



Schneider-Picard, Gisela, Coles, Jonathan A., and Girardier, Lucien (1985) *Alpha- and beta-adrenergic mediation of changes in metabolism and Na/K exchange in rat brown fat*. *Journal of General Physiology*, 86 (2). pp. 169-188. ISSN 0022-1295

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Deposited on: 14 July 2014

α - and β -Adrenergic Mediation of Changes in Metabolism and Na/K Exchange in Rat Brown Fat

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ABSTRACT Double- and triple-barreled ion-sensitive microelectrodes were used to measure changes in extracellular K^+ and Na^+ concentrations ($[K^+]_o$, $[Na^+]_o$) in brown fat. Redox states of different respiratory enzymes were measured simultaneously in order to correlate ion movements with metabolic activity. Trains of stimuli applied to the efferent nerves evoked two distinct increases in $[K^+]_o$. A first, small, rapid increase occurred within 10 s and accompanied a first, rapid membrane depolarization. A second, slow increase of $[K^+]_o$ occurred several minutes after stimulation and accompanied a second, slow depolarization. A few seconds after stimulation onset, while the membrane was repolarizing and shifts in redox states indicated increases in lipolysis and respiration, $[K^+]_o$ decreased. The $[K^+]_o$ decrease was accompanied by an increase in $[Na^+]_o$, and could be partly blocked by ouabain. Phentolamine, an α -antagonist that blocks the first depolarization, also blocked the first, rapid $[K^+]_o$ increase and part of the subsequent decrease. Propranolol, a β -antagonist, had little effect on the first depolarization and the first increase in $[K^+]_o$, but blocked part of the subsequent $[K^+]_o$ decrease and the second, slow $[K^+]_o$ increase. The changes in $[K^+]_o$ were almost completely abolished in the presence of both antagonists. It is concluded that brown adipocytes take up K^+ and simultaneously lose Na^+ in response to the interaction of noradrenaline with α - and β -receptors, and this indicates a very early stimulation of the Na^+ pump.

INTRODUCTION

Brown fat in small homeotherms is a site of regulatory heat production during cold exposure (Smith and Horwitz, 1969) and may be involved in the regulation of body weight (Trayhurn and James, 1978; Himms-Hagen and Desautels, 1978; Rothwell and Stock, 1979; Seydoux et al., 1981). The heat production is controlled mainly by sympathetic innervation (Girardier and Seydoux, 1971a). Neither the thermogenic mechanisms that account for the more than 10-fold increase of respiration (Williamson, 1970; Foster and Frydman, 1978; Bukowieki

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et al., 1980) nor the intracellular events that control them are fully understood (recently reviewed by Horwitz, 1979; Girardier, 1983; Nicholls and Locke, 1984). It is known that both α - and β -receptors are involved (Flaim et al., 1977) and that the latter activate the adenylate cyclase pathway. This stimulates lipolysis and furnishes the free fatty acids that are the main substrate for oxidation in the mitochondria. Both receptors may be involved in transmembrane ion movements, as indicated by the α - and β -adrenergic-mediated depolarizations of the membrane induced by adrenergic agonists or nerve stimulation (Fink and Williams, 1976; Girardier and Schneider-Picard, 1983; Horwitz and Hamilton, 1984). In the present work, we have investigated ion movements further by monitoring extracellular K and Na concentrations ($[K^+]_o$, $[Na^+]_o$) while the tissue was activated, and simultaneously measuring changes in the redox states of respiratory enzymes as an index of metabolic activity (Schneider-Picard and Girardier, 1982). α - and β -adrenergic antagonists and the specific blocker of the Na^+ pump, ouabain, were used to analyze the different components of the response.

METHODS

Tissue Preparation

Male Sprague-Dawley rats (bred in the laboratory), housed at 23°C and weighing 230–290 g, were used. The interscapular brown fat was removed together with four of its five intercostal nerves and superfused in a Perspex chamber at 30°C as previously described (Seydoux et al., 1977). The composition of the Krebs-Ringer solution was (mM): 116.9 NaCl, 5.9 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4 , 1.5 or 2.5 $CaCl_2$, 24.9 $NaHCO_3$, and 5 glucose, equilibrated with 95% O_2 , 5% CO_2 , pH 7.4.

Recording of Optical Signals and Membrane Potential

The method of recording the two principal optical signals was as described previously (Schneider-Picard and Girardier, 1982; or, in more detail, Schneider-Picard, 1983). The preparation was illuminated at 465 nm (12 nm half-bandwidth) and, with the aid of a beam chopper, reflected light at 465 nm and fluorescence at 540 nm were measured. The intensity of the reflected light depends on the absorption (A): at 465 nm, a decrease in absorption indicates a reduction of the flavoproteins involved in the first step of β -oxidation of fatty acids (Garland et al., 1967; Schneider-Picard and Girardier, 1982). An increase in fluorescence (F) at 540 nm is attributed to oxidation of flavoproteins linked to NADH dehydrogenation (Garland et al., 1967; Schneider-Picard and Girardier, 1982). In some experiments, a third signal was obtained by illumination at 550 nm (25 nm half-bandwidth) and measurement of the reflected intensity, also at 550 nm (12 nm half-bandwidth) (Schneider-Picard, 1983). A decrease in absorption at 550 nm indicates oxidation of cytochrome c . A and F were recorded continuously on a multichannel pen recorder. The optical signals are expressed as percentages of the basal state signals (A_o , F_o ; i.e., $\Delta A/A_o \times 100$ for changes in absorption, and $\Delta F/F_o \times 100$ for changes in fluorescence). Membrane potential was recorded continuously with a conventional 3 M KCl electrode as described in Girardier and Schneider-Picard (1983).

Ion-sensitive Microelectrodes

Ion-sensitive microelectrodes were of the liquid membrane type, in which a column of ion sensor is lodged in the tip of a glass micropipette (Walker, 1971). Micropipettes were pulled on the puller described in Coles and Orkand (1983), from tubing with one or two

partitions (R & D Scientific Glass Co., Spencerville, MD). The barrels that were to be ion-sensitive were washed with acid and silanized, and the tips were filled with sensor as described in Munoz et al. (1983).

In preliminary experiments, the K⁺-sensitive barrels contained a classical ion-exchanger solution (3% by weight K tetra-*p*-chlorophenylborate in 2,6-dimethylnitrobenzene [Fluka, Buchs, Switzerland]). This has the advantage of a relatively low resistivity, but it is very sensitive to certain biological ions such as choline (Scholer and Simon, 1972; Kriz and Syková, 1981). In subsequent experiments, a much more selective sensor was used that was based on valinomycin (Oehme and Simon, 1976). Its composition was 5% valinomycin (Calbiochem AG, Lucerne, Switzerland) and 2% K tetra-*p*-chlorophenylborate in 2,6-dimethylnitrobenzene (Ammann, D., personal communication). To measure extracellular Na⁺, the sensors most widely used were unsuitable because they are sensitive to Ca²⁺ in the millimolar concentrations of the Krebs-Ringer solution, and extracellular [Ca²⁺] is known to change during stimulation in several tissues (see Orkand et al., 1984). We therefore used a sensor based on the neutral ligand ETH 157 (Ammann et al., 1976). Its composition was 10% ligand and 0.5% Na tetraphenylborate in 2-nitrophenyl-octyl-ether and it was a gift from Dr. D. Ammann, ETH, Zurich, Switzerland. This sensor is, however, sensitive to K⁺. Some of the ion-sensitive electrodes were usable for up to weeks, and we suggest that a factor that may have contributed to this long lifetime was the acid treatment of the micropipette (Coles et al., 1985).

