Regulation of Na^+ transport in brown adipose tissue

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In order to test the hypothesis that Na^+,K^+-ATPase (Na^+,K^+-dependent ATPase) is involved in the noradrenaline-mediated stimulation of respiration in brown adipose tissue, the effects of noradrenaline on Na^+,K^+-ATPase in isolated brown-fat-cell membrane vesicles, and on $^{22}Na^+$ and K^+ ($^{86}Rb^+$) fluxes across the membranes of intact isolated cells, were measured. The ouabain-sensitive fraction of the K^+-dependent ATPase activity in the isolated membrane-vesicle preparation was small and was not affected by the presence of noradrenaline in the incubation media. The uptake of $^{86}Rb^+$ into intact hormone-sensitive cells was inhibited by 80% by ouabain, but it was insensitive to the presence of noradrenaline. $^{22}Na^+$ uptake and efflux measured in the intact cells were 8 times more rapid than the $^{86}Rb^+$ fluxes and were unaffected by ouabain. This indicated the presence of a separate, more active, transport system for Na^+ than the Na^+,K^+-ATPase. This is likely to be a Na^+/Na^+ exchange activity under normal aerobic conditions. However, under anaerobic conditions, or conditions simulating anaerobiosis (2 mM-NaCN), the unidirectional uptake of Na^+ increased dramatically, while efflux was unaltered.

INTRODUCTION

The primary function of white adipose tissue is to store fat, whereas the primary function of brown adipose tissue is to generate heat during periods of acute cold stress (Nicholls & Locke, 1984; LaNoue, 1986). Brown adipose tissue oxidizes fatty acids at rates regulated by the sympathetic nervous system (Himms-Hagen, 1976), and then noradrenaline released at the nerve endings increases respiration rates and fatty oxidation by 20–40-fold (Foster & Frydman, 1978*a*,*b*, 1979; Wickler *et al.*, 1984).

In most tissues, respiration rates are regulated by tissue ATP requirements, and, as a result, the cytosolic ratio of ATP/ADP is an important determinant of respiration (Davis & Davis-van Thienen, 1978; Kunz *et al.*, 1981). Under normal conditions, respiration rates are tightly coupled to rates of ADP phosphorylation. However, in brown fat, there is a unique protein, which is part of the mitochondrial inner membrane, which uncouples respiration from phosphorylation when the cells are hormonally activated (Nicholls, 1976; Heaton & Nicholls, 1977; Heaton *et al.*, 1978; Rial & Nicholls, 1984; LaNoue *et al.*, 1986).

Until the discovery of the uncoupling protein, numerous lines of evidence had led investigators to believe that regulation of respiration in this tissue was achieved by hormonal regulation of the cell membrane Na^+,K^+-ATPase (Girardier *et al.*, 1968; Horwitz, 1979). Hormone binding to its receptor was thought to activate the Na^+,K^+-ATPase, and the resultant increase in ATP utilization and decrease in cytosolic ATP/ADP ratios were thought to stimulate respiration. Evidence from several different laboratories supported this hypothesis. Cell-membrane vesicles from brown-fat cells exhibited a K^+-dependent ATPase which could be stimulated by noradrenaline *in vitro* (Herd *et al.*, 1970; Horwitz & Eaton, 1975; Rothwell *et al.*, 1981; Hettinger & Horwitz, 1983). The extent of stimulation appeared to correlate with the thermogenic state of the animal (Rothwell *et al.*, 1981).

Also, cells treated with ouabain, a specific inhibitor of the Na^+,K^+-ATPase, exhibited poor responses to noradrenaline (Herd *et al.*, 1973; Horwitz, 1973). Additionally, microelectrode studies demonstrated that the hormone caused a depolarization of the cell membrane which was attributed to an increase in Na^+ permeability. This depolarization was demonstrated *in situ* (Horwitz *et al.*, 1969; Flaim *et al.*, 1977) and in perifused blocks of excised tissue (Williams & Matthews, 1974*a*,*b*; Fink & Williams, 1976; Girardier & Schneider-Picard, 1983).

Several years ago we suggested that the uncoupling protein was regulated *in situ* by the concentration of ATP in the cytosol, and that a small change in cytosolic ATP could trigger the activation of the uncoupling protein (LaNoue *et al.*, 1982). The mechanism includes the hormonal activation of a cellular ATPase to activate the uncoupling protein via a decrease in cytosolic ATP. It seemed likely, in view of the evidence linking the sodium pump to the hormonal response, that this triggering ATPase could be the Na^+,K^+-ATPase. For this reason, the problem of ion fluxes was re-examined in brown-fat cells. The objective was to measure unidirectional Na^+ and K^+ ($^{86}Rb^+$ was used as an analogue for K^+) fluxes across the outer membranes of intact cells in steady state, with and without hormones present. Because of technical difficulties inherent in the low intracellular aqueous volume of brown-fat cells, Na^+ fluxes have not been measured previously and $^{86}Rb^+$ fluxes have only been reported for brown-fat cells depleted of K^+ by overnight incubation at 4 °C (Nanberg *et al.*, 1984). Rb^+ is used by Na^+,K^+-ATPase in place of K^+. Ouabain-sensitive $^{86}Rb^+$ fluxes have been measured in white adipocytes, where it was shown that $^{86}Rb^+$ uptake was stimulated by insulin (Resh *et al.*, 1980).

