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Ruthenium Red-insensitive calcium transport in ascites-sarcoma 180/TG cells

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1. Ruthenium Red-insensitive Ca^{2+} transport in the mouse ascites sarcoma 180/TG is enriched in a 'heavy' microsomal fraction (microsomes) sedimented at 35000 **g** for 20min. The subcellular distribution of this Ca^{2+} transport differed from that of Ruthenium Red-sensitive Ca^{2+} transport and $(Na^+ + K^+)$-dependent ATPase activity, but was similar to that of glucose 6-phosphatase. 2. The affinity of this transport system for 'free' Ca^{2+} is high (K_m approx. 6μM) and that for MgATP somewhat lower (K_m approx. 100μM). Ca^{2+} transport by the tumour microsomes, by contrast with that by liver microsomes, was greatly stimulated by low concentrations of P_i. 3. Although incubation of intact ascites cells with glucagon led to an increase in intracellular cyclic AMP, no stable increase in the initial rate of Ca^{2+} transport in the subsequently isolated 'heavy' microsomes could be detected as in similar experiments carried out previously with rat liver cells. Reconstitution experiments suggest that a deficiency exists in the tumour microsomal membrane such that an action of glucagon that is normally present in rat liver microsomes is not evoked.

Much evidence suggests that a redistribution of cytoplasmic Ca^{2+} is an important event in the action of a number of hormones and in many aspects of the regulation of cell metabolism (Rasmussen & Goodman, 1977). It is therefore potentially of some clinical significance that a range of tumour tissues, whose patterns of metabolic regulation are considered to be aberrant, reportedly contain abnormally high concentrations of Ca^{2+} (see, e.g., Lanoue *et al.*, 1974). Moreover, mitochondria isolated from a range of tumour tissues are known to be resistant to the uncoupling action of high concentrations of Ca^{2+} (for a review see Bygrave, 1981). These observations provide the basis for suggesting that mechanisms involving control by mitochondria of intracellular Ca^{2+} differ between normal and tumour tissue (Bygrave, 1976).

A redistribution of intracellular Ca^{2+} in non-muscle tissues may also occur through the action of the system located in a fraction derived from the rough endoplasmic reticulum (Reinhart & Bygrave, 1981). The insensitivity of this latter system to Ruthenium Red readily distinguishes it from the mitochondrial Ca^{2+}-transport system. Ruthenium Red-insensitive transport may be considered to be of physiological significance in liver tissue, because of (*a*) the rapidity with which Ca^{2+} is sequestered by the microsomal vesicles and the affinity for free Ca^{2+} (Bygrave, 1978) and (*b*) the observation that the system is subject to hormonal control (Bygrave & Tranter, 1978; Waltenbaugh & Friedmann, 1978; Reinhart & Bygrave, 1981) and undergoes marked changes in activity in the perinatal period of liver development (Bygrave & Tranter, 1978; Reinhart & Bygrave, 1981).

In light of these features and of the above-mentioned findings that tumour mitochondria handle Ca^{2+} *in vitro* differently from rat liver mitochondria, we decided to investigate the properties of Ruthenium Red-insensitive Ca^{2+} transport in fractions isolated from a rapidly growing ascites tumour. We wished principally to determine whether the mechanism of hormonal control of such transport, described in rat liver, exists in the tumour.

Experimental

Ascites-tumour cells

The material used in these studies was ascites-sarcoma 180/TG cells. The tumour was propagated by weekly intraperitoneal inoculation of 0.1ml of ascitic fluid into male mice of a mixed laboratory strain. After 10–18 days mice were killed by cervical fracture and the ascitic fluid was allowed to

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drain from an abdominal incision into a conical flask prewashed with 250mM-sucrose/1mM-EDTA, pH6.8 (Thorne & Bygrave, 1973). A reasonable yield of cell protein could be obtained from two or three mice, from which was drained a total volume of approx. 20ml of creamy-coloured ascitic fluid. The cells were packed by low-speed centrifugation in a bench centrifuge, and the cell-free ascitic fluid was drawn off with a vacuum aspirator and discarded. They were then washed four times in the wash medium by resuspension and centrifugation. Care was taken when resuspending not to disturb any of the packed erythrocytes. Histological analysis of three representative preparations with May–Grünwald strain revealed that $98 \pm 0.5\%$ of cells present were tumour cells. The mean viability of seven different preparations of tumour cells determined with Trypan Blue strain was 93–100%.

