α-Adrenergic Stimulation Activates a Calcium-sensitive Chloride Current in Brown Fat Cells

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ABSTRACT The first response of brown adipocytes to adrenergic stimulation is a rapid depolarizing conductance increase mediated by α-adrenergic receptors. We used patch recording techniques on cultured brown fat cells from neonatal rats to characterize this conductance. Measurements in perforated patch clamped cells showed that fast depolarizing responses were frequent in cells maintained in culture for 1 d or less, but were seen less often in cells cultured for longer periods. Ion substitution showed that the depolarization was due to a selective increase in membrane chloride permeability. The reversal potential for the depolarizing current in perforated patch clamped cells indicated that intracellular chloride concentrations were significantly higher than expected if chloride were passively distributed. The chloride conductance could be activated by increases in intracellular calcium, either by exposing intact cells to the ionophore A23187 or by using pipette solutions with free calcium levels of 0.2–1.0 μM in whole-cell configuration. The chloride conductance did not increase monotonically with increases in intracellular calcium, and going whole cell with pipette-free calcium concentrations ≥10 μM rapidly inactivated the current. The chloride currents ran down in whole-cell recordings using intracellular solutions of various compositions, and were absent in excised patches. These findings imply that cytoplasmic factors in addition to intracellular calcium are involved in regulation of the chloride conductance. The chloride currents could be blocked by niflumic acid or flufenamic acid with IC₅₀s of 3 and 7 μM, or by higher concentrations of SITS (IC₅₀ = 170 μM), DIDS (IC₅₀ = 50 μM), or 9-anthracene carboxylic acid (IC₅₀ = 80 μM). The chloride conductance activated in whole cell by intracellular calcium had the permeability sequence PNO₃ >PBr >PI >PCl >P_Cl, measured from either reversal potentials or conductances. Instantaneous current-voltage relations for the calcium-activated chloride currents were linear in symmetric chloride solutions. Much of the current was time and voltage independent and active at all membrane potentials between −100 and +100 mV, but an additional component of variable amplitude showed time-dependent activation with depolarization. Volume-sensitive chloride currents were also present in brown fat cells, but differed from the calcium-activated currents in that they responded to cell swelling, re-
quired intracellular ATP in whole-cell recordings, showed no sensitivity to intracellular or extracellular calcium levels, and were relatively resistant to block by niflumic and flufenamic acids. We conclude that the intracellular calcium increases resulting from adrenergic stimulation in brown fat activate a chloride-selective membrane conductance distinct from the volume-sensitive chloride conductance also present in the cells.

INTRODUCTION

Brown adipose tissue is present in many mammals and is especially prominent in hibernators and neonates. It has long been known that brown fat generates heat for thermoregulation in a cold environment and arousal from hibernation (for review see Horwitz, 1989). Recent work has shown that the energy-wasting activity of brown fat is also used to burn off excess food energy in animals fed an unbalanced diet, and that this diet-induced activation of brown fat is impaired in genetically obese animals (reviewed in Himms-Hagen, 1989; Stock, 1989; Trayhurn, 1989). Thermogenesis in brown fat is normally activated by adrenergic stimulation through the sympathetic terminals innervating the tissue. The activation of β-adrenergic pathways mobilizes fatty acids and uncouples mitochondria through activation of a brown fat-specific mitochondrial protein, leading to as much as 40-fold increases in heat production within a few minutes of stimulation. Other immediate responses of sympathetic stimulation are mediated by the α-adrenergic receptors also present in the tissue, and include changes in the membrane electrical potential (Girardier and Schneider-Picard, 1983; Horwitz and Hamilton, 1984) and ion fluxes (Schneider-Picard, Coles, and Girardier, 1985; Dasso, Connolly, and Nedergaard, 1990) across the cell membrane, cytoplasmic alkalinization (Giovannini, Seydoux, and Girardier, 1988; Lee, Hamilton, Trammell, Horwitz, and Pappone, 1994), and increases in intracellular free calcium levels through release from intracellular stores and plasma membrane fluxes (Lee, Nuccitelli, and Pappone, 1993). Long-term stimulation of brown fat in vivo induces tissue growth, as a result of increases in both the number of brown fat cells and their size. In addition, the heat-generating capacity of the cells increases, through increased numbers of mitochondria and increased expression of the mitochondrial uncoupling protein. Both α- and β-adrenergic receptors are thought to play a role in the profound effects of sustained adrenergic stimulation on tissue properties in brown fat.

Changes in membrane potential are the first measurable effects of hormonal stimulation in brown fat, but the roles of the electrical responses are not known. Norepinephrine (NE) applied either in vivo (Horwitz and Hamilton, 1984) or in vitro (Girardier, Seydoux, and Clausen, 1968; Girardier and Schneider-Picard, 1983; Schneider-Picard, Coles, and Girardier, 1985) induces a complex membrane potential response, consisting of a rapid depolarization and hyperpolarization, followed by a sustained depolarization. The initial depolarization and hyperpolarization are mediated by α-adrenergic receptors and precede any metabolic activation of the tissue. The slow depolarizing response is concurrent with increases in metabolism (Girardier and Schneider-Picard, 1983; Schneider-Picard et al., 1985). Our previous experiments have shown that all three phases of the membrane electrical response are due to membrane conductance increases (Lucero and Pappone,
We have demonstrated that the hyperpolarization is mediated by a voltage-independent potassium conductance activated by increases in internal Ca\(^{2+}\) released by intracellular stores (Lucero and Pappone, 1990; Lee, Nuccitelli, and Pappone, 1993). The second messengers eliciting the depolarizing responses have not been described previously. The initial depolarization could be blocked by amiloride, leading to the suggestion that pH regulatory mechanisms or changes in pH\(_i\) could be involved (Giovannini, Seydoux, and Girardier, 1988), although amiloride may have acted through interference with receptor activation in these experiments (Howard, Mullen, and Insel, 1987). Microelectrode measurements in intact brown fat tissue suggest that Ca\(^{2+}\) influx is not responsible for the fast depolarization, because neither removing external Ca\(^{2+}\) nor adding calcium channel blockers altered it (Horwitz, Hamilton, Lucero, and Pappone, 1989). The slow depolarization is probably mediated by the nonspecific cation channel that is a prevalent feature in patches from brown fat cell membranes. Although the normal cellular mechanisms regulating the nonspecific cation conductance are not known, it has been suggested that changes in cytoplasmic calcium or ATP levels (Siemen and Reuhl, 1987; Weber and Siemen, 1989; Koivisto, Dotzler, Russ, Nedergaard, and Siemen, 1993) or cytoplasmic redox state (Koivisto, Siemen, and Nedergaard, 1993) are involved.

The work presented here characterizes the conductance underlying the fast depolarizing response of brown fat cells. We show that the fast adrenergic depolarization is mediated by a chloride-selective membrane conductance increase. The chloride conductance is activated by increases in intracellular calcium, but is influenced by other cytoplasmic factors as well. Preliminary accounts of this work have been presented previously (Pappone and Lee, 1994; Pappone, Lee, and Ortiz-Miranda, 1994).

**MATERIALS AND METHODS**

**Cells**

Brown adipocytes were isolated from the interscapular fat pads of 1–7-day old Sprague-Dawley rat pups by collagenase digestion and plated on collagen-coated glass coverslips as previously described (Lucero and Pappone, 1990). Cells were incubated at 37°C and 5% CO\(_2\) in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf/horse serum, 0.2 U/ml insulin, 100 μg/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were maintained in culture for 0–14 d before patch clamping.

**Patch Clamp**

Whole-cell currents were measured using either nystatin permeabilized perforated-patch (Horn and Marty, 1988) or standard whole-cell patch clamp techniques (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) as described previously (Lucero and Pappone, 1990). Thick-walled borosilicate capillaries (Sutter Instruments, Inc., Novato, CA) were used to manufacture pipettes with resistances of 1–4 MΩ. The voltage offset between the patch pipette and the bath solution was nulled immediately before patch formation. No correction was made for junction potentials between the bath solution and the agar bridge containing 1 M KCl connecting it to the bath electrode. Junction potentials were <7 mV for all solutions used, determined by direct measurement.
against a low resistance electrode containing 3 M KCl or from the shift in zero current potential measured in macropatches. Membrane currents were recorded and membrane capacity currents nulled with a patch clamp amplifier (model EPC-7, List Electronic, Darmstadt, Germany; or model 3900, Dagan Corp., Minneapolis, MN) connected to a computer via a Basic23 interface (INDEC Systems, Sunnyvale, CA). Pulse protocols were delivered and data collected and analyzed using software developed by R.S. Lewis, Stanford University. Unless stated otherwise, membrane conductance was measured from a linear least-squares fit of currents recorded during voltage ramps of 500-ms duration. Data are reported as means ± SEM.

