Norepinephrine-induced sustained inward current in brown fat cells: α₁-mediated by nonselective cation channels

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Norepinephrine (NE) stimulation led to a whole cell current response consisting of two phases: a first inward current, lasting for only 1 min, and a sustained inward current, lasting as long as the adrenergic stimulation was maintained. The nature of the sustained current was here investigated. It could be induced by the α₁-agonist cirazoline but not by the β₁-agonist CGP-12177A. Reduction of extracellular Cl⁻ concentration had no effect, but omission of extracellular Ca²⁺ or Na⁺ totally eliminated it. When unstimulated cells were studied in the cell-attached mode, some activity of ~30 pS nonselective cation channels was observed. NE perfusion led to a 10-fold increase in their open probability (from ~0.002 to ~0.017), which persisted as long as the perfusion was maintained. The activation was much stronger with the α₁-agonist phenylephrine than with the β₁-agonist CGP-12177A, and with the Ca²⁺ ionophore A-23187 than with the adenyl cyclase activator forskolin. We conclude that the sustained inward current was due to activation of ~30 pS nonselective cation channels via α₁-adrenergic receptors and that the effect may be mediated via an increase in intracellular free Ca²⁺ concentration.

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membrane was placed on the surface of the medium. The floating brown fat cells spontaneously adhered to the hydrophilic side of this membrane. The medium was exchanged after 24 h, and the cells on the membrane were then examined, as we will describe. Routinely, cells were examined during the first 1–3 days, but occasionally they were kept in the well for as many as 7 days; we did not observe any difference in response during this time, nor did the cells change their multilocular appearance.

**Patch-Clamp Techniques**

For both the perforated-patch studies and the cell-attached-mode studies, the biofoil with the attached brown fat cells was placed in a chamber with a volume of ∼300 μl and was perfused continuously with extracellular solution (see below) at room temperature (22–25°C) with a flow rate of ∼300 μl/min; the perfusate was removed at the same rate by a peristaltic pump. The perfusion system consisted of a 4-channel electromagnetic valve-controlled local application holder, fed by gravity. The extracellular solution consisted of (in mM): 134 NaCl, 6 KCl, 1.2 MgCl₂, 1.2 CaCl₂, 5 glucose, and 10 HEPES (pH 7.4 adjusted with NaOH, corresponding to ∼5 mM Na⁺). Where indicated, modified solutions were used, and/or agents were dissolved in this medium.

Pipettes were pulled from borosilicate glass and had resistances between 6 and 3 MΩ. The pipette solution consisted of (in mM): 60 KCl, 80 K gluconate, 10 NaCl, 1 CaCl₂, 1 EGTA, and 10 MOPS (pH 7.2 adjusted with KOH, corresponding to ∼5 mM Na⁺). The choice of the rather high level of Cl⁻ was based on implications from 36Cl⁻ efflux experiments indicating that the cytosolic Cl⁻ level is higher than the equilibrium level for the resting membrane potential (4) (which would be −50 mM) and from experiments (21) showing that the Cl⁻ currents induced by norepinephrine stimulation had a reversal potential of ∼−20 mV, corresponding to an estimated cytosolic concentration of ∼70 mM. The free Ca²⁺ concentration in the pipette was calculated to be 10 μM, according to the computer program BAPS; the presence of 80 mM gluconate, which may have Ca²⁺-chelating properties, may have lowered this level further. To the pipette solution, 0.24–0.32 μg of amphotericin B (dissolved in DMSO) was added per milliliter. In a few cases, pluronic F-127 (0.08%) was also filtered with an 8-pole filter. In the recordings in the cell-attached mode, the number of extracellular solution was experimentally determined to be −50 mV; i.e., the potential is 50 mV more negative than that indicated on the axes. The junction potentials in the buffers with altered Cl⁻ concentration were not markedly different. Routinely, the cell capacitance in the resting state (∼30 pF) was compensated by the circuitry in the amplifier, and no further capacitance compensation was performed during the experiment. Each cell responded qualitatively in the same way to repeated agonist stimulation for up to 2 h, but in general there was a tendency to a decrease in the magnitude of the responses on successive stimulations.

For the cell-attached-mode studies, the pipettes had resistances between 8 and 12 MΩ, and the pipette medium was here identical to the extracellular solution. Each experiment was finalized by excising the patch and studying it as an inside-out patch; these excised patches were thus formed and studied in the extracellular solution.

**Recordings and Data Analysis**

The currents were recorded with an L/M EPC 7 patch-clamp amplifier. During the whole cell experiments, voltage ramps were frequently run under manual initiation. In control experiments (not shown), we found that the current at each voltage tested was stable with time, except for those at very high depolarizations (more positive than 0 mV), which became inactivated, in accordance with our earlier observations (23). Therefore, we surmise that the voltage-dependent conductance changes observed during voltage ramps represented time-independent values. In each voltage ramp, the holding potential of −50 mV was increased to −100 mV for 10 ms; during the next 600 ms, it was linearly decreased to +20 mV and then directly returned to −50 mV. The command voltage ramps were generated and saved together with the resulting currents by the Clampfit of the pCLAMP 6.03 program.

The data were used for calculation of cellular zero current potentials (i.e., membrane potentials) and conductances. Conductances at −50 mV (G_{m,−50}) were calculated from linear fits between −60 and −40 mV, and conductances at +15 mV (G_{m,+15}) were calculated from linear fits between +10 and +20 mV. Agonist-induced current characteristics were determined from voltage ramp-induced currents in the presence of agonist minus the currents in the absence of agonist, as indicated. After the current signal had been digitized with a modified pulse code modulator, the data were stored on video cassettes. For the later off-line analysis, the current signal from the cassettes was in the whole cell studies sampled at a frequency of 1 or 3 kHz and was low-pass filtered with a −3-dB frequency of 200 Hz by an 8-pole filter with Bessel characteristics. In the cell-attached mode, the current signal from the cassettes was sampled at a frequency of 5 kHz and was low-pass filtered with a −3-dB frequency of 0.5 kHz. The signals were transferred by a 12-bit interface to a computer and analyzed with the pCLAMP 6.03 program (Axon Instruments).

In the recordings in the cell-attached mode, the number of simultaneously open channels was low (1–3), making it possible routinely with the pCLAMP program to calculate the open and closed times and the observed open probability (P_o,obs) directly from lists of events, according to a 50% amplitude threshold crossing criterion. To obtain the P_o,obs values, data were collected over 20 mV. Agonist-induced current characteristics were determined from voltage ramp-induced currents in the presence of agonist minus the currents in the absence of agonist, as indicated. After the current signal had been digitized with a modified pulse code modulator, the data were stored on video cassettes. For the later off-line analysis, the current signal from the cassettes was sampled at a frequency of 5 kHz and was low-pass filtered with a −3-dB frequency of 0.5 kHz. The signals were transferred by a 12-bit interface to a computer and analyzed with the pCLAMP 6.03 program (Axon Instruments).

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brane potential was not simultaneously measured). In the displayed recordings, downward deflections denote currents corresponding to cellular inward currents.

**Chemicals**

EGTA, K-gluconate, L-norepinephrine bitartrate, Na-aspartate, and L-phenylephrine HCl were directly dissolved in the extracellular solution, and amphotericin B, forskolin, and A-23187 were stock-dissolved in DMSO (0.1% in final solution); all were from Sigma. CGP-12177A was a gift from Ciba-Geigy, cirazoline was from Research Biochemicals International, and pluronic F-127 (dissolved in DMSO) was from Molecular Probes. The adrenergic agonists were protected from light in the perfusion system.