Preparation of cell fractions

The washed cells were resuspended in 4vol. of 5mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH6.8, and homogenized with 15 vigorous up-and-down strokes of a Dounce tissue homogenizer. An equivalent volume of ice-cold hyperosmotic medium (500mM-sucrose/5mM-Hepes/1mM-EGTA/1mM-dithiothreitol, pH 6.8) was then added. All manipulations were performed at 0–4°C. In the experiments presented in Table 1, where the supernatant fraction was added to a Ca^{2+}-transport-assay medium, EGTA was omitted from the hyperosmotic medium.

The crude homogenate was centrifuged at 300*g* for 5min in an SS-34 rotor of a Sorvall RC-2B refrigerated centrifuge, to remove any unbroken cells. The resulting supernatant was then fractionated by differential centrifugation into six fractions exactly as described by Reinhart & Bygrave (1981) for liver tissue. The centrifugation forces and times employed were 1085*g* for 5min (fraction 1), 4340*g* for 5min (fraction 2), 7710*g* for 10min (fraction 3), 34888*g* for 20min (fraction 4) and 105000*g* for 60min (fraction 5). The supernatant obtained at this last step was designated fraction 6. All pellets were resuspended in 250mM-sucrose/5mM-Hepes/1mM-dithiothreitol/10mM-KCl (pH6.8) by three or four gentle up-and-down strokes of a Thomas homogenizer (size AA). In experiments where it was necessary to measure total protein recovery, the volume of the homogenate and each of the fractions was measured. The protein content of each sample was measured by the method of Lowry *et al.* (1951). Crystalline bovine serum albumin (Sigma fraction 5) was used for the preparation of the standard.

Incubation of tumour cells in vivo with glucagon or cyclic AMP

Tumour cells were resuspended (5%, w/v) in Krebs–Henseleit bicarbonate buffer pregassed with O_2/CO_2 (19:1) and the tightly capped incubation vessel was placed in a 37°C water bath. After a 5min preincubation with continuous shaking, the appropriate agent was added and the agitation of the vessel resumed. When the concentration of cyclic AMP was to be measured, samples (0.5ml) were removed at specific intervals, placed in 1ml of boiling distilled water and boiled for a further 10min with intermittent agitation to aid dispersal of cell debris. Most of the denatured proteins were pelleted by centrifugation in an Eppendorf Microfuge for 4min. When Ca^{2+} transport was to be measured, the incubation was stopped after 10min by low-speed centrifugation in a bench centrifuge. The incubation medium was drawn off with a vacuum aspirator and discarded. The cell pellet was then homogenized and fraction 4 isolated as described above.

$^{45}Ca^{2+}$ transport

Initial rates of Ca^{2+} transport were measured, by using $^{45}CaCl_2$ and the Millipore-filtration technique exactly as described by Reinhart & Bygrave (1981). The medium contained 100mM-KCl, 20mM-Hepes, 2mM-P_i, 3.3mM-ATP, 3.3mM-$MgCl_2$, 1.4μM-Ruthenium Red and 0.5–1mg of protein in a final volume of 1.5ml. The pH was 6.8 and the temperature 37°C. The reaction was initiated by adding 50nmol of $CaCl_2$, containing 0.3μCi of ^{45}Ca.