Solutions

For most experiments examining agonist or ionophore responses the cells were continuously perfused with a bicarbonate-buffered Krebs solution equilibrated with 95% O₂/5% CO₂. The Kreb's solution contained (in millimolar): 120 NaCl, 4.5 KCl, 0.5 MgCl₂, 2 CaCl₂, 0.7 Na₂HPO₄, 1.3 NaH₂PO₄, 25 NaHCO₃, 10 glucose, pH 7.4. The composition of the Aspartate Kreb's, NMG Kreb's, and TEA Kreb's were the same except that 120 mM Na-aspartate, N-methyl-D-glucamine chloride, or tetraethylammonium chloride replaced 120 mM NaCl. Most whole-cell experiments, including selectivity and osmotic response measurements, were made in a potassium-free Ringer's solution (0K Ringer's) to minimize currents through potassium channels. The control 0K Ringer's solution contained (in millimolar): 145 NaCl, 2 CaCl₂, 1 MgCl₂, 10 Na-MOPS, pH 7.4. Solutions for selectivity measurements had the same composition but with 145 mM NaBr, NaI, NaNO₃, or Na-aspartate replacing the NaCl. The solutions usually used to activate the volume-sensitive currents had the same composition as 0K Ringer except the NaCl concentration was reduced to 75 mM to give a solution of 60% normal tonicity. In other experiments, volume-sensitive currents were activated in Kreb's solution diluted with water or by going while-cell with pipette solution made hypertonic by addition of extra Na aspartate. Normal tonicity solutions had measured osmolarities of 285–310 mOsm.

Table 1

| No. | Cl  | Ca²⁺ | NaCl  | KCl | Na-Asp | K-Asp | CaCl₂ | EGTA | HEDTA |
|-----|-----|------|-------|-----|--------|-------|-------|------|-------|
|     | mM  | µM   | mM    | mM  | mM     | mM    | mM    | mM   | mM    |
| 1   | 10  | —    | 10    | —   | 140    | —     | —     | —    | —     |
| 2   | 15  | 0.5  | —     | 125 | —      | 7.5   | 10    | —    | —     |
| 3   | 15  | —    | 15    | —   | 187    | —     | 2     | —    | —     |
| 4   | 30  | 0.05 | 25    | —   | 92.5   | 3.5   | 15    | —    | —     |
| 5   | 30  | 0.5  | 7.4   | —   | 100.8  | 11.5  | 15    | —    | —     |
| 6   | 30  | 0.5  | 7.4   | —   | 100.8  | 11.5  | 15    | —    | —     |
| 7   | 30  | 1    | 24    | —   | 87.5   | 3     | 15    | —    | —     |
| 8   | 30  | 10   | 8.6   | —   | 112    | 10.7  | —     | 15   | —     |
| 9   | 30  | 100  | 1     | —   | 118    | 14.5  | —     | 15   | —     |
| 10  | 35  | —    | 10    | 25  | —      | 115   | —     | —    | —     |
| 11  | 129 | 2    | 120   | —   | —      | 4.5   | —     | 10   | —     |
| 12  | 140 | 0.5  | 125   | —   | —      | 7.5   | 10    | —    | —     |

All solutions contained 10 or 15 mM MOPS, titrated to pH 7.2 with NaOH or KOH. Osmolarities were 290–310 mOsm. Free calcium levels shown (Ca²⁺) were calculated from the solution composition and pH using the MAXCHELA program (C. Patton, Stanford University, Stanford, CA).
Table I shows the composition of the pipette solutions used. CaCl₂, EGTA, and HEDTA concentrations were varied to produce the desired free Ca²⁺ concentrations calculated from the MAXCHELA program (C. Patton, Stanford University). Perforated-patch experiments generally were done using solution Nos. 8 or 10 with ~0.25 mg/ml nystatin and 0.1% Pluronic F127 added.

**Chemicals**

Norepinephrine and phenylephrine were made up as 1-10 mM aqueous stock solutions. Nystatin (50 mg/ml), A23187 (10 mM), and Pluronic F127 (0.2 g/ml) stock solutions were made up in DMSO. These stock solutions were stored frozen, and thawed and diluted into the appropriate solution immediately before use. Niflumic and flufenamic acids were made up as 1-10 mM aqueous solutions, neutralized with NaOH, and stored refrigerated. 4-acetamido-4'-isothiocyanostilbene-2-2'-disulfonic acid (SITS), 4,4'-diisothiocyanostilbene 2,2'-disulfonic acid (DIDS), and 9-anthracene carboxylic acid (9ACA) were made up as 1 mM solutions in Ringer shortly before use. A23187 was from Calbiochem (LaJolla, CA) and Pluronic F127 was from Molecular Probes, Inc. (Eugene, OR). All other chemicals were from Sigma Chemical Co., (St. Louis, MO).

**RESULTS**

**Adrenergic Stimulation Activates a Depolarizing Conductance in Brown Fat**

Our previous work has shown that brown fat cells have at least three distinct conductance responses to adrenergic stimulation: a fast depolarization and a fast hyperpolarization activated by α-adrenergic stimulation, and a slow, sustained depolarization activated by prolonged exposure to β-adrenergic agonists (Lucero and Pappone, 1990). The fast hyperpolarization is mediated by a calcium-activated, apamin and TEA sensitive potassium-selective current, I_{K,ca}. The slow depolarizing current is carried by a nonselective cation conductance with a reversal potential near 0 mV, and its activation was usually irreversible. For the present experiments we generally used brief agonist exposures, in order to avoid activating the slow depolarizing current and to permit study of the fast depolarizing responses in isolation. We could readily discern when the currents contained a slow depolarizing component from the noisiness of the currents and their more positive reversal potential. Such records were excluded from the analysis presented here.

Our first concern then was to demonstrate that we could effectively isolate fast depolarizing current responses from the other current components present in brown fat cells. In many cells, exposure to norepinephrine (NE) can lead to a rapid increase in membrane conductance with a constant depolarizing reversal potential. Fig. 1 A shows currents measured in such a cell during ramps of membrane potential applied before, during, and after a brief exposure to NE. Within a few seconds, NE induced an almost fivefold conductance increase over the unstimulated level. While the conductance varied, the reversal potential of the conductance increase remained constant, near ~16 mV in this cell. In 36 cells showing purely or clearly resolvable fast depolarizing responses to NE under similar conditions, the average reversal potential of the conductance determined from the intersection of ramp currents recorded before and during the NE response was 20 ± 1 mV. The consistency of the reversal potential of the activated currents indicates that they were due to activation of a single conductance mechanism, while the negative value of
the reversal potential shows that this conductance is different from the slowly activating depolarizing conductance. The average zero current potential in these same cells before exposure to NE was $-38 \pm 2$ mV, so activation of the conductance would have depolarized unclamped cells.

The cell in Fig. 1 responded with two oscillating conductance increases to a single NE stimulation in both this and a subsequent exposure to NE, as shown in Fig. 1 B. The form of the fast depolarizing NE response was highly variable from cell to cell in these experiments, ranging from a simple conductance increase and decrease to as many as five oscillations in membrane conductance. However, the responses in any one cell to repeated applications of NE tended to be similar. These features—oscillations in the conductance, variability in the response properties between cells, and consistency in the form of the response within a single cell—

![Figure 1](image.png)

**Figure 1.** Fast depolarizing response to adrenergic stimulation in a perforated-patch clamped brown fat cell. (A) Currents recorded during 275 ms ramps of potential from $-100$ to $+20$ mV before, during, and after the maximum response to $1 \mu$M NE. Reversal potential of the membrane current in the unstimulated cell was $-36$ mV. NE transiently activated a current with a reversal potential of $-16$ mV, determined from the intersection of the currents in the presence and absence of NE. (B) Membrane conductance in the same cell as a function of time after initiation of the experiment. Membrane conductance, $g$, was determined from a linear fit of the membrane currents between $-80$ and $-30$ mV, recorded during potential ramps like those in A. The cell was continuously perfused with Kreb's solution throughout the experiment. At the times indicated the cell was briefly exposed to a 100 µl bolus of 1 µM NE. Pipette solution No. 8.

resemble the response characteristics of the calcium-activated potassium current, $I_{K,Ca}$, in brown fat cells. This pattern of activation and the fact that the fast depolarizing conductance increase is elicited by $\alpha$-adrenergic stimulation (Horwitz and Hamilton, 1984; Girardier and Schneider-Picard, 1983; Lucero and Pappone, 1990) suggested that the conductance is regulated by intracellular calcium levels.

