

Voltage-gated Potassium Channels in Brown Fat Cells

MARY T. LUCERO and PAMELA A. PAPPONE

From the Department of Animal Physiology, University of California, Davis, California 95616

ABSTRACT We studied the membrane currents of isolated cultured brown fat cells from neonatal rats using whole-cell and single-channel voltage-clamp recording. All brown fat cells that were recorded from had voltage-gated K currents as their predominant membrane current. No inward currents were seen in these experiments. The K currents of brown fat cells resemble the delayed rectifier currents of nerve and muscle cells. The channels were highly selective for K⁺, showing a 58-mV change in reversal potential for a 10-fold change in the external [K⁺]. Their selectivity was typical for K channels, with relative permeabilities of K⁺ > Rb⁺ > NH₄⁺ » Cs⁺, Na⁺. The K currents in brown adipocytes activated with a sigmoidal delay after depolarizations to membrane potentials positive to -50 mV. Activation was half maximal at a potential of -28 mV and did not require the presence of significant concentrations of internal calcium. Maximal voltage-activated K conductance averaged 20 nS in high external K⁺ solutions. The K currents inactivated slowly with sustained depolarization with time constants for the inactivation process on the order of hundreds of milliseconds to tens of seconds. The K channels had an average single-channel conductance of 9 pS and a channel density of ~1,000 channels/cell. The K current was blocked by tetraethylammonium or 4-aminopyridine with half maximal block occurring at concentrations of 1–2 mM for either blocker. K currents were unaffected by two blockers of Ca²⁺-activated K channels, charybdotoxin and apamin. Bath-applied norepinephrine did not affect the K currents or other membrane currents under our experimental conditions. These properties of the K channels indicate that they could produce an increase in the K⁺ permeability of the brown fat cell membrane during the depolarization that accompanies norepinephrine-stimulated thermogenesis, but that they do not contribute directly to the norepinephrine-induced depolarization.

INTRODUCTION

Brown adipose tissue is an important site of heat production in mammals and plays an essential role in cold acclimation, arousal from hibernation, and prevention of hypothermia (Smith and Horwitz, 1969; Foster and Frydman, 1978; Nedergaard and Lindberg, 1982; Nicholls and Locke, 1984). Recent findings have also implicated brown fat as playing a role in diet-induced thermogenesis, indicating that its

Address reprint requests to Dr. Pamela A. Pappone, Department of Animal Physiology, University of California, Davis, CA 95616.

metabolic activity is relevant to obesity and energy balance (Rothwell and Stock, 1979). The thermogenic capabilities of brown fat are tightly regulated by the sympathetic nervous system. Release of norepinephrine by sympathetic neurons onto α - and β -adrenergic receptors on the brown fat cell membrane initiates two responses; a triphasic membrane electrical response, consisting of a rapid depolarization and hyperpolarization followed by a sustained depolarization (Girardier et al., 1968; Williams and Mathews, 1974*a, b*; Fink and Williams, 1976; Girardier and Schneider-Picard, 1983; Horwitz and Hamilton, 1984; Schneider-Picard et al., 1985) and a metabolic response, in which the cell's oxygen consumption and heat production increase 10–40-fold (Foster and Frydman, 1978; Wickler et al., 1984).

While the metabolic responses of brown adipose tissue have been fairly well characterized, there has been much controversy about the ionic basis of the changes in membrane potential and the role that these changes play in transduction of the thermogenic signal across the brown fat cell membrane. On the basis of flux measurements and experiments modifying the Na/K pump activity, increased membrane permeability to Na⁺ and subsequent Na⁺ influx followed by K⁺ efflux have been postulated to play a role in brown fat activation (Herd et al., 1973; Horwitz, 1973; Nedergaard, 1981; Connolly et al., 1984, 1986). However, measurements of ionic fluxes in brown fat are difficult because the nonlipid portion of the intracellular cytosolic volume is small, and time resolution of data collection may be slow compared with ion movements. Thus controversy exists over measurement and interpretation of the flux data (Nanberg et al., 1984; Connolly et al., 1986; LaNoue et al., 1986).

In the present experiments, we used the patch-clamp technique (Hamill et al., 1981) on primary cultures of brown fat cells isolated from neonatal rats to examine membrane permeability changes directly. Previous patch clamp studies of brown adipocytes isolated from adult hamster and rat have demonstrated the presence of a nonselective cation channel that may play a role in the depolarizing phases of the triphasic response to norepinephrine (Siemen and Reuhl, 1987; Siemen and Weber, 1988). In this paper, we present evidence for a voltage-gated K channel in the membrane of isolated brown fat cells similar to delayed rectifier channels of nerve. We have characterized this channel and, based on selectivity, kinetics, pharmacology, and single-channel properties, we propose that it may be responsible for the hyperpolarizing phase of the triphasic response of brown fat cells to norepinephrine. Since the hyperpolarizing phase occurs simultaneously with increases in metabolism, this K channel may be important in the modulation of the norepinephrine-induced thermogenic response. A preliminary account of this work has appeared in abstract form (Lucero and Pappone, 1988).

METHODS

Cell Isolation and Culture Conditions

Brown fat cells were isolated from neonatal Osborne-Mendel rats, with a procedure similar to that described by Fain et al. (1967). We used primary cultures of brown adipocytes isolated from neonatal rats for the following reasons: (*a*) neonatal brown fat is capable of a vigorous thermogenic response (Skala, 1983), (*b*) younger brown fat cells are better suited for whole-

cell voltage-clamp experiments because they are smaller than adult cells, and (c) neonatal brown adipocytes contain less lipid than do adult cells and sink rather than float, making them easier to grow in culture and to patch. The 1–2 d old rats were kept for 1 h at 5°C to deplete brown fat cells of stored lipid and to induce analgesia. The rats were then swabbed with 75% ethanol and killed by decapitation. The interscapular and subscapular brown fat pads were carefully dissected under sterile conditions and placed in 5 ml of isolation buffer that consisted of Hanks balanced salt solution (HBSS), 2% bovine serum albumin (BSA), and 10 μ l/ml antibiotic antimycotic solution (ABAM) consisting of 100 μ g/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μ g/ml amphotericin B. All chemicals were from Sigma Chemical Co., St. Louis, MO unless stated otherwise.

Brown fat pads from four to six neonatal rats were pooled, minced, and placed in a sterile 15-ml centrifuge tube. The tissue was rinsed with 10 ml isolation buffer, which removed the majority of red blood cells, floating tissue fragments, and cell debris from the mincing. Most of the tissue fragments sank to the bottom and were resuspended in isolation buffer containing 2.6 mg/ml collagenase (Worthington Biochemical Co., Freehold, NJ) and 0.7 mg/ml trypsin inhibitor. The tissue-enzyme solution was sealed and incubated in a shaking 37°C water bath for 30 min. Upon removal from the bath, the undigested tissue fragments were allowed to settle for 2 min. The enzyme supernatant containing brown fat cells was placed in a new centrifuge tube and centrifuged at 1,500 rpm for 4 min. The enzyme supernatant was then placed back on the undigested tissue fragments and returned to the shaking water bath while the cell pellet was rinsed twice in isolation buffer containing 0.2 mg/ml DNase. The DNA released from broken cells causes the intact cells to form a pellet that had to be dissociated by DNase before the cells could be plated. After a final centrifugation for 2 min at 1,500 rpm the cells were plated on acid-cleaned glass coverslips in 35-mm plastic culture dishes in 2.5 ml of culture medium consisting of Dulbecco's modified Eagle medium (DMEM) with 5% fetal bovine serum (Gibco Laboratories, Grand Island, NY) and ABAM at 10 μ l/ml. Tests of cell growth in serum concentrations from 0.5 to 20% showed that 5% serum was optimal for maintaining brown fat cells without promoting fibroblast growth. The steps outlined above were repeated on the tissue fragments that had been returned to the shaking water bath which resulted in a substantial increase in the total yield of isolated brown fat cells. Cells were maintained at 37°C in a humidified atmosphere of 95% O₂ and 5% CO₂. After 24 h, 1 ml/dish of the media was replaced with fresh media containing 2 μ M cytosine arabinoside to inhibit fibroblast growth. The cytosine arabinoside was removed after 24 h, and the culture medium was replaced every 2 d thereafter. Cells were used for experiments after 2–22 d in culture.