In the triple-barreled electrodes, one of the outer barrels contained valinomycin, the other contained the Na⁺ sensor, and the reference was in the middle. A Krebs-Ringer solution that did not contain CaCl₂ was used to backfill the ion-sensitive barrels and to fill the reference. The barrels were connected via Ag/AgCl wires to the inputs of operational amplifiers (OPA 104 CM, Burr-Brown, Tucson, AZ, or AD 515 LH, Analog Devices, Norwood, MA) that were selected for input bias currents of $<20 \times 10^{-15}$ A. All electrodes were bevelled to tip diameters of ~ 1.5 – 2.5 μm and had resistances of 50–100 M Ω for the reference barrels and up to 5×10^{11} Ω for the barrels filled with the ion sensors. The voltages measured between the different barrels and a Ag/AgCl pellet in the superfusion chamber were amplified with the suppression of 50 Hz with a notch filter. The Na⁺ signal was passed through a low-pass filter with a cut-off frequency of 1 Hz. To obtain the ion signals, the electrical voltage of the reference barrel was subtracted from the voltages of the ion-sensitive barrels and recorded together with the ion signals on a pen recorder. The electrodes were calibrated before and after each experiment in a chamber of smaller volume than the superfusion chamber. In the calibration chamber, the voltages were measured between the different barrels and an agar–3 M KCl–Ag/AgCl bridge, and the reference voltage and the ion signals were recorded as described above. The calibration solutions were a Krebs-Ringer solution (6 mM KCl without CaCl₂) and two modifications of it, with 3 and 12 mM KCl substituting for equimolar NaCl. In some experiments, calibrations were done with the tip of the electrode in extracellular locations within the tissue (Fig. 8D): the response to an increase of [K⁺] from 6 to 12 mM was smaller by ~ 1 mV than the 17–18-mV response in the calibration chamber.

Tissue Stimulation and Drugs

The tissue was stimulated by applying biphasic electrical pulses (± 5 mA, 2 ms) between two Ag/AgCl wires placed on the nerve trunks. Noradrenaline (Arterenol, Hoechst AG, Frankfurt, Federal Republic of Germany [FRG]) was injected from a syringe in the superfusion medium close to the tissue region from which signals were recorded. Ouabain (Merck, Darmstadt, FRG), phentolamine (Regitine, Ciba, Basel, Switzerland), and DL-propranolol (Sigma Chemical Co., St. Louis, MO) were added directly to the Krebs-Ringer solution.

Experimental Procedure

After equilibration of the tissue in the superfusion chamber (for ~40 min), a basal value for reflected light was obtained. The ion-sensitive electrode was advanced under microscopic control until its tip touched the tissue. It was then advanced until the reference barrel showed an abrupt negative voltage deflection of ~20–50 mV. The K⁺ signal showed a positive deflection of ~40–70 mV. To test the integrity of the preparation, the nerve was given 100 stimuli at 4 Hz and the optical and voltage signals were recorded. By the time the tissue recovered, the reference and the K⁺ potentials had fallen to a bath value of ±1.5 mV and the electrode responded to changes in bath [K⁺] (Fig. 8D). The electrode was left in this position for the 4–7 h necessary for the experiments. This technique for locating the electrode in an “extracellular” position was chosen in preference to advancing the electrode into the tissue and then withdrawing it to find an extracellular position as judged by a voltage on the reference barrel within 5 mV of that in the bath. The latter method would probably not have distinguished between a site between adipocytes and the lumen of one of the numerous capillaries that traverse the tissue: it was found to give less reproducible results, and in only one of eight experiments was the first, rapid increase of [K⁺]_o observed (see below). Further, the amplitude of the subsequent [K⁺]_o decrease was only ~50% of that determined in the later experiments with the other method.

Ion Concentrations and Presentation of the Data

The voltage signals of the ion-sensitive electrodes were transformed to ion concentrations in the following way. For the valinomycin electrodes, it was verified that the selectivity against other ions was so high that the calibration voltage was close to that given by the Nernst equation, and $\Delta V = S \log\{[K^+]_2/[K^+]_1\}$, where $S = 60$ mV under our experimental conditions and [K⁺]₁, the prestimulus value of [K⁺]_o, was taken as the concentration in the bath. (In 20 experiments, the differential voltage deflection on the K⁺ barrel was usually less than ±1.5 mV when the electrode was withdrawn from its intratissular location into the bath.) Electrodes with the classical K⁺ exchanger have a finite sensitivity to Na⁺, and the calibration curve varies from one electrode to another (e.g., Fig. 14 of Coles and Tsacopoulos, 1979). Therefore, [K⁺] was obtained from the voltage signal by reading off from the calibration curve. Although the Na⁺ sensor is somewhat sensitive to K⁺, tests confirmed that the measurements of [Na⁺]_o were little affected by changes in [K⁺]_o: in the presence of constant [Na⁺]_o, a decrease of [K⁺] from 12 to 6 mM caused a steady state shift of the Na⁺ signal of less than -0.1 mV. Moreover, as will be shown, physiological changes in [K⁺]_o and [Na⁺]_o are roughly reciprocal, so the small correction necessary was taken care of by our choice of calibration solutions. The fractional changes in [Na⁺]_o were so small that the change in voltage signal was proportional to the change in [Na⁺]_o. Since ion-sensitive electrodes are affected by changes in temperature, we made tests to see whether heat generation by the brown fat might contribute to the Na⁺ signal. Stimulation caused the tissue temperature to change by no more than 0.01 °C (presumably because of heat transfer to the superfusate). Further, artificially raising the temperature at the tip of the electrode by 1 °C caused the potential to shift by -1 mV; the stimulus-induced Na⁺ changes to be described were in the opposite direction.

Mean values are given ± SEM. The numbers of preparations are given with the results. *P* values were determined by the χ^2 test.