Overall, fast depolarizing conductance increases were observed in approximately half the cells showing membrane responses to agonist and were seen both in the presence and absence of hyperpolarizing conductance increases due to $I_{K,CA}$ activation. $I_{K,CA}$ activation could be seen as a potential-independent increase in membrane current with a more negative reversal potential. The hyperpolarizing current was sensitive to block by apamin or TEA, as shown for example in Fig. 5. Agonist responses were less frequent in Ringer's solutions than in Kreb's (29 vs 87% responsive cells), but the proportion of responsive cells showing depolarizing and/or hyperpolarizing responses did not differ between the two solutions.
The prevalence of depolarizing and hyperpolarizing responses to NE stimulation was highly dependent on how long the cells had been cultured. The proportion of depolarizing responses decreased with time in culture, while the incidence of hyperpolarizing responses increased, as shown in Fig. 2. The depolarizing conductance was most often seen in newly cultured brown adipocytes, with 89% of freshly isolated or 1-d cultured cells responding with depolarization, as opposed to only 45% of cells after six or more days in culture. Hyperpolarizing currents showed the opposite trend, increasing in frequency in the older cultures. Thus, purely depolarizing responses were seen most frequently in freshly isolated cells, purely hyperpolarizing responses were most often present in cultures older than six days, and cells from cultures of intermediate age tended to show mixed responses. There was no correlation with the age of the pups (1-7 d) from which the cells were isolated. These results indicate that the expression and/or sensitivity of both depolarizing and hyperpolarizing conductance mechanisms may be regulated by the presence or absence of factors in the culture media.

The Fast Depolarizing Current Results from a Selective Increase in Chloride Permeability

Three lines of evidence indicate that the fast depolarizing current elicited by NE in perforated patch clamped cells is carried largely by chloride ions. First, replacing half the Kreb's solution bathing the cells with isotonic sucrose shifted the reversal potential of the NE-activated current to more positive potentials in two cells. Second, replacing all the Na⁺ in the Kreb's solution bathing the cells with NMG (three cells) or replacing half the Na⁺ with TEA (three cells) had no effect on the reversal potential of the hormone-elicited current. Fig. 3B shows the response of the cell shown in Fig. 1 to a subsequent NE exposure in NMG-substituted Kreb's. The reversal potential of the NE-activated current is unchanged, indicating that the depolar-
izing conductance is not significantly permeant to cations. The magnitude of the conductance change is substantially smaller than that seen with the earlier exposures to NE in normal Kreb's solution, probably due to the rundown in responsiveness usually seen in these experiments. Third, replacing 145 mM of the Cl\textsuperscript{-} in the Kreb's solution with aspartate ions shifted the reversal potential of the NE-activated current towards more positive potentials, as shown in Fig. 3, C and D. In five cells in which extracellular Cl\textsuperscript{-} was replaced by aspartate, the extrapolated reversal potential measured in normal Kreb's solution. (C and D) Replacement of extracellular Cl ions with aspartate shifts the reversal potential of the fast depolarizing conductance to more positive potentials. Currents recorded before, during, and after NE stimulation in another perforated-patch clamped cell in normal Kreb's solution (C, 151 mM Cl\textsuperscript{-}) and in a solution in which 145 mM of the Cl was replaced by aspartate ions (D, 6 mM Cl\textsuperscript{-}). Pipette solution No. 10.

Figure 3. Ionic basis of the fast depolarizing current. (A) The fast depolarizing current is insensitive to block by apamin. Currents from a perforated-patch clamped cell during ramps of potential before and during brief exposures to 100 μM NE in the absence and presence of 200 nM apamin. Neither the magnitude nor the reversal potential of the NE-elicited conductance increase were significantly affected by apamin. Pipette solution No. 8. (B) Replacement of extracellular Na\textsuperscript{+} with NMG\textsuperscript{+} does not affect the reversal potential of the fast depolarizing current. Shown are currents recorded from the same cell as in Fig. 1 after all the Na in the perfusing Kreb's solution was replaced with NMG. Stimulation with 1 μM NE activated a conductance increase with a reversal potential of -19 mV, similar to the -16 mV reversal potential measured in normal Kreb's solution. (C and D) Replacement of extracellular Cl ions with aspartate shifts the reversal potential of the fast depolarizing conductance to more positive potentials. Currents recorded before, during, and after NE stimulation in another perforated-patch clamped cell in normal Kreb's solution (C, 151 mM Cl\textsuperscript{-}) and in a solution in which 145 mM of the Cl was replaced by aspartate ions (D, 6 mM Cl\textsuperscript{-}). Pipette solution No. 10.

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The observation that $I_{K_{Ca}}$ is present in many of the cells showing a depolarizing response (see Fig. 2), and the indication that both currents are regulated by intracellular calcium levels (see below), raised the possibility that the depolarizing conductance increases were contaminated by activation of $I_{K_{Ca}}$. We tested this possibility by examining the effects of apamin, an effective blocker of $I_{K_{Ca}}$ (Lucero and Pappone, 1990) on the depolarizing conductance. In four cells showing what appeared to be a purely depolarizing hormone response, that is conductance increases with a constant reversal potential near $-20 \text{ mV}$, NE responses were essentially the same in the presence and absence of 200 nM apamin, as shown for one example in Fig. 3 A. Apamin shifted the reversal potential of the NE-activated current by only $+3 \pm 4 \text{ mV}$ ($n = 4$), and in three out of the four cells the magnitude of the depolarizing conductance increase was the same in the presence and absence of apamin. Thus, $I_{K_{Ca}}$ did not corrupt our reversal potential determinations for the fast depolarizing conductance increase.

Our reversal potential measurements indicate that Cl$^{-}$ ions are not equilibrated across the resting membrane of intact brown fat cells, but are present in much higher than passively distributed intracellular concentrations. The $-20 \text{ mV}$ reversal potential of the depolarizing current measured in our standard perforated patch conditions would predict an intracellular Cl$^{-}$ ion concentration of $\sim 70 \text{ mM}$ for a Cl-selective conductance, significantly higher than the 35 mM Cl$^{-}$ in the pipette and the 15 mM Cl$^{-}$ that would be at equilibrium with the $-60 \text{ mV}$ holding potential. The reversal potential we measured for the NE-activated conductance was influenced by the concentration of chloride in the pipette solution, because reducing the pipette Cl$^{-}$ to 10 mM (Solution No. 1, Table 1) shifted the average reversal potential of the NE-elicited conductance to $-30 \text{ mV}$ ($\pm 3 \text{ mV}$, $n = 8$). This change in $E_R$ would correspond to a 23 mM decrease in the intracellular chloride concentration for a purely Cl-selective conductance. There was no corresponding change in the unstimulated zero current potential of the cells under the two recording conditions ($-40 \pm 4 \text{ mV}$ with 35 mM Cl$^{-}$, $-42 \pm 4 \text{ mV}$ with 10 mM Cl$^{-}$), indicating that the differences in reversal potential were not due to a potential offset across the permeabilized patch. Thus, we conclude that brown fat cells are capable of maintaining relatively high intracellular Cl$^{-}$ concentrations through some form of active transport.