**RESULTS**

In the present experiments, the response of brown fat cells to sustained adrenergic stimulation was analyzed first as whole cell currents, with the perforated-patch technique. To directly identify the channels responsible for the currents observed, the cell-attached patch-clamp technique was subsequently used.

**Whole Cell Currents**

In the perforated-patch whole cell studies, the cell membrane potential was clamped at \(-250 \text{ mV}\) (except during the voltage ramps); this is a voltage similar to that observed in resting brown fat cells at 37°C (5, 30) but somewhat hyperpolarized when compared with the actual resting potential of the brown fat cells studied under the present conditions (see below).

The perfette solution was composed so as to be close to expected cytosolic ion levels. Because amphotericin makes the membrane permeable to Na\(^+\), K\(^+\), and Cl\(^-\) ions (22), the monovalent ions from the perfette probably diffused sufficiently to in reality determine the intracellular levels. [Because amphotericin does not make the patch permeable to Ca\(^{2+}\) (22), the perfette Ca\(^{2+}\) concentration was not expected to influence cytosolic Ca\(^{2+}\) levels.] The expected equilibrium potentials were therefore \(-82 \text{ mV}\) for K\(^+\), +68 mV for Na\(^+\), and \(-18 \text{ mV}\) for Cl\(^-\).

Whole cell currents were determined when a stable membrane resistance had been attained. The resting cell capacitance was 35 ± 1 pF (\(n = 26\)). During the experiments, we frequently followed the current response to voltage ramps (as is seen, for example, in Fig. 1). These ramps allowed for determination of membrane potential (i.e., the zero current potential) and were also used to measure total cellular conductance at \(-50 \text{ mV}\) (referred to as \(G_{m,-50}\) (i.e., the conductance at the clamped potential) and at +15 mV (\(G_{m,+15}\)).

**The unstimulated state.** In unstimulated cells, a stable resting current (Fig. 1A) was observed. On the basis of voltage ramps performed during this period (as exemplified in Fig. 1B, current 1), the mean resting membrane potential was estimated to be \(-21 \text{ mV}\) (Table 1); this was also the voltage observed in the current clamp mode immediately before the setup was switched to the voltage clamp mode. The membrane conductance at

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**Fig. 1. Norepinephrine-induced membrane currents in a rat brown fat cell.** A: whole cell currents followed with the perforated-patch technique. Where indicated, 1 µM norepinephrine (NE) was present; the washout phase is not shown. Voltage ramps were initiated manually. Initial cell capacitance of 34 pF was electronically canceled out, but alterations in cellular capacitance occurred during norepinephrine stimulation of the cell (here just observable as a short thickening of the line from the voltage ramps close to the main current curve). B: currents observed during voltage ramps from \(-100\) to \(+20\) mV: 1) in the resting state, 2) during the first inward current, 3) during the sustained inward current, 4) after norepinephrine washout. C: a family of norepinephrine-induced currents during voltage ramps run during the first inward current (i.e., the actual currents minus current 1 in B). Currents are from the first 4 ramps during the first inward current (a,b,c,d) and from the indicated ramp (e) during inactivation of this current. D: norepinephrine-induced current during a voltage ramp during the sustained inward current (i.e., current ramp 3 minus current ramp 1 in B).
Table 1. Characterization of the response to norepinephrine

<table>
<thead>
<tr>
<th></th>
<th>Resting</th>
<th>First Inward Current</th>
<th>Sustained Inward Current</th>
<th>Washout</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta I ), pA</td>
<td></td>
<td>367 ± 45</td>
<td>44 ± 7</td>
<td></td>
</tr>
<tr>
<td>( E_m ), mV</td>
<td>-21 ± 1</td>
<td>-6 ± 1 *</td>
<td>-13 ± 1 *</td>
<td></td>
</tr>
<tr>
<td>( G_{m,-50} ), nS</td>
<td>0.69 ± 0.09</td>
<td>6.84 ± 0.97 *</td>
<td>1.36 ± 0.16 *</td>
<td>0.67 ± 0.09 NS</td>
</tr>
<tr>
<td>( G_{m,+15} ), nS</td>
<td>13.8 ± 0.9</td>
<td>17.1 ± 1.0 *</td>
<td>13.2 ± 0.9 NS</td>
<td>13.0 ± 1.2 NS</td>
</tr>
</tbody>
</table>

Data are from experiments such as that shown in Fig. 1. Change in current (\( \Delta I \)) is obtained directly from current traces. Membrane potential (\( E_m \)) and conductance (\( G_m \)) values are based on current curves from voltage ramps, with the ramp chosen to represent the first inward current phase being the ramp with the highest conductance. Results are means ± SE from 15 experiments (12 for washout data). NS, not significant. Comparison with resting values (NS \( P > 0.05 \) and * \( P < 0.001 \), respectively; Student’s paired \( t \)-test).

†Estimation of \( \Delta I \) would be uncertain because of the long time period between resting and washout, with possible baseline drift.

Followed during the entire response. Membrane potential changes are depicted in Fig. 2A and summarized in Table 1. As seen, after norepinephrine had reached the cell, a depolarization occurred during the first inward current, from -21 mV down to -6 mV, i.e., a depolarization of ~15 mV. After this, \( E_m \) slowly but steadily repolarized, until it reached and became stable at about -13 mV, i.e., still ~8 mV depolarized compared with the resting state. \( E_m \) fully returned to initial values upon norepinephrine washout (Table 1).

Membrane conductances. The alterations in \( E_m \) would be expected to result from alterations in cell membrane conductance. As seen in Fig. 2B and in

the holding potential of -50 mV (\( G_{m,-50} \)) was 0.7 nS (Table 1). As is also seen from the current ramp in Fig. 1B, the membrane conductance at positive membrane potentials was much higher; at +15 mV (i.e., \( G_{m,+15} \)), it was 14 nS.

Effects of norepinephrine. After initiation of perfusion with 1 \( \mu \)M norepinephrine, the cells responded with characteristic successive alterations in transmembrane current, despite the constant presence of norepinephrine. Two major response types could be distinguished. In ~75% of the cells, a relatively simple type of response, such as that illustrated in Fig. 1, was observed. In the other cells, an oscillatory response was observed (not shown). The present analysis concerns only the response pattern of the majority of cells.

Currents. In the current response elicited by norepinephrine, there were two phases (Fig. 1). Both phases consisted of inward currents, i.e., both currents would probably be depolarizing in the unclamped cell. The “first inward current” (referred to throughout in this way) was transient, with a rapid initiation and a rather slow inactivation. It was large, with a mean maximal current value of 367 pA (Table 1). The mean total length of this current phase, i.e., the time from the current deviation from resting level to its attainment of new plateau corresponding to the next phase, was 58 ± 5 s (n = 13). This current was followed by a “sustained inward current” (referred to throughout in this way), which persisted unabated and nonoscillatory for as long as the norepinephrine stimulation lasted. This sustained inward current amounted to 44 pA. When norepinephrine was washed out, the cell membrane current returned to the initial resting levels. Because the sustained phase (which is the one under investigation here) of necessity always was preceded by the first inward current, our results concerning the first inward current are also briefly reported here, but a full analysis is not attempted.

Membrane potentials. On the basis of the voltage ramps, the alterations in membrane potential (\( E_m \)) under the present conditions and in conductances were
Table 1, the first inward current was associated with a 10-fold increase in the conductance at −50 mV ($G_{m,-50}$). However, maximal depolarization was obtained already at the time of the first ramp, when the increase in conductance was only about threefold, and the further increase in conductance (seen here during the subsequent three voltage ramps) was actually occurring while the membrane potential was repolarizing toward the resting level. During the sustained inward current, the $G_{m,-50}$ was still twice as high as it was in the resting state. $G_{m,-50}$ remained elevated until norepinephrine had been washed out.