RESULTS

Fig. 1 shows simultaneous records of membrane potential (V_m , top trace) and two optical signals. The preparation was illuminated at 465 nm. The fourth trace shows reflected intensity and indicates changes in absorption ($\Delta A/A_o$) by flavo-

proteins involved in fatty acid oxidation (Schneider-Picard and Girardier, 1982). An upward deflection of the trace indicates decreased absorption and flavoprotein reduction. The fifth trace shows changes in fluorescence at 540 nm ($\Delta F/F_0$)

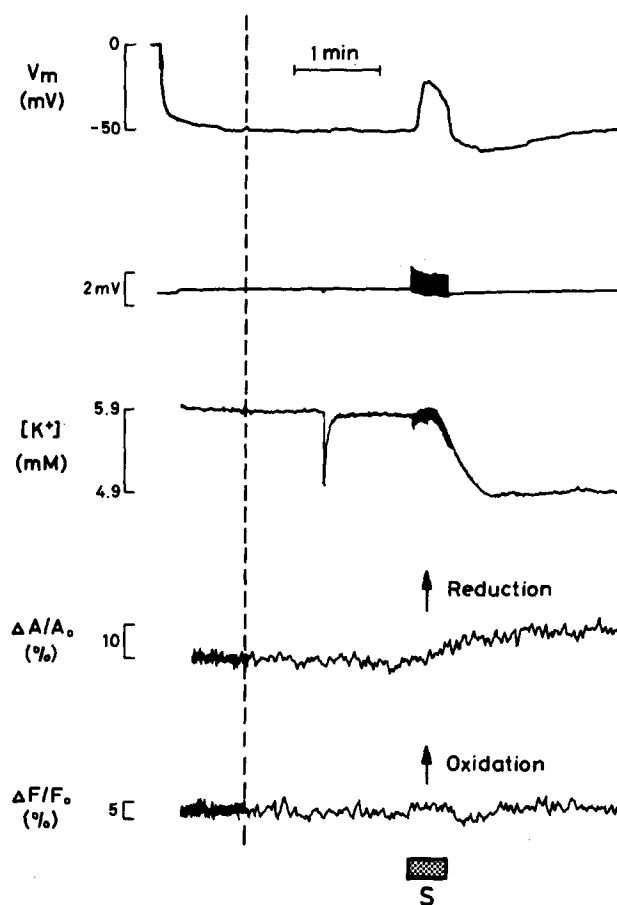


FIGURE 1. Simultaneous recording of the variations of membrane potential, $[K^+]_o$, and flavoprotein redox states evoked by nerve stimulation. During the period indicated by *S*, the nerve was stimulated by 100 stimuli at 4 Hz. The top trace shows membrane potential recorded with a 3 M KCl electrode. The second trace shows the voltage recorded by the reference barrel of the double-barreled K^+ -sensitive microelectrode. The rapid deflections recorded during stimulation are artifacts caused by pulse application between the stimulating electrodes. The third trace shows the K^+ signal, i.e., the voltage recorded with the ion-sensitive barrel from which the voltage of the reference barrel has been subtracted. The K^+ concentration scale gives the values of maximum and minimum values of $[K^+]_o$. The ion sensor was based on valinomycin. The fourth trace shows the fractional changes in reflected light intensity at 465 nm ($\Delta A/A_0$): an upward deflection indicates a decrease in absorption, i.e., an increase in reduction of flavoproteins. The lowest trace shows fractional changes in emitted fluorescence at 540 nm ($\Delta F/F_0$): an upward deflection indicates an increase in fluorescence, i.e., oxidation of flavoproteins. Between the dashed lines, the chart recorder speed was increased by a factor of five.

and an upward deflection indicates oxidation of flavoproteins linked to NADH dehydrogenation (Schneider-Picard and Girardier, 1982). As described previously (Girardier and Schneider-Picard, 1983), nerve stimulation (in this case, 100 stimuli at 4 Hz) caused the cell to depolarize within seconds, before any changes in tissue absorption or fluorescence could be observed. After the end of such a train of stimulation, the membrane hyperpolarized. The figure also shows simultaneous records from a double-barreled, K^+ -sensitive, intratissular microelectrode. The second trace is the potential recorded by the reference barrel, whose steady state potential was within 0.5 mV of the bath potential; the positive-going deflections are artifacts caused by pulse application between the stimulating electrodes on the nerve trunks. The third trace is the K^+ signal. It will be argued in the Discussion that this signal is at least a qualitative indication of changes in

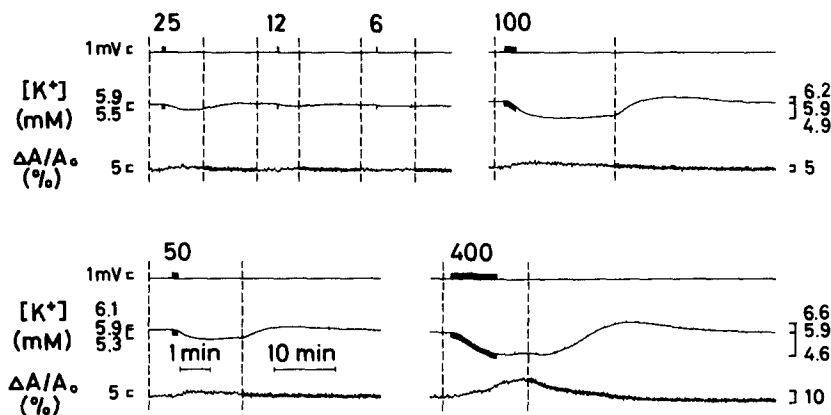


FIGURE 2. The variations of $[K^+]_o$ (middle trace) and flavoproteins at absorption ($\Delta A/A_o$, lower trace) as a function of stimulation duration. The duration of the nerve stimulation at 4 Hz is indicated by the duration of the artifactual voltage deflection on the reference barrel of the double-barreled electrode (top trace) and the number of stimuli is indicated above the record. The ion-sensitive barrel contained K^+ exchanger. Note the increase in recording speed between the dashed lines.

$[K^+]_o$, the K^+ concentration in the extracellular clefts. It is seen that after ~ 20 s of stimulation, $[K^+]_o$ began to fall, from 5.9 to 4.9 mM. Before the fall, there was a hint of a first, rapid increase: this was observed in 64 of the 106 recordings (34 preparations). Its amplitude seemed to depend on the intratissular location of the electrode tip, as described in Methods, and we suggest that it is genuine.

Fig. 2 shows that the amplitudes of the slow $[K^+]_o$ variations, as well as the absorption decrease, depend on the duration of the tissue stimulation at a fixed frequency. It also shows that there was a change in $[K^+]_o$ in response to six stimuli, but not in reflected light intensity; this may have been simply because the optical signals were noisier than the K^+ signal. For trains of fewer than 400 stimuli, the maximum decrease in $[K^+]_o$ occurred after termination of stimulation, at ~ 1 min after stimulus onset. For ≥ 100 stimuli, the $[K^+]_o$ decrease was followed

by a second, slow $[K^+]_o$ increase beyond the prestimulus value, several minutes after the end of stimulation (right-hand panels in Fig. 2). If the stimulation duration was prolonged further, as shown in Fig. 3A, $[K^+]_o$ increased beyond the prestimulus value during stimulation (five experiments). This increase in $[K^+]_o$ could be followed by a second undershoot, which was not examined further in these experiments. Fig. 3B shows that decreases in $[K^+]_o$ could also be observed after tissue activation by exogenous noradrenaline. The second decrease in