Measurement of the effects of addition of cyclic AMP in vitro on Ca^{2+} uptake

Experiments involving the addition of cyclic AMP to fraction 4 *in vitro* and the subsequent assay of Ca^{2+}-transport activity were performed as follows. Between 0.5 and 1mg of protein from fraction 4 was placed in 1.4ml of incubation medium containing 100mM-KCl, 20mM-Hepes, 5mM-ammonium oxalate, 0.5mM-3-isobutyl-1-methylxanthine, 0.1% trypsin inhibitor, 0.5mM-ATP, and a volume of fraction 6 containing approx. 3mg of protein. The final pH of this medium was 7.4. For some experiments fraction 6 was replaced by EGTA-free isolation medium. This medium was allowed to preincubate at 25°C for 7min before addition of dibutyryl cyclic AMP (final concn. 50μM). The incubation vessel was immediately transferred to a 37°C water bath and allowed to incubate for 1min before the addition of ATP (final concn. 3.3mM), 4μl of 2M-HCl and 50nmol of $CaCl_2$ containing 0.3μCi of $^{45}Ca^{2+}$. Samples were taken then for measurement of the initial rate of Ca^{2+} transport.

Enzyme assays

$(Na^+ + K^+)$-dependent ATPase was determined

by measuring ouabain-sensitive P_i production from ATP (Wilson, 1978, Evans, 1979).

Glucose 6-phosphatase was assayed by a method suitable for the measurement of low enzyme activity (K. C. Reed, personal communication). Subcellular fractions (2.5 ml, containing 3.5 mg of protein) were activated by mixing with 0.31 ml of ammonia solution (1 M-NH_3 adjusted to pH 10.3 with 2 M-HCl) and overnight freezing. The suspensions were then thawed and adjusted to pH 6.5 with 1 M-maleic acid. The reaction was started by addition of 2 ml of incubation medium (60 mM-sodium cacodylate adjusted to pH 6.5 with 1 M-maleic acid, 5.5 mM-EDTA, 10 mM-tartaric acid, 20 mM-glucose 6-phosphate) and incubated at 37°C with continuous shaking. Samples (0.4 ml) were removed at successive 10 min intervals into 1 ml of 14% (w/v) trichloroacetic acid, vigorously mixed and centrifuged for 2 min in an Eppendorf Microfuge. Samples (1 ml) were then assayed for the presence of P_i. The activity of the glucose 6-phosphatase was determined by the amount of P_i released /h per mg of protein. P_i was measured by the procedure of Baginski *et al.* (1967). Cytochrome *c* oxidase was assayed by the method of Tolbert (1974).

Materials

Glucagon was kept under nitrogen until placed into solution, when it was dissolved in 1 mM-HCl. 3-Isobutyl-1-methylxanthine, kindly given by Dr. N. H. Hunt of the Australian National University, was dissolved in equal volumes of ethanol and 1 mM-KOH. Membrane filters were obtained from Gelman Instrument Co., Ann Arbor, MI, U.S.A. All fine chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All reagents used were of analytical grade.

Results and discussion

Subcellular distribution of Ruthenium Red-insensitive Ca^{2+} transport in ascites-sarcoma 180/TG cells

We have shown previously that Ruthenium Red-insensitive Ca^{2+} transport in rat liver co-fractionates with glucose 6-phosphatase activity in vesicles that sediment at approx. 35000 *g* for 20 min (Bygrave & Tranter, 1978; Reinhart & Bygrave, 1981). The available evidence indicates that these 'heavy' vesicles are derived from the rough endoplasmic reticulum (Reinhart & Bygrave, 1981). By using a subfractionation procedure identical with that employed for rat liver (Reinhart & Bygrave, 1981), the subcellular distribution of Ruthenium Red-insensitive Ca^{2+} transport was measured in the ascites cells and compared with Ruthenium Red-sensitive Ca^{2+} transport, glucose 6-phosphatase and ouabain-sensitive $(Na^+ + K^+)$-dependent ATPase activities. The latter enzymes reflect the presence of microsomal and plasma membranes respectively (see, e.g., Fleischer & Kervina, 1974).