Abbreviation used: Na^+,K^+-ATPase, Na^+,K^+-dependent ATPase.

METHODS AND MATERIALS

Cell and membrane preparations

Isolated brown-fat cells were prepared from golden hamsters raised at 20–25 °C as described by Nedergaard & Lindberg (1979). After isolation, the cells were maintained at 37 °C in Krebs–Ringer bicarbonate solution, containing 142.6 mM-Na^+, 6.9 mM-K^+, 1.9 mM-Ca^{2+}, 1.7 mM-Mg^{2+}, 126.5 mM-Cl^-, 24.6 mM-HCO_3^-, 1.5 mM-HPO_4^{2-}, 1.7 mM-SO_4^{2-}, 10 mM-glucose, 10 mM-fructose and 4% (w/v) fatty-acid-free bovine serum albumin (medium A) in an atmosphere of O_2/CO_2 (19:1). They remained viable for over 6 h under these conditions. The quality of the cells was judged by the extent of stimulation of O_2 consumption on addition of 1 μM-noradrenaline to the cell suspension in medium A. Routinely, an 8–10-fold respiratory stimulation was then observed, and no cell preparation with a stimulation of under 5-fold was used.

Isolated cell membrane vesicles were prepared as described by Rothwell *et al.* (1981). They were stored frozen in the isolation medium. When they were stored thus, the activity did not decrease for at least 2 weeks.

Quantification of cells and vesicles

Cells were quantified by assaying citrate synthase in samples of sonicated cell suspensions. The sonication procedure has been described previously (LaNoue *et al.*, 1982). Citrate synthase was assayed as described by Idell-Wenger *et al.* (1978). Citrate synthase is a mitochondrial marker enzyme, and the activity of citrate synthase in isolated brown-fat mitochondria is known (LaNoue *et al.*, 1982). Therefore the procedure allows us to report all cellular data in units per mg of mitochondrial protein, since:

$$\frac{\text{units of citrate synthase}}{\text{ml of cell suspension}} \times \frac{\text{mg of mitochondrial protein}}{\text{units of citrate synthase}} = \frac{\text{mg of mitochondrial protein}}{\text{ml of cell suspension}}$$

Protein content cannot be used to quantify cells, because cells lyse when they are removed from suspending media that contains less than 4% bovine serum albumin.

The membrane-vesicle preparation was assayed for protein by the procedure of Lowry *et al.* (1951). K^+-dependent ATPase was assayed in the membrane fraction as described by Horwitz & Eaton (1975). The method involves incubating the vesicle preparation with ATP in the presence and absence of K^+ and assaying the rate of production of P_i; the latter was assayed by the method of Baginski *et al.* (1967). Samples were taken at 0, 1, 2, 3, 4 and 5 min to determine initial, linear, rates of ATP hydrolysis.

O_2 consumption of the cells was assayed polarographically with a Clark electrode. Radioactivity was measured with a Beckman liquid-scintillation counter model LS 250.

Na^+ uptake into intact cells

Cells (6–10 units of citrate synthase/ml) were incubated with $^{22}Na^+$ (15 μCi/ml) in medium A saturated with O_2/CO_2 (19:1). At 15 s and at 1 min intervals thereafter, 50 μl samples were taken and diluted 70-fold into 3.5 ml of ice-cold Krebs–Ringer bicarbonate buffer containing 4% bovine serum albumin, [^{14}C]sucrose (0.1 μCi/ml) and ^3H_2O (1.0 μCi/ml), but no $^{22}Na^+$. A 3 ml sample of the diluted suspension was placed in the bottom of a 3 ml silicone-treated glass vial with a constricted neck. Silicone oil with a density lower than the cell incubation medium was prepared by mixing 4.6 parts of Dow-Corning silicone fluid 200 (viscosity 2 cSt) with 13 parts of Dow-Corning silicone fluid 550 (viscosity 125 cSt). This was used to fill the neck of the vial (0.3 ml) and 500 μl of iso-osmotic saline (0.9% NaCl) was layered above the oil. The vials were centrifuged in a table-top centrifuge for 1 min, and the upper saline layer containing the cells was removed quantitatively with a 200 μl wash and placed in 10 ml of scintillation cocktail for radioactivity counting. The ^{22}Na content of the cell fraction was corrected for adhering extracellular $^{22}Na^+$ by using the volume of extracellular fluid and the $^{22}Na^+$ c.p.m./ml in the extracellular fluid. The volume of extracellular fluid and intracellular aqueous volume in each sample were determined from the [^{14}C]sucrose and ^3H_2O contents of the cellular fractions and a knowledge of the [^{14}C]sucrose c.p.m./ml and ^3H_2O c.p.m./ml in the diluting media.

Rb^+ uptake

This was measured by the same technique used to measure $^{22}Na^+$ uptake. However, the initial incubation medium contained less cells (approx. 3 units of citrate synthase/ml). The initial incubation medium was similar, except that 0.5 μCi of $^{86}Rb^+$/ml was used rather than 15 μCi of $^{22}Na^+$/ml. At 1 min intervals, the cells were diluted 10-fold with ice-cold Krebs–Ringer bicarbonate buffer containing 4% bovine serum albumin, [^{14}C]sucrose (0.1 μCi/ml) and ^3H_2O (1 μCi/ml) and centrifuged, as in the $^{22}Na^+$-uptake experiments, in silicone-treated glass vials. The cell fraction was washed quantitatively off the top of the silicone oil and placed in a scintillation cocktail and counted for radioactivity.