The conductance at +15 mV ($G_{m,+15}$), which probably results mainly from voltage-gated K$^+$ channels (14, 23), was marginally altered during the norepinephrine response (Fig. 2C). Although not clearly evident from Fig. 2C, $G_{m,+15}$ was significantly increased during the first inward current (Table 1). There was a tendency toward a reduced $G_{m,+15}$ during the sustained inward current in many experiments (as is evident in Fig. 2C) (Table 1).

**Voltage ramp currents.** For a first analysis of the ionic character of the changes in membrane conductance, the character of the currents during the voltage ramps was studied.

As seen in Fig. 1B, the currents of the ramp obtained in the resting state (trace 1) reversed at fairly low potential, here at about −18 mV, i.e., far from the expected K$^+$ equilibrium potential of −83 mV. At positive membrane potentials, large outward currents of the type earlier described for voltage-gated K$^+$ channels in these cells (14, 23) were observed.

The norepinephrine-induced currents were obtained by subtracting this resting-state voltage ramp current from the subsequent ones. In Fig. 1C, a family of norepinephrine-induced voltage ramp currents obtained during the first inward current is shown; these curves thus represent Δ-currents. These induced-current curves were complex, implying that the induced currents consisted of several ionic fluxes, and they were not further analyzed here. The induced curves (i.e., the Δ-curves) obtained during voltage ramps during the sustained inward current phase (exemplified in Fig. 1D) were comparably simple to analyze. The curves were time independent (not shown) and close to ohmic when negative potentials were applied. Zero current was reached between −10 mV and 0 mV [mean value $−0.4 ± 1.1$ mV ($n = 15$)]. That the zero current potential was close to 0 mV eliminated the possibility that this current represented currents through specific Na$^+$ channels but would principally be in agreement with characteristics of currents through nonselective cation (NSC) channels. Therefore, activated NSC channels could be responsible for the inward current during the sustained current phase. The absence of outward current at positive membrane potential (Fig. 1D) is, however, not in accordance with general characteristics of NSC channels (which are conducting also at positive membrane potentials). We suggest that this deviation may be due to an unrelated norepinephrine-induced decrease in the activity of the voltage-depen-
dent K$^+$ channels (cf. Fig. 2C). This decrease may be of sufficient magnitude (a <10% inhibition of the K$^+$ current would be needed) to more than counteract the expected outward current of activated NSC channels at positive membrane potentials. (Attempts to eliminate this K$^+$ current by exchanging K$^+$ with Cs$^+$ in the pipette were principally successful but had to be abandoned because the ion substitution was otherwise poorly tolerated by the cells.)

**Effects of the adrenergic subtype-selective agonists cirazoline and CGP-12177A.** To determine which subtype of adrenergic receptor was responsible for the different phases in the norepinephrine-induced current response, we studied the effects of the α$_1$-selective agonist cirazoline and the β$_3$-specific agonist CGP-12177A, known selective activators of the indicated adrenergic subtypes in brown fat cells (31). We compared the responses to each of these selective agonists with the responses to the endogenous agonist norepinephrine described above; norepinephrine is expected in itself to be able to activate all types of adrenergic receptors.

**Cirazoline.** The α$_1$-adrenergic agonist cirazoline could qualitatively mimic the action of norepinephrine; both the first and the sustained inward currents could clearly be identified (not shown, but compare with Table 2). Quantitatively, however, cirazoline did not seem to be able to mimic fully the effect of norepinephrine. As seen in Table 2, the cirazoline-induced first inward current was only two-thirds of that induced by norepinephrine, and the induced membrane depolarization during this phase was only 10 mV, i.e., also only two-thirds of that induced by norepinephrine. However, the sustained inward current was the same whether it was induced by cirazoline or by norepinephrine, as was the depolarization during this phase (8 mV). Thus, whereas a β-adrenergic component of the first inward current could not be excluded, α$_1$-adrenergic stimulation seemed to be competent to induce the sustained inward current to the same extent as did norepinephrine.

CGP-12177A. The clear responses to cirazoline could be contrasted with those to β$_3$-stimulation induced by CGP-12177A. In cells that responded well to norepinephrine or cirazoline, 1 or 10 μM CGP-12177A was in some cases fully without effect, and in other cases it

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**Table 2. Effect of cirazoline on plasma membrane parameters in brown fat cells**

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>First Inward Current</th>
<th>Sustained Inward Current</th>
<th>Washout</th>
</tr>
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<tbody>
<tr>
<td>$I_m$, pA</td>
<td></td>
<td>238 ± 40$^{\dagger}$</td>
<td>35 ± 7$^{\text{NS}}$</td>
<td></td>
</tr>
<tr>
<td>$E_{mV}$</td>
<td>−26 ± 3$^{\text{NS}}$</td>
<td>−14 ± 2$^{\text{NS}}$</td>
<td>−18 ± 2$^{\text{NS}}$</td>
<td>−21 ± 3$^{\text{NS}}$</td>
</tr>
<tr>
<td>$G_{m,-50}$ sS</td>
<td>0.96 ± 0.10$^{\text{NS}}$</td>
<td>6.4 ± 1.0$^{\text{NS}}$</td>
<td>1.4 ± 0.1$^{\text{NS}}$</td>
<td>0.94 ± 0.06$^{\text{NS}}$</td>
</tr>
<tr>
<td>$G_{m,+15}$ sS</td>
<td>12.0 ± 1.0$^{\text{NS}}$</td>
<td>14.0 ± 1.4$^{\text{NS}}$</td>
<td>10.4 ± 1.3$^{\text{NS}}$</td>
<td>11.5 ± 1.8$^{\text{NS}}$</td>
</tr>
</tbody>
</table>

Data shown are from 5–6 experiments with cirazoline, principally similar to that shown with norepinephrine in Fig. 1A. Data were analyzed as described in legend to Table 1. $^{\ast,\dagger}$ Comparison with norepinephrine effects (Table 1); $^{\ast}P < 0.001$, $^{\dagger}P = 0.1$, and $^{\text{NS}}P > 0.1$, respectively; Student’s unpaired t-test.
adrenergic stimulation in a buffer that did not contain Ca\(^{2+}\) but instead contained 1 mM of the Ca\(^{2+}\)-chelator EGTA. Such conditions lead to a diminished norepinephrine-induced increase in [Ca\(^{2+}\)]\(_i\). (see Ref. 29).

In the absence of extracellular Ca\(^{2+}\) during the entire response (Fig. 3A), the resting conductance was somewhat reduced (not shown). As seen, the first inward current could be induced even in the absence of extracellular Ca\(^{2+}\), but the current was only one-half of that seen in the same cell in the presence of Ca\(^{2+}\). However, Ca\(^{2+}\) omission led to complete disappearance of the sustained inward current. In agreement with this, the sustained current was also eliminated when Ca\(^{2+}\) was temporarily omitted from the medium during this current phase (Fig. 3B). In Table 3, results from several experiments in which Ca\(^{2+}\) was omitted during the sustained current are summarized. It is clear that, under these conditions, no sustained inward current was observable; for unknown reasons this did not lead to a significant repolarization [note, however, the activation under these circumstances of a large-conductance NSC channel (described below), which may be responsible for the maintained membrane depolarization under these conditions]. However, the \(G_{m,-30}\) was halved, down to the normal resting level (cf. Table 1), whereas \(G_{m,+15}\) was statistically unchanged.

Although this experiment in itself does not allow us to differentiate between the proposals that Ca\(^{2+}\) has a signaling role or that Ca\(^{2+}\) actually should be the ion carrying the charge, it is most likely that we have interfered with the signaling mechanism. Thus an increase in [Ca\(^{2+}\)]\(_i\) is probably a step in the mediation of the signal between the \(\alpha_1\)-receptor and the channel carrying the sustained inward current.