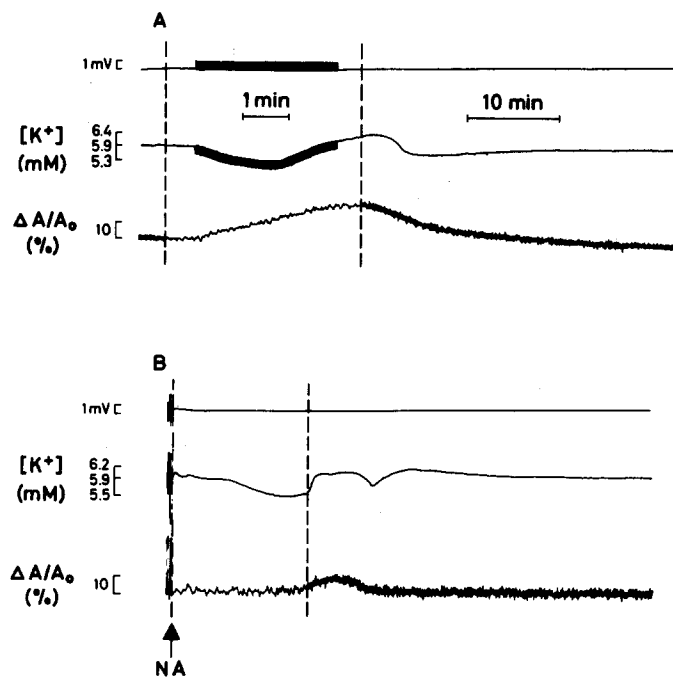


FIGURE 3. Variations of $[K^+]_o$ and flavoprotein redox states evoked by prolonged nerve stimulation or by an injection of noradrenaline. In A, the nerve was stimulated at 4 Hz for ~ 3 min, as can be seen from the artifactual voltage deflection on the reference barrel (upper trace). In B, injection of a small volume of noradrenaline (10^{-6} M) near the tissue is indicated by the arrow. The flavoproteins at absorption ($\Delta A/A_o$) are shown in the lower traces; an upward deflection indicates decreased absorption. Note the increase in recording speed between the dashed lines.

$[K^+]_o$ 10–15 min after noradrenaline injection was observed in three out of four cases.

A well-established way in which transient decreases in $[K^+]_o$ below the baseline can be produced is by activity of the Na^+/K^+ exchange pump (e.g., Heinemann and Lux, 1975; Kriz et al., 1975; Kunze, 1977; Morad and Martin, 1981; Tsacopoulos et al., 1983). In the present work, we have investigated this possibility, first, by looking for an associated increase in $[Na^+]_o$ and, second, by observing the effect of ouabain.

Simultaneous Measurement of Changes in $[K^+]_o$ and $[Na^+]_o$ in Response to Nerve Stimulation

Fig. 4 shows recordings made with a triple-barreled electrode that measured both $[Na^+]_o$ and $[K^+]_o$. It can be seen that the $[K^+]_o$ decrease (third trace) was accompanied by a $[Na^+]_o$ increase (second trace), and the second small $[K^+]_o$ increase was accompanied by a hint of a $[Na^+]_o$ decrease. In response to 12 of 16 stimulation trains (six preparations), the decrease of $[K^+]_o$ was accompanied by a detectable increase in $[Na^+]_o$. For the four trials where no change in $[Na^+]_o$ was observed, the maximum $[K^+]_o$ decrease was unusually small (0.3 ± 0.1 mM). The means of the maximum concentration changes for the 16 trials were -0.8 ± 0.1 mM for $[K^+]_o$ and $+0.4 \pm 0.1$ mM for $[Na^+]_o$. We conclude that the $[K^+]_o$

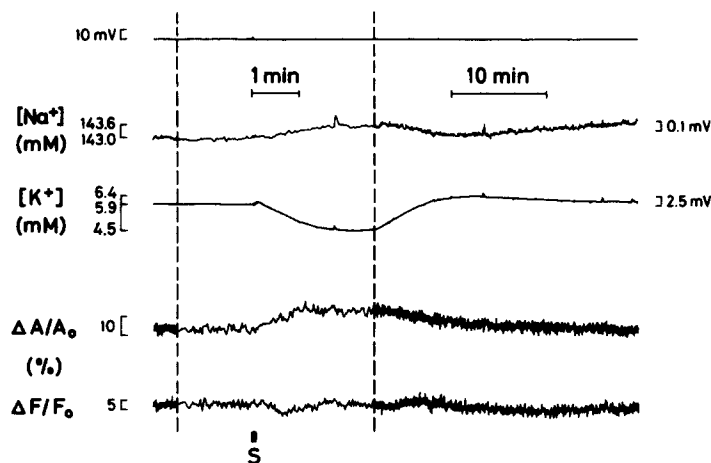


FIGURE 4. Simultaneous recording of the variations of $[Na^+]_o$ and $[K^+]_o$ and flavoprotein redox states evoked by nerve stimulation. The ion concentrations were measured with a triple-barreled electrode. The voltage of the reference barrel (top trace) was subtracted from the voltages of the ion-sensitive barrels to give the ion signals for Na^+ (second trace) and K^+ (third trace). During the period indicated by *S*, the nerve was stimulated by 100 stimuli at 32 Hz. The lower traces show flavoproteins at absorption ($\Delta A/A_o$) and at fluorescence ($\Delta F/F_o$). Note the increase in recording speed between the dashed lines.

decrease is associated with an increase in $[Na^+]_o$, as would be the case if Na^+ were pumped out of cells in exchange for K^+ .

Effect of Ouabain on the Changes of $[K^+]_o$

In this series of experiments, when the tissue had recovered from a control stimulation (Fig. 5A), it was exposed to ouabain. The bar in Fig. 5B indicates the period of 5 min during which the solution supplied to the peristaltic pump contained 10^{-4} M ouabain. Tests made with a fluorescent dye showed that a drug applied in this way reached the surface of the tissue with a delay of ~ 3 min, reached its full concentration ~ 6 min later, maintained this for ~ 1 min, and then was washed out with a half-time of ~ 4 min. Fig. 5B shows that ouabain caused

$[K^+]_o$ to increase in the resting tissue. In experiments during 20 min exposure to ouabain (not shown), it was found that $[K^+]_o$ reached its maximum ~10 min after the switching of the solutions. Fig. 5B shows the effect of stimulation at

