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Sodium-induced calcium release from mitochondria in brown adipose tissue

(nonshivering thermogenesis/brown adipocytes/Ca^{2+} ionophore A23187/monensin)

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ABSTRACT **Coupled mitochondria of brown adipose tissue can accumulate Ca^{2+} if a substrate is present. The Ca^{2+} is released by addition of 20 mM Na^+, but not by addition of K^+ or choline$^+$. Energy-dissipating Na^+-induced Ca^{2+} cycling occurs maximally with 20 mM Na^+ and 10 μM Ca^{2+}. In brown adipocytes, the Ca^{2+} ionophore A23187 and the Na^+ ionophore monensin increase respiration if substrate is added, and incubation in a low-Na^+ buffer decreases norepinephrine-induced respiration. Thus Na^+-induced Ca^{2+} release takes place in brown adipose tissue; released Ca^{2+} could have a regulatory or thermogenic role or both.**

The initiator of thermogenesis in brown adipose tissue, norepinephrine, induces a persistent depolarization of the cellular membrane potential (1) and an increased membrane conductance (2). This increased conductance is presumably reflected in an entry into the cell of extracellular sodium, and thus in an increased cytosolic sodium concentration.

Carafoli and coworkers (3–5) have shown that Na^+ leads to an efflux of sequestered Ca^{2+} from mitochondria isolated from some sources (e.g., heart, brain) but not from others (e.g., liver, kidney). They suggest that this Na^+-induced Ca^{2+} efflux may be involved in regulating the intracellular Ca^{2+} concentration in the Na^+-responsive tissues. They have also demonstrated that, when this process is coupled to Ca^{2+} uptake, Ca^{2+} cycling occurs, which leads to energy dissipation (4, 5).

When mitochondria of brown adipose tissue are transferred to an energy-conserving state, they are capable of taking up Ca^{2+} from the external medium (6). We have investigated the nature of this uptake in isolated mitochondria and also the possible influence of Na^+ on Ca^{2+} efflux. Further, we have studied the effect of these ions on isolated brown adipocytes to see if they could have a possible regulatory role in thermogenesis.

MATERIALS AND METHODS

Brown Fat Mitochondria. These mitochondria were prepared from the pooled brown adipose tissue of cold-adapted (3 weeks at 5°C) golden hamsters (*Mesocricetus auratus*), essentially as described (7), except that the isolation medium was 250 mM sucrose/20 mM Tris·HCl/0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA)-/0.1% bovine serum albumin (fatty acid free), pH 7.2. Albumin was not included in the final washing medium, or in the suspension medium. Protein was determined by the biuret method (8).

Brown Adipocytes. Cells were isolated by collagenase digestion in Krebs–Ringer phosphate buffer of the pooled brown adipose tissue of golden hamsters maintained at 21°C, as described (9, 10).

Calcium Uptake and Release. Uptake and release were followed in a dual-wavelength spectrophotometer (Aminco-DW-2) by using arsenazo III {2,2′-[1,8-dihydroxy-3,6-bisulfo-2,7-naphthalene-bis(azo)]-dibenzenearsonic acid} as indicator at 675–685 nm with a 3-nm slit, essentially under conditions described by Harris (11). The medium was 125 mM sucrose (batch-purified with Dowex 50 WX8, H^+ form)/20 mM Tris·HCl, pH 7.2, and contained 2 μM rotenone, 1 mM GDP, 0.1% bovine serum albumin (fatty acid free), and 5 mM L-glycerol 3-phosphate, dicyclohexylammonium salt. Mitochondria were added to give a concentration of 0.5 mg of protein per ml and the volume was 2 ml.

Oxygen Consumption. Consumption was monitored polarographically with a Yellow Springs Instrument 4004 Clark oxygen probe. Oxygen tension and rate of oxygen consumption were recorded simultaneously. Conditions are described in legends to figures.

Chemicals. Crude collagenase and arsenazo III (grade 1) were obtained from Sigma; carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) from Pierce Eurochemie (Rotterdam, Holland); fatty-acid-free bovine serum albumin, fraction V, from Miles; L-glycerol 3-phosphate, dicyclohexylammonium salt, from Boehringer Mannheim. The Ca^{2+} ionophore A23187 and the Na^+-ionophore monensin were gifts from Eli Lilly (Stockholm, Sweden).