Identification of ions involved. To establish which ion(s) carry the inward current, especially the sustained inward current, experiments were performed with omission of extracellular Cl\(^-\) or Na\(^+\).

EFFECTS OF CL\(^-\) REMOVAL. Extracellular Cl\(^-\) was reduced from 144.8 mM to 30 mM by partially substituting it with aspartate. The calculated Cl\(^-\) reversal potential would now be +31 mV, i.e., an expected shift of +49 mV. The resting membrane potential was not altered by partial Cl\(^-\) substitution (-24 mV before induced membrane current oscillations (not shown). However, the sustained inward current was always totally absent in the response induced by CGP-12177A.

Thus \(\alpha_1\)-receptors were primarily responsible for the overall current response. Through \(\alpha_1\)-stimulation, most of the first inward current response and the entire sustained inward current response could be elicited. \(\beta_2\)-Receptors may participate in the first inward current response but could not induce at all the sustained inward current.

Effects of extracellular Ca\(^{2+}\) removal during \(\alpha_1\)-stimulation. \(\alpha_1\)-Adrenergic stimulation is associated with an increase in [Ca\(^{2+}\)]\(_i\) in brown fat cells (1, 13, 27, 29), and this increase is believed to be one of the intracellular mediators of \(\alpha_1\)-adrenergic stimulation. To investigate to what extent the observed \(\alpha_1\)-effects were dependent on Ca\(^{2+}\), we examined the responses to

![Figure 3. Effect of extracellular Ca\(^{2+}\) removal on the cirazoline-induced whole cell currents. A: a brown fat cell was first stimulated with 1 \(\mu\)M cirazoline (Cira). The cell was then perfused with Ca\(^{2+}\)-free, EGTA-containing medium for 3 min and then with the same medium with cirazoline (curve labeled EGTA). Currents induced by voltage ramps have been eliminated in the presentation. B: effect of temporary omission of Ca\(^{2+}\) on the sustained inward current. A brown fat cell was perfused with normal medium with norepinephrine and, where indicated (EGTA), with Ca\(^{2+}\)-free, EGTA-containing medium together with NE. Note the presence of a large conductance channel (cf. Fig. 5) and the increase in its opening frequency during Ca\(^{2+}\) omission. As an effect of this channel, the length of the currents from the negative voltage ramps is varying during the sustained phase (cf. Fig 5B).](http://ajpendo.physiology.org/)

Table 3. Effect of Ca\(^{2+}\) omission on the sustained inward current

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>+EGTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\Delta I), pA</td>
<td>38 ± 8</td>
<td>2 ± 1*</td>
</tr>
<tr>
<td>(E_{m}, mV)</td>
<td>-12 ± 2</td>
<td>-12 ± 4NS</td>
</tr>
<tr>
<td>(G_{m,-50}, nS)</td>
<td>1.40 ± 0.22</td>
<td>0.56 ± 0.12a</td>
</tr>
<tr>
<td>(G_{m,+15}, nS)</td>
<td>13.2 ± 3</td>
<td>11.4 ± 3.1NS</td>
</tr>
</tbody>
</table>

Data are from experiments such as that illustrated in Fig. 3 during norepinephrine or cirazoline perfusion. +EGTA indicates omission of Ca\(^{2+}\) and presence of 1 mM EGTA in the medium. Data are means ± SE from 3 experiments, with EGTA present either during the entire experiment or during only a part of the sustained inward current phase. Comparisons between values in control conditions and during Ca\(^{2+}\) omission in the same cell: *P < 0.05 and NSP > 0.05, respectively; Student’s paired t-test.
substitution, −25 mV after; mean from 2 experiments), implying that Cl− permeability in this state was low compared with that of other ions. Accordingly, there was no marked effect of Cl− substitution on Gm,−50 or Gm,+,15 in the resting state (not shown). However, the first inward current was diminished (not shown), and the first depolarization was now only −8 mV (not shown). In contrast, the sustained inward current was not diminished by Cl− substitution, and the induced current obtained from the voltage ramps at negative voltages was not markedly affected by Cl− substitution (not shown), implying that this phase of the response was not mediated by Cl− currents.

**Effects of Na+ removal.** If Na+ was replaced by N-methyl-d-glucamine (NMDG+) already at the start of the experiment, the resting membrane potential was increased from −20 to −40 mV, and the Gm,−50 was halved (not shown). In the absence of Na+, the first inward current could still be observed (Fig. 4A). The voltage ramp-induced current had characteristics similar to those observed in normal buffer (not shown). However, Na+ omission completely eliminated the norepinephrine-induced sustained inward current (Fig. 4A). Similarly, when Na+ was temporarily omitted during the time of the expected sustained inward current (Fig. 4B and Table 4), it acutely eliminated the inward current; thus the absence of inward current in Na+-free buffer was not due to secondary effects of prolonged omission of Na+. The sustained inward current reappeared when NMDG+ was again replaced with Na+; similarly, the membrane potential depolarized 8 mV when Na+ was reintroduced, and the Gm,−50 was doubled. In the absence of extracellular Na+, there was no norepinephrine-induced current during the voltage ramps (contrast the Δ-curve in Fig. 4C with that in Fig. 1D), but it may be noted that there was still an apparent induced inward current at positive membrane potentials, again probably a manifestation of an independent inhibitory effect of norepinephrine on K+ channels (cf. Fig. 1C). This effect was thus clearly not mediated through an Na+ influx.

Thus Na+ was not involved in the first inward current, but the sustained inward current was entirely mediated by a Na+ influx.

**A Large-Conductance NSC Channel**

During norepinephrine perfusion, we often (in 14 of the 21 cells tested) observed currents apparently resulting from single channel activity. Such currents are quite prominent in Fig. 3B and are discernible in Fig. 1A. In Fig. 5A, we show an enlargement of such current events. The events clearly represented inward currents from a single but large ion channel. In most cases, only one active channel per cell was detected, maximally two. A preliminary characterization of these channels could be made from their response to the conditions tested above.

### Table 4. Effect of temporary Na+ omission during the norepinephrine-induced sustained inward current phase

<table>
<thead>
<tr>
<th></th>
<th>+NMDG</th>
<th>After NMDG washout</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔI, pA</td>
<td>−16 ± 16</td>
<td>−48 ± 28*</td>
</tr>
<tr>
<td>Em, mV</td>
<td>−26 ± 4</td>
<td>−18 ± 0 NS</td>
</tr>
<tr>
<td>Gm,−50, nS</td>
<td>1.31 ± 0.44</td>
<td>2.45 ± 0.33 NS</td>
</tr>
<tr>
<td>Gm,+15, nS</td>
<td>9.2 ± 1.4</td>
<td>9.7 ± 1.3 NS</td>
</tr>
</tbody>
</table>

Data are from experiments such as those shown in Fig. 4 and are means ± SE from 4 experiments. Comparisons between the values in the presence and the absence of NMDG in the same cell (*P < 0.05 and NSP > 0.05, respectively; Student’s paired t-test).
The large-conductance channel currents were never observed in unstimulated cells but were frequently observed during norepinephrine and cirazoline stimulation. CGP-12177A (tested in 4 cells) was not able to activate the channel, even in a cell in which both norepinephrine and cirazoline could activate it. The activity disappeared upon washout of the adrenergic agonists. It was occasionally possible to observe activity during a voltage ramp (Fig. 5B); when it opened during the sustained inward current phase, it temporarily doubled the current. Extrapolation to zero current voltage indicates that the cell depolarized ~10 mV during each such opening. It is also clear from this and similar recordings (not shown) that the activity was not observable at potentials more positive than ~10 mV.