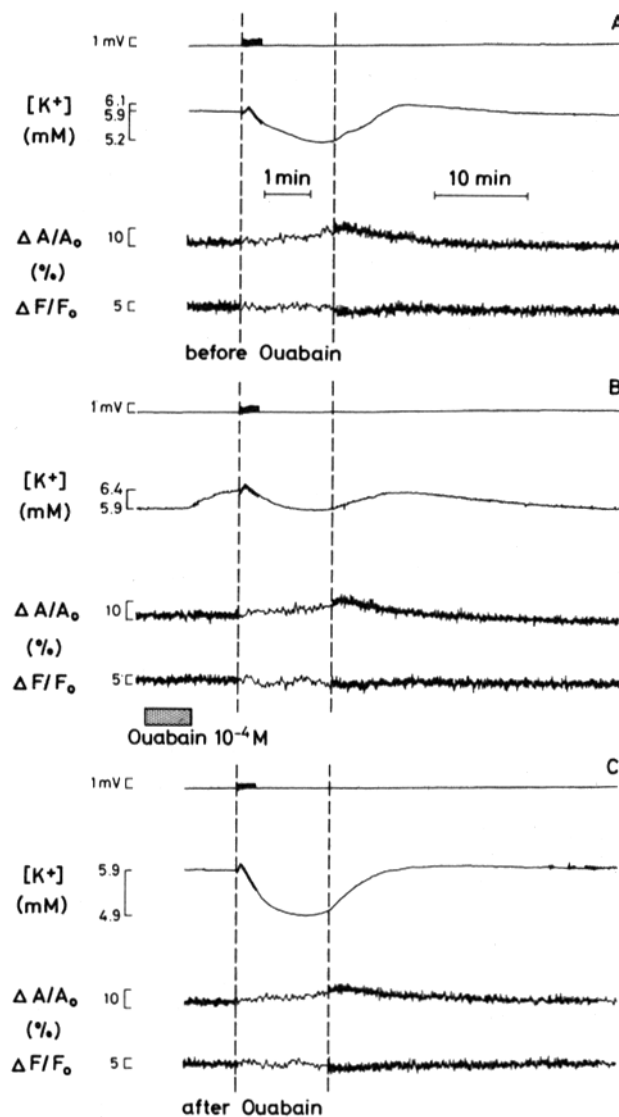


FIGURE 5. The effect of ouabain on the variations of $[K^+]_o$ and flavoprotein redox states evoked by nerve stimulation. During the periods of voltage deflection on the reference barrel (upper traces), the nerve was stimulated at 4 Hz by 100 stimuli. The control stimulation is shown in A. During the period indicated in B, the solution supplied to the peristaltic pump included ouabain (10^{-4} M). In C, the nerve stimulation was ~60 min after ouabain exposure. Note the increase in recording speed between the dashed lines.

this time: the decrease in $[K^+]_o$ was reduced. About 60 min after exposure to ouabain, the nerve was stimulated again, and Fig. 5C shows that the effect of ouabain was reversible. During a prolonged (20 min) exposure to ouabain, the effect on the stimulation-induced decrease in $[K^+]_o$ was comparable to that of a short (1 min) exposure. During washout of ouabain (20 min exposure), $[K^+]_o$ could fall under the resting level for ~20 min. With the same experimental protocols as in Fig. 5, in six preparations, 1 min exposure to ouabain 10^{-4} M caused $[K^+]_o$ to increase by 0.7 ± 0.3 mM; 20 min exposure caused it to increase by 1.0 ± 0.3 mM. The amplitude of the $[K^+]_o$ decrease in response to 100 stimuli at 4 Hz was 0.8 ± 0.1 mM in the control stimulation. A short exposure to ouabain decreased this amplitude by 40% and prolonged exposure by 30% ($P < 0.001$ control vs. ouabain). These ouabain exposures did not produce any consistent effects on the amplitudes of absorption decrease and fluorescence increase. We conclude that the $[K^+]_o$ decrease can be at least partially inhibited by ouabain, and this result, like the measurement of $[Na^+]_o$, suggests that the decrease depends on the activity of the Na^+/K^+ pump.

Different Patterns of Stimulation

To understand better the relation between the various parameters that were measured, we examined the effect of stimulation with a series of 2.5-s bursts at 4 Hz given every 24 s. As with a continuous train (Figs. 1–6A), the major changes in $[K^+]_o$ were a decrease, followed by a second, slow increase that overshoot the baseline (Fig. 6, A and B). At the onset of a continuous train, $[K^+]_o$ began to decrease within 10 s, as in Fig. 6A. With the stimulation in bursts, it could be seen that the events leading to the decrease could occur in <5 s (Fig. 6B). With such a stimulation, it was also observed that the rapid depolarization and the rapid $[K^+]_o$ increase evoked by each burst diminished in amplitude with successive bursts (Fig. 6B). In further experiments (five preparations) in which only V_m was measured, this progressive diminution in the amplitude of the rapid depolarization was observed consistently. Fig. 6 also illustrates that there was no obvious and constant relation between the changes in $[K^+]_o$ and in V_m . However, it can be seen that whenever V_m was slowly becoming more negative (membrane polarization), as during the later part of the stimulation in Fig. 6A (or, more visibly, in Fig. 1) or during the intervals between the first three stimulation bursts in Fig. 6B, then $[K^+]_o$ was falling. $[K^+]_o$ was also falling whenever the membrane hyperpolarized beyond the resting level (Figs. 1 and 6, A and B).

Summary of Optical Signals

In the steady state, the pump-mediated flux of K^+ into the cell must be balanced by a passive efflux, and either an increase in pump activity or a decrease in passive K^+ efflux would cause a decrease in $[K^+]_o$ (e.g., Matsuura et al., 1978; Shimazaki and Oakley, 1984). The former possibility, stimulation of the pump, would involve increased ATP consumption, whereas the latter would not. We therefore examined the optical signals for signs of increased mitochondrial respiration.

Stimulation of brown fat is known to cause a decrease in partial pressure of O_2 within seconds (Prusiner et al., 1968; Williamson, 1970; Seydoux et al., 1977), but it is difficult to deduce the time course of O_2 consumption from this parameter (see Mahler, 1978; Tsacopoulos and Poitry, 1982). Absorption at 465 nm, which is attributed to flavoproteins of fatty acid oxidation (Garland et al., 1967; Schneider-Picard and Girardier, 1982), gives information about substrates