Na^+ efflux

Cells (5–10 units of citrate synthase) were incubated in medium A containing 15 μCi of $^{22}Na^+$/ml for 20 min. At that time, the entire suspension was diluted 70-fold in additional medium containing [^{14}C]sucrose (0.1 μCi/ml) and ^3H_2O (1 μCi/ml) at 37 °C. In some cases, as noted in the Figure legends, the ionic composition of medium A was altered to test the effects of external Na^+ or Ca^+. The incubation was continued in the diluted suspensions and 3.0 ml samples were taken at rapid intervals (~45 s). The samples were centrifuged as described for the uptake experiments, and the cell fractions were counted for radioactivity to determine $^{22}Na^+$, and intra- and extracellular-fluid volumes.

RESULTS

Reports from several laboratories have indicated that cell membrane vesicle preparations contain a noradrenaline-sensitive K^+-dependent ATPase (Herd *et al.*, 1970; Horwitz & Eaton, 1975; Rothwell *et al.*, 1981; Hettinger

Table 1. Effect of noradrenaline on K^+-dependent ATPase in brown-fat vesicles

Vesicles (approx. 0.1 mg of protein/ml) were added (A) to an incubation medium containing 20 mM-Mops, 5 mM-$MgCl_2$, 4 mM-NaCN, 27 mM-sucrose, 8.7 mM-KCl, 70 mM-NaCl and 2.7 mM-EDTA, pH 7.2, or (B) to a buffer which was similar to the buffer of (A), except that NaCl and KCl were replaced with 190 mM-sucrose. Samples were taken at $t = 0$, 1, 2, 3 and 4 min to determine changes in P_i. Ouabain, when present, was at 1 mM and noradrenaline 1 μM. Results are means ± S.E.M. of six experiments.

	ATPase activity (nmol of P_i/min per mg of membrane protein)		
	A	B	K^+-dependent activity (A − B)
Control	1151 ± 104	871 ± 151	304 ± 28
+ Noradrenaline	1193 ± 105	860 ± 122	333 ± 42
+ Ouabain	1018 ± 101	813 ± 105	210 ± 18
+ Ouabain plus noradrenaline	1010 ± 103	783 ± 121	226 ± 34

& Horwitz, 1983). However, most of these reports did not specify whether or not vanadium-free ATP was used in the assay. It has been shown that some commercial preparations of ATP are contaminated with vanadium and that noradrenaline will bind to this potent Na^+,K^+-ATPase inhibitor (Josephson & Cantley, 1977; Cantley *et al.*, 1978; Sawas & Gilbert, 1981). Therefore, when vanadium is present in the ATP, noradrenaline produces an apparent stimulation of the ATPase. Only one recent publication (Hettinger & Horwitz, 1983) reports use of vanadium-free ATP for measuring the ATPase in brown-fat membrane-vesicle preparations, and in that case the stimulatory effect of noradrenaline was less dramatic, but still significant. It seemed worthwhile to re-examine this phenomenon, with vanadium-free ATP. Previous workers examined only the K^+-sensitivity of the vesicular ATPase and assumed that all the K^+-sensitivity represented Na^+,K^+-ATPase activity. Cell membrane K^+-dependent ATPases other than the Na^+,K^+-ATPase have been reported (Faller *et al.*, 1982) and, since ouabain-sensitivity is a much more definitive probe for the ubiquitous Na^+ pump, it seemed more important to examine the ouabain-sensitivity of the K^+-dependent ATPase activity.

The results of the experiments are shown in Table 1. Membrane vesicles were prepared from golden hamsters which were not cold-adapted. The assay of K^+-dependent ATPase was carried out as described above. However, samples were taken every 1 min for 5 min for phosphate assay rather than as in previous studies, which employed a single 10 min time point. In our hands, the ATPase assay was linear over the time period studied. The activity could not be stimulated by Triton X-100 (0.01–0.1%) or digitonin (0.1–1.0 mg/mg of protein). As shown in Table 1, the K^+-sensitive fraction of the Mg^{2+}-dependent ATPase is quite small, and only a fraction of the K^+-dependent ATPase is sensitive to ouabain. Stimulation by noradrenaline could not be detected. These experiments were repeated with vesicles from both warm- and cold-adapted rats, with essentially identical results. The results suggest that the activity of the sodium pump is very low in these vesicles, and insensitive to noradrenaline.

