pads was markedly lower than the rate in isolated fat-cells (Table 4).

General discussion

The results presented support the view that in common with other cell types (Borle, 1970, 1972) the uptake of ^{45}Ca by isolated fat-cells consists of a rapid phase representing low-affinity binding to the surface of the cell and a slow phase representing a carrier-mediated transport across the cell membrane. The slow phase appears to represent exchange with more than one intracellular pool.

Possible sites of accumulation of Ca^{2+} within the cell are binding sites on membrane surfaces and proteins, the endoplasmic reticulum, which has been shown by Siddle *et al.* (1973) to accumulate Ca^{2+} , and the mitochondria, which are capable of

accumulating large amounts of Ca^{2+} (Severson *et al.*, 1974). The results of the present study support the view that accumulation into cellular organelles, particularly the mitochondria, may have a dominant role in the exchange of ^{45}Ca . The capacity of the mitochondria to accumulate and retain Ca^{2+} is dependent on energy production or the availability of ATP. When this was impaired by the addition of 2,4-dinitrophenol or other uncouplers the accumulation of ^{45}Ca was inhibited in both whole fat-pads and isolated cells. Conversely the release of ^{45}Ca from preloaded pads and cells was stimulated by uncouplers.

Studies with mitochondria isolated from a number of tissues including fat-cells have shown that the accumulation of Ca^{2+} by mitochondria is dependent on the availability of P_i (Lehninger, 1974; Severson *et al.*, 1974). Isolated fat-cell mitochondria in the presence of P_i and SO_4^{2-} can accumulate Ca^{2+} to concentrations of 200 ng-atoms/mg of protein. This is equivalent to approx. 800 ng-atoms/ml of cells, which is sufficient to account for the total fat-cell content of calcium (Severson *et al.*, 1974). Borle (1972, 1973) and Kurokawa & Rasmussen (1973) have suggested that the availability of P_i in the cytoplasm may be important in determining the Ca balance of the cell and the ability of the cell to accumulate Ca. The results of the present study give support to this view since the accumulation and retention of ^{45}Ca in isolated fat-cells has been shown to be markedly dependent on the concentration of P_i in the medium. The accumulation of $^{32}\text{P}_i$ is also dependent to a smaller extent on the presence of Ca^{2+} in the medium. $^{32}\text{P}_i$ is known to be rapidly incorporated into organic phosphates, in particular nucleoside phosphates (Hepp *et al.*, 1968), phospho-

lipids, and presumably the mitochondria as $\text{Ca}_3(\text{PO}_4)_2$. The inhibition of the mitochondrial accumulation of P_i provides the most obvious explanation of the decrease in ^{32}P uptake seen in the absence of Ca^{2+} but it cannot be excluded that the relative rates of synthesis and degradation of ATP are directly influenced by Ca^{2+} in a manner which might in part account for the observed changes. However, the observation that hyperosmolarity stimulates the accumulation of both ^{32}P and ^{45}Ca in both whole pads and isolated cells gives further support for the hypothesis that Ca^{2+} and P_i are interrelated.

There is a striking discrepancy between the results obtained with isolated fat-cells and with whole fat-pads in that in pads the accumulation and release of ^{45}Ca is not affected by changes in the medium P_i concentration. A possible explanation of this is that the process of collagenase digestion used in isolating the fat-cells leads to marked changes in the accessibility of the cytoplasm to external P_i . This view is supported by the observation that the rate of ^{32}P accumulation in isolated cells is markedly higher than in whole fat-pads. It appears that the uptake and release of ^{45}Ca and ^{32}P in fat-cells takes place by a closely interdependent process and that the mitochondria play a dominant role in determining the distribution of Ca^{2+} and P_i within the cell. In the whole fat-pads an interrelationship between Ca^{2+} and P_i distribution is indicated by the stimulation of both ^{45}Ca and ^{32}P uptake in the presence of 200mM-mannitol or if the medium Na^+ is substituted by Li^+ , but the rate of transport of these ions across the cell membrane may exert a much greater influence than in the isolated cell.

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