The majority of brown fat cells isolated from cold-exposed neonatal rats under these conditions contained very little fat and sank to the bottom of the culture dish. The sinking brown fat cells adhered tightly to the glass coverslips, divided rapidly for the first 3 d, and accumulated numerous lipid droplets. Some brown fat cells grew as single cells, as small clusters of two to four cells, as shown in Fig. 1, or as dense patches of explant-type growth of several hundred cells. Voltage-clamp experiments were performed only on isolated cells. Cells isolated and cultured under these conditions retained the ability to respond to norepinephrine for at least 8–10 d as measured by fatty acid release and decreased size of their lipid droplets, apparently they lose their hormonal sensitivity when they become confluent (Kuusela et al., 1986).

Solutions and Channel Blockers

The control external solution used in electrophysiological studies on cultured brown fat cells was a mammalian Na-Ringers consisting of 150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, and 10 mM tetramethylammonium (TMA) HEPES buffer. The K-Ringers solution used in the selec-

tivity experiments consisted of 155 mM KCl, 2 mM CaCl₂, and 10 mM TMA-HEPES. The 80 and 40 mM K-Ringers solutions were made by appropriate mixtures of the K- and Na-Ringers. To test the permeability of other monovalent cations, 155 mM RbCl, CsCl, NH₄Cl, or NaCl, were substituted for KCl in the K-Ringers solution. For cell-attached patches, HBSS and 0.1% fatty acid-free BSA were used. All external solutions had a pH of 7.4 and osmolarities of 290–300 mosmol.

Tetraethylammonium (TEA) chloride (Kodak Chemical Co., Rochester, NY) Ringers solution was similar to Na-Ringers except that 150 mM TEA was substituted for Na. Various concentrations of TEA solutions were obtained by appropriate dilutions of TEA-Ringers with Na-Ringers. The 4-aminopyridine (4AP) solutions were made by dilutions of a 100-mM stock solution in water with appropriate amounts of Na-Ringers. A 20 μM apamin solution was



FIGURE 1. Cultured brown fat cells. Brown fat cells grown in primary culture 9 d in DMEM + 5% fetal bovine serum contain numerous lipid droplets. Scale bar represents 50 μm.

made up by adding crystalline apamin to Na-Ringers. This stock solution was aliquoted and frozen until just before further dilution and use. Purified charybdotoxin in an 18-μM solution (gift from E. Moczydlowski, Department of Pharmacology, Yale University) was added directly to the bath, which contained Na-Ringers and 0.1% BSA, to reach a final concentration of ~150 nM. BSA helps prevent loss of charybdotoxin activity that is due to nonspecific binding to plastics and glass.

The main internal pipette solutions used in these studies were either KF-BAPTA, consisting of 155 mM KF, 3 mM MgCl₂, 0.5 mM CaCl₂, 5.5 mM BAPTA, and 10 mM TMA-HEPES; or KF-EGTA, consisting of 140 mM KF, 1 mM CaCl₂, 2 mM MgCl₂, 11 mM K₂EGTA, and 10 mM TMA-HEPES. In addition, a KCl or K-aspartate internal solution was used that consisted of 150 mM KCl or K-aspartate, 10 mM K-HEPES, 2 mM NaATP, 3 mM MgCl₂, 0.6 mM

Na-cAMP, and 1.4 mM Na-cGMP. A CsCl internal solution, used to block outward K currents, was the same as the KCl internal solution except that 150 mM CsCl was substituted for KCl. Norepinephrine (*l*-arterenol bitartrate) was made up in a 3-mM stock solution with 1 mg/ml ascorbic acid to prevent oxidation, and added directly to the bath to give a final concentration of $\sim 5 \mu\text{M}$.

Voltage-Clamp Recordings

A small piece of coverslip with adherent brown fat cells was placed in a glass-bottomed chamber having a volume of $\sim 200 \mu\text{l}$. The chamber was thoroughly flushed with Na-Ringers. Patch recording pipettes were pulled from Dow-Corning glass (7052; Garner Glass, Claremont, CA), coated with Sylgard, and fire polished to have a resistance of 2–4 M Ω when filled with internal solution. A 3-cm H₂O positive pressure was applied to the interior of the patch pipette during the immersion and approach to the cell to prevent clogging or contamination of the pipette tip. Upon touching the cell membrane, the pressure was switched to a gentle suction of 3 cm H₂O until a gigaohm seal formed. Both spherical and flattened brown fat cells formed high-resistance seals with similar success. After gigaohm seal formation, the pipette capacitance of $\sim 5 \text{ pF}$ was nulled electronically, and a strong pulse of suction from a 50-ml syringe was applied to rupture the patch of membrane under the pipette and to achieve the whole-cell configuration. In all experiments the current signal was balanced to zero when the pipette was immersed in the bath solution, but the 0–6-mV junction potential between the pipette and bath solutions was not corrected for. In the experiments which examined kinetics of membrane currents, 25–40% of the resistance in series with the cell membrane was compensated by positive feedback. Experiments were done at room temperature ($\sim 22^\circ\text{C}$).

Data Acquisition and Analysis

Voltage-clamp experiments were run on-line using a Cheshire Data Interface and LSI 11/73 computer system (INDEC Systems, Sunnyvale, CA) to deliver command voltages, and sample and store current data. The membrane current signal was sampled by a 12-bit A/D converter and filtered as described in the figure legends using an eight-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA). Linear leak currents and residual uncompensated current through the membrane capacitance were measured using the P/4 procedure (Armstrong and Bezanilla, 1974), stored on disk, and subsequently digitally subtracted before further analysis of the data. All of the current records in this paper have been leak subtracted.

Single-channel currents were recorded in the cell-attached configuration with 2 s voltage steps from -60 mV negative to the cell's resting potential incrementing by 10-mV steps to $+100 \text{ mV}$ positive to rest. The sample interval was 4,208 μs with the filter set at 48 Hz. Amplitude histograms were made from records containing fully resolved channel openings. Single-channel conductance was determined from the slope of a line fitted by linear regression to the current data. Only high resistance seals ($\geq 20 \text{ G}\Omega$) were used for cell-attached single-channel recordings. All averages in this paper are reported as the mean \pm the standard deviation.

RESULTS

Passive Electrical Properties of Isolated Brown Fat Cells

The passive properties of brown fat cells voltage clamped in the whole-cell configuration were measured by averaging membrane currents from 32 depolarizing pulses of 8-ms duration and 10-mV amplitude from the holding potential of -60 mV . The pipette capacitance of 4–5 pF was nulled before breaking into the cell. The cell

membrane capacitance, calculated by integration of the transient current response to the pulses, averaged 23 ± 7 pF ($n = 35$) with corresponding cell diameters ranging from 15 to 35 μm . The input resistance, determined from the steady-state current at the end of the leak pulse averaged 1.3 ± 1.1 G Ω ($n = 44$), ranging from 0.1 to 4.7 G Ω . Assuming a specific membrane capacitance of 1 $\mu\text{F}/\text{cm}^2$, this corresponds to a specific resting membrane resistance of ~ 30 k Ω cm^2 .

Whole-Cell Currents

Whole-cell voltage-clamp experiments were performed on 163 cultured brown fat cells from 27 separate cell isolations 2–22 d after their isolation. Outward currents similar to those shown in Fig. 2 were seen in every cell tested. The currents activated in response to depolarizing voltage steps with a sigmoidal time course were sustained for the duration of the 200-ms depolarizing pulse, and were carried by K^+ ions (see below). Some cells had currents that exhibited greater degrees of inactivation during the pulse although most appeared similar to the currents in Fig. 2.

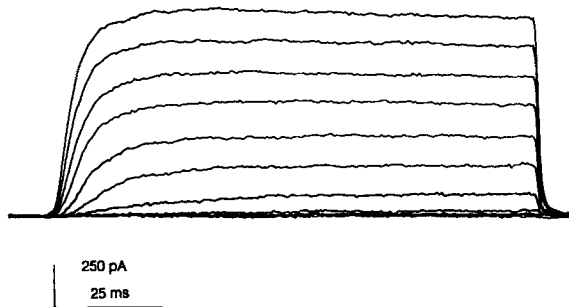


FIGURE 2. Voltage-dependent outward currents from an isolated brown fat cell patch clamped in the whole-cell configuration. The cell was bathed in Na-Ringers with KF-BAPTA in the pipette. Shown are superimposed currents recorded every 5 s during 200-ms voltage steps in 10-mV increments from -50 mV to $+50$ mV, applied from a holding potential of -60 mV, filtered at 380 Hz.