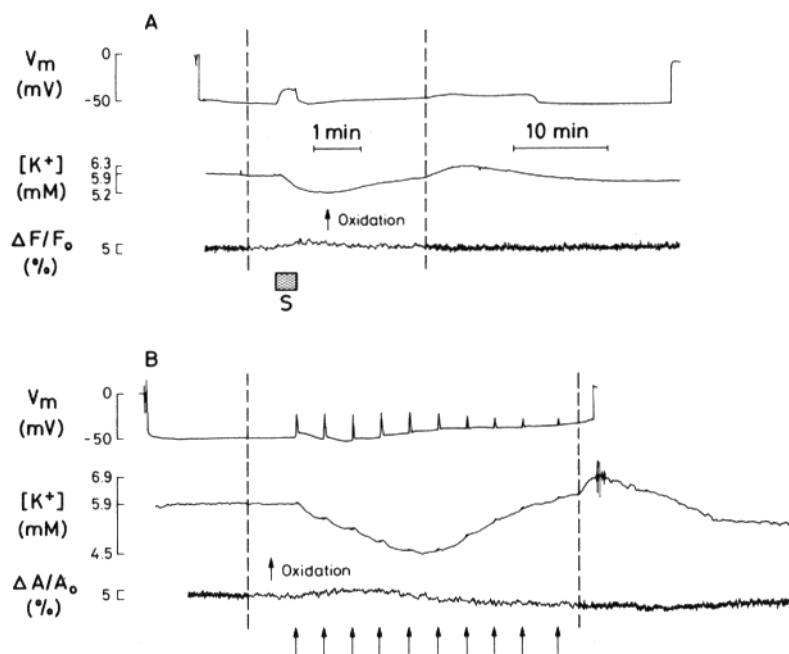


FIGURE 6. Variations of membrane potential, $[K^+]_o$, and redox states of flavoprotein and cytochrome *c* evoked by two different patterns of stimulation. As indicated, in *A* the nerve was stimulated by a continuous train of 100 stimuli at 4 Hz (*S*) and in *B* by bursts of 10 stimuli at 4 Hz (arrows), each separated by an interval of 24 s. The top traces in *A* and *B* show the membrane potential. The middle traces show $[K^+]_o$. The ion-sensitive barrel of the double-barreled electrode contained (*A*) the K^+ exchanger and (*B*) the sensor based on valinomycin. The lower trace in *A* shows flavoprotein fluorescence ($\Delta F/F_o$); the lower trace in *B* shows cytochrome *c* absorption ($\Delta A/A_o$). The arrows indicate oxidation. Note the increase in recording speed between the dashed lines. The record in *A* was made with the help of Mr. P. Dulguerov.

derived from lipolysis and entering the respiratory chain at the region of ubiquinone (Garland et al., 1967). In response to 18 stimulations of the types illustrated by Figs. 5*A*, 7*A*, and 8*A*, tissue absorption at 465 nm showed only monophasic reductions when $[K^+]_o$ decreased (e.g., Figs. 1–5, 7*A*, and 8*A*); this indicates that reducing equivalents derived from β -adrenergic-stimulated lipolysis are supplied to the respiratory chain. Absorption at 550 nm and fluorescence

at 540 nm indicate the redox states of cytochrome *c* and the flavoproteins linked to NADH dehydrogenation (Garland et al., 1967; Schneider-Picard, 1983), two members in the main stream of the respiratory chain. In Fig. 6*B*, the decrease in $[K^+]_o$ is seen to be paralleled by a decreased absorption at 550 nm, which indicates oxidation of cytochrome *c*. When $[K^+]_o$ increased above the baseline toward the end of the stimulation, the absorption signal indicated a small reduction of cytochrome *c*. A similar parallelism was observed with eight of nine stimulations (two preparations). In the ninth case, no change was detected. For fluorescence at 540 nm, the results were less clear-cut. In response to 18 stimulations of the types illustrated by Figs. 5*A*, 7*A*, and 8*A*, flavoprotein fluorescence showing monophasic oxidation with a time course similar to the decrease in $[K^+]_o$ was observed in six cases (e.g., Fig. 6*A*). In five cases, initial oxidation at the onset of the $[K^+]_o$ decrease was followed by a reduction when $[K^+]_o$ started to increase toward baseline (e.g., Fig. 7*A*). In one case, no change in fluorescence was detected. In three cases, oxidation was preceded by a short-lived reduction during $[K^+]_o$ decrease (e.g., Fig. 4). In three cases, there was only a reduction. In other words, in eight out of nine cases, the changes in absorption at 550 nm, and in 14 out of 18 cases, the changes in fluorescence at 540 nm, indicated oxidation and a transition from a resting to an active metabolic state (Chance and Williams, 1955). To summarize, the oxidation of two enzymes in the main stream of the respiratory chain was usually observed when $[K^+]_o$ decreased but appeared not to be obligatory.

Effect of α - and β -Adrenergic Antagonists on the Changes of $[K^+]_o$

Observations in several tissues suggest that catecholamines may stimulate the Na^+/K^+ exchange pump (Herd et al., 1970; Rogus et al., 1977; Clausen and Flatman, 1977; Hougan et al., 1981; Wu and Phillis, 1981; Gadsby, 1984) through both α - and β -adrenergic receptors (see Phillis and Wu, 1981). We examined the effect of adrenergic antagonists on the $[K^+]_o$ decrease in brown fat.

In this series of experiments, the control stimulation (Fig. 7*A*) was applied in bursts of 10 stimuli in order to determine the latency of the $[K^+]_o$ decrease. The stimulation frequency was 32 Hz. Fig. 7*B* shows the changes of $[K^+]_o$ when the α -antagonist phentolamine was added. The rapid increases in $[K^+]_o$ were absent and the amplitude of the $[K^+]_o$ decrease was diminished. The second, slow increase in $[K^+]_o$ and the amplitudes in reflected light intensities were not affected. Fig. 7*C* shows that in the presence of α - and β -antagonists (phentolamine and propranolol), the changes in $[K^+]_o$, as well as in reflected light intensities, were almost completely blocked. In some preparations, the reversibility of the antagonists was tested after a washout of ~2 h (e.g., Fig. 7*D*). Blocking of the rapid increase in $[K^+]_o$ was not seen to be reversible. This is probably due to the long duration of these experiments: it has been shown that the greatest decline in tissue responses after 6 h is in the amplitude of the first cell depolarization (Girardier and Schneider-Picard, 1983). With the same experimental protocol as in Fig. 7, in six preparations, no rapid increase in $[K^+]_o$ was observed in the presence of phentolamine. Phentolamine diminished the amplitude of the sub-

sequent $[K^+]_o$ decrease (1.3 ± 0.3 mM for the control stimulations) by 35% ($P = 0.1$, $n = 6$). In five of the six experiments, partial inhibition of the $[K^+]_o$ decrease was observed (mean 45%, $P = 0.06$); however, in one experiment, the $[K^+]_o$ decrease was increased by 40%, perhaps because of a dominant action of phentolamine on presynaptic α_2 receptors (Exton, 1981). Phentolamine had no significant effect on the amplitudes of the second, slow $[K^+]_o$ increase (0.7 ± 0.2 and 0.6 ± 0.2 mM) and of the absorption decrease (17 ± 2 and $22 \pm 1.5\%$). Phentolamine increased the latency of the $[K^+]_o$ decrease from 5 ± 1 to 11 ± 2 s, and, as already observed, the time to peak of the maximum absorption decrease was prolonged by ~ 1 min (Girardier and Schneider-Picard, 1983). In the pres-