In some experiments, we recorded the conductance and the activity of the large-conductance channel at different membrane potentials. An example of this is given in Fig. 5A, and data from one such study are collected in Fig. 5, C and D. In Fig. 5C, the current-voltage relationship is shown. The activity corresponded to a single-channel conductance of 270 pS, which was fully ohmic within the voltages tested and which had a reversal potential very close to 0 mV. This would indicate that this large-conductance channel was an NSC channel. The open probability \((P_o)\) showed an unusual dependence on the membrane potential, being, if anything, activated by hyperpolarization (Fig. 5D).

Omission of extracellular \(\text{Ca}^{2+}\) did not affect the conductance of the channel (the current was 13 pA at ~50 mV both before and during \(\text{Ca}^{2+}\) removal) but impressively increased the open probability sevenfold, from 0.05 to 0.33 (not shown but cf. Fig. 3B). Reduction of extracellular \(\text{Cl}^-\) to 30 mM did not affect the single-channel amplitude or the activity of the channel (not shown). However, removal of extracellular \(\text{Na}^-\) reversibly abolished the large-conductance channel activity (not shown), demonstrating that it was a \(\text{Na}^-\)-conducting channel.

Thus the large-conductance channel was identified as an NSC channel with unusual regulatory properties. The physiological role of this type of channel in brown fat cells remains unknown, but a similar type of channel has been observed in rat basophilic leukemia cells; that channel was suggested to regulate exocytosis (20).

The properties of the large-conductance channel, especially the unusual \(\text{Ca}^{2+}\) dependence characteristics, clearly indicated that it was not this channel that carried the current during the sustained inward current phase. We therefore proceeded to the cell-attached mode to identify the ion channels carrying the depolarizing current.

**Adrenergic Activation of the 27 pS NSC Channel**

To identify the ion channels responsible for the sustained depolarization of brown fat cells during adrenergic stimulation, patch-clamp experiments in the cell-attached mode were performed. In the whole cell studies above, the current to be sought was established to be elicited by \(\alpha_1\)-adrenergic stimulation and have a delay of ~1 min, but then to be persistent for as long as the adrenergic stimulation persisted. It was a \(\text{Ca}^{2+}\)-dependent \(\text{Na}^+\) current and was implied to have characteristics compatible with it being mediated via a channel that is nonspecific in character (e.g., with a reversal potential close to 0 mV). Because the membrane potential in these cells is only about ~20 mV (see above), we chose to clamp the holding potential to +20 mV to enhance the single-channel current through the NSC channel we searched. The expected transmembrane potential across the patch was therefore ~40 mV.

**Effects of norepinephrine on single channel activity.** Norepinephrine activates single channel activity. Figure 6A shows a trace of the current through the cell-attached pipette after a gigaohm seal had been formed. The recording was started while the cell was perfused...
direct contact with the channels investigated, it was clear that the activation must have been mediated through an intracellular mediator.

As is observable in Fig. 6A, but as is more clear in the open probability ($P_o$) chart in Fig. 6B, the response was weak during the first minute after norepinephrine perfusion was started. On the basis of the whole cell current studies above and earlier investigations (4, 18, 21, 23), stimulation with norepinephrine should, during this time, lead to activation of other ion channels (especially Cl⁻ channels and Ca²⁺-activated K⁺ channels) in the brown fat cells, but we did not observe prominent channel activity from any other channel types. This may, however, be understandable on the basis of characteristics of these channels: they are probably low-conductance channels and were here monitored under conditions fairly close to their respective equilibrium potential.

After the delay of ~1 min, the ion channel activity in the patch became more intense, although not fully uniform; it had a tendency to fluctuate with an interval of 1–2 min between periods of highest $P_o$. The response was, however, persistent and did not show any tendency to inactivate during the routine recordings, which were continued for ≥5 min. Thus the observed ion channel activity had the current direction and time course that would be required for a current mediating the sustained inward current.

In Fig. 7, a quantification of results is shown from a series of studies performed like those illustrated in Fig. 6 A and B. Because of the fluctuating behavior of the channel, the $P_o$ was calculated for an extended time period (≥4 min). As seen, norepinephrine very significantly increased the observed open probability ($P_{o,obs}$) from 0.005 to 0.038; the mean increase was >10-fold when calculated from each experiment.

The increase in $P_o$ caused by norepinephrine could be due to an extended time spent in the open state, to a higher frequency of openings, or to more channels due to an extended time spent in the open state, to a uniform; it had a tendency to fluctuate with an interval of 1–2 min between periods of highest $P_o$. The response was, however, persistent and did not show any tendency to inactivate during the routine recordings, which were continued for ≥5 min. Thus the observed ion channel activity had the current direction and time course that would be required for a current mediating the sustained inward current.

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The increase in $P_o$ caused by norepinephrine could be due to an extended time spent in the open state, to a higher frequency of openings, or to more channels.

with only extracellular solution; as seen, some spontaneous ion channel activity was observed during this time. Such channel activity in unstimulated brown fat cells has earlier been observed and demonstrated to be mediated via 27 pS NSC channels (28) (and see below). During the time indicated by the horizontal line, the cell was perfused with the same solution, now containing 1 μM norepinephrine; as seen, this resulted in a dramatic increase in channel activity in the patch. It was considered likely that this prominent activity could be responsible for the sustained inward current; further analysis detailed below supported this view. Because the perfused norepinephrine did not come in
being recruited. Because of the low number of events occurring in the cell-attached mode, a full kinetic analysis of open and closed times was not feasible. Instead, we calculated the arithmetic mean of the time spent in the open state before and during perfusion. As seen in Table 5, the mean time spent in the open state was significantly increased during perfusion with norepinephrine, from 52 to 101 ms. However, as this increase in mean open time was only 2-fold, whereas the increase in \( P_o \) was >10-fold, the prolongation of mean open time only partly explains the increase in \( P_o \); i.e., the time between openings was also decreased.

**Single channel conductance.** To obtain the single channel conductance and the reversal potential of the channel observed, the holding potential was altered before and during the perfusion with norepinephrine. An example of such an analysis is shown in Fig. 8. In this particular cell-attached patch, sufficient spontaneous channel activity occurred to allow for estimation of channel conductance in the unstimulated state. As seen, the channel had an ohmic conductance within the voltage range studied; the slope corresponded to a conductance of 33 pS. This value was in very good agreement with earlier observations of spontaneous channel activity in brown fat cells (28).

The data obtained during norepinephrine stimulation yielded a straight line that was not significantly different in slope (now 35 pS), but the currents were systematically 0.15 pA smaller for each applied voltage. This probably meant that the endogenous membrane potential had become reduced, in this particular experiment by \( \sim 4 \) mV.