Further experiments were carried out to measure the effect of noradrenaline on Na^+,K^+-ATPase fluxes in intact cells. In preliminary experiments (LaNoue *et al.*, 1982), we could detect no differences in Na^+ fluxes between control and noradrenaline-treated cells. Therefore we used $^{86}Rb^+$ uptake into the cells as a means of estimating the activity of the sodium pump. This method has been used successfully in white (Resh *et al.*, 1980) and brown adipocytes (Nanberg *et al.*, 1984). The previous study with brown adipocytes used $^{86}Rb^+$ as a tracer for K^+ uptake in cells that had been depleted of K^+ by overnight storage at 4 °C. The present experiments were done with freshly prepared cells, which were stored for no more than 3 h at 37 °C in Krebs–Ringer bicarbonate medium. In some experiments (Fig. 1*b*), tracer $^{86}Rb^+$ was used and the ratio of the concentration of $^{86}Rb^+$ inside to that outside was recorded as a function of time. In other incubations (Fig. 1*a*), the K^+ (6.9 mM) in the normal medium was replaced with Rb^+ (6.91 mM), so that it was possible to calculate Rb^+ fluxes in absolute terms. The rate of Rb^+ uptake in the absence of ouabain was 0.85 nmol/min per mg of mitochondrial protein, and the rate in the presence of ouabain was 0.12 nmol/min per mg. Therefore one can conclude that the ouabain-sensitive Rb^+ flux is 0.73 nmol/min per mg, and ATP utilization by the sodium pump is approx. 0.37 nmol/mg. This is in agreement with the very low ouabain-sensitivity of the cell membrane ATPase. These rates compare with rates of O_2 consumption of over 1000 ng-atoms of O/min per mg in the hormone-stimulated state and 100 ng-atoms/min per mg in the basal state. Therefore, ATP utilization by the sodium pump constitutes a very small fraction of the total energy balance in these cells. Fig. 1 also shows that noradrenaline has no effect on the ouabain-sensitive $^{86}Rb^+$ fluxes. From this, it is possible to conclude that noradrenaline does not affect the activity of the Na^+,K^+-ATPase. Ouabain appears to be maximally effective within 1 min after addition of the cells to the incubation media. When the effect of noradrenaline on O_2 consumption was measured in the presence and absence of ouabain, no effect of ouabain could be observed unless the cells were preincubated for more than 30 min in the presence of ouabain (results not shown). This is in agreement with a recent report (Nanberg *et al.*, 1984), but not with older reports (Herd *et al.*, 1973; Horwitz, 1973). The older data were obtained by a manometric procedure for measuring O_2 consumption, and this required a prolonged incubation to obtain the

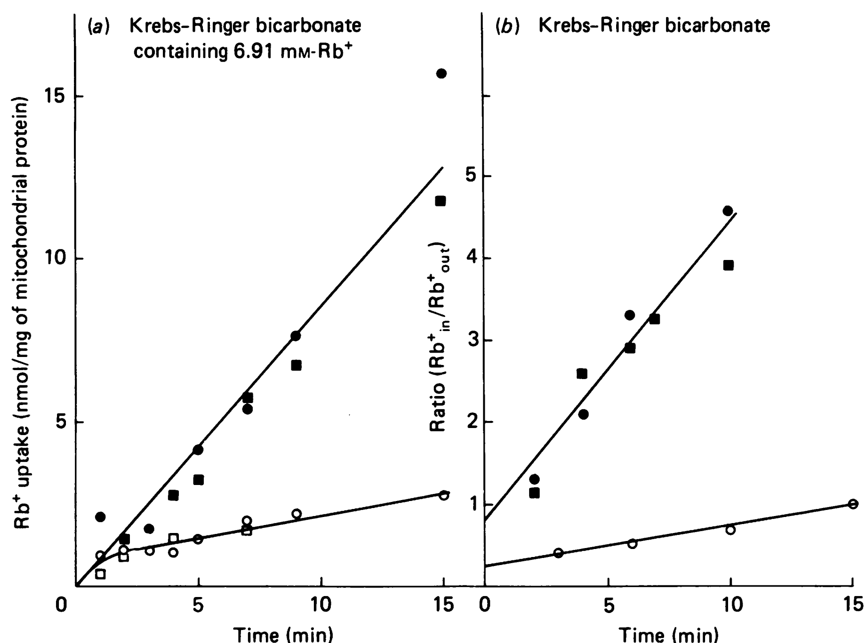

Fig. 1. Effect of noradrenaline and ouabain on Rb^+ fluxes in brown adipocytes

Cells (1.5 mg of mitochondrial protein/ml) were incubated in Krebs–Ringer bicarbonate medium containing 4% fatty-acid-free bovine serum albumin and 0.3 μCi of $^{86}Rb^+$/ml. In panel (*a*), the K^+ in the usual buffer was replaced with 6.91 mM-RbCl. In panel (*b*), only tracer amounts of $^{86}Rb^+$ were present. At the times shown, 0.35 ml samples were removed and diluted to 3.5 ml with ice-cold Krebs–Ringer bicarbonate. The cold diluting media contained [^{14}C]sucrose (0.1 μCi/ml) and ^3H_2O (1 μCi/ml). The control condition (●) did not include ouabain or noradrenaline. Ouabain, when present (○, □), was 1 mM and noradrenaline (■, □) was 1 μM. The diluted cells were centrifuged in silicone-treated vials containing a lighter layer of silicone oil as described in the Methods and materials section. The radioactivity in the upper cellular fraction was measured to determine $^{86}Rb^+$ content and intra- and extra-cellular aqueous volumes.