**The Depolarizing Conductance Can Be Activated by Intracellular Ca$^{2+}$**

Activation parallels changes in intracellular calcium. Both the fast depolarizing conductance and increases in intracellular calcium are activated by selective $\alpha$-adrenergic stimulation (Lucero and Pappone, 1990; Lee et al., 1993). Fast depolarizing currents activated by the $\alpha$-adrenergic agonist phenylephrine (PE) were similar in reversal potential and dependence on time in culture to the currents activated by NE. With both NE and PE the level of chloride current activation varied in perforated-patch experiments even in the presence of a constant concentration of agonist. Fig. 4 A shows the current response of a day 1 cell before, during, and after perfusion with Kreb’s solution containing 10 $\mu$M PE. PE stimulation produced a purely depolarizing conductance increase in this cell that reached a maximum
within <1 min and rapidly decreased thereafter. Although increased conductance was sustained in the presence of PE in this cell, the steady state response was only ~25% of the conductance increase seen during the peak of the response. Similar declines in the response were seen with sustained NE stimulation as well. In eight perforated patch clamped cells exposed to NE or PE for long periods, the depolarizing conductance decayed to a steady level that averaged 16 ± 6% of the peak response. The conductance declined rapidly following peak activation, averaging 43 ± 20 s to reach half its maximum value. This time course of the depolarizing conductance response, a rapidly activating and inactivating peak followed by a sustained plateau, closely resembles the time course of intracellular calcium changes measured by fura-2 imaging experiments (Lee et al., 1993). Thus, both the agonist sensitivity and the time course of the response to maintained agonist are consistent with the hypothesis that the fast depolarizing chloride conductance is regulated by intracellular calcium levels. Based on these findings and the results presented be-

![Figure 4](image-url)

**Figure 4.** Response to a sustained exposure to phenylephrine. (A) Ramp currents recorded before (1), during (2 and 3) and after (4) a 6.4-min exposure to 10 μM phenylephrine. Cell was perfused with Kreb's solution throughout. (B) Membrane conductance as a function of recording time in the same cell. The numbers correspond to the current records in A. The perfusion solution contained 10 μM phenylephrine during the time indicated by the bar. Conductance was calculated from the slope of a linear fit of the ramp current records between -80 and -30 mV. Pipette solution No. 7.

...low, we will henceforth refer to the fast depolarizing current as a calcium-activated chloride current, $I_{CaCl}$. 

**Calcium ionophore activates $I_{CaCl}$.** Exposure of perforated patch clamped cells to 0.25 to 1 μM of the calcium ionophore A23187 activates increases in membrane chloride conductance. Activation and deactivation of $I_{CaCl}$ with ionophore was somewhat slower than with agonist, but in other respects the currents were quite similar. If a cell gave a depolarizing, hyperpolarizing, or mixed response to NE stimulation, it usually gave the same type of response to ionophore (16 of 18 cells). In accordance with this finding, depolarizing responses to ionophore were most frequent in cells that had been in culture 1–2 d as opposed to cells cultured longer. In addition, the reversal potentials of the ionophore-activated currents averaged −21 ± 1 mV ($n = 13, 35$ mM pipette Cl), close to that of the agonist-activated current. Thus, it seems likely that ionophore-mediated increases in intracellular calcium activate $I_{CaCl}$, the same current activated by NE.
FIGURE 5. Activation of $I_G$ and $I_{K Ca}$ by calcium ionophore. Membrane currents and conductance in a perforated patch clamped cell before and during exposure to 0.3 μM A23187. (A) Membrane conductance measured from a fit of ramp currents between −20 and −80 mV as a function of time after gaining access to the cell interior. The numbered marks denote the times at which the corresponding currents in B and C were recorded. Ionophore was added to the bath solution at 10.7 min, immediately before the first mark, and was present throughout the rest of the experiment. 40 mM TEA was present during the time indicated by the bar. (B) Membrane currents recorded before (1) and during exposure to ionophore in the same cell showing variations in both magnitude and reversal potential of the ionophore-activated currents. Number labels correspond to the time the current trace was recorded, as shown in A. (C) Net currents remaining after subtracting a linear leak fit to the current between −80 and −20 mV in 1 before ionophore exposure. Shown are currents measured immediately before (7) and after (8) adding 40 mM TEA to the bath solution. The TEA-blocked current has a reversal potential of −71 mV, near that expected for $I_{K Ca}$. The remaining unblocked, ionophore-activated current has a reversal potential of −16 mV, consistent with $I_G$ activation. Extracellular solution Hank’s, pipette solution No. 11.
The time courses of $I_{Ca,\text{aq}}$ activation by agonist and ionophore are consistent with a requirement for relatively high levels of intracellular calcium for activation. Fig. 5, A and B, shows current and conductance responses before and during exposure of a cell to calcium ionophore. In this cell ionophore initially activated a small hyperpolarizing current, reversing at $-62\,\text{mV}$ (trace 2), then briefly activated a very large depolarizing current with a reversal potential of $-15\,\text{mV}$ (trace 4), followed by a sustained, fairly constant activation of currents that reversed near $-44\,\text{mV}$ (traces 5 and 6). The steady state conductance increase in the presence of ionophore was likely due to simultaneous activation of both $I_{K,\text{cl}}$ and $I_{Ca,\text{aq}}$. Fig. 5 C shows ionophore-activated currents with a linear leak current fitted to the pre-ionophore currents subtracted from the records. TEA, which blocks $I_{K,\text{cl}}$, blocked only part of the ionophore-activated current, with the blocked current showing a reversal potential of $-71\,\text{mV}$, near $E_K$. The remaining ionophore-activated current (trace 8) reversed at $-16\,\text{mV}$, near $E_{Ca}$ and close to the reversal potential of the large early depolarizing conductance. Thus, it seems that both $I_{K,\text{cl}}$ and $I_{Ca,\text{aq}}$ were activated by ionophore in this experiment, and that the level of activation of each varied with time, and presumably with intracellular calcium levels, throughout the course of the ionophore exposure. If both $I_{K,\text{cl}}$ and $I_{Ca,\text{aq}}$ levels were directly determined by intracellular calcium, this experiment would indicate that $I_{Ca,\text{aq}}$ requires higher calcium for maximum activation than does $I_{K,\text{cl}}$, because higher conductance levels were always associated with more positive reversal potentials when both currents were present. Further, these results demonstrate that $I_{Ca,\text{aq}}$ can remain activated for long periods (e.g., 40 min in Fig. 5) with sustained elevated intracellular calcium in intact cells.

$I_{Ca,\text{aq}}$ can be activated by pipette calcium in whole-cell configuration. Fig. 6 shows membrane conductance and currents recorded upon going whole cell with a pipette solution containing 500 nM free $\text{Ca}^{2+}$, 30 mM $\text{Cl}^-$, with $\text{K}^+$, aspartate, HEPES, and EGTA as its only other constituents (solution No. 6 in Table I). The current that is activated by this procedure has a reversal potential near the calculated $E_C$ of $-41\,\text{mV}$, as determined from the “pivot point” of the superimposed ramp currents during activation (see e.g., Fig. 1). Going whole cell with a pipette solution buffered to low free $\text{Ca}^{2+}$ (50 nM, solution No. 4, Table I) did not lead to any current activation.

The activated membrane conductance is largely permeable to chloride ions, as shown by the decreased conductance and positive shift in reversal potential of the membrane current when aspartate replaces extracellular chloride in Fig. 6, A and B. In contrast, replacement of extracellular sodium by NMG had negligible effects on the membrane currents (Fig. 6 C). Membrane chloride conductance remained high in this 25 pF cell for the entire almost 60-min duration of the experiment, even though we expect that most diffusible substances would have dialyzed out through the 2 MΩ pipette by this time (Pusch and Neher, 1988). This result supports the hypothesis that $I_{Ca,\text{aq}}$ is activated by intracellular calcium levels.