RESULTS AND DISCUSSION

Ca^{2+} uptake and release in brown adipose tissue mitochondria

Serum albumin and purine nucleotides transfer mitochondria from brown adipose tissue from the state of low energy conservation in which they are isolated to a state of high energy conservation (for review see ref. 12). If the mitochondria are then provided with a substrate, they take up Ca^{2+} competently (6). Fig. 1 shows the uptake of Ca^{2+} after addition of L-glycerol 3-phosphate. The maximal uptake rates are 40–50 nmol of Ca^{2+} per min per mg of mitochondrial protein. The uptake is entirely inhibited by ruthenium red (0.4–1 nmol per mg of mitochondrial protein) and thus appears to proceed via an influx system similar to that reported for other mitochondria (13). The uptake rate can be stimulated by, but is not entirely dependent upon, exogenous phosphate, perhaps because of the presence of an endogenous pool. The Ca^{2+} uptake is also inhibited by Mg^{2+}, maximally at concentrations of 0.5 mM and above. In this respect it resembles the influx system in heart mitochondria (14).

Ca^{2+} accumulated by *heart* mitochondria is released by addition of Na^+, and the efflux occurs in a ruthenium-red-insensitive manner—i.e., not via the uptake system (4). A va-

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Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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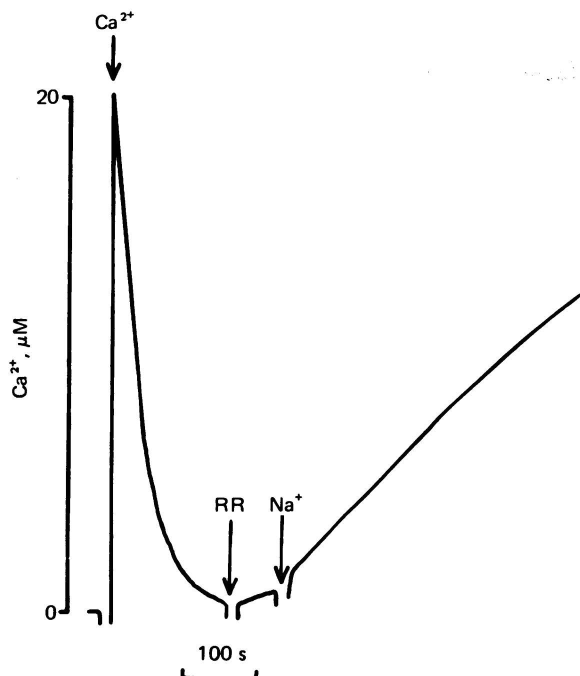

FIG. 1. Uptake and release of Ca^{2+} in brown adipose tissue mitochondria. The reaction was started by addition of 40 nmol of Ca^{2+}. The medium, 2 ml, contained 90 μM arsenazo III. RR, ruthenium red, 0.4 nmol/mg of mitochondrial protein; Na^+, 10 mM NaCl.

riety of other mitochondria possess this system, and as such have been termed "Na^+-responsive" (5). Because, as shown above, the Ca^{2+} *influx* system in brown adipose tissue mitochondria resembles that in heart mitochondria, and because changes in cytosolic Na^+ concentration are also expected in this tissue in the thermogenic state, we have tested the influence of Na^+ on the Ca^{2+} *efflux* in the isolated mitochondria. Fig. 1 demonstrates the stimulation of Ca^{2+} efflux by Na^+ (10 mM), in the presence of ruthenium red. No stimulation of efflux is found upon using 20 mM K^+ or choline$^+$. Li^+ has only a slight effect at 20 mM.

Fig. 2 shows the influence of Na^+ concentration on the initial rate of Ca^{2+} efflux from the brown adipose tissue mitochondria. A sigmoidal relationship is obtained, as has been observed with all other Na^+-responsive mitochondria (5). A Na^+ concentration of approximately 10 mM is required for half-maximal Ca^{2+} efflux rate, and an apparent maximum Na^+-induced Ca^{2+} efflux rate of 5.5 nmol per min per mg of mitochondrial protein can be observed under these conditions.