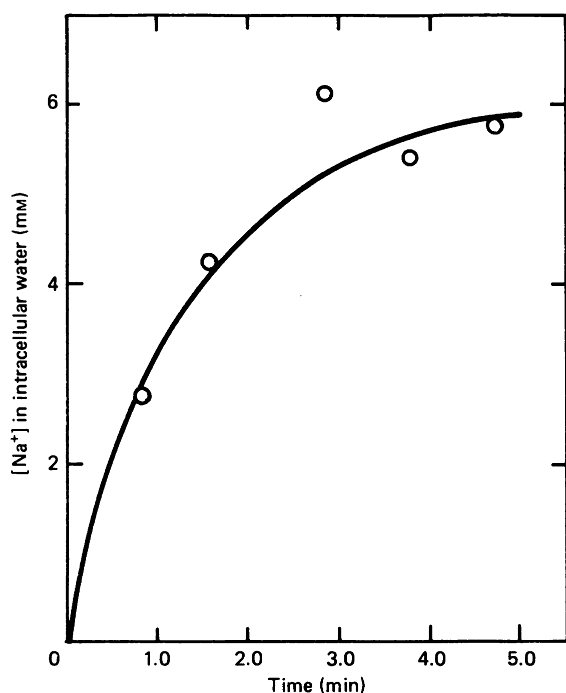

Fig. 2. Uptake of $^{22}Na^+$ into hamster brown adipocytes

Isolated cells (9.5 units of citrate synthase/ml) were incubated for the indicated times in medium A containing 15 μCi of ^{22}NaCl/ml. The method of quantifying intracellular $^{22}Na^+$ is described in the Methods and materials section.

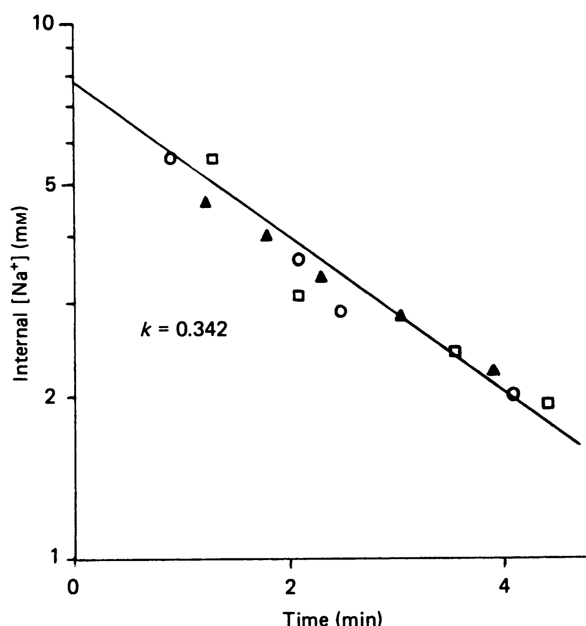

Fig. 3. Effect of ouabain and noradrenaline on rates of efflux of $^{22}Na^+$ from $^{22}Na^+$-pre-loaded brown adipocytes

Isolated cells (10.3 units of citrate synthase/ml) were preloaded with $^{22}Na^+$ (approx. 0.1 μCi/μmol) in medium equilibrated with O_2/CO_2 (19:1) and then diluted 1:70 with more medium A at 37 °C. Rates of efflux and concentrations of intracellular $^{22}Na^+$ were measured as described in the Methods and materials section. The control condition (○) did not include ouabain or noradrenaline. When present, ouabain was 1 mM (□) and noradrenaline was 1 μM (▲).

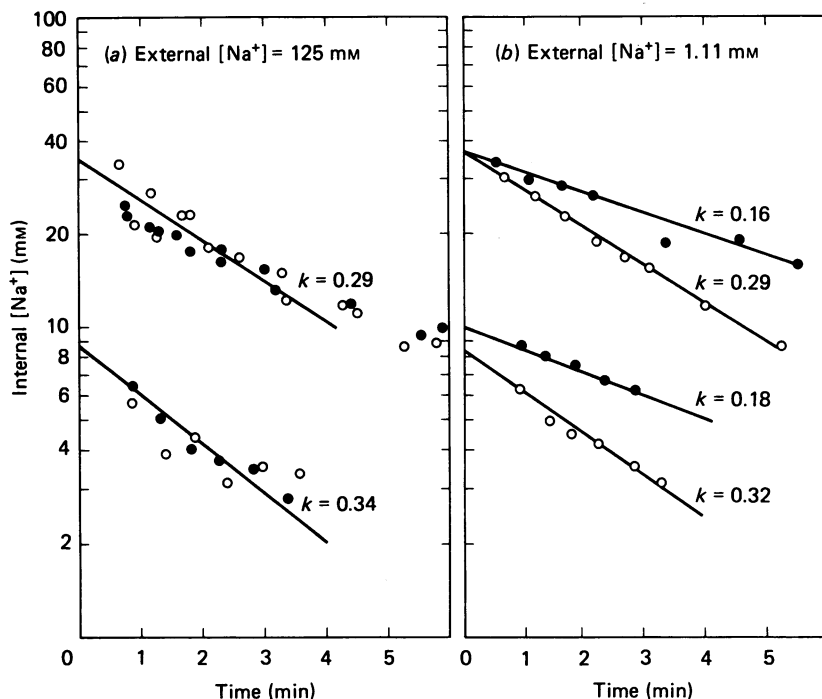

Fig. 4. Effect of ouabain and extracellular Na^+ on Na^+ efflux from brown adipocytes

Isolated cells (8.5 units of citrate synthase/ml) were preincubated in medium A saturated with air/CO_2 (19:1) (upper curves) or O_2/CO_2 (19:1) (lower curves) containing 15 μCi of $^{22}Na^+$/ml. Cells were diluted 1:100 (*a*) into a buffer similar to medium A, but in which the $NaHCO_3$ was replaced with Hepes buffer (25 mM), or else (*b*) into medium B. Medium B is similar to medium A, but the NaCl was replaced with choline chloride and the $NaHCO_3$ was replaced with Hepes buffer (40 mM). In some incubations ouabain (1 mM) was included (●) and in others it was absent (○). The method of measuring efflux is described in the Methods and materials section.

measurement. Taken together, the data strongly suggest that the Na^+,K^+-ATPase is not involved in the hormonal response.