Additional Factors Influence $I_{Ca,\text{aq}}$ Activation

The results presented so far are all consistent with the idea that $I_{Ca,\text{aq}}$ is activated by high concentrations of intracellular calcium ions. We attempted to test this hypoth-
esis more directly by changing free calcium levels at the cytoplasmic membrane surface by intracellular perfusion in the whole-cell configuration. However, the more or less rapid rundown of $I_{Ca,Cl}$ in whole-cell experiments (see below) confounded interpretation of the results. We also attempted measurement of $I_{Cl,Cl}$ in inside-out macropatches where we could more rapidly and confidently control calcium levels. We were unable to activate any detectable $I_{Cl,Cl}$ in 11 macropatch recordings with patches from 1-d cultured cells pulled off into 500 nM Ca$^{2+}$ solution, even though $I_{Cl,Cl}$ was present in 5/5 whole-cell recordings from the same preparations with the same cytoplasmic solution. Increasing the calcium concentration to 1 or 10 μM also failed to activate any $I_{Cl,Cl}$ in the patches. This may reflect the rapid loss of some essential cytoplasmic factors in the cell-free patches. These findings indicate that calcium levels are not the sole determinant of $I_{Cl,Cl}$ activity. Although it was possible to get sustained activation of $I_{Cl,Cl}$ in whole-cell recordings, such as seen in Fig. 6, such long-standing activation of $I_{Cl,Cl}$ was rare. In most whole-cell experiments with 500 nM free pipette calcium $I_{Cl,Cl}$ disappeared within 5–20 min. The time course for $I_{Cl,Cl}$ decay was similar using a wide variety of pipette solutions. Intracellular solutions containing added ATP, GTP, Mg, glutathione, or with phosphate buffer replacing the usual MOPS or HEPES, chloride replacing aspartate, sodium replacing potassium, or iodide replacing chloride, all failed to slow the rapid

**Figure 6.** Activation of $I_{Cl}$ by intracellular calcium. Conductance and current records from whole-cell recordings with a pipette solution containing 500 nM free calcium (solution No. 6). (A) Membrane conductance determined from a linear fit to ramp current records between $-80$ and $-40$ mV. Whole-cell recording configuration was achieved at 3.3 min. At the times indicated 150 mM of the Cl in the perfusion solution was substituted with aspartate ions (ASP) or 150 mM of the sodium was replaced with N-methyl-D-glucamine (NMG). The dip in conductance before the NMG application was caused by a glitch in the perfusion system. (B and C) Ramp currents in the same cell immediately before and during the second exposure to aspartate (B) or NMG (C).
rundown of $I_{Ca}$. Rundown rates were similar when cells were bathed in Ringer solution or oxygenated Kreb's solution with a CO$_2$/bicarbonate buffer.

Since our experiments with agonists and ionophore in intact cells seemed to indicate that $I_{Ca}$ required relatively high intracellular calcium for activation, we considered the hypothesis that the time course of $I_{Ca}$ in whole-cell experiments reflected changes in intracellular calcium levels as the cytoplasm equilibrated with the pipette solution. The initial activation of $I_{Ca}$ could have resulted from calcium release from intracellular stores upon going whole cell with high free calcium in the pipette. This might initially raise intracellular calcium levels well above the pipette concentration, activating $I_{Ca}$. Then as the cell contents were dialyzed out, the cell would lose the ability to regulate calcium and levels would approach those in the pipette and the proportion of $I_{Ca}$ activated would decline. In contrast to the prediction of this hypothesis, $I_{Ca}$ ran down even more rapidly when we went whole cell with higher pipette calcium concentrations, as shown in Fig. 7, A and B. Both the rate and the extent to which $I_{Ca}$ declined seemed to increase with in-

![Figure 7](image-url)
creasing pipette-free calcium, such that it was often not possible to resolve any $I_{\text{Cl},\text{Ca}}$ activation with 100 $\mu$M pipette calcium. The discrepancy between the results in intact cells, which indicate that high intracellular calcium levels are required to activate $I_{\text{Cl},\text{Ca}}$, and those in whole-cell experiments, in which high calcium seems to inactivate the current, implies that $I_{\text{Cl},\text{Ca}}$ regulation involves additional factors.

**Pharmacology**

We examined the effects of several chloride channel blockers on $I_{\text{Cl},\text{Ca}}$ activated by going whole cell with 500 nM free intracellular Ca$^{2+}$ in the pipette. Fig. 8A shows total membrane currents recorded during voltage ramps without blocker and in the presence of 3 and 100 $\mu$M niflumic acid (NFA). More than half of the current in this cell was blocked by 3 $\mu$M NFA and $I_{\text{Cl},\text{Ca}}$ was completely blocked by 100 $\mu$M NFA, leaving only a small, almost linear voltage-independent leak current. The un-

**Figure 8.** (A) Total membrane current in whole-cell measured during ramps of applied potential in the absence of blocker and in the presence of 3 $\mu$M or 100 $\mu$M niflumic acid (NFA). $I_{\text{Cl},\text{Ca}}$ was activated with 500 nM pipette Ca$^{2+}$ (solution no. 2). K$^+$ was absent from both the pipette and bath solutions to eliminate currents through K channels. (B) NFA-sensitive currents from the same cell isolated by subtracting the record in 100 $\mu$M NFA from the control and 3 $\mu$M currents. Also shown is the 3 $\mu$M current scaled by a factor of 3.3 to match the control current at 0 mV. The rectification of the remaining current is identical to the control. (C) Dose-response curves for block of $I_{\text{Cl},\text{Ca}}$ by niflumic acid (NFA, open squares), flufenamic acid (FFA, filled squares), DIDS (open circles), and SITS (filled circles). Ramp currents at high concentrations of blocker were subtracted from the current records to isolate $I_{\text{Cl},\text{Ca}}$. Each point is the percent of the chloride conductance blocked, measured from the slope of the subtracted ramp currents between 0 and +30 mV at each concentration, relative to the net conductance measured before blocker exposure. Symbols overlap at 1 mM. Lines are binding isotherms with IC$_{50}$'s of 3 $\mu$M for NFA, 7 $\mu$M for FFA, 50 $\mu$M for DIDS, and 170 $\mu$M for SITS. $I_{\text{Cl},\text{Ca}}$ was activated in whole-cell recordings with 0.5 $\mu$M calcium, solution No. 2.
changing reversal potential of the blocked currents with different levels of block indicates that \( I_{\text{Cl,ca}} \) is the only current present affected by NFA. Fig. 8B shows the NFA-sensitive currents in the absence of blocker and in the presence of 3 \( \mu \text{M} \) NFA. Also plotted is the 3 \( \mu \text{M} \) NFA-sensitive current scaled 3.3-fold. The correspondence of the scaled and unblocked control current records indicates that NFA reduced the current to the same extent at all membrane potentials, further indicating that the block is of a single current species. The fenamates niflumic acid and flufenamic acid were the most potent of the compounds tested, with half-blocking concentrations of 3 (±0.5, \( n = 13 \)) and 7 \( \mu \text{M} \) (±1, \( n = 8 \)) respectively, as shown in Fig. 8C. SITS, DIDS and anthracene-9-carboxylic acid (9ACA) were 1-2 orders of magnitude less potent, with measured \( IC_{50} \)'s of 170 (±20, \( n = 12 \)), 50 (±15, \( n = 3 \)), and 80 \( \mu \text{M} \) (±40, \( n = 4 \)). When tested in the same cell, saturating concentrations of each blocker reduced the ramp currents to the same level, indicating that each was blocking the same current. Block of \( I_{\text{Cl,ca}} \) by flufenamic acid, SITS, and DIDS, like NFA block, was independent of membrane potential, but block of \( I_{\text{Cl,ca}} \) by 9ACA was greater at more positive membrane potentials, as has been reported for \( I_{\text{Cl,ca}} \) in smooth muscle cells (Hogg, Wang, and Large, 1993). All blockers were relatively quick to act and recovery of \( I_{\text{Cl,ca}} \) on washout was rapid but often incomplete, especially with high concentrations of the fenamates. Given the substantial rates of rundown \( I_{\text{Cl,ca}} \) shows in whole-cell recordings, it seems likely that the blocker effects were reversible.

**Anion Selectivity of \( I_{\text{Cl,ca}} \)**

We activated \( I_{\text{Cl,ca}} \) with intracellular Ca in the whole-cell configuration to test the selectivity of \( I_{\text{Cl,ca}} \) for several other anions. Selectivity measurements were made with zero K\(^+\) in both intracellular and extracellular solutions and with Na\(^+\) as the major intracellular cation to minimize currents through K-selective channels. Fig. 9A shows the total membrane current measured during potential ramps with a pipette solution containing 15 mM Cl and 125 mM aspartate as the major anions. Under these conditions there is a large, somewhat outwardly rectifying membrane current with a negative reversal potential. Replacing extracellular Cl with aspartate ions both reduces the membrane current and makes it linear with voltage, indicating that the current was carried largely by Cl ions.