These outward currents were the only voltage-dependent currents apparent in any of the brown fat cells we recorded from, even when we used a more physiological internal solution than the F^- solution used in Fig. 2, internal solutions with little calcium or pH buffering capacity, or solutions that eliminated the voltage-gated K currents (see below). Neither the types of membrane currents present in the cells nor the properties of the K currents varied with the length of time the cells had been in culture. Membrane currents measured in several acutely isolated brown fat cells were indistinguishable from those seen in cultured cells.

Selectivity

To test if the channels responsible for the voltage-dependent outward currents were K^+ -selective, external Na^+ was replaced with increasing concentrations of K^+ . Fig. 3, A and B shows that the inward K current appeared as the reversal potential shifted to more positive potentials with increasing external K^+ concentration. To measure reversal potentials, even at low external K^+ concentrations, peak instantaneous tail currents were used to determine the reversal potential, as described in the legend of Fig. 3. The open-channel current-voltage relation for three concentrations of exter-

nal K^+ is shown in Fig. 3 C. Averaged reversal potentials measured from data like that in Fig. 3 C were plotted against the log of external K^+ concentration in Fig. 3 D. The predicted slope for a K-selective channel at 22°C of 58.6 mV/10-fold change in external K^+ is shown as a straight line superimposed on the data in Fig. 3 D. The

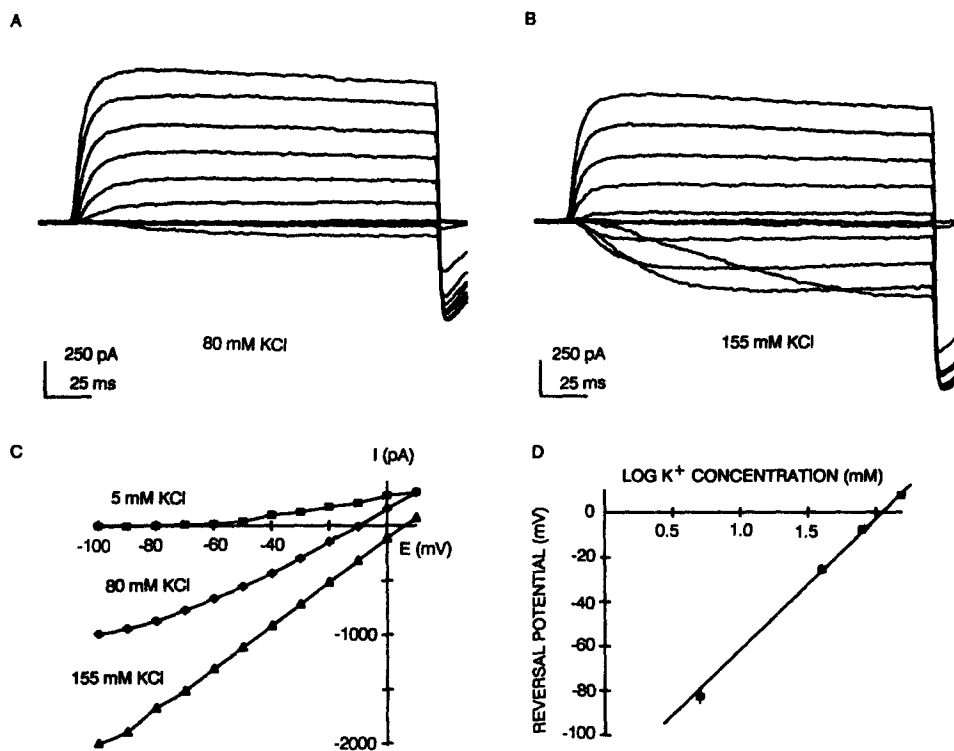


FIGURE 3. Appearance of inward K^+ currents as external Na^+ was replaced with increasing amounts of external K^+ ; the same voltage-clamp protocol was used as in Fig. 2. (A) 80 mM of the bath Na^+ was replaced with 80 mM K^+ . (B) 155 mM of the bath Na^+ was replaced with 155 mM K^+ . (C) Instantaneous current-voltage relation measured by fitting a single exponential function to the tail currents recorded at each voltage and extrapolating to determine the current amplitude at the time of the step. Tail currents were elicited by applying, at 15-s intervals, a depolarizing prepulse from the holding potential of -60 to $+20$ mV for 20 ms followed by a test pulse to the voltages shown for 100 ms. Currents were recorded with 5 mM KCl (squares), 80 mM KCl (diamonds), and 155 mM KCl (triangles) replacing NaCl in the bath solution. Records A–C are from the same cell. (D) Plot of averaged reversal potential measured from the instantaneous current-voltage relation vs. the log of the K^+ concentration. Standard deviations are in some cases smaller than the symbol. The slope of the straight line is 58.6 mV/decade which is the Nernst-derived slope for a K-selective channel at 22°C. Each point is the average for data collected from three different cells from two separate cell isolations.

close fit of the data to the predicted line indicates that these channels are highly selective for K^+ .

We tested the selectivity of the K channels in brown fat for other monovalent cations by measuring the shift in reversal potential when all of the external K^+ in the

K-Ringers solution was replaced by different test cations. Examples of currents recorded in Rb-, NH₄-, and Cs-Ringers are shown in Fig. 4. Permeability ratios for each test cation relative to K⁺, P_{X^+}/P_{K^+} , were calculated from the averaged change in reversal potential measured from tail currents using a modified Goldman (1943), and Hodgkin and Katz (1949) equation for equal external monovalent test ion concentrations:

$$P_{X^+}/P_{K^+} = e^{\Delta E_R F/RT} \quad (1)$$

where ΔE_R is the shift in reversal potential and F , R , and T are the Faraday constant, gas constant, and absolute temperature, respectively. Fig. 4 shows that Rb⁺ ions

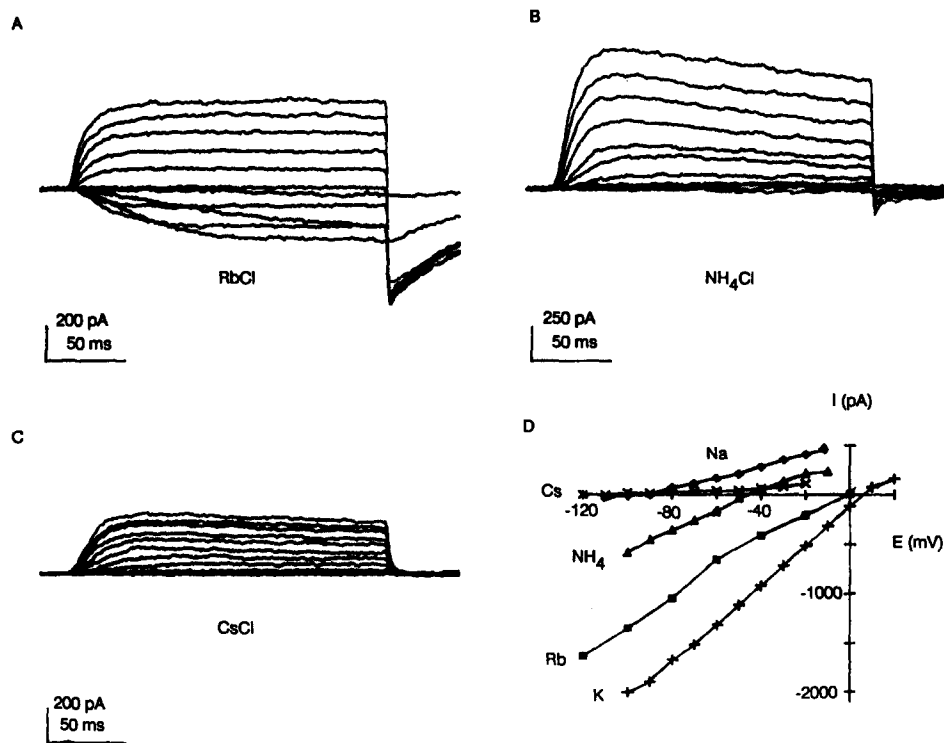


FIGURE 4. Selectivity of the voltage-dependent K channel for monovalent cations. (A–C) Whole-cell current records with 155 mM KCl in the bath solution replaced by 155 mM RbCl (A), NH₄Cl (B), or CsCl (C); same pulse protocol as in Fig. 2. Current records filtered at 800 Hz. (D) Instantaneous current-voltage relations measured as in Fig. 3 after replacing 155 mM KCl (crosses) with 155 mM Na (diamonds), 155 mM Cs (Xs), 155 mM NH₄ (triangles), or 155 mM Rb (squares).