Data from several such experiments are shown in Table 5. As seen, the predominant ion channel ob-

**Table 5. Effect of adrenergic agonists on channel mean open time and single channel conductance in intact cells**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mean Open Time, ms</th>
<th>Single Channel Conductance, pS</th>
<th>Single Channel Current, pA</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>52 ± 6</td>
<td>~33</td>
<td>1.36 ± 0.05</td>
<td>22</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>101 ± 21 NS</td>
<td>~30</td>
<td>1.19 ± 0.08</td>
<td>6</td>
</tr>
<tr>
<td>CGP-12177</td>
<td>59 ± 15 NS</td>
<td>~33</td>
<td>1.59 ± 0.12</td>
<td>6</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>202 ± 97 NS</td>
<td>~27</td>
<td>1.14 ± 0.10</td>
<td>5</td>
</tr>
<tr>
<td>Forskolin</td>
<td>76 ± 24 NS</td>
<td>~28</td>
<td>1.32 ± 0.09</td>
<td>5</td>
</tr>
<tr>
<td>A-23187</td>
<td>182 ± 19 NS</td>
<td>~25</td>
<td>1.26 ± 0.12</td>
<td>3</td>
</tr>
</tbody>
</table>

Data are from the same experiments as those analyzed in Table 6. Because of a low no. of prestimulation (basal) events, data from all recordings have been combined. Mean open time is the mean (±SE) of the arithmetic mean open time calculated for each experiment. Correlation analysis indicated that the mean open time was an exponential function of open probability (\( P_o \)) (not shown). Single channel conductance was estimated from mean current values from \( \geq 2 \) holding potentials (of +20 and +40 mV) from 1–4 experiments for each agent. Single channel currents (means ± SE) were calculated from histogram analysis of all data points for 4 min after stimulation (or for 1 min before stimulation for the basal value). Differences between mean open times observed in the presence of the agent and in the basal condition: \( \text{NS} P > 0.05, ^* P < 0.01, \) and \( ^{+} P < 0.001, \) respectively. Analysis of variance of the single channel current indicated significant differences between the means (\( P < 0.05 \)).

The single channel current was measured in several experiments at the holding potential of +20 mV before and during norepinephrine perfusion (Table 5). The mean current observed before norepinephrine perfusion was 1.36 pA. During norepinephrine perfusion, the current was reduced to 1.19 pA, i.e., by 0.17 pA. On the basis of a conductance of 30 pS, the driving force for the current was in the mean reduced by 6 mV, i.e., the cells had become depolarized. The depolarization determined in this way is in remarkably good agreement with the depolarization (change in zero current potential, 8 mV) measured during the sustained inward current phase in the whole cell study above (Table 1). The extrapolated reversal potential observed in Fig. 8 and similar studies was in the range of +15 mV in the control state and +10 mV in the norepinephrine-stimulated cells. These values are very close (but with reversed sign) to the measured cell membrane potentials in the two conditions (Table 1). This means that the experienced reversal potential of these currents in the patch would be very close to 0 mV, as would be expected for a NSC channel. There is, therefore, good reason to conclude that the \( \sim 30 \) pS conductance channel activated by norepinephrine stimulation of brown fat cells represents an NSC channel.

Thus the norepinephrine-induced single channel activity observed in cell-attached patches had the time course and reversal potential required for the channel mediating the sustained membrane depolarization in brown fat cells.
Effects of selective \(\alpha_1\) - and \(\beta_2\)-adrenergic agonists on ion channel activity. On the basis of the data shown (above) from whole cell current studies, the channel responsible for the sustained inward current should be \(\alpha_1\)-adrenergically activated. To examine whether this was the case for the single channel activity observed here, we examined the activity of two subtype-selective adrenergic agonists, the \(\beta_2\)-agonist CGP-12177A and the \(\alpha_1\)-agonist phenylephrine, to elicit a channel activation similar to that elicited by norepinephrine.

CGP-12177A. CGP-12177A only doubled channel activity (Fig. 7), in contrast to the 10-fold increase caused by norepinephrine, and CGP-12177A had no effect on mean open time (Table 5). The channels observed during CGP-12177A perfusion also had a single channel conductance of \(\approx 27\) pS (Table 5) but, in contrast to the case for norepinephrine, the single channel current was not lower than in unstimulated cells (if anything, it was higher). Thus, in agreement with our observations in whole cell studies, CGP-12177A perfusion did not seem to lead to measurable membrane depolarization. Because the 10-fold increase in channel activity observed with norepinephrine (Table 5) led only to a doubling in total cell conductance (Table 1), the doubling in channel activity observed with CGP-12177A would only be expected to lead to a \(< 10\%\) increase in total cellular conductance, which would hardly be observable as a depolarization.

Phenylephrine. Phenylephrine, just like norepinephrine, increased channel activity about an order of magnitude (Fig. 7). The channel activity was, if anything, higher than that observed during norepinephrine perfusion. The mean open time (Table 5) was even longer than that seen during norepinephrine perfusion, but the estimated single channel conductance was the same (\(\approx 27\) pS) (Table 5). In accordance with the tendency to a more intense effect of phenylephrine than of norepinephrine, the single channel current was also more reduced during phenylephrine perfusion, by 0.22 pA, corresponding to a membrane depolarization of 8 mV.

Thus much more dramatic effects on channel activity were seen after phenylephrine (i.e., \(\alpha_1\)-stimulation) than after CGP-12177A (i.e., \(\beta_2\)-stimulation); in fact, phenylephrine could fully mimic the effect of norepinephrine. This is thus in accordance with the adrenergic receptor requirements from the whole cell studies for the channel activity mediating the sustained membrane depolarization in these cells.

Identity of the second messenger regulating the ion channel activity. On the basis of the fact that the sustained inward current observed in the whole cell studies was \(\alpha_1\)-adrenergic and \(\Ca\) dependent, it would be expected that the single channel activity responsible for the mediation of this current would be activated, directly or indirectly, through an increase in \([\Ca]\). To investigate whether this was the case, we examined whether an increase in cAMP, or, as would be expected, an increase in \([\Ca]\), would be able to increase the single channel activity. We used the adenyl cyclase activator forskolin to increase intracellular cAMP levels and the ionophore A-23187 to increase \([\Ca]\).

Forskolin. Forskolin only doubled channel activity, just like CGP-12177A (Fig. 7). The effect on mean open time (Table 5) was nonsignificant, and forskolin did not affect the amplitude of the single channel current, implying that it did not lead to membrane depolarization. These weak effects were observed despite the fact that this concentration of forskolin leads to cAMP levels higher than those induced by 1 \(\mu\)M norepinephrine (27, 31). Thus the response to forskolin was similar to the response to CGP-12177, and much less intense than that to norepinephrine or phenylephrine.

\(\Ca\) ionophore. With the \(\Ca\) ionophore A-23187 we saw a very marked activation of the NSC channel (Fig. 7). The mean open time (Table 5) was increased as much as it was during phenylephrine perfusion, and a reduced single channel current was observed. Thus, it would seem that A-23187 application also led to membrane depolarization. Together with the data above, this would indicate that the \(\alpha_1/\Ca\) pathway had a much better ability than the \(\beta/\Ca\) pathway to activate the channel activity, an observation fully in accordance with the demands for the channel mediating the sustained whole cell current.

Identity of the channel mediating the sustained inward current. It is clear from the patch-clamp studies in the cell-attached mode that the single channel activity observed fulfilled the criteria for being the channel activity behind the sustained inward current observed in the whole cell studies: time course of activation, nonselective nature of current, adrenergic receptor involvement, and \(\Ca\) dependence of activation. With respect to \(\Ca\) dependence, and especially on consideration of the observed channel size of \(\approx 30\) pS, it is very notable that, in excised patches from these cells, an NSC channel with exactly these properties has been well characterized (11, 12, 23, 26, 28). It is therefore very likely that it is this NSC channel, the 27 pS channel, that mediates the sustained inward current.