In contrast to the effects of aspartate substitution in perforated-patch experiments, in which there was substantial NE-activated inward current in the presence of bath aspartate (e.g., Fig. 3D), extracellular aspartate blocked both inward and outward currents in these whole-cell measurements. The difference in current characteristics in aspartate solutions in the two kinds of experiments is not due to the different intracellular cation composition, i.e., largely Na\(^+\) in these whole-cell measurements as opposed to largely K\(^+\) in the perforated-patch experiments (data not shown). The aspartate bath solution blocked \( I_{\text{Cl,ca}} \) essentially completely in whole-cell experiments, as evidenced by comparison of the membrane conductance in aspartate, \( g_{\text{Asp}} \), and the conductance with \( I_{\text{Cl,ca}} \) blocked by 100 \( \mu \text{M} \) niflumic or flufenamic acid, \( g_{\text{FA}} \) in the same cells. The average conductance ratio, \( g_{\text{Asp}}/g_{\text{FA}} \), was 0.8 ± 0.1 (\( n = 5 \)), not significantly different from unity. The current-voltage relations of the cells were linear at potentials negative to ~50 mV and reversed near 0
mV with $I_{\text{Ca,Cl}}$ blocked by either aspartate or the fenamates. The block of both inward and outward $I_{\text{Ca,Cl}}$ by extracellular aspartate may result from a requirement for extracellular permeant ions for channel gating, as has been reported for *Torpedo* CIC-0 chloride channels expressed in oocytes (Pusch, Ludewig, Rehfeldt, and Jentsch, 1995). If brown fat chloride channels have a similar requirement for permeant anions, these results may indicate that the bicarbonate present in our intact cell experiments can activate chloride channel gating.

We subtracted a linear leak component derived from a least squares fit to the current measured in the presence of aspartate or fenamate to separate currents through $g_{\text{Ca,Cl}}$ from other membrane conductances. Fig. 9, B–D shows chloride currents isolated in this way with $\text{Cl}^-$, $\text{Br}^-$, $\text{I}^-$, or $\text{NO}_3^-$ as the major anion in the bath solution. $g_{\text{Ca,Cl}}$ conducts all of these test ions better than $\text{Cl}^-$, as shown by the negative shift in the reversal potential of the currents and the increase in magnitude of the outward currents in the test solutions. Data like these were used to calculate the permeability of $g_{\text{Ca,Cl}}$ to test ions relative to $\text{Cl}^-$ from the shift in reversal potential of the current using a modified GHK equation:

$$
\frac{P_x}{P_{\text{Cl}}} = \frac{([\text{Cl}^-]_o + P_C/P_{\text{Cl}}[\text{C}^+]) \exp((E_{\text{Cl}} - E_x)F/RT)}{-[\text{Cl}^-]_o - P_C/P_{\text{Cl}}[\text{C}^+]/[X^-]_o}.
$$

**Figure 9.** Selectivity of the Cl conductance. (A) Total membrane currents recorded with aspartate as the major extracellular anion, and after returning to Cl Ringer. (B) $I_{\text{Cl}}$ currents isolated by subtraction of the current measured in aspartate solution from the total membrane currents recorded in Br Ringer and subsequently in Cl Ringer. Same cell as in A. (C and D) $I_{\text{Cl}}$ currents in another cell isolated by subtraction of the current measured in 100 $\mu$M FFA from the total membrane currents in Cl, I or NO$_3$ Ringer. Pipette solutions all contained 500 nM free Ca. Pipette chloride concentration was 15 mM in A and B (solution No. 2), 30 mM in C and D (solution No. 5).
[Cl\(^-\)]\text{Cl} and [Cl\(^-\)]\text{X} are the bath concentrations of Cl\(^-\) in the Cl and test ion solutions respectively, \(E_\text{Cl}\) and \(E_\text{X}\) are the reversal potentials measured in the chloride and test solutions, [X\(^-\)]\text{o} is the concentration of the test anion in the bath solution, [C\(^+\)]\text{i} is the pipette cation concentration, and \(P_c/P_\text{Cl}\) is the relative cation permeability of the conductance. \(P_c/P_\text{Cl}\) was estimated to be \(-0.1\) from the measured \(-9.9\) mV (±0.8 mV, \(n = 3\)) shift in reversal potential upon replacing 70 mM of the NaCl in the bathing solution with 140 mM sucrose. Table II shows the average shifts in reversal potential and the relative permeabilities calculated from them, as well as the relative conductances for each anion. For both the reversal potential and conductance measurements the permeability sequence was \(P_{\text{NO}_3} > P_\text{I} > P_\text{Br} > P_\text{Cl}\). Aspartate was not measurably permeant through the conductance. \(P_{\text{NO}_3}/P_\text{Cl}\) was calculated to be near zero from the reversal potential measured for \(I_{\text{Cl},c}\) in 0K Cl Ringer with intracellular solutions of either 15 mM Cl/125 mM aspartate (\(E_\text{R} = -43.4 \pm 2.3\) mV, \(n = 17\)) or 30 mM Cl/100.8 mM aspartate (\(E_\text{R} = -39.0 \pm 2.7\) mV, \(n = 9\)), consistent with the lack of measurable aspartate influx with bath substitution.

| Ion (n) | \(\Delta E_\text{R}\) | \(P_c/P_\text{Cl}\) | \(g_c/g_\text{Cl}\) |
|--------|----------------|-----------------|----------------|
| Nitrate (8) | \(-23.2 \pm 2.7\) | 2.8 ± 0.3 | 1.5 ± 0.2 |
| Iodide (5) | \(-20.9 \pm 1.8\) | 2.4 ± 0.2 | 1.2 ± 0.1 |
| Bromide (7) | \(-8.0 \pm 3.1\) | 1.5 ± 0.2 | 1.1 ± 0.3 |

\(\Delta E_\text{R}\) is the change in reversal potential of the \(I_{\text{Cl},c}\) in the test ion solution relative to that measured in 0K Cl Ringer solution. \(I_{\text{Cl},c}\) was isolated from other membrane currents by subtraction of the currents measured with \(I_{\text{Cl},c}\) blocked by extracellular fenamates or aspartate. \(P_c/P_\text{Cl}\) was calculated from \(\Delta E_\text{R}\) using Eq. 1. \(g_c/g_\text{Cl}\) is the ratio of the conductances measured from a linear fit of the membrane currents between 0 and +30 mV in the test ion and Cl Ringer solutions. All values are shown ±SEM.

**\(I_{\text{Cl},c}\) Rectification Results from Asymmetric Chloride Concentrations and Voltage-dependent Gating**

Under our usual whole-cell experimental conditions for recording \(I_{\text{Cl},c}\)—that is, ramp currents measured with zero potassium inside and out and an intracellular solution with low chloride and high aspartate—\(I_{\text{Cl},c}\) usually showed a high degree of outward rectification. The amount of rectification seen varied greatly from cell to cell, and often decreased with time in a single cell. Rectification ratios determined from linear fits of inward and outward \(I_{\text{Cl},c}\) recorded from positive-going potential ramps ranged from 1.4 to 3.8 with 30 mM intracellular chloride. The mean rectification ratios determined from cells with 15, 30, or 140 mM intracellular Cl concentrations were not significantly different from each other \((t\text{ test}, P > 0.14)\), indicating that the outward rectification was not due to block of chloride efflux by intracellular aspartate ions. Outward current rectification was much less apparent in agonist-activated \(I_{\text{Cl},c}\) in intact, perforated patch clamped cells. Indeed,
the fast depolarizing current in intact cells often appeared to carry inward current more effectively (e.g., the cell in Fig. 1). However, this apparent inward rectification seemed to be due to changes in potassium currents during the hormone response, because agonist-activated $I_{\text{Cl,Ca}}$ showed clear outward rectification, similar to that seen in whole-cell experiments, in the presence of TEA (three experiments).

Some of the apparent rectification in $I_{\text{Cl,Ca}}$ seen in our ramp current records is due to a time- and voltage-dependent component of the currents. Fig. 10, A and B, show the niflumic acid-sensitive $I_{\text{Cl,Ca}}$ currents recorded during 440 ms voltage steps to potentials from $-100$ to $+100$ mV in whole cell configuration. The instantaneous jump in current with the voltage steps shows that there is substantial $I_{\text{Cl,Ca}}$ active at the holding potential of $-60$ mV. Some of the initial current deactivated when the potential was stepped negative, and additional $I_{\text{Cl,Ca}}$ activated at more positive membrane potentials. The time courses of activation and deactivation of $I_{\text{Cl,Ca}}$ with membrane potential were slow, averaging ~50 ms, and were independent of membrane potential or intracellular chloride concentration, as shown in Table III. The amount of voltage-sensitive $I_{\text{Cl,Ca}}$ present was highly variable, ranging from 5 to 80% of the total NFA-sensitive current activated by 500 nM Ca$^{2+}$ in these cells. In addition, there was often a decrease in the proportion of voltage-gated $I_{\text{Cl,Ca}}$ with time in whole-cell recordings. Although the average amount of voltage-gated $I_{\text{Cl,Ca}}$ we measured at positive potentials was higher with lower intracellular chloride concentrations as shown in Table III, the difference was not significant given the high degree of variability. Comparison of the initial current with the current at the end
of the voltage pulses in Fig. 10 shows that the time-dependent current is substantial only at extreme negative and positive potentials, well outside the physiological range of membrane potentials for brown fat cells.