The reported cell-membrane depolarization produced by noradrenaline has been studied extensively and shown to be due to an increased conductance across the cell membrane (Horowitz *et al.*, 1971). It has been speculated that the increased conductance relates to an increase in Na^+ permeability (Horowitz *et al.*, 1971). Previous studies (LaNoue *et al.*, 1982) of Na^+ fluxes across brown-fat-cell membranes indicated that noradrenaline did not change the steady-state concentration of Na^+ in the cell, but that the unidirectional fluxes were too fast to measure accurately. Since the Rb^+ fluxes were slow, it seemed unusual that the Na^+ fluxes would be too rapid to measure. Therefore, further attempts were made to measure the Na^+ fluxes accurately and to document the effects of noradrenaline and ouabain. Fig. 2 is an example of data obtained during Na^+-uptake experiments with control cells. Similar data were obtained in the presence and absence of noradrenaline. It is apparent that accurate determination of fluxes of Na^+ is difficult, since it is possible to obtain only one or two points in the uptake curve before the internal Na^+ equilibrates with the external, the two pools have the same specific radioactivity and the rate of influx of $^{22}Na^+$ is equal to the rate of efflux of $^{22}Na^+$. Therefore an alternative approach was used. It is known that the concentration of Na^+ inside the cells does not change during incubation with noradrenaline (LaNoue *et al.*, 1982). Therefore, an estimate of the efflux of $^{22}Na^+$ at steady state would allow us to estimate the uptake fluxes more accurately than by direct uptake measurements. At steady state, the rate at which Na^+ enters the cells must equal the rate at which it leaves. To estimate flux at steady state, one can label either the internal or the external pool. In this particular experimental set-up, the external pool is much larger than the internal pool, and therefore, whether the internal or the external pool is labelled initially, the specific radioactivity of the internal pool will change and approach that of the larger external pool. The change in specific radioactivity in the internal pool in this experiment is equivalent to the change in $^{22}Na^+$ c.p.m., because the amount of internal Na^+ stays constant. Then:

$$\frac{d(^{22}Na^+)}{dt} = k'(^{22}Na^+)_{out} - k'(^{22}Na^+)_{internal}$$

If one starts with $(^{22}Na^+)_{out}$ and $(^{22}Na^+)_{in} = 0$ and follows its equilibration with the internal pool, within a few minutes $(^{22}Na)_{out} = (^{22}Na)_{in}$ and $d(Na^+)/dt = 0$. The value of k' can be determined as described previously (LaNoue *et al.*, 1983) from uptake data and can be estimated as approx. 0.3 min^{-1} from Fig. 2. However, if the $(^{22}Na^+)_{out}$ specific radioactivity is zero, it remains approx. 0 throughout the course of the experiment, and $d(^{22}Na^+)_{in}/dt = k'(^{22}Na^+)_{internal}$. Integrating this equation gives $\ln(^{22}Na^+) = k'\Delta t$, and one can obtain k' from a semi-logarithmic plot of $(^{22}Na^+)_{in}$ versus time. The value obtained from Fig. 3 is 0.342, in good agreement

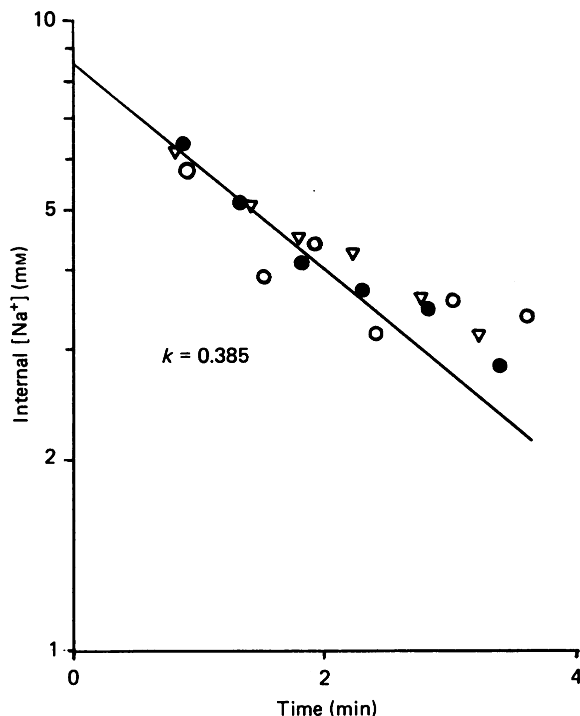

Fig. 5. Effect of Ca^{2+} on the rate of efflux of Na^+ from brown adipocytes

Cells (9.1 units of citrate synthase/ml) were incubated in medium A containing 15 μCi of $^{22}Na^+$/ml. At 20 min, the cells were diluted into medium A (○) or into a medium similar to medium A from which the Ca^{2+} had been omitted and which also contained 2 mM-EGTA (●). Alternatively the cells were diluted into medium B, which contains no Na^+ (△). Efflux was measured as described in the Methods and materials section.