Fig. 1 shows that, as in experiments with rat liver, the initial rate of Ruthenium Red-insensitive Ca^{2+} transport, which is attributable to mitochondria (see Ash & Bygrave, 1977), is enriched in fraction 3 of the ascites cells (Fig. 1*a*). This was confirmed by experiments (results not shown) demonstrating that the distribution of cytochrome oxidase was identical with that of mitochondrial Ca^{2+} transport in these fractions. The distribution of $(Na^+ + K^+)$-dependent ATPase differs sufficiently from that of Ruthenium Red-insensitive Ca^{2+} transport to allow the conclusion that this Ca^{2+} transport is not located in the plasma membranes. This conclusion was reached also in the experiments undertaken with rat liver (Reinhart & Bygrave, 1981).

Thus it seems from these data that, as in liver, Ruthenium Red-insensitive Ca^{2+} transport is associated with the 'heavy' vesicles derived from the endoplasmic reticulum.

Properties of Ruthenium Red-insensitive Ca^{2+} transport in fraction 4 from ascites-tumour cells

Effect of permeant weak acids. The permeant weak acid oxalate stimulates the transport of Ca^{2+} into microsomal vesicles isolated from many tissues (Martonosi, 1972). A similar effect was demonstrated in the present study, with near-maximal stimulation of the initial rate of Ca^{2+} transport occurring at 2 mM-oxalate (results not shown).

Fig. 2 shows the influence of the permeant weak acid P_i on the initial rate of Ruthenium Red-insensitive Ca^{2+} transport. Whereas P_i had no effect on Ca^{2+} transport by fraction 4 isolated from rat liver, 5 mM-P_i potentiated by up to 500% the initial rate of Ruthenium Red-insensitive Ca^{2+} transport by the corresponding fraction from the ascites cells. P_i (5 mM) has been shown to stimulate only slightly the initial rate of Ca^{2+} transport by rabbit myometrial microsomal fraction (Batra, 1978).

Affinity for Ca^{2+} and for MgATP. The affinity for free Ca^{2+} was determined from initial-rate experiments (results not shown) and calculated to be 6 μM, a value only marginally greater than the 1–2 μM seen with liver microsomal fraction (Bygrave, 1978). The K_m of 6 μM was the same whether P_i or oxalate was the permeant weak acid. The affinity for MgATP was calculated from initial-rate experiments (results not shown) to be approx. 0.1 mM, as compared with 0.5 mM in the liver experiments (Bygrave, 1978). Again the K_m was unaffected by the nature of the permeant weak acid. Maximal stimulation of approx. 300% occurred at 0.25 mM-MgATP, and higher concentrations were inhibitory.