The asymmetric chloride concentrations we usually used in these experiments also contributed to the outward rectification of ramp currents. Fig. 10 C shows instantaneous current-voltage relations measured for the same cells as in Figs. 10, A and B. In normal high Cl Ringer, outward rectification of the current-voltage relation is present when the intracellular chloride concentration is low, but the relation is close to linear when intracellular chloride is also high. Equivalent instantaneous current-voltage data were obtained for six other cells, four with low and two with high internal Cl⁻ (not shown).

The time-dependent and the time-independent components of Ia,ca had indistinguishable sensitivity to the blockers we tested. Fig. 8 B shows that Ia,ca partially blocked by NFA had the same degree of rectification as the unblocked currents, as would be expected if blocked and unblocked currents came from the same chan-

| TABLE III |
| Voltage-gated Current |
|-----------------------|
| Time Constant         | Percent of voltage-gated at +60 mV |
| [Cl]ᵢ [mM] | −100 mV | +60 mV | +100 mV |%
| 15 mM                | 56 ± 19 (3) | 60 ± 9 (8) | 57 ± 13 (4) | 46 ± 8 (8) |
| 140 mM               | 67 ± 15 (4) | 67 ± 14 (5) | 60 ± 15 (5) | 30 ± 6 (5) |

Time constants for activation or deactivation of IcLca were determined from a single exponential function fit by eye to the niflumic acid-sensitive currents recorded during 440-ms voltage steps to the potentials shown. Percent of voltage-gated current was determined from the values of the niflumic acid-sensitive currents at the beginning and end of a 440 ms pulse to +60 mV from the holding potential of −60 mV. Values are given ±SEM with the number of cells in parentheses.

Similar results were seen in currents partially blocked by FFA. In addition, the degree of rectification seen in anion selectivity experiments did not vary with the test ion within a single cell. Although different cells had very different degrees of rectification, they all showed the same selectivity sequence. Thus, our results indicate that brown fat cells have a single calcium-activated chloride conductance that shows a variable amount of shallow, slow voltage dependence.

Volume-activated Chloride Currents Differ from the Calcium-activated IcLca

As reported for many other cell types, osmotic gradients can activate increased membrane chloride conductance in brown fat cells. Fig. 11 A shows the effects on ramp currents of replacing the normal bath solution with hypotonic solution in a perforated patch clamped cell. Reducing bath solution osmolarity by diluting normal Ringer by 40–50% with water activated a membrane current with a reversal po-
potential near -20 mV. Activation of the current resulted from the osmotic gradient across the cell membrane, because it rapidly deactivated when the cell was exposed to Ringer similarly diluted with isotonic sucrose rather than water (not shown). In addition, the level of activation of the conductance paralleled the hypotonicity of the bath solution. The conductance activated slowly following the switch to hypotonic solution, as shown in Fig. 11B. No increase in membrane current was seen until almost 5 min in hypotonic solution, at which time the cell was visibly swollen. In other cells currents took 1–5 min to begin activation. Steady state levels of activation of the current took almost 10 min to attain following the solution switch in this cell, and an average of 9 min in 8 other cells. Upon return to normosmotic solution the current shut off somewhat more rapidly, within 1–4 min. The time courses of activation and deactivation of the current were similar with repeated exposures to hypotonic solution, as shown in Fig. 11.

Several lines of evidence show that the volume-activated current in brown fat is carried by chloride ions. First, the reversal potential of the current was sensitive to the pipette chloride concentration in perforated patch recordings, averaging -41
$\pm 2 \text{ mV} (n = 8)$ with 15 mM Cl and $-23 \pm 3 \text{ mV} (n = 3)$ with 30 mM chloride in the pipette. Second, substituting aspartate ions for most of the chloride in the extracellular solution decreased the volume-activated conductance and shifted its reversal potential positive. Finally, the volume-activated current was sensitive to block by NFA (10–100 μM) in hypotonic solutions.

Although the volume-activated current and $I_{\text{Cl,ca}}$ are superficially similar, several differences in the currents argue against their mediation by the same conductance mechanism. First, the volume-activated current is insensitive to calcium. Activation of the current in nominally zero calcium hypotonic solution was the same as in the normal hypotonic media (three cells), suggesting that calcium influx is not necessary for activation of the conductance. In addition, the current could be activated by an osmotic gradient in 10 out of 12 whole-cell recordings with intracellular cal-

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**Figure 12.** $I_{\text{Cl,ca}}$ and volume-activated currents are different. (A) Calcium-activated chloride currents are sensitive to block by NFA but insensitive to removal of the osmotic gradient. Membrane conductance as a function of time recorded from ramp currents made in whole-cell configuration with a 400 mosm pipette solution containing 500 nM free Ca$^{2+}$ and no ATP (solution No. 2 with 50 mM additional Na aspartate). The cell was exposed to 10 μM NFA or 160% normal tonicity (500 mosM, normal Ringer + sucrose) solution during the times shown by the bars. (B) Volume-activated currents are sensitive to osmotic gradient but not fenamates. Shown is membrane conductance as a function of time from a whole-cell recording with a hyperosmotic pipette solution (solution No. 3, Table I) supplemented with 3 mM MgCl$_2$ and 3 mM NaATP (400 mosM, total 21 mM Cl$^{-}$). During the times shown by the bars the cell was exposed to 10, 50, or 100 μM NFA or 10 μM FFA, or to a Ringer solution having 160% normal tonicity.
cium levels buffered to <10 nM with EGTA. Second, the volume-activated currents required intracellular ATP for activity, as seen in other systems (Lewis, Ross, and Cahalan, 1993). Sustained volume-activated currents could be elicited in whole cell configuration if ATP was included in the pipette solution (10/12 cells), but the currents were absent or small and extremely short-lived in whole-cell experiments without pipette ATP (five cells). As shown above, $I_{\text{Cl},\text{Ca}}$ does not require ATP for sustained activation. Third, $I_{\text{Cl},\text{Ca}}$ is not sensitive to the osmotic gradient. Fig. 12 A shows conductances measured in a cell with a hypertonic pipette solution containing 500 nM Ca$^{2+}$ and no ATP (solution 2 in Table I with 50 mM Na-aspartate added). Going whole-cell with this solution activated a conductance increase with a reversal potential of $-42$ mV in this cell and averaging $-38 \pm 2$ mV in six cells with 15 mM pipette Cl, consistent with $I_{\text{Cl},\text{Ca}}$ activation. Switching from the normal bath solution to a hypertonic solution did not affect the conductance, but exposure to 10 $\mu$M NFA substantially reduces it, suggesting that significant $I_{\text{Cl},\text{Ca}}$ was activated. Fourth, the volume-activated current is less sensitive than $I_{\text{Cl},\text{Ca}}$ to block by fenamates. Fig. 12 B shows conductances measured in whole cell with a hypertonic pipette solution containing ATP and zero free Ca$^{2+}$. With normosmotic bath solution there was a slow activation of a conductance with a reversal potential of $-30$ mV in this experiment. In 9/12 similar experiments, the reversal potential of the activated currents averaged $-30 \pm 3$ mV with ~24 mM pipette Cl. The current once activated ran down throughout the course of this 1-h experiment, as was seen for all osmotically activated currents in whole-cell experiments. Exposure to NFA or FFA (10 or 100 $\mu$M) in normosmotic bath solution had little effect on the current, but it could be completely shut down by switching to a hypertonic bath solution that removed the osmotic gradient. Presumably the higher sensitivity of the volume-activated current to fenamates seen in hypotonic solutions is due to the decreased ionic strength of the solutions. These experiments show that under almost identical experimental conditions $I_{\text{Cl},\text{Ca}}$ and the volume-activated current differ dramatically in their sensitivity to osmotic gradients and blockers. These differences make it extremely unlikely that $I_{\text{Cl},\text{Ca}}$ and the volume-activated current are mediated by the same conductance mechanism.