with k' estimated from Fig. 2. The data show that noradrenaline and ouabain have no effect on Na^+ fluxes. The rate of Na^+ efflux at steady state can be calculated from the data. Since the average Na^+ concentration at steady state is 6.5 mM and the intracellular aqueous space is 3 μl/mg of mitochondrial protein, the cell Na^+ content at steady state is 19.5 nmol/mg of mitochondrial protein. The Na^+ flux can be calculated by multiplying the Na^+ content by the first-order rate constant determined from experiments illustrated in Fig. 3. This value is 6.67 nmol/min per mg, or about 8 times the Rb^+ fluxes. The dissimilarity between the Rb^+ fluxes and the Na^+ fluxes and the lack of ouabain-sensitivity suggest the presence of a separate, more active transport system for Na^+ than the Na^+,K^+-ATPase. Since a normal Na^+ gradient is maintained in these cells, the transporter should be energy-linked or catalyse a Na^+/Na^+ exchange. To determine whether external Na^+ promotes the efflux of internal Na^+, the $^{22}Na^+$-loaded cells were incubated in the presence and absence of external Na^+. The Na^+ in the external Krebs–Ringer bicarbonate medium was replaced by choline chloride. An initial 1:100 dilution of the ^{22}Na-loaded cells resulted in a concentration of external Na^+ of 1.11 mM-$^{22}Na^+$ as measured by atomic-absorption spectrophotometry. This Na^+ had the same specific radioactivity as the intracellular Na^+. The rate of efflux of $^{22}Na^+$ in the presence and absence of ouabain is shown in Fig. 4. In the absence of high external Na^+, total intracellular Na^+ decreases, and the rate of influx of labelled and unlabelled Na^+ is negligible. Net efflux of Na^+ is proportional to efflux of $^{22}Na^+$ and can be calculated from the $^{22}Na^+$ specific radioactivity. When external non-radioactive Na^+ was present at 145 mM, there was no inhibition of Na^+ efflux by ouabain, but ouabain-sensitivity was apparent in the absence of external Na^+. Thus a Na^+-exchange activity could be demonstrated, but only in the presence of ouabain. In the experiment of Fig. 4, some cells were loaded with $^{22}Na^+$ in a medium saturated with air/CO_2 (19:1) rather than O_2/CO_2 (19:1). Apparently, owing to hypoxia, these cells initially had much higher contents of Na^+, but the rate constants of efflux were unchanged. The Na^+ contents undoubtedly decreased to control values during the normoxic (efflux) incubation. This hypoxic phenomenon was studied in later experiments (Fig. 6). The fact that k is the same suggests that, at the concentration of Na^+ observed here, efflux is not only first-order with respect to changing specific radioactivity in steady-state measurements, but first-order with respect to Na^+ concentration.

Since it seemed possible that the Na^+ could actively exchange for Ca^{2+} (Reeves & Sutko, 1979) while maintaining a physiological Na^+ gradient, the effect of omitting Ca^{2+} from the external medium and adding 2 mM-EGTA was measured. The results of this experiment are illustrated in Fig. 5, which shows that lack of Ca^{2+} in the external media has no effect on Na^+ efflux. In other experiments the effect of a combination of 2 mM-EGTA and low Na^+ (1.11 mM) was measured as well as the effect of amiloride (1 mM). The rate of efflux of Na^+ was not effected by any of these treatments.

Cells were normally loaded with $^{22}Na^+$ by incubating concentrated suspensions of cells in Krebs–Ringer bicarbonate saturated with O_2/CO_2 (19:1) for 20 min. Although 1 mM-ouabain had no effect on the concentration of $^{22}Na^+$ achieved after this time interval, incubation in an air/CO_2 atmosphere produced much higher contents of Na^+, as shown in Fig. 4. For this reason, it seemed possible that the cells might contain a sodium pump distinct from the Na^+,K^+-ATPase, but linked to the hydrolysis of ATP. To test this hypothesis, cells were incubated and Na^+ fluxes measured in 2 mM-NaCN and 2 mM-iodoacetate in order to inhibit both oxidative and glycolytic ATP formation. The results are shown in Fig. 6. Although Na^+ efflux did not appear to be affected by the presence of CN^- and iodoacetate, the rate of Na^+ entry increased. This implies the presence of a Na^+ transporter, perhaps a channel, which is closed when the cell contains normal concentrations of ATP and NAD^+, but which can open when ATP decreases or perhaps when NADH increases.

DISCUSSION

Measurements of ouabain and noradrenaline effects on all membrane-vesicular ATPases and on respiration in intact cells, and direct measurements of ion fluxes in intact cells, carried out in this study, lead to the conclusion that the cell membrane Na^+,K^+-ATPase is not involved in the response of brown adipose tissue to noradrenaline. Therefore, the results of previous workers who measured decreases in the plasma-membrane electrical potential gradient on addition of noradrenaline to brown adipose tissue are puzzling and require some discussion.