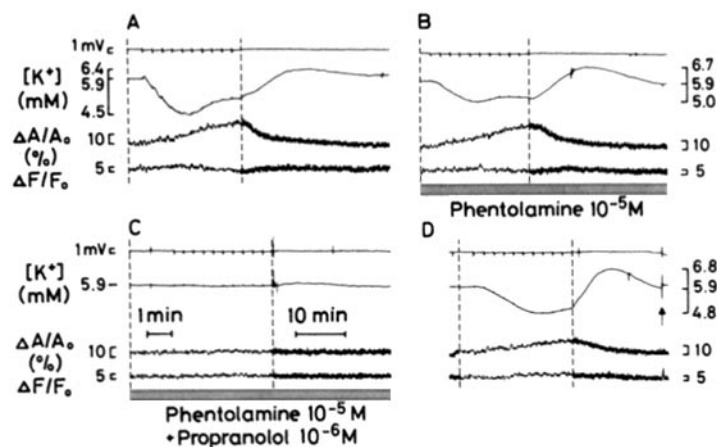


FIGURE 7. The effect of α - and β -adrenergic antagonists on the variations of $[K^+]_o$ and flavoprotein redox states evoked by nerve stimulation. The deflections on the reference barrel (upper traces) indicate when the nerve was stimulated by trains of 10 stimuli at 32 Hz separated by intervals of 24 s. Control stimulation is shown in *A*. Phentolamine (10^{-5} M) was added ~ 70 min before the second period of stimulation (*B*). Propranolol (10^{-6} M) was added ~ 60 min before the third period of stimulation (*C*), while phentolamine was continued. In *D*, a fourth stimulation is shown 150 min after removal of the antagonists. The arrow indicates when the electrode was withdrawn into the bath. The lower traces show flavoproteins at absorption ($\Delta A/A_o$) and at fluorescence ($\Delta F/F_o$). Note the increase in recording speed between the dashed lines.

ence of both antagonists, all amplitudes of the slow signals were diminished by 90% ($P < 0.005$).

In a further series of experiments, the tissue was exposed first to propranolol. Fig. 8*B* shows how the rapid $[K^+]_o$ increase occurring in response to each stimulation burst did not decrease in amplitude with repeated stimulation. This is in contrast to control stimulation, where after about five to six bursts the amplitude of the rapid $[K^+]_o$ increase diminished (Fig. 8*A*; see also Fig. 6*B*). With the same experimental protocol as in Fig. 8, in six preparations, propranolol decreased the amplitude of the $[K^+]_o$ decrease (1.1 ± 0.2 mM for the control

stimulations) by 55% ($P < 0.05$), that of the second, slow $[K^+]_o$ increase (0.5 ± 0.1 mM) by 60% ($P < 0.05$), and that of the absorption decrease ($12 \pm 2.5\%$) by 80% ($P < 0.005$). Fig. 8D shows that the K^+ -sensitive electrode could be calibrated with its tip in an "extracellular" location within the tissue. Small increases in reflected intensity at absorption and fluorescence (lower traces) during superfusion with the high-K solution can also be seen. We conclude that the multiphasic changes in extracellular ion concentration seem to be mediated by both α - and β -adrenergic receptors. The first, rapid $[K^+]_o$ increase is secondary to α -receptor activation; the second, slow $[K^+]_o$ increase results from β -receptor activation. Both α - and β -receptors seem to contribute to the early $[K^+]_o$ decrease.

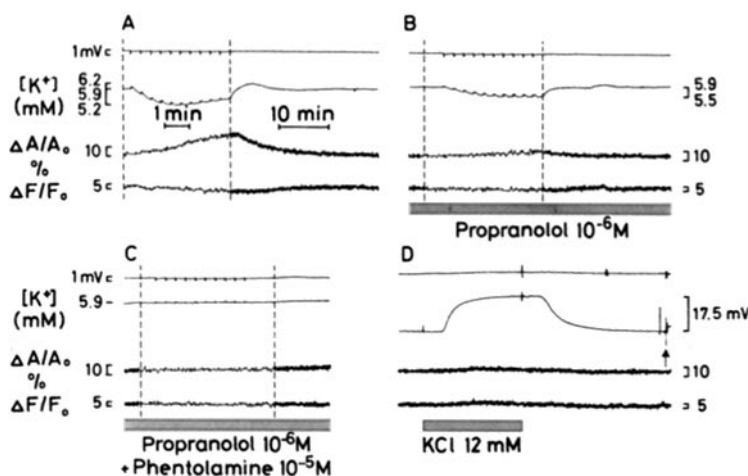


FIGURE 8. The effect of β - and α -adrenergic antagonists on the variations of $[K^+]_o$ and flavoprotein redox states ($\Delta A/A_o$ at absorption and $\Delta F/F_o$ at fluorescence) evoked by nerve stimulation. The deflections on the reference barrel (upper trace) indicate when the nerve was stimulated. The control stimulation is shown in A. The tissue was first exposed to propranolol (10^{-6} M) ~ 50 min before the second period of stimulation (B). Phentolamine (10^{-5} M) was added ~ 80 min before the third period of stimulation (C), while propranolol was continued. D shows the K^+ signal in response to a solution change with the tip of the electrode within the tissue. During the period indicated, the preparation was superfused with a modified Krebs-Ringer solution containing 12 mM KCl. For the last minute of the record, the amplification of the K^+ signal was increased by a factor of 2.5. The electrode was withdrawn to the bath (arrow) and a deflection of 1.3 mV was recorded. Note the increase in recording speed between the dashed lines.

DISCUSSION

Validity of the Ion Measurements

Our choice of unconventional ion sensors for most of the measurements ruled out the two known possibilities for artifacts caused by interfering ions (quaternary ammonium ions for the classical K^+ exchanger, and Ca^{2+} for the widely used

Na⁺ sensor based on ligand ETH 227 [Scholer and Simon, 1972; Steiner et al., 1979]). Another question is what it means to put a 2- μm electrode tip into an extracellular space that was originally $\sim 0.1\text{--}0.3\ \mu\text{m}$ wide as judged from freeze-fracture replicas of brown fat. The electrode tip was initially located at a site ($\sim 100\ \mu\text{m}$ from the tissue surface) where potential and $[\text{K}^+]_o$ suggested a damaged cell, but after ~ 40 min these parameters came close to those in the bath and followed changes in bath concentrations (Fig. 8D), which indicates free diffusional communication with the bath. Measurements of a variety of ions in a variety of tissues have given results compatible with the idea that an "extracellular" electrode does measure changes in concentrations in extracellular clefts (Coles and Tsacopoulos, 1979; Nicholson and Phillips, 1981; Orkand et al., 1984), perhaps with some delay (<5 s) and probably with an underestimation of the amplitude of the first, rapid $[\text{K}^+]_o$ increase (see Newman and Odette, 1984, and section 2 in Coles, 1985). It is likely that the diffusion of Na⁺ and K⁺ was similarly affected and therefore the measured ratio $\Delta[\text{Na}^+]_o/\Delta[\text{K}^+]_o$ was independent of the electrode position. The small changes in electrical potential and K⁺ signal that were often seen when the electrode was withdrawn to the bath are to be expected in a relatively large mass of syncytial tissue (e.g., Mathias and Rae, 1983; Gardner-Medwin et al., 1981).