**DISCUSSION**

Among the first responses to adrenergic stimulation in brown fat cells is a rapid transient depolarization of the cell membrane (Girardier et al., 1968; Girardier and Schneider-Picard, 1983; Horwitz and Hamilton, 1984). Our patch clamp results presented here demonstrate that the fast depolarization results from a selective increase in membrane chloride permeability. This finding is in accord with our previous studies showing that the $\alpha$-adrenergic depolarization is present in intact brown fat superfused with Na$^{+}$-free or nominally Ca$^{2+}$-free solutions (Horwitz et al., 1989), and with other work showing that Cl$^{-}$ efflux is increased by $\alpha$-adrenergic stimulation in isolated brown fat cells (Dasso, Connolly, and Nedergaard, 1990). The fact that increased chloride permeability depolarizes brown fat indicates that intracellular chloride is maintained at significantly higher than equilibrium concentrations in brown fat cells, as had been suggested previously (Dasso et al., 1990).
Regulation of $I_{CaCL}$

Adrenergic stimulation activates increases in intracellular calcium levels with a time course and agonist sensitivity similar to what we observe for the chloride conductance (Wilcke and Nedergaard, 1989; Lee et al., 1993). Our results clearly indicate that the $\alpha$-adrenergic chloride conductance can be turned on by increases in intracellular free calcium, either through exposure of intact cells to calcium ionophore or dialysis with calcium-containing solutions in whole-cell recordings. In addition, washout of calcium ionophore results in deactivation of the chloride conductance. Hence, we have referred to the current as a calcium-activated chloride current, $I_{CaCL}$.

We find that factors other than the intracellular calcium concentration must modulate $I_{CaCL}$ activation as well. $I_{CaCL}$ ran down over the course of 5–20 min in whole-cell experiments with our usual intracellular solutions containing a relatively high (30 mM) concentration of EGTA. It is possible that intracellular calcium levels were not well-controlled by the EGTA buffer (Evans and Marty, 1986), but $I_{CaCL}$ rundown was even faster with solutions buffered to higher free calcium levels with the more effective calcium buffer HEDTA. This result makes it unlikely that rundown was due to gradually decreasing free calcium levels. The rapid inactivation of $I_{CaCL}$ in high intracellular calcium may explain why Dasso et al. (1990) failed to measure an increased chloride flux from brown fat cells exposed to very high concentrations of calcium ionophore. In addition, we were unable to activate $I_{CaCL}$ with cytoplasmic calcium increases in cell-free inside-out patches or in internally perfused whole cells, as would be predicted for a direct, concentration-dependent activation and inactivation of $I_{CaCL}$ by intracellular calcium. Thus, we conclude that other factors must influence $I_{CaCL}$ activity.

Modulation by additional factors has been a frequently observed feature of calcium-activated chloride conductances. Candidate modulators elicited by adrenergic stimulation of brown fat include cytoplasmic alkalinization (Giovannini et al., 1988; Lee, Hamilton, Trammell, Horwitz, and Pappone, 1994) and activation of protein kinase C (Schimmel, Dzierzanowski, Elliott, and Honeyman, 1986). We have not determined whether intracellular pH modulates the conductance, but experiments switching between bath solutions with and without CO$_2$ strongly affected the level of $I_{CaCL}$ activation in brown fat (not shown), suggesting that this may be the case. It is impossible to determine without direct measurement whether intracellular pH affects the conductance directly or through changes in free calcium levels or some other consequence of the pH changes. In epithelial cells PKC activates calcium-activated chloride conductances at low calcium concentrations and inhibits the conductance at high calcium concentrations (McCann and Welsh, 1990; Kachintorn, Vonkovit, Vajanaphanich, Dinh, Barrett, and Dharmathaphorn, 1992). Similar regulatory mechanisms could explain the complex calcium dependence of $I_{CaCL}$ activation we see in brown fat cells, where going whole cell with pipette calcium concentrations $\leq$ 1 $\mu$M activated $I_{CaCL}$, while concentrations $\geq$ 10 $\mu$M rapidly inactivate the conductance. Clarification of this point will require further experimentation.

In addition to the immediate regulation by intracellular calcium, maintenance of
responsive \( I_{\text{cl,ca}} \) in the brown fat cell membrane seems to require some factor that is absent under our culture conditions, because depolarizing responses became infrequent in cells cultured more than a few days. Many features of brown fat differentiation are highly regulated by activity of the tissue's sympathetic innervation (reviewed in Himms-Hagen, 1991), and the apparent down-regulation of \( I_{\text{cl,ca}} \) levels in culture may reflect the lack of sympathetic input.

Comparison to Calcium-activated Chloride Currents in Other Cells

We find that activation of \( I_{\text{cl,ca}} \) in brown fat cells is sensitive both to intracellular calcium and to membrane potential. Our whole-cell results indicate that \( I_{\text{cl,ca}} \) is activated by free calcium levels in the submicromolar to micromolar range (0.2–1.0 \( \mu \)M), similar to the concentration sensitivity reported for \( I_{\text{cl,ca}} \) in muscle (Sipido, Callewaert, and Carmeliet, 1993; Ohta, Ito, and Nakazato, 1993), neuronal (Taleb, Feltz, Bossu, and Feltz, 1988), hepatic (Koumi, Sato, and Aramaki, 1994), and secretory cells (Evans and Marty, 1986; Kasai and Augustine, 1990; Osipchuk, Wakui, Yule, Gallacher, and Petersen, 1990). \( I_{\text{cl,ca}} \) in brown fat is somewhat voltage sensitive, slowly activating with depolarization and deactivating with hyperpolarization from the normal resting potential. The amount of voltage-dependence shown by \( I_{\text{cl,ca}} \) varied greatly from cell to cell, and often decreased with time in a single cell in whole-cell recordings. There was no apparent difference in selectivity or sensitivity to blockers of the voltage-dependent and voltage-independent components of \( I_{\text{cl,ca}} \). Thus, it seems that the two components of \( I_{\text{cl,ca}} \) represent differently modulated states of the same channel as has been seen for the potential dependence and rectification of \( I_{\text{cl,ca}} \) in lacrimal (Evans and Marty, 1986), kidney (Marunaka and Eaton, 1990), and olfactory cells (Kleene and Gesteland, 1991).

Permeability properties of \( I_{\text{cl,ca}} \) in brown fat cells differed from many other chloride currents in that instantaneous current-voltage relations for \( I_{\text{cl,ca}} \) in symmetric chloride solutions were linear. Thus, it seems that the outward rectification we saw in intact cells was due to the asymmetric chloride gradient and to the current's slight voltage dependence. \( I_{\text{cl,ca}} \) is apparently carried by small-conductance channels, because there was no readily discernable increase in membrane noise associated with activation of \( I_{\text{cl,ca}} \). Consistent with this conclusion, in additional experiments not presented here we were unable to resolve single-channel currents attributable to \( I_{\text{cl,ca}} \) channels in on-cell patches from cells exposed to ionophore or agonist. The selectivity sequence we measured for \( I_{\text{cl,ca}} \), \( \text{NO}_3^- > I^- > \text{Br}^- > \text{Cl}^- >> \text{aspartate} \), indicates that selectivity occurs at a low field strength site, as reported for \( I_{\text{cl,ca}} \) from several other cell types (Evans and Marty, 1986; Amedee, Large, and Wang, 1990; Ohta et al., 1993).

Functions of \( I_{\text{cl,ca}} \) in Brown Fat

The functions of intracellular chloride and the adrenergic chloride conductance in brown fat are not known. \( I_{\text{cl,ca}} \) does not seem to participate in osmoregulation, because we find that it is not affected by osmotic gradients and is distinct in its requirements for activation and pharmacology from the volume-regulated chloride conductance also present in these cells. Activation of \( \alpha \)-adrenergic pathways is not
required for a thermogenic response in brown fat, but does contribute to the pro-
liferative and differentiative responses of the tissue to sustained adrenergic stimula-
tion (Himms-Hagen, 1991). Adrenergic activation of brown fat cells increases the
membrane transport of ions, glucose, and fatty acids (Nedergaard and Lindberg,
1982), and fat cells secrete several endocrine and paracrine factors (Ailhaud, Grimal-
di, and Negrel, 1992). I_\text{Ca,L} in other electrically inexcitable cells has been associ-
ated with transport and secretory functions, and this may be true of the I_\text{Ca,L} in
brown fat as well.

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