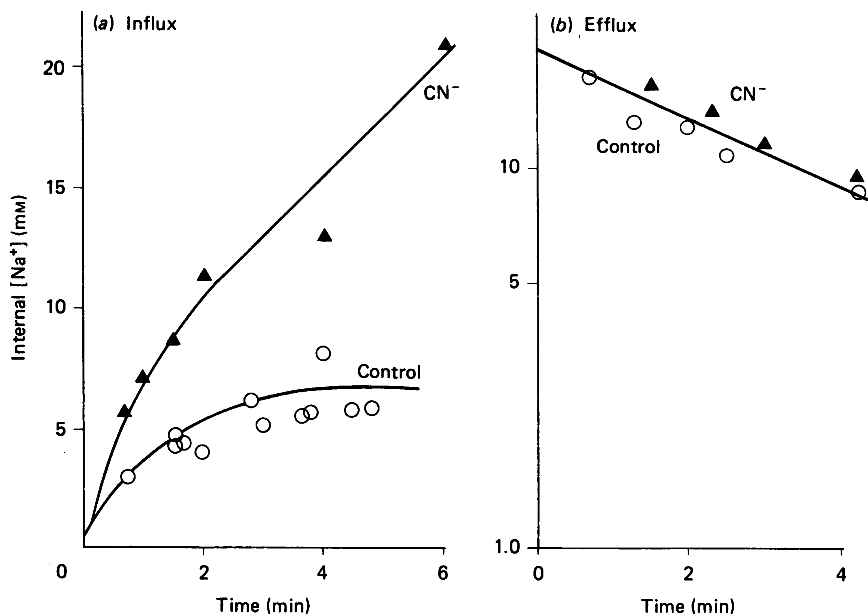

Fig. 6. Effect of CN^- and iodoacetate on Na^+ fluxes in brown adipocytes

(*a*) Influx study. Cells (8 units of citrate synthase/ml) were incubated in medium A containing 15 μCi of $^{22}Na^+$/ml and saturated with O_2/CO_2 (19:1). In some cases (▲), medium A also contained 2 mM-CN^- and 2 mM-iodoacetate. The control experiments were repeated three times and the CN^- experiments twice. All the data points from each type of experiment are shown. The method of measuring intracellular $^{22}Na^+$ is described in the Methods and materials section. (*b*) Efflux study. Cells (9.5 units of citrate synthase/ml) were incubated in medium A containing 15 μCi of $^{22}Na^+$/ml and saturated with O_2/CO_2 (19:1). After 20 min, in some cases (○) the cells were diluted 1:70 into additional medium A at 37 °C. In other incubations (▲), the diluting media A contained 2 mM-CN^- and 2 mM-iodoacetate.

Most studies of plasma-membrane depolarizations caused by addition of noradrenaline to brown fat have been carried out with excised tissue perifused with oxygenated buffer (Williams & Matthews, 1974*a*,*b*; Fink & Williams, 1976). Microelectrodes were inserted into surface cells to measure cell membrane potential differences before and after noradrenaline was added to the perfusate. It seems likely that these cells became anaerobic when stimulated. Girardier *et al.* (1968) reported that ouabain caused a very slow depolarization over a period of about 2 h, whereas noradrenaline and anaerobiosis produced a rapid large (30 mV) depolarization. Other workers (Williams & Matthews, 1974*b*), however, found more rapid depolarizations with ouabain. Measurements have also been made *in vivo* (Flaim *et al.*, 1977). A pulse of noradrenaline caused a rapid, early, depolarization of about 10 mV, which lasted only a few minutes, whereas temperature in the brown-fat pad rose and slowly fell for a period of about 15 min after the injection of hormone. It seems plausible that noradrenaline initially stimulated metabolism in the tissue, producing an anaerobiosis and thus an increase in Na^+ conductance. Homoeostatic mechanisms would increase the blood supply, restoring the potential to normal because of the increased availability of O_2. This view is supported by the fact that the temperature in the tissue increased only after the return of the cell membrane potential to normal. In more recent studies (Girardier & Schneider-Picard, 1983) measurements of cell membrane potential difference have been combined with measurements of tissue surface fluorescence. This provides a measure of the oxidation/reduction state of the tissue NAD(P)(H). The data indicate that when the excised tissue is perifused with noradrenaline there is an initial small transient depolarization, followed by a larger sustained depolarization which corresponds in time to a large increase in the surface fluorescence (i.e. the $NADH/NAD^+$ ratio). This increase in the redox state of the tissue does not occur in brown-fat pads *in situ* when exposed to noradrenaline (Prusiner *et al.*, 1968), nor does it occur in well-oxygenated brown adipocytes exposed to noradrenaline (LaNoue *et al.*, 1982). Separate studies with α-adrenergic-specific antagonists and β-antagonists indicate that the early transient depolarization is due to an α-adrenergic component of the noradrenaline response (Girardier & Schneider-Picard, 1983). Nanberg *et al.* (1984) also concluded, on the basis of antagonist sensitivities, that the noradrenaline-induced decrease in the K^+ diffusion potential was due to an α-adrenergic receptor, probably mediated by increases in cytosolic free Ca^{2+}. Thus further work needs to be done in order to clarify the physiological role of the putative Na^+ channel, which appears to be regulated by the ATP content of the cells, and the role of the other ion channel, which may be regulated by α-adrenergic hormones.

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