were very permeant with a permeability ratio relative to K⁺ of 0.81 (± 0.08 , $n = 4$); and NH₄⁺ ions were less permeant with a permeability ratio of 0.18 (± 0.01 , $n = 4$). Na⁺ and Cs⁺ were almost impermeant, with an upper limit for their permeability ratios of 0.03 and 0.02, respectively. This selectivity sequence of K⁺ > Rb⁺ > NH₄⁺ > Na⁺ = Cs⁺ is similar to that found in delayed rectifier and many other K channels (Hille, 1984; Pappone and Cahalan, 1986).

Gating

Activation. The peak current-voltage relationship for a cell with K-Ringers in the bath and KF-BAPTA in the pipette is shown in Fig. 5 A. An inward K current that was activated with depolarizations positive to -50 mV reversed at $+6$ mV, close to E_K for these solutions, and reached a substantial magnitude of $1,215$ pA at $+50$ mV. The average current in brown fat cells at $+50$ mV was $1,630 \pm 695$ pA in high K solutions ($n = 28$). The series resistance in these experiments averaged 10 ± 18 M Ω ($n = 19$). This would produce an error of up to 16 mV in our uncompensated voltage measurements at $+50$ mV. In the kinetic experiments described

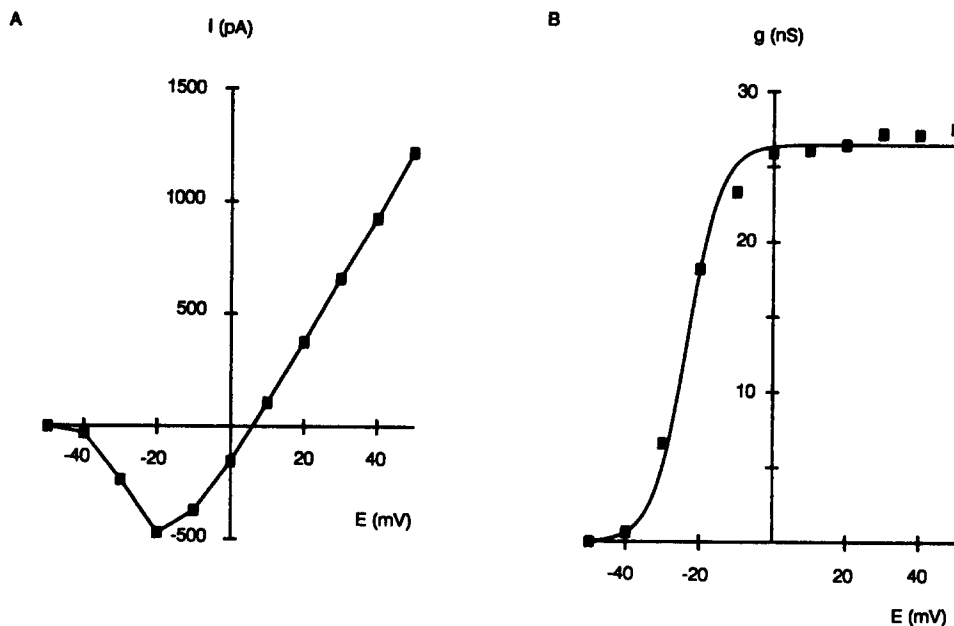


FIGURE 5. (A) Peak current-voltage relationship for a cell with a K-Ringers bath solution and a KF-BAPTA internal solution. The pulse protocol was the same as described in Fig. 2. Currents were activated at -40 mV and reversed direction at $+6$ mV near E_K for these solutions. The tip potential was not corrected in this and other records. (B) Conductance-voltage relation for the same cell. A Boltzmann relation (Eq. 2) fitted to the data (smooth line) gives a maximum conductance of 27 nS, $E_{1/2} = -23$ mV, and $k = 5$ mV.

below, 30–40% of the series resistance was compensated for by positive feedback. The instantaneous current-voltage relation in symmetrical K solutions was roughly linear as shown in Figs. 3 C and 4 D, thus the membrane permeability changes could be described as conductances under these conditions. The conductance-voltage relationship for these data from the maximum K currents at each potential, is shown in Fig. 5 B. The peak conductance increased sigmoidally with depolarization and saturated at potentials positive to 0 mV. The smooth line is a Boltzmann equation fitted to the peak K conductances (g_K), of the form:

$$g_K(E_m) = g_{K,max} / [1 + e^{(E_{1/2} - E_m)/k}] \quad (2)$$

The maximum conductance in this cell was 27 nS, and the average maximum conductance in these experiments was 20 ± 9 nS ($n = 14$) in K-Ringers. In Na-Ringers the maximum conductance was about half that in K-Ringers, averaging 9.5 ± 4.2 nS ($n = 11$). When normalized to the membrane capacitance of 23 ± 7 pF, and assuming a specific capacitance of $1 \mu\text{F}/\text{cm}^2$, this yields a specific conductance of $870 \mu\text{S}/\text{cm}^2$ in K-Ringers and $413 \mu\text{S}/\text{cm}^2$ in Na-Ringers. The voltage for half activation of the channels, $E_{1/2}$, was -23 mV in this example and averaged -28 ± 5 mV ($n = 14$). The steepness, k , averaged 5 ± 1 mV ($n = 14$), which indicates that the channels are very steeply voltage dependent. The magnitude of the K conductance and the voltage-dependence of K current activation were similar with F^- , Cl^- , or aspartate as the major anion in the internal solution. In addition, we examined the effect of permeant external monovalent cations on activation kinetics and found that, as in squid K channels (Matteson and Swenson, 1986), neither NH_4^+ nor Rb^+ had significant effects on brown adipocyte K channel activation.

K currents in brown adipocytes show a voltage dependence that is similar to the delayed rectifier K current in nerve (Hille, 1984), and it may have a similar role in repolarization of the membrane after depolarization. The activation kinetics described above suggest that the voltage-dependent K channels in brown adipocytes could be strongly activated by the initial membrane depolarization in response to norepinephrine, resulting in an outward current that could be large enough to repolarize and hyperpolarize the cell.

Permeant cations affect channel closing. The effects of external monovalent cations on channel closing kinetics are shown in Fig. 6. The tail currents in NH_4 -Ringers and Rb-Ringers were scaled to match the peak amplitude of the tail current in K-Ringers. The current in Rb-Ringers deactivates much more slowly than when K^+ is the current-carrying ion (Fig. 6 A). In contrast, the scaled tail current in NH_4 -Ringers declines more rapidly than when K^+ is in the external solution (Fig. 6 B). This effect is in the opposite direction from that expected from uncompensated series resistance error. To examine the closing kinetics at a whole range of membrane potentials, single exponential functions were fitted to tail currents measured during voltage steps from -120 to -20 mV. Fig. 6 C shows the time constants for channel closing plotted as a function of membrane potential for a representative cell in K-Ringers, Rb-Ringers, and NH_4 -Ringers. As is seen in squid axons, highly permeant Rb^+ slows while relatively impermeant NH_4^+ speeds channel closing (Matteson and Swenson, 1986). Time constants in Rb-Ringers were 1.2–2 times slower than in K-Ringers and time constants in NH_4 -Ringers were two to five times faster than in K-Ringers. This dependence of closing kinetics on the ion-carrying current through the channel has been explained by the “occupancy hypothesis” in which the channel is thought to be prevented from closing when a permeant cation occupies a binding site within the channel (Marchais and Marty, 1979; Swenson and Armstrong, 1981).