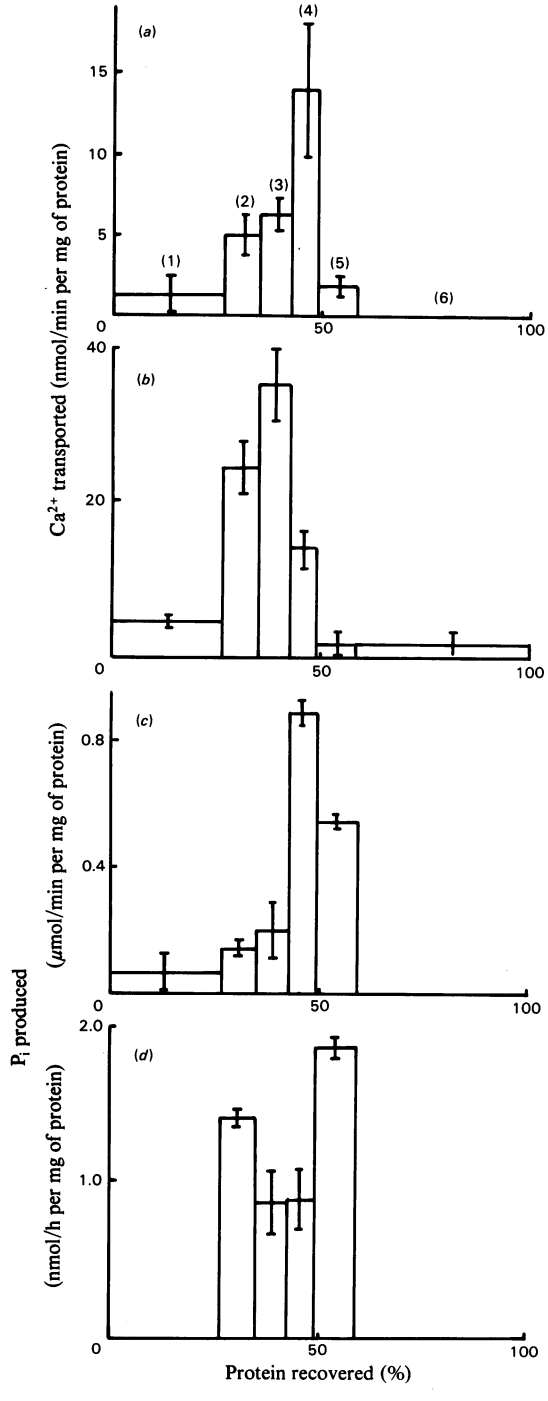

Fig. 1. *Subcellular distribution of Ruthenium Red-insentive (a) and -sensitive (b) Ca^{2+} transport, glucose 6-phosphatase (c) and ($Na^+ + K^+$)-dependent ATPase (d) in fractions isolated from ascites-tumour cells*

Subcellular fractions were isolated from the tumour cells as described in the Experimental section, and Ca^{2+} transport and the indicated enzymes assayed as described also therein. Values shown are means ± S.E.M. for three separate experiments. The numbers in parentheses (1–6) refer to the six fractions defined in the Experimental section. In (*a*) Ruthenium Red was present at 1.4 μM.

Studies to assess the influence of glucagon on Ruthenium Red-insensitive Ca^{2+} transport in ascites-tumour cells

An important feature of Ruthenium Red-insensitive Ca^{2+} transport in liver tissue is that its activity can be enhanced when measured *in vitro* after the exposure of intact liver *in situ* (Bygrave & Tranter, 1978; Reinhart & Bygrave, 1981; Waltenbaugh & Friedmann, 1978) or hepatocytes (Taylor *et al.*, 1979, 1980) to glucagon. The effects may

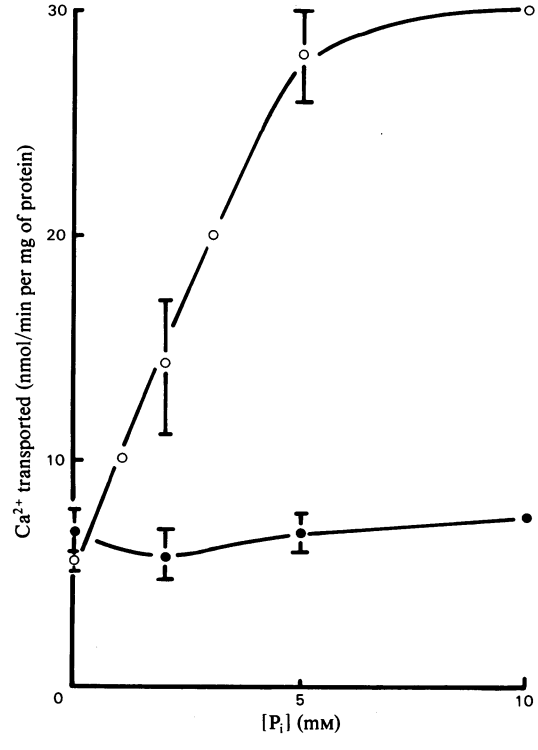

Fig. 2. *Effect of varying the P_i concentration on the initial rate of Ruthenium Red-insensitive Ca^{2+} transport in fraction 4 isolated from rat liver (●) and ascites-tumour cells (○)*

Experiments were performed as described in the Experimental section, except that the indicated concentrations of P_i replaced oxalate in the incubation medium. Values with bars are means ± S.E.M. for at least three experiments performed in triplicate. Those without bars are means of two experiments.