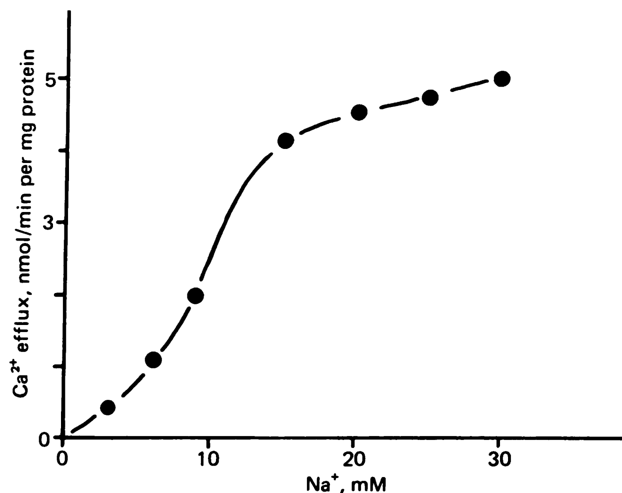

FIG. 2. Increase in rate of Ca^{2+} efflux from brown adipose tissue mitochondria as a function of Na^+ concentration. Conditions as in Fig. 1.

Energy-dissipative Na^+-induced Ca^{2+} cycling in mitochondria of brown adipose tissue

In the absence of ruthenium red, Ca^{2+} released from the mitochondria by Na^+ can be reaccumulated through the ruthenium-red-sensitive uptake system in an energy-requiring manner. This leads to a stimulation of respiration as Ca^{2+} is cycled across the inner membrane (4, 5). In Fig. 3 we demonstrate that addition of Na^+ to mitochondria that have accumulated Ca^{2+} leads to a stimulation of respiration in brown adipose tissue mitochondria. In the presence of 10 μM Ca^{2+}, maximal stimulation of respiration is achieved with 20–40 mM Na^+; stimulation commences at 4 mM. When testing with 20 mM Na^+, we found that maximal stimulation is obtained at Ca^{2+} concentrations between 10 and 20 μM (20–40 nmol per mg of mitochondrial protein). A stimulation of up to 40% of the initial rate can be obtained, being equivalent to 25 nmol of O per min per mg of mitochondrial protein at 25°C. This is 5 times the rate reported for heart mitochondria (4). As expected, this Na^+ stimulation is prevented by the prior addition of ruthenium red (1 nmol per mg of mitochondrial protein). K^+ and choline$^+$ are unable to replace Na^+. Although this Ca^{2+}-cycling system can thus induce a stimulation of respiration, it does not have a capacity equivalent to that of the respiratory chain, because subsequent addition of the artificial uncoupler FCCP is able further to stimulate respiration.

Possible effects of Na^+-induced Ca^{2+} release within brown adipocytes

When norepinephrine binds to the plasma membrane of brown adipocytes, there is an increase in cyclic AMP concentration (9), which is presumably responsible for the increased rate of lipolysis that has been observed (10, 15). This reaction sequence provides the mitochondria with a substrate, fatty acids, for thermogenesis. Simultaneously with the provision of a substrate, mitochondrial respiration is stimulated. While a model exists

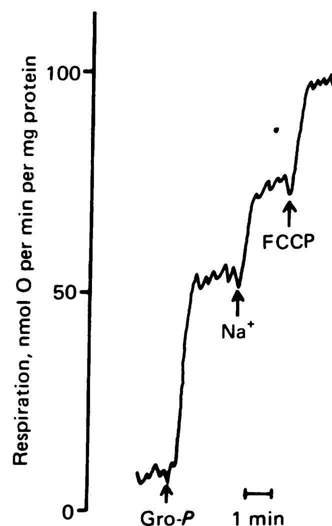

FIG. 3. Stimulation of glycerol 3-phosphate respiration in brown adipose tissue mitochondria by Na^+ in the presence of Ca^{2+}. The medium was 125 mM sucrose/20 mM Tris-HCl, pH 7.1, and contained 2 μM rotenone, 1 mM GDP, 0.1% bovine serum albumin, and 10 μM $CaCl_2$. There was 0.5 mg of mitochondrial protein in a volume of 1 ml at 25°C. Gro-P, L-glycerol 3-phosphate, dicyclohexylammonium salt, 5 mM; Na^+, 20 mM NaCl; FCCP, 10 μM. Note that 1 nmol O = ½ nmol O_2.