Inactivation. K currents inactivated completely with test pulses positive to -20 mV that were sustained for more than 1.5 min. To study the voltage dependence of steady-state inactivation at more negative potentials, two voltage protocols were used. Fig. 7 A shows the steady-state inactivation curve obtained using a 15-s pre-pulse to voltages from -90 to -30 mV in 10-mV increments followed by a 100-ms

test pulse to +30 mV. The peak current during the test pulse for each prepulse potential was normalized to the peak current after the prepulse to -90 mV. In the second protocol, the holding potential was varied from -120 to -40 mV, and the peak K⁺ current in response to a short test pulse of +50 mV given every 30 s was recorded until the current amplitude reached a steady value. To control for current

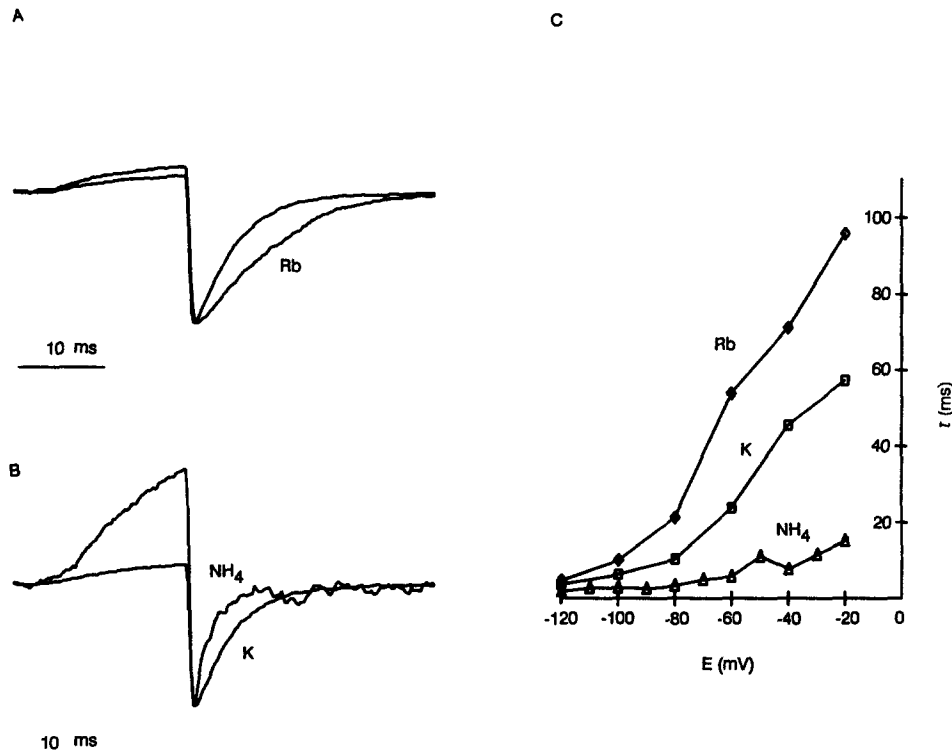


FIGURE 6. Permeant cations affect K channel closing. Tail currents were recorded as described in Fig. 3. (A) Tail current from a test pulse to -100 mV in 155 mM Rb-Ringers bath solution was scaled by a factor of 1.5 to match the corresponding tail current in K-Ringers (KF-BAPTA internal solution). The current in Rb-Ringers deactivates more slowly than in K-Ringers. (B) Tail current from a test pulse to -100 mV in NH₄-Ringers was scaled by a factor of 3.4 to match the peak of the tail current in K-Ringers. The current deactivates faster when NH₄ is the current-carrying ion. The filter was at 800 Hz in A and B. (C) Single exponential functions were fitted to tail currents from the same cells at potentials from -120 to -20 mV. The corresponding time constants (τ) were plotted against membrane potential. Diamonds, Rb-Ringers; triangles, NH₄-Ringers; squares, K-Ringers.

rundown, each holding potential was bracketed by test pulses at a holding potential of -60 mV. We found that 20–30% of the current ran down during 20 min of inactivation experiments. A Boltzmann equation (Eq. 2) was fitted to the data from experiments using either voltage protocol. The voltage of half inactivation was -46 mV in the cell of Fig. 7 A, and ranged from -35 to -60 mV ($n = 4$), which suggests that less than half of the channels are inactivated at the cell's normal poten-

tial of -55 mV. Inactivation was slightly less voltage dependent than was activation, with a 9 ± 1 mV ($n = 4$) change in membrane potential resulting in an e-fold change in inactivation.

There were two components to the time course of development of inactivation: a fast component on the order of hundreds of milliseconds and a slow component on

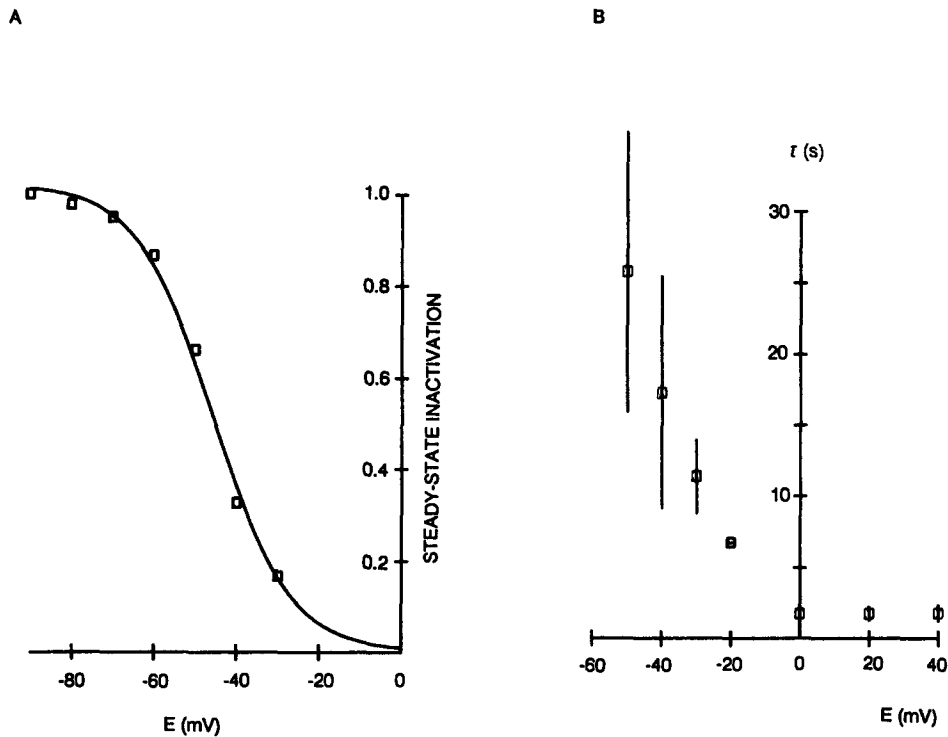


FIGURE 7. (A) Steady-state inactivation of brown fat K channels measured by stepping from the holding potential of -60 mV to a 15-s prepulse ranging from -90 to -30 mV. The prepulse was followed by a test pulse to $+30$ mV for 100 ms before the potential was stepped back to the holding potential of -60 mV. The pulses were applied at 30-s intervals. The current of the test pulse for each prepulse was normalized to the peak current at -90 mV and plotted against the prepulse potential. The smooth curve is a Boltzmann relation (Eq. 2) fit to the data with $E_{1/2} = -46$ mV, and $k = -9$ mV. (B) The time course of inactivation is voltage dependent. Averaged time constants (τ) were obtained at negative potentials by fitting single exponential functions to the peak currents measured during 50-ms test pulses to $+30$ mV at 30-s intervals after setting the holding potential to -50 , -40 , or -30 mV ($n = 6$). Time constants at more positive potentials (-20 to $+40$ mV) were obtained by fitting a single exponential function to the inactivating phase of the current during a 6-s pulse to each potential ($n = 3$).

the order of seconds. The voltage dependence of the slow component of inactivation is shown in Fig. 7 B. The squares represent the time constants measured by fitting a single exponential function to the peak current of test pulses given during the approach to steady-state using the second voltage protocol described above. Inactivation becomes more rapid with depolarization until it reaches a plateau of

~2 s at 0 mV. Recovery from inactivation also consisted of fast and slow components. The fast recovery of 20% of the current occurred with a time constant of 300 ms at -60 mV, while the remainder of the current recovered with a time constant of 13 s.