Table 1. *Stimulation of Ruthenium Red-insensitive Ca^{2+} transport by dibutyryl cyclic AMP in a reconstituted system*
Subcellular fractions were prepared from liver or tumour tissue as described in the Experimental section. Incubations contained the indicated combinations of fraction 6 (approx. 3 mg of protein) and fraction 4 (approx. 1 mg of protein) and were carried out as described in the Experimental section, except that 5 mM-oxalate was used in place of 2 mM-P_i. Where shown, results are means ± S.E.M. for three experiments performed in triplicate; others are results of one experiment performed in triplicate.

Tissue from which membrane fraction was prepared	Tissue from which supernatant fraction was prepared	Stimulation of the initial rate of Ca^{2+} transport induced by cyclic AMP (%)
Liver	Liver	44 ± 5
Liver	Tumour	55
Liver	Isolation medium	0
Tumour	Liver	0 ± 5
Tumour	Tumour	0

involve the action of cyclic AMP-dependent protein kinase(s) (Taylor *et al.*, 1980) and are seen to be of physiological importance in the control of cell Ca^{2+} (Reinhart & Bygrave, 1981).

In view of the apparent imbalance in the regulation of Ca^{2+} concentrations in tumour tissues (see the introduction), we investigated the ability of glucagon to induce stable changes to Ruthenium Red-insensitive Ca^{2+} transport in the ascites-tumour cells. The initial experiments involved incubating the intact cells with glucagon for different times, then sedimenting the cells, isolating fraction 4 (see the Experimental section) and assaying for Ruthenium Red-insensitive Ca^{2+} transport exactly as done previously with hepatocytes (Taylor *et al.*, 1979). No enhancement of Ca^{2+} transport was observed in any of the ten experiments performed (results not shown). In some experiments the concentration of cyclic AMP was measured after incubation of ascites cells with 1 μM-glucagon. A 255% increase in cyclic AMP concentration was detected after 1.5 min (from 2.9 ± 0.3 to 7.4 ± 1.8 pmol of cyclic AMP/10^6 cells: S.E.M., n = 3) and a 275% increase after 4 min (from 3.3 ± 0.3 to 9.0 ± 1.6 pmol of cyclic AMP/10^6 cells; $n = 3$). This extent of increase is similar to that observed with hepatocytes (Taylor *et al.*, 1980) and indicates the existence of a fully functional glucagon-sensitive adenylate cyclase in plasma membrane of these ascites-tumour cells. Thus, despite an ability to increase the intracellular concentrations of cyclic AMP, glucagon appeared unable to induce any increase in Ruthenium Red-insensitive Ca^{2+} transport.

Further information on this apparent lesion was obtained by making use of the prior demonstration in our laboratory that, in a reconstituted system, co-incubation of fractions 4 and 6 from rat liver with cyclic AMP and ATP leads to a stimulation in the initial rate of Ca^{2+} transport (Taylor *et al.*, 1980; Reinhart & Bygrave, 1981). Data in Table 1 confirm this observation for liver and show also that fraction 6 from the ascites cells is as effective as that from rat liver in bringing about such a stimulation. However, replacement of fraction 4 from rat liver with that from the tumour does not result in any stimulation of Ruthenium Red-insensitive Ca^{2+} transport. It thus appears from these reconstitution experiments that the lesion lies at a site in or on a component present in the endoplasmic reticulum in the tumour cells involved in the transport of Ca^{2+}.

Concluding comments

Experiments in this study provide new information about the regulation of Ca^{2+} in tumour tissue. They reveal (*a*) the existence of a Ca^{2+}-transport system insensitive to inhibition by Ruthenium Red and thus not attributable to mitochondria and (*b*) that many of the properties of this system, including subcellular location, affinity for Ca^{2+} and requirement for MgATP, are similar to those previously described for rat liver.

Two significantly different properties also are revealed: (*a*) the potentiation by P_i of the initial rate of Ca^{2+} transport and (*b*) the inability of glucagon to induce a stable increase in Ca^{2+} transport after its incubation with ascites cells. It remains to be determined whether these apparent differences are common to tumour tissues in general and whether they contribute to the abnormally high concentrations of Ca^{2+} seen in such tissues.

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