As is evident in Fig. 6A, only a small number (1–2) of simultaneously open channels was normally seen in the patch before and during norepinephrine perfusion. However, the recordings were terminated by excision of the patch into the bath solution and the formation of an inside-out patch. This led to a very dramatic activation of ion channel activity in the patch (Fig. 6C). These channels had a conductance of 24–29 pS (Figs. 6C and 7), and it had earlier been amply demonstrated that these “spontaneously” activated (i.e., due to high \(\Ca\) in the medium) channels also are the 27 pS NSC channels (26, 28). The number of channels present in excised inside-out patches, \(N_{\text{tot}}\), was always higher than the maximal number observed in cell-attached recordings. Thus a mean of 2.8 channels was observed in the inside-out patches in the experiments in which the cells had been perfused with norepinephrine before excision, during which time a mean of only 1.2 channels was observed in the cell-attached mode with the same pipette patch.
Two interpretations of this are possible: either the excision process activated NSC channels that were fully inactive when the patch was part of the cell membrane, or the observed activity in the cell-attached mode was really the collective activity of all channels observed in the excised patch. However, each would then have a lower \( P_o \) than that earlier estimated, based on the presence of only 1.2 channels in each patch. There is no simple way to distinguish between these two possibilities. However, if all the channels are considered equally active, it is possible to recalculate the data of the observed open probabilities (\( P_{o,obs} \)) to obtain \( P_{o,tot} \). Thus the \( P_{o,obs} \) for norepinephrine was 0.038 \( \pm \) 0.009, but when calculated on the basis of all channels revealed by excision, the \( P_{o,tot} \) was only 0.017 \( \pm \) 0.004. The activation by norepinephrine then corresponds to an increase in \( P_{o,tot} \) from 0.002 to 0.017; this is then the activity level of the NSC channel to be looked for in direct studies of channel activity in excised patches.

**DISCUSSION**

We show here that norepinephrine induced two inward current phases in brown fat cells: a transient current, which lasted for \( \sim 1 \) min, and a sustained current that persisted as long as norepinephrine stimulation persisted. The experiments conducted had as their main goal to further the understanding of the nature of the sustained current, which we could characterize fairly well, and which we conclude is an \( \alpha_1 \)-adrenergically induced \( \text{Ca}^{2+} \)-dependent activation of 27 pS NSC channels.

Concerning the first inward current phase, it was, of course, unavoidable to obtain experimental results during the experiments conducted here; they were also briefly reported above. The elucidation of these events was not the prime goal of the present investigations, and a full understanding of the events has not been reached here. However, on the basis of these observations and literature data, a tentative interpretation of the plasma membrane events after norepinephrine stimulation of brown fat cells is given below, in which we discuss in succession the resting state, the first inward current, and the sustained inward current (Fig. 9).

**The Resting State**

The mean capacitance of the brown fat cells investigated here was 35 pF (Fig. 8A). Based on the general value of 1 \( \mu \text{F/cm}^2 \) cell membrane, this capacitance corresponds to the surface of a sphere with a diameter of 33 \( \mu \text{m} \).

The \( G_{m,-50} \) value of 0.7 nS was much smaller than whole cell conductances observed in classical microelectrode experiments in brown adipose tissue (\( \sim 700 \) nS (7)) and even somewhat smaller than those earlier observed with patch-clamp techniques, in classical whole cell studies (14), or in perforated-patch studies (2–8 nS) (15).

On the basis of the zero current during voltage ramps, the resting membrane potential was estimated to be about \(-21 \) mV. Because the cells studied probably had a clamped intracellular ion level determined by the pipette concentration, it may be questioned whether this potential represents that present under innate conditions. However, the estimates in the cell-attached mode, in which intracellular ion concentrations were not affected, confirmed this rather low value under these conditions.

The identity of the ion permeabilities that determine the resting membrane potential is not immediately evident. The measured potential is far from the expected \( \text{K}^+ \) equilibrium potential of \(-83 \) mV. This deviation could be due to fairly high permeabilities for \( \text{Cl}^- \) or \( \text{Na}^+ \). Alterations in extracellular \( \text{Cl}^- \) concentration did not markedly influence the resting membrane potential, but the membrane potential became more negative when extracellular \( \text{Na}^+ \) was exchanged with NMDG\(^+\). However, in the presence of mefenamic acid,
which fully inhibits the 27 pS NSC channel (11), the observed resting $E_m$ did not come closer to $-85$ mV. This also implies that the low basal activity of the NSC channels (cf. Table 5) is not sufficient to significantly influence the resting membrane potential. Thus the identities of the ion permeabilities responsible for the low resting membrane potential are not known, but a Na$^+$ permeability not mediated by the 27 pS NSC channels is likely involved.

The First Inward Current

Adrenergic receptor type involved. The selective $\alpha_1$-adrenergic agonist cirazoline was able to induce the first inward current in a manner qualitatively similar to norepinephrine, but the response was only two-thirds of that observed with norepinephrine (Fig. 8, B and C). The difference may be due to effects mediated via $\beta_3$-receptors, because CGP-12177A generally elicited some membrane currents during this phase. The $\alpha_1$-induced inward current became much smaller in Ca$^{2+}$-free buffer, implying that the $\alpha_1$-signal is intracellularly mediated via an increase in Ca$^{2+}$ levels.

Ionic characteristics of the first inward current. The first inward current phase probably involves currents of more than one ion species, mediated through more than one channel. The fact that the currents during voltage ramps were changed as an effect of reduction of extracellular Cl$^-$ levels is in agreement with results from earlier ion flux (4) and patch-clamp (21) studies concluding that Cl$^-$ currents probably were induced in this phase.

It is likely that K$^+$ currents are also induced during the first inward current phase. Indications for this are the fact that the current and the cell membrane permeability increase at the time when the cell is repolarizing. The existence of an $\alpha_1$-adrenergic, Ca$^{2+}$-dependent K$^+$ current is in agreement with earlier ion flux and and electrophysiological studies (15, 17, 18). In our voltage-clamped cell system, the putative K$^+$ current never became sufficiently large to induce a net outward current, but under nonclamped conditions, the voltage-sensitive K$^+$ channels (14, 23) may also be activated secondarily to the depolarization caused by the increased Cl$^-$ permeability, leading to the hyperpolarization observed in non-voltage-clamped cells (9, 14).

A tentative interpretation of the events during the first inward current in the voltage-clamped cell is therefore that $\alpha_1$-adrenergic stimulation, via increases in cytosolic Ca$^{2+}$, first activates a Cl$^-$ conductance that is responsible for the immediate depolarization observed (Fig. 9B). However, nearly at the same time, but with a slight delay, there is an activation of K$^+$ channels, probably also Ca$^{2+}$ dependent; thus the total conductance of the cell increases vastly (Fig. 9C). The activation of K$^+$ channels tends to counteract the depolarization and help in the repolarization event; thus the time of maximal depolarization precedes the time of maximal conductance. Both the Cl$^-$ and K$^+$ currents are inactivated (through unknown mechanisms) after ~1 min.

That both Cl$^-$ and K$^+$ currents are involved during the first inward current is supported by the earlier observations of ionic fluxes from these cells. Within the first minute after $\alpha_1$-adrenergic stimulation, both Cl$^-$ (4) and K$^+$ (17, 18) ions are lost from the cells. If only Cl$^-$ conductance had increased, the cell would have depolarized, but because a new electrochemical equilibrium would have been reached, a large $^{36}$Cl$^-$ efflux could hardly have been expected. Thus a net loss of KCl from the cell probably occurs during the 1st min of adrenergic stimulation. This may mean that volume changes occur in the cells in this phase.

Ion channels involved. The identities of the ion channels involved in the first inward current have not been established. A 50 pS Cl$^-$ channel has been observed in brown fat cells (24), but there is no functional evidence to associate it with the norepinephrine-induced Cl$^-$ current. At the single channel level, a Ca$^{2+}$-activated K$^+$ channel has not been identified in the tissue.

The Sustained Inward Current

Norepinephrine could consistently activate a sustained inward current (membrane depolarization) in the rat brown fat cells (Fig. 9D). The existence of a norepinephrine-induced sustained depolarization is well established from classical microelectrode studies in brown adipose tissue (5–7, 9, 10, 30). The events during this phase have now become clear.