The onset of inactivation is slow enough to allow the K channels to repolarize the brown fat cell membrane after the initial norepinephrine-induced depolarization. The resulting hyperpolarization would remove inactivation and return the channels to a resting state. The time course for development of inactivation indicates that the majority of the channels would inactivate during the second slow depolarizing phase of the norepinephrine-induced triphasic membrane response, allowing the observed sustained depolarization.

K Channel Blockers

The effects of several K channel blockers were studied. Two blockers of voltage-dependent K channels, TEA and 4AP, had similar affinities for brown fat K channels, with a half blocking concentration of 1–2 mM. Fig. 8 A shows a control current during a 200-ms depolarization to +50 mV in Na-Ringers. When 5 mM TEA was added to the bath solution the superimposed current trace showed >50% block of the outward K⁺ current. When the TEA was washed off, currents returned to 93% of control levels. When 4 mM 4AP was subsequently added to the bath >50% of the current was blocked. 4AP changed K channel kinetics, suggesting that the block was partially relieved during the 200-ms depolarization (Thompson, 1982). We were unable to test 4AP at concentrations >10 mM because the cells irreversibly became leaky. Block by TEA or concentrations of 4AP <10 mM was almost completely reversed after 2 min of washout in most cells. Fig. 8 B shows a semilog plot of the dose-response data for TEA and 4AP measured during depolarizations to +50 mV. The line is given by:

$$\%block = [blocker]/(K_D + [blocker]) \quad (3)$$

with K_D set equal to 1.4 mM. The peak current during the pulse was used for the dose-response measurements; thus the affinity of 4AP for the channel may be slightly higher than indicated in the dose-response curve. We also tested *Pandinus imperator* scorpion venom, which blocks voltage-dependent K channels in a pituitary tumor cell line (Pappone and Lucero, 1988) and in frog node of Ranvier (Pappone and Cahalan, 1987). 200 μ g/ml of the crude venom had no effect on the brown fat K channels.

Fig. 8 C shows that K currents were the only voltage-activated membrane currents apparent in these experiments even in the presence of 50 mM TEA, which has blocked 93% of the outward current in this cell. In other experiments there was no evidence of inward currents when we replaced internal F⁻ with Cl⁻ or aspartate, substituted EGTA for BAPTA, or added nucleotides to the internal solution (ATP, cAMP, cGMP, GTP). The membrane currents observed in these experiments do not appear to be gated by Ca²⁺. We have not seen the characteristic "N-shaped" current-voltage relation of Ca²⁺-activated K currents (Meech and Standen, 1975) nor do we see any difference in the currents when we use a highly Ca²⁺-buffered KF-BAPTA internal solution vs. a solution with very little Ca²⁺ buffering. We could not

record currents in the absence of Ca^{2+} buffering because the cells would reseal shortly after going to the whole-cell recording mode. Raising external Ca^{2+} from 2 to 10 mM did not change the shape of the current-voltage relation. In addition, we tested whether the voltage-dependent K channel was pharmacologically similar to Ca^{2+} -activated K channels by applying the Ca^{2+} -activated K channel blockers apamin

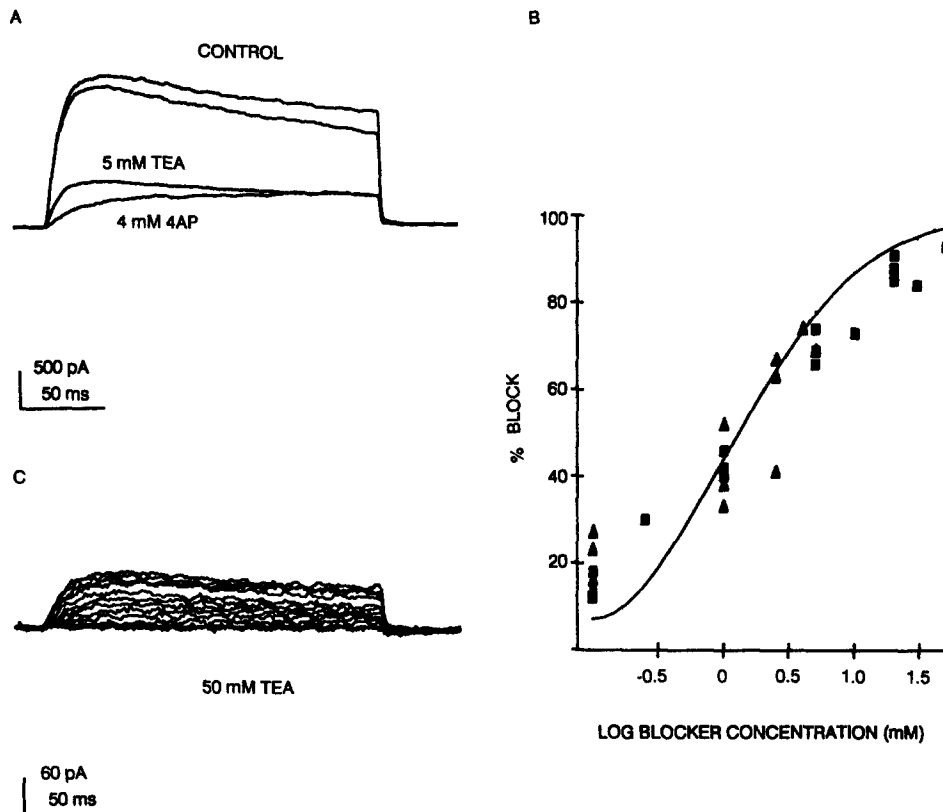


FIGURE 8. (A) Whole-cell currents recorded during a 200-ms voltage step from -60 to $+50$ mV in Na-Ringers (control). Superimposed on this trace is the current trace that was produced after replacing the bath solution with 5 mM TEA Na-Ringers. The TEA Na-Ringers was removed and currents returned to 93% of control values before the addition of 4 mM 4AP Na-Ringers to the bath (lowest trace). KF-BAPTA was the internal solution. (B) Semilog plot of dose response to TEA and 4AP measured from the peak amplitude of currents during 200-ms voltage steps to $+50$ mV. K channels show similar sensitivity to TEA (diamonds) and 4AP (triangles). The line was drawn using Eq. 3 with a K_D of 1.4 mM. Each point represents a measurement in a single cell. (C) After the addition of 50 mM TEA Na-Ringers to the bath, 93% of the outward currents were blocked. There was no evidence of inward currents. Filter was at 380 Hz in A and C.

and charybdotoxin to brown fat cells. Neither 150 nM charybdotoxin, which blocks the maxi-K Ca^{2+} -activated channel (Miller et al., 1985), nor 500 nM apamin, which blocks the small Ca^{2+} -activated K channel (Blatz and Magelby, 1986), had any effect on the voltage-gated K currents. These results suggest there is little, if any, contribution from Ca^{2+} -activated K channels to our whole-cell current records and that

these K channels are pharmacologically distinct from Ca^{2+} -activated K channels in other preparations.