for the mechanism of this stimulation in isolated mitochondria (12, 16), the molecular mechanism for the norepinephrine-induced respiratory increase has not as yet been demonstrated in the intact tissue or in isolated adipocytes. We have, therefore, considered in this respect the probability that norepinephrine also induces an increased Na^+ concentration within brown fat cells. From the experiments with isolated mitochondria shown above, it may be deduced that Na^+ should be able to alter the cytosolic Ca^{2+} concentration and could perhaps in addition also induce some respiratory stimulation via Ca^{2+} cycling.

Fig. 4*A* shows that the Ca^{2+} ionophore A23187 stimulates respiration in isolated brown adipocytes, provided that a substrate is added. This can be interpreted as oxygen consumption coupled to Ca^{2+} uptake, when the cytosolic Ca^{2+} concentration is increased.

By addition of the Na^+ ionophore monensin, the cytosolic Na^+ concentration should be artificially increased; this may bring about a respiratory stimulation similar to that shown with A23187 by altering the cytosolic Ca^{2+} concentration. Fig. 4*B* shows that, in the presence of pyruvate, monensin addition leads to a transitory respiratory stimulation. FCCP can subsequently stimulate respiration further. Without added substrate no monensin stimulation is seen. The respiratory stimulation observed with monensin is blocked by the prior addition of 10 μM ruthenium red, although this does not impair the FCCP response. This monensin stimulation is dependent upon extracellular Na^+; if 75% of this is replaced with choline$^+$, monensin inhibits basal respiration. The effect of monensin is not due to a simple uncoupling of respiration, because monensin addition to coupled brown fat mitochondria in the presence of glycerol 3-phosphate and Na^+ does not lead to a stimulation of oxygen consumption. The experiment indicates that the monensin effect observed in isolated cells is mediated by an additional component. The inhibition of the stimulation by ruthenium red suggests that this component is Ca^{2+}, although this assumes a specific site of action of the inhibitor.

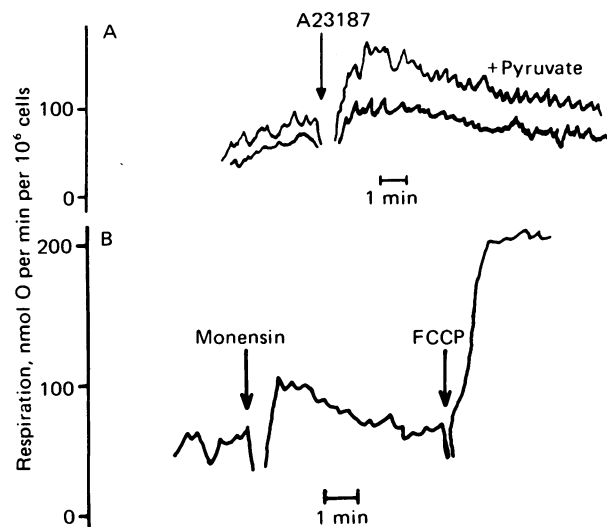

FIG. 4. (*A*) Stimulation of exogenous pyruvate respiration in brown adipocytes by the Ca^{2+} ionophore A23187. Brown adipocytes were incubated in Krebs–Ringer bicarbonate buffer (10) containing 10 mM glucose, 10 mM fructose, and 4% albumin at 37°C in the presence (+ pyruvate) and in the absence of 10 mM pyruvate. At the arrow, A23187 was added to 20 μg/ml. (*B*) Stimulation of exogenous pyruvate respiration in brown adipocytes by the Na^+ ionophore monensin. Brown adipocytes (150,000/ml) were incubated as in *A*, including 10 mM pyruvate. At the arrows, 200 μM monensin and 20 μM FCCP were added.