Adrenergic receptor involved. The sustained inward current and the depolarization could be induced by the $\alpha_1$-agonist cirazoline to the same extent as by norepinephrine; $\beta_3$-stimulation was without effect. As mentioned in the introductory comments, the third-phase sustained depolarization in brown fat cells has generally been thought to be $\beta$-adrenergic in nature (reviewed in Refs. 2 and 8), and the present data are therefore in contrast to that tenet. However, a reexamination of the literature prompted by the present results revealed that the $\beta$-adrenergic nature of the sustained-phase depolarization is not well established experimentally. In several earlier investigations, a poststimulation recovery response, after a short pulse of adrenergic or nervous stimulation, is in reality what has been analyzed (6, 9), i.e., a situation that is principally different from the persistent stimulation studied here. In contrast, several investigations with persistent adrenergic stimulation implied both an $\alpha$- and a $\beta$-induced depolarization of about equal magnitude (5, 10). The published $\alpha$-results are thus in good agreement with the outcome of our studies. The question is therefore why a marked $\beta$-induced depolarization has often been observed previously, whereas in our studies, the $\beta$-adrenergic pathway seems to be of no importance.

The studies indicating a $\beta$-adrenergic response have in general been performed in excised pads of brown adipose tissue (5, 7, 8, 30). In these pads, there is a risk of an inadequate O$_2$ supply during norepinephrine stimulation. The low O$_2$ level may lead to a decrease in mitochondrial membrane potential and an ensuing re-
lease of mitochondrial Ca\(^{2+}\) into the cytosol, where, in an apparently β-adrenergic way, it could induce the depolarizing current. This could therefore explain why a β-component is readily observable in excised and perfused brown fat pads but not in cells with an adequate O\(_2\) supply, such as in brown fat cells in situ or, as here, in single brown fat cells in a well-oxygenated medium.

Ionic character of the sustained current. Partial substitution of aspartate for Cl\(^-\) was without effect on the sustained inward current, but the current was completely abolished when extracellular Na\(^+\) was replaced with NMDG\(^+\). Our observations therefore also agree with the observation that the corresponding depolarization is Na\(^+\) dependent (8). The current is thus clearly a Na\(^+\) current, but because it is α\(_1\)-adrenergic, it cannot be related to the β-adrenergically induced Na\(^+\) influx earlier reported in brown fat cells (3). Because the α\(_1\)-induced sustained current had a zero current potential close to 0 mV, it is unlikely that it represented a current through selective Na\(^+\) channels. This zero current potential is rather what is expected for a current mediated through an NSC channel.

The sustained depolarization may be ascribed to the activation of the 27 pS NSC channels. Based on the studies in the cell-attached mode, we concluded that the NSC channels with a conductance of ~27 pS, earlier observed in brown fat cells (11, 12, 23, 26, 28), had all the properties required to ascribe to them the role of being the channels responsible for the adrenergically induced sustained inward current in voltage-clamped brown fat cells and the sustained membrane depolarization in nonclamped cells. Their activity was increased as an effect of norepinephrine stimulation; there was a 1-min delay before full activation was reached, but the adrenergically induced NSC channel activity then persisted as long as the adrenergic stimulant was present. The adrenergically induced NSC channels were originally described in brown fat cells (11, 12, 23, 26, 28), had a high slope, and the reversal potential in the cell-attached configuration was identical to the known membrane potential of the brown fat cells under these conditions. Furthermore, the adrenergically induced NSC channels activity then persisted as long as the adrenergic stimulation lasted. The activated channels had an ~30 pS slope, and the reversal potential in the cell-attached configuration was identical to the known membrane potential of the brown fat cells under these conditions. Furthermore, the activation was mainly α\(_1\)-adrenergic in nature and occurred through a second messenger, probably [Ca\(^{2+}\)]\(_i\). In all these respects, the NSC channel exhibited exactly the properties required if these channels should be the mediators of the sustained current.

This also means that we suggest that it is through activation of these NSC channels that the norepinephrine-induced long-lasting depolarization recorded originally (7), and amply confirmed since then, is mediated. It thus confirms the suggestion put forward when the NSC channels were originally described in brown fat cells, that these could be the channels mediating the sustained depolarization (26, 28).

Is the NSC channel activity sufficient? In the whole cell studies, the total cell conductance (at the clamped voltage of ~50 mV) was increased by 0.7 nS during the sustained inward current phase (Table 1). If the NSC channels observed here are to be the sole channels responsible for this increase in conductance, the sum of their conductances must be of this magnitude. From the values obtained here, it is possible to estimate this combined conductance. With a single channel conductance of 30 pS (Table 6) and the increase in \(P_o\) of 0.03 in the norepinephrine-stimulated state (Table 5), each norepinephrine-activated channel corresponds to a virtual conductance of 0.9 pS. To estimate the corresponding whole cell conductance, a number of 1.2 observed channels per patch, an estimated patch area of 1 μm\(^2\) (25), and the estimate of the cell being a sphere with a diameter of 33 μm were used. Thus, on the basis of the single channel activities, the estimated increase in total cell conductance during the sustained phase would be 3.7 nS (or ~2 nS if it is supposed that channels on the foil-adhering part of the cell are not functional). This estimated value thus even exceeds the conductance increase actually observed in the whole cell system (0.7 nS), and this calculation therefore indicates that the NSC channels together have the capacity to be the channels mediating the sustained inward current.

The Physiological Role of the Sustained Depolarization

The immediate effect of the increased membrane conductance underlying the sustained current described here would be a decrease in membrane potential, as was indeed observed. It may be that this depolarization in itself is the major physiological significance of the sustained increase in membrane conductance. Many cellular uptake or release processes are dependent on the magnitude of the membrane potential, and it has, for example, been demonstrated that depolarization of the brown fat cell membrane (with high extracellular K\(^+\)) diminishes the [Ca\(^{2+}\)]\(_i\) level observed in the cytosol during adrenergic stimulation (13). Thus the sustained increase in membrane conductance may protect the cells from excessively high increases in [Ca\(^{2+}\)]\(_i\).

Because the described increase in conductance is associated with inward Na\(^+\) currents, a direct connection between Na\(^+\) influx and thermogenesis could be postulated, as was indeed originally suggested by Girardier et al. (7). However, this is unlikely, because substitution of NMDG\(^+\) for Na\(^+\) has only marginal effects on norepinephrine-induced thermogenesis (3). However, the sustained inward current would represent a Na\(^+\) influx that, in the new steady state, would have to be counteracted by the Na\(^+\)-K\(^+\)-ATPase. In agreement with this, a fraction of α\(_1\)-adrenergically stimulated respiration can be inhibited by the Na\(^+\)-K\(^+\)-ATPase inhibitor ouabain (16). It should, however, be realized that this fraction represents only a very small fraction (~5%) of the total oxygen consumption (thermogenesis) that is induced by norepinephrine; the main part is through β-adrenergic pathways. Furthermore, as an effect of the increased Na\(^+\) permeability, a new steady-state cytosolic Na\(^+\) level would be established, somewhat higher than the resting level; these
could be direct effects of such alterations in cytosolic Na$^+$ levels.

The above effects are all of an acute nature. However, there is now extensive evidence that adrenergic stimulation of the brown fat cell promotes cell proliferation and cell differentiation (19). In these processes, $\alpha_1$-adrenergic stimulation may be important. Indeed, the expression of c-fos, for example, which is supposedly involved in regulation of cell differentiation, is under strong synergistic control by $\alpha_1$- and $\beta$-adrenergic pathways (27). It is an interesting possibility that it is in processes like these that the $\alpha_1$-induced depolarization, the ensuing influx of Na$^+$ ions, and the possible increase in cytosolic Na$^+$ levels are of importance.

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