Single-Channel Measurements

Single K channel currents were recorded in the cell-attached mode with HBSS and 0.1% fatty acid-free BSA in the bath and pipette. Fig. 9 A shows such currents

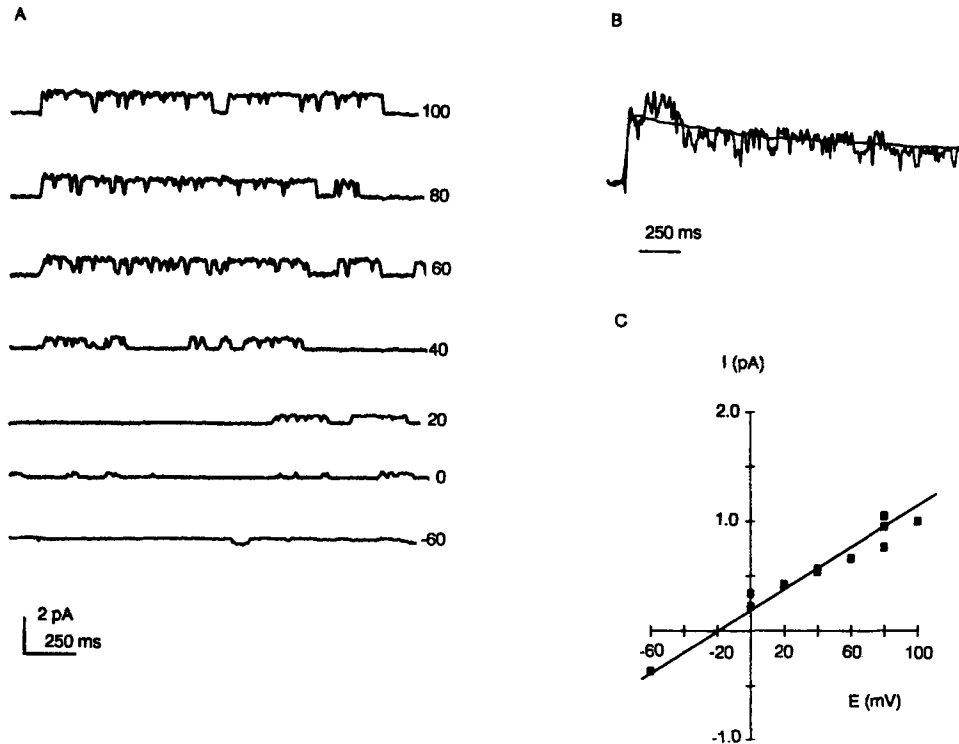


FIGURE 9. (A) Single-channel currents recorded from a cell-attached patch with HBSS + 0.1% BSA in the patch pipette and bath solution. The cell membrane under the patch was given 2-s voltage steps, incrementing by 10 mV from -60 mV hyperpolarized to the cell's resting potential to 100 mV depolarized from rest. Outward current is upward. Averages of currents during pulses without any channel openings were subtracted from the current records to remove the linear components of the current records. Filter was at 48 Hz. (B) Ensemble average of leak subtracted single-channel events from the same cell as A during nine 2-s pulses 80 mV positive to rest. Superimposed is a whole-cell current trace recorded from another cell during a 2-s pulse 80 mV positive to the holding potential of -60 mV scaled to match the ensemble average. (C) Single-channel current-voltage relation for the same cell as in A. The conductance measured from the slope of the line fit by linear regression to the data was 10 pS.

recorded during a 2-s pulse to potentials from 100 mV positive to 60 mV negative to the cell's resting membrane potential. As in the macroscopic currents, channel activation was voltage dependent; the occurrence of channel opening increased with depolarization and decreased with hyperpolarization. Fig. 9 B shows an ensemble average of cell-attached single-channel currents from nine 2-s voltage steps 80 mV

positive to the cell's resting potential. Superimposed on the ensemble average is a single whole-cell current trace recorded during a 2-s depolarization to +20 mV, 80 mV positive to the holding potential of -60 mV. Even with the relatively small number of current traces in the average there is a remarkably good match between the single-channel ensemble average and whole-cell current. Fig. 9 C shows the single-channel current-voltage relationship for the cell in 9 A. A straight line fit by linear regression gives a single-channel conductance of 10 pS. The average single-channel conductance was $9 \text{ pS} \pm 2 \text{ pS}$ ($n = 13$) in HBSS. From the maximum K conductance calculated in Na-Ringers of $\sim 9 \text{ nS/cell}$, and the single-channel conductance of 9 pS, we calculate an average of $\sim 1,000$ K channels/cell. The channel in Fig. 9 A had a reversal potential of -20 mV relative to the cell's resting membrane potential. The average reversal potential of $-31 \text{ mV} \pm 13 \text{ mV}$ ($n = 13$) was close to the E_K for these solutions, assuming a resting membrane potential of -50 to -60 mV and an internal K concentration of 155 mM. The single-channel currents could be inactivated by holding the patch of membrane at depolarized potentials, by replacing the bath solution with a high K-Ringers solution to depolarize the entire cell, or by applying $10 \mu\text{M}$ norepinephrine to the bath solution, presumably causing transmitter-induced membrane depolarization. Inactivation could be removed by holding the membrane under the patch pipette at more negative potentials. The voltage dependence, reversal potential, ensemble average, and inactivation suggest that the 9-pS channel recorded from cell-attached patches is responsible for the macroscopic voltage-dependent K currents described in this paper.

Effects of Norepinephrine

Our cultured brown fat cells were capable of responding metabolically to norepinephrine as evidenced by the decrease in number and size of their lipid droplets when norepinephrine was added to the culture media. Indirect evidence for a membrane response to norepinephrine in intact cells is that K channels recorded in the cell-attached mode were inactivated when $10 \mu\text{M}$ norepinephrine was applied to the bathing solution ($n = 5$), presumably by the norepinephrine-induced depolarization. In addition, preliminary experiments examining membrane responses using microelectrode recording or patch current-clamp recordings showed that our cultured cells can respond to norepinephrine with changes in membrane potential under some circumstances. However, we saw no direct electrophysiological effects of norepinephrine on whole-cell current records, as shown in Fig. 10. The effects of $5 \mu\text{M}$ norepinephrine in Na-Ringers on whole-cell voltage-dependent K currents were studied with F^- , Cl^- , or aspartate as the major internal anion, with BAPTA or EGTA to buffer Ca^{2+} , and in the presence or absence of nucleotides (ATP, cAMP, cGMP, GTP). There was no observable effect of norepinephrine on the voltage-gated K currents under any of these conditions. This is demonstrated, for example, by the superimposed current traces and $I-V$ relations before and after the addition of $5 \mu\text{M}$ norepinephrine to a cell with KCl and nucleotides in its internal solution, as shown in Fig. 10 A. Our experiments suggest that the voltage-gated K channels in brown fat are not directly affected by norepinephrine and do not contribute directly to the norepinephrine-induced depolarization of brown fat.

We did not observe any norepinephrine effect on remaining whole-cell membrane currents when the outward K currents were blocked by internal CsCl (Fig.

10 B). However, components needed to modulate cell currents might be dialyzed out during whole-cell recording, and because our internal solutions were highly buffered against pH and Ca^{2+} changes, it is possible that this buffering prevented a norepinephrine-mediated membrane response (Horwitz and Hamilton, 1986; Giovannini et al., 1988). Use of internal solutions with less Ca^{2+} buffering was not possible because the cells would have resealed during the experiment.