When Na^+ in the buffer is replaced with choline$^+$, virtually no increase in oxygen consumption is observed upon addition of norepinephrine (Fig. 5). A partial inhibition of norepinephrine stimulation in a medium with reduced Na^+ content has been reported (18, 19). Addition of FCCP after norepinephrine leads to an increased oxygen consumption, whereas no effect of FCCP is found without norepinephrine in this buffer. This indicates that, in a Na^+-free buffer, norepinephrine is still able to induce lipolysis to provide substrate for mitochondrial respiration, but that the respiratory stimulation normally seen cannot occur. We have also observed a rapid and significant inhibition (60%) of norepinephrine-induced respiration in a Na^+-containing buffer upon addition of 10 μM ruthenium red. FCCP or FCCP plus pyruvate is able to stimulate respiration after the ruthenium red inhibition. Taken together, the results demonstrate the possible occurrence of Na^+-induced Ca^{2+} cycling in brown adipocytes during norepinephrine-mediated thermogenesis.

The Na^+ dependence of norepinephrine-stimulated respiration has earlier been discussed in terms of a stimulation of the Na^+,K^+-dependent plasma membrane ATPase (20). Energy-dissipative Ca^{2+} cycling as mentioned here has the advantage that it does not involve initial mitochondrial synthesis of ATP, a process known to occur only at a low rate in hamster brown fat (21). We are, however, not able at this stage to give any indication of the possible quantitative importance of Na^+-induced Ca^{2+} cycling in thermogenesis.

The Na^+-induced leakage of Ca^{2+} from the mitochondria can lead to an increased cytosolic Ca^{2+} concentration. In this case, Ca^{2+} could mediate other responses within the cell. In this connection, we have studied whether Ca^{2+} is able to influence the action of purine nucleotides in brown fat mitochondria. In isolated mitochondria purine nucleotides bind to a polypeptide of molecular weight 32,000 and in so doing inhibit an energy-dissipating ion "channel" (22). At equimolar concentrations (50 μM) of Ca^{2+} and GDP, we have found that Ca^{2+} does not influence GDP-limited passive swelling in 100 mM KCl plus valinomycin, nor does it influence binding of [^{3}H]GDP to brown fat mitochondria (David Herron and Ulf Sundin, personal communication). Other effects of an elevated cytosolic Ca^{2+} concentration cannot, however, be excluded.

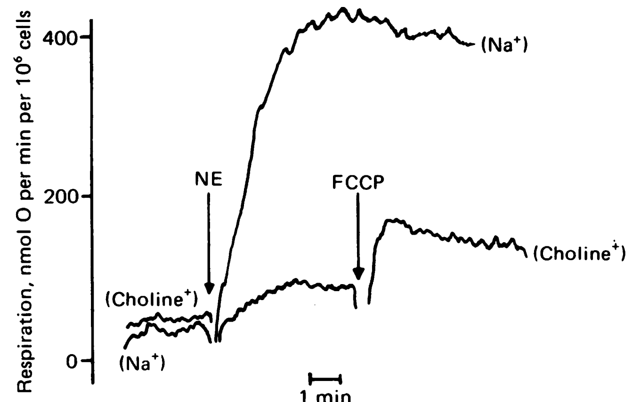

FIG. 5. Influence of lack of sodium on norepinephrine-stimulated respiration in brown adipocytes. Brown adipocytes were incubated (100,000/ml) in a medium consisting of 118.7 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 24.6 mM Hepes buffer (17); 4% albumin was added. The buffer was bubbled with 5% CO_2 in air and adjusted to pH 7.4 during bubbling. For the trace labeled choline, choline chloride was substituted for NaCl in the above buffer; the Na^+ concentration resulting from the addition of concentrated cells in the preparation buffer to the incubation medium was less than 2 mM. NE, 1 μM norepinephrine; FCCP, 20 μM.

In conclusion, we have demonstrated that brown fat mitochondria belong to the class of mitochondria termed Na^+-responsive in having a Na^+-induced Ca^{2+} efflux process and respiration-stimulated reuptake of this Ca^{2+} (Ca^{2+} cycling). It is not clear if this Ca^{2+} cycling is of quantitative importance for thermogenesis, but even if the quantitative contribution to heat production were small, Na^+-mediated changes in intracellular Ca^{2+} distribution could nonetheless be of greater significance in the mechanism of thermogenesis.

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