Time Courses of the Different Signals

The simultaneous recordings of the multiphasic changes in redox states, membrane potential, and extracellular ion concentrations provide information about the metabolic and electrical events occurring in brown fat after the interaction of noradrenaline with α - and β -receptors. The present work confirms that in response to nerve stimulation, brown fat cells depolarize within seconds, before any change in optical signals is detected (Girardier and Schneider-Picard, 1983). At ~ 10 s, when shifts in the redox states of the respiratory enzymes indicate increased metabolic activity, the cells start to repolarize. Minutes after the end of stimulation, a second, slow depolarization occurs (Fig. 6A; see also Fig. 2 in Girardier and Schneider-Picard, 1983). The simultaneous recording of $[\text{K}^+]_o$ shows that the two temporally distinct membrane depolarizations are accompanied by two temporally distinct increases in $[\text{K}^+]_o$. The main conclusions from the simultaneous recording of the different signals are: nerve stimulation by trains of 100 stimuli is followed by a long-lasting (30–50 min) increase in lipolysis and respiration, as indicated by the optical signals. This change in metabolic activity is accompanied by complex multiphasic changes in membrane potential and extracellular ion concentrations. After a few seconds (as will be discussed below), stimulation of both respiration and active Na⁺/K⁺ exchange occurs and the latter leads to a decrease of $[\text{K}^+]_o$. The subsequent increase of $[\text{K}^+]_o$ over minutes might be the consequence of increased passive fluxes that lead to the increase in $[\text{Na}^+]_i$ and the decrease in $[\text{K}^+]_i$ observed in previous studies (Girardier and Seydoux, 1971b). The restoration of such perturbed ion gradients by the Na pump is perhaps the cause of the second decrease in $[\text{K}^+]_o$ and membrane repolarization (Fig. 3).

Evidence That Stimulation of Na⁺/K⁺ Exchange Contributes to the Decrease in [K⁺]_o

The changes in [K⁺]_o that we have observed correspond to transmembrane ion fluxes many times larger than those necessary to produce the changes of charge on the membrane capacity that correspond to the observed changes in membrane potential (e.g., for cells 40 μm in diameter, with an extracellular volume fraction of 10%, a 50-mV change in membrane potential requires a transmembrane ion movement that would change the extracellular concentration of that ion by ~0.01 mM). Hence, to maintain electroneutrality, ions other than K⁺ must also cross the membrane. There are three processes that could, in principle, cause a decrease in [K⁺]_o and an increase in [Na⁺]_o, such as we observed: (a) Entry of K⁺ and Cl⁻ into the cells. To maintain osmolarity, there would also be a movement of water into the cell, which would cause shrinking of extracellular space and an increase in [Na⁺]_o (see, e.g., Orkand et al., 1984). Since [K⁺]_o starts to fall while the cell is depolarized (e.g., Fig. 6A), this initial part of the fall, at least, could not be due to a straightforward passive entry of K⁺. (b) Increased pumping by a Na⁺/K⁺ exchange pump. (c) A Na⁺/K⁺ exchange pump that works at a constant rate in the presence of passive fluxes, and these passive fluxes are reduced by stimulation.

The permeability of the brown adipocyte cell membrane to Na⁺ seems relatively high (Girardier et al., 1968), and our observation that resting [K⁺]_o increases in the presence of ouabain (Fig. 5) is further evidence for a Na⁺ leak that is normally balanced by the continuous working of a Na⁺/K⁺ exchange pump. In both process *b* and process *c*, the stimulation-induced decrease in [K⁺]_o depends on the Na⁺/K⁺ exchange pump; the fact that the rate of decrease is reduced in the presence of ouabain shows that process *b* or *c* or both must be present. Since a Na⁺/K⁺ exchange pump hydrolyzes ATP, the observation that stimulation increases respiration makes process *b* the more likely. We cannot exclude a role for process *c*, and, indeed, the time courses of the changes in [K⁺]_o and V_m cannot be explained in any straightforward way just by changes in Na⁺/K⁺ pump rate. But if *c* were the only process, then the changes in membrane permeability would have to be complex and unusual. At the onset of stimulation, the membrane depolarizes without any fall in [K⁺]_o; this might be due to an entry of Na⁺. Then [K⁺]_o starts to fall, which would require a decrease in K⁺ permeability. Then the membrane hyperpolarizes with no change in the rate of fall of [K⁺]_o (e.g., Fig. 1), and so on. We suggest that a contribution from the activation of the pump is a more promising working hypothesis.

The Mechanisms of the Early Activation of the Na⁺ Pump

In excitable cells, the Na⁺ pump is stimulated mainly by the increase in [Na⁺]_i that occurs during action potentials (e.g., Thomas, 1972). In brown fat, stimulation of the Na⁺ pump by this mechanism occurs several minutes after stimulation (Girardier et al., 1968; Girardier and Seydoux, 1971*b*). However, the initial decrease of [K⁺]_o was of much greater amplitude and was longer lasting than the preceding [K⁺]_o increase. This poses the question of what can stimulate the Na⁺ pump at this time. During the [K⁺]_o decrease, [K⁺]_o and, presumably, [Na⁺]_i

changed in the directions that would inhibit rather than stimulate the Na⁺ pump. Other mechanisms, such as those reviewed by Phillis and Wu (1981), must be considered. Hettinger and Horwitz (1983) studied the effects of adrenergic agonists and antagonists on Na⁺/K⁺ ATPase in brown fat membrane preparations and suggested that one way by which both α - and β -adrenergic agonists could stimulate the Na⁺ pump is by increasing the affinity for K⁺ at its substrate binding site. Other observations in different tissues suggest that β -adrenergic activation of the Na⁺ pump can also be secondary to the increase in cAMP (Rogus et al., 1977; Clausen and Flatman, 1977; Heinemann et al., 1978; Scheid and Fay, 1984). In resolving the question of how α - and β -receptors mediate Na⁺ pump stimulation in brown fat, a factor to be taken into account is that the latencies for the α - and β -adrenergic-mediated [K⁺]_o decreases were different (5 and 10 s, respectively).

To conclude, we have shown that nerve stimulation causes [K⁺]_o in brown fat to decrease and we argue that this is because the Na⁺ pump is stimulated. It appears that this occurs in a complex way as a result of at least two mechanisms, and that the mechanism mediated by β -receptors has a longer latency than the one mediated by α -receptors. For the α -receptor, in particular, nothing is known about the intracellular events occurring after the activation of the receptor or about their role in the metabolic response. The continuous recordings of membrane potential, redox shifts, and ion concentrations show that the α -receptor plays a role in the initial membrane events and affects the time course of lipolysis stimulated by β -receptors. Finally, the noradrenergic-stimulated Na⁺ pump could play two distinct roles in brown fat heat production: in addition to the heat produced directly by its functioning, the Na⁺ pump may cause a decrease in [ATP] that will stimulate mitochondrial respiration and perhaps contribute to the uncoupling of the respiration (Klingenberg, 1984).

We thank Dr. M. Tsacopoulos for critical comments on the manuscript. Mr. J. L. Munoz made most of the electrodes, Mr. R. Tièche built the amplifier, Mrs. A. Jousson helped with the brown fat preparation, and Mr. O. Zulauf prepared the illustrations. Mr. P. Dulguerov participated in preliminary experiments.

This work was supported by grant 3.071.081 from the Swiss National Science Foundation and U.S. Public Health Service grant EY 03504.

Original version received 5 November 1984 and accepted version received 19 March 1985.

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