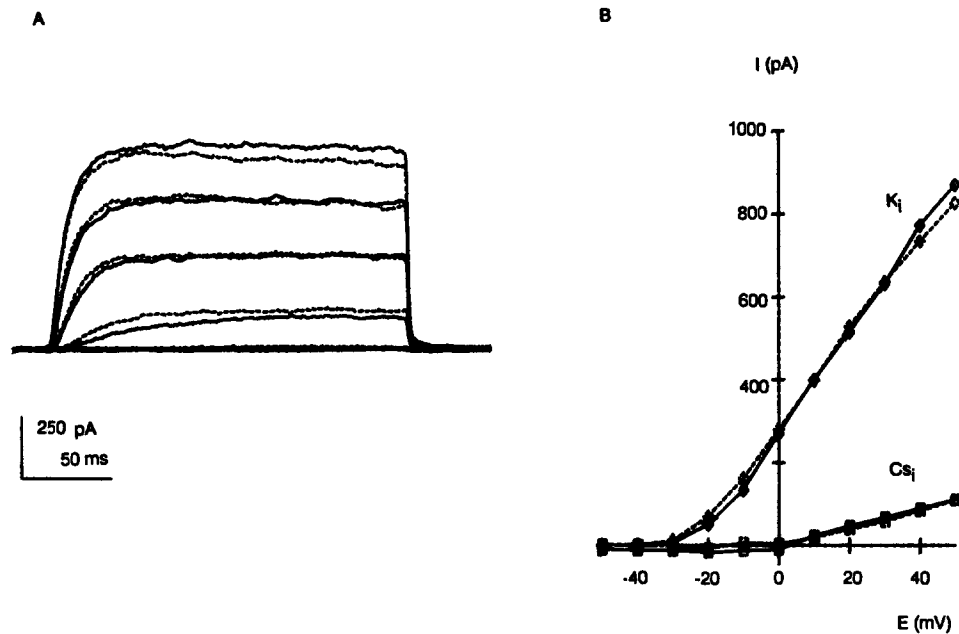


FIGURE 10. (A) Effects of norepinephrine on whole-cell K currents recorded in Na-Ringers with an internal solution containing 150 mM KCl, 10 mM TMA-HEPES, 2 mM Na-ATP, 3 mM MgCl_2 , 0.6 mM Na-cAMP, and 1.4 mM Na-cGMP. Superimposed are current traces before (smooth line) and after (dashed line) adding 5 μM norepinephrine in ascorbic acid to the bath. Current traces are in response to 200-ms voltage steps from a holding potential of -60 mV, incrementing by 20 mV, from -50 to $+50$ mV. Filter was at 380 Hz. (B) Peak current-voltage relations for the currents shown in A (diamonds) before (solid line) and after (dashed line) the addition of norepinephrine. On the same graph is the peak current-voltage relation for a cell in Na Ringer with Cs substituted for K in the internal solution (squares) before (solid line) and after (dashed line) the addition of 5 μM norepinephrine in ascorbic acid to the bath.

DISCUSSION

In this work, we have demonstrated that brown fat cells isolated from neonatal rats and grown in culture possess voltage-dependent K^+ channels. Activation of the outward currents carried by these channels is highly voltage dependent, occurring with membrane depolarization to potentials positive to ~ -50 mV and being half maximal at a membrane potential of ~ -30 mV. Activation of the K current does not require significant concentrations of internal Ca^{2+} ions. K channels in brown fat also inactivate with $\sim 30\%$ of the channels inactivated near the normal resting poten-

tial of -55 mV. The channels are highly selective for K^+ and show a selectivity typical of K channels with a permeability sequence of $K^+ > Rb^+ > NH_4^+ \gg Na^+ = Cs^+$. Brown fat K channels are sensitive to block by both TEA and 4AP with a K_D of 1–2 mM for each but are insensitive to apamin or charybdotoxin. Single-channel currents recorded from cell-attached patches had an average conductance of 9 pS, and showed characteristics of the macroscopic current such as voltage-dependent activation and inactivation. The K^+ channels of brown fat cells resemble delayed rectifier K^+ channels of nerve (Hille, 1984) and voltage-dependent K^+ channels in other nonneuronal cells such as avian hepatocytes (Marchetti et al., 1988), human lymphocytes (Cahalan et al., 1985), and bovine chromaffin cells (Marty and Neher, 1985) with respect to their voltage dependence, selectivity, relatively slow inactivation, and single-channel conductance.

Norepinephrine, either released by adrenergic neurons innervating brown fat or applied extracellularly, causes an increase in brown fat metabolism and a triphasic membrane potential response. The changes in membrane potential have been observed both *in vivo* (Horwitz and Hamilton, 1984) and *in vitro* (Girardier and Schneider-Picard, 1983; Schneider-Picard et al., 1985; Hamilton et al., 1988). The first phase of the membrane potential response is characterized by a transient 25 mV depolarization occurring within 2 s of stimulation and lasting 10–30 s. The initial depolarization precedes the tissue's increase in metabolism. The second phase of the response is a repolarization and 5–10 mV hyperpolarization of the membrane potential lasting 30–120 s and occurring simultaneously with the increase in metabolism (Girardier and Schneider-Picard, 1983). The third phase consists of a second 20–25 mV depolarization that is sustained for the duration of the metabolic response.

From the voltage dependence of activation and inactivation of the brown fat cell K currents measured in these experiments, $\sim 0.3\%$ of the K channels would be open in the steady state at a normal resting potential of -55 mV. Thus, the channels would provide ~ 66 pS of resting K^+ conductance, which would be $\sim 15\%$ of the normal resting cell conductance. Therefore, it seems unlikely that the depolarization seen when brown fat cells are stimulated with norepinephrine could be caused by a decreased activity of these channels. This view is consistent with the lack of effect of norepinephrine on the K currents found in the present experiments and with the results of microelectrode measurements that indicated that the depolarization was associated with a conductance increase (Horowitz et al., 1971).

The voltage-gated K^+ channel described in this paper could be responsible for the norepinephrine-induced hyperpolarization in the triphasic membrane potential response and may modulate the sustained depolarization. Based on their properties measured under voltage clamp, more than half of the channels would be activated during the initial depolarization by norepinephrine. Their activation could therefore cause the hyperpolarizing phase of the response. Recent evidence that supports this suggestion is that an addition of 30 mM TEA to the bath of superfused brown fat preparations, which would block the voltage-gated K channels almost completely, inhibits the hyperpolarizing phase of the triphasic membrane response to norepinephrine (Hamilton et al., 1988). Hyperpolarization has two effects on voltage-dependent K channels in brown fat: it closes the channels and it removes inacti-

vation. Thus, more voltage-gated channels would be in the closed resting state at the end of the hyperpolarizing phase than before stimulation. This increase in the number of K channels available for activation may account for the slow time course of the second depolarization. As the membrane depolarizes, more K channels are recruited to slow the depolarization. The second depolarization is sustained, so that the K channels open and then most inactivate, resulting in the maintenance of depolarization. Our results indicate that during a normal sustained depolarization to -30 mV, $\sim 6\%$ of the K channels would be open in the steady state. This would be a twentyfold increase over the resting K conductance through these channels, and may account for the second phase K^+ efflux described by Schneider-Picard et al. (1985) and the increased $^{86}\text{Rb}^+$ fluxes measured with norepinephrine stimulation (Nanberg et al., 1984). These results indicate that the sustained depolarizing current induced by norepinephrine must be $> \sim 60$ pA to overcome the outward current carried by voltage-gated K channels.

It has been hypothesized based on indirect evidence that Ca^{2+} -activated K channels (Nanberg et al., 1984, 1985) or increased activity of the Na/K pump (Schneider-Picard et al., 1985) play a role in the hyperpolarization of the brown fat cell membrane. We saw no evidence of Ca^{2+} -activated K currents in our experiments, and believe that activity of the voltage-gated K channels could readily explain the results that led to this hypothesis. However, we were not able to make stable recordings without strongly buffering the internal Ca^{2+} concentration, and so may have failed to activate any Ca-dependent currents present in the cells. We probably would not be able to discern changes in pump activity were they present under our experimental conditions.

We cannot as yet make quantitative predictions about the activation of the voltage-gated K channels in brown fat cells during the response to norepinephrine, because the mechanism and properties of neither the initial nor the sustained depolarization are known. Our whole-cell voltage-clamp experiments did not show any voltage-dependent currents or norepinephrine-induced changes in membrane currents that could account for the membrane depolarizations. As discussed above, modulation of the voltage-gated K currents does not seem likely as a mechanism of the norepinephrine-induced depolarization. Siemen and Reuhl (1987) found a Ca^{2+} -activated nonselective cation channel using the patch-clamp technique on inside-out patches of cultured brown fat cells. Activation of this channel required internal Ca^{2+} concentrations of ~ 1 μM . We did not see this current in our experiments, probably because of our highly buffered low internal Ca^{2+} concentrations. In addition, we cannot rule out the possibility that an essential component for channel activation may have been dialyzed out of our cells.

The role of K currents in the normal physiology of brown fat cells is not known. The intracellular concentration of K^+ ions affects lipolysis and Ca^{2+} reuptake by mitochondria in brown fat cells (Nedergaard, 1981). In addition, voltage-gated K channels are essential for mitogenic responses in some cells (DeCoursey et al., 1984; Lee et al., 1986; Chiu and Wilson, 1988). These results suggest that voltage-gated K channels can be important in regulating ion distributions during increases in cell metabolism. Since the brown fat cell's increase in metabolism appears to occur simultaneously with membrane hyperpolarization, the voltage-dependent K^+ chan-

nels described in this paper may modulate the acute thermogenic response of brown fat to norepinephrine or play a role in long term norepinephrine effects on cell proliferation during cold acclimation.

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