Indirectly Gated Cl\textsuperscript{−}-Dependent Cl\textsuperscript{−} Channels Sense Physiological Changes of Extracellular Chloride in the Leech

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\textsuperscript{1}Fakultät für Biologie, Universität Konstanz, D-78457 Konstanz, Germany; \textsuperscript{2}Stazione Zoologica “Anton Dohrn,” I-80121 Naples, Italy; and \textsuperscript{3}Department of Biology, Emory University, Atlanta, Georgia 30322

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\textbf{Wenning, Angela, Christian F. J. Erxleben, and Ronald L. Calabrese.} Indirectly gated Cl\textsuperscript{−}-dependent Cl\textsuperscript{−} channels sense physiological changes of extracellular chloride in the leech. \textit{J Neurophysiol} 86: 1826–1838, 2001. The maintenance of ion homeostasis requires adequate ion sensors. In leeches, 34 nephridial nerve cells (NNCs) monitor the Cl\textsuperscript{−} concentration of the blood. After a blood meal, the Cl\textsuperscript{−} concentration of leech blood triples and is gradually restored to its normal value within 48 h after feeding. As previously shown in voltage-clamp experiments, the Cl\textsuperscript{−} sensitivity of the NNCs relies on a persistent depolarizing Cl\textsuperscript{−} current that is turned off by an increase of the extracellular Cl\textsuperscript{−} concentration. The activation of this Cl\textsuperscript{−}-dependent Cl\textsuperscript{−} current is independent of voltage and of extra- and intracellular Ca\textsuperscript{2+}. The transduction mechanism is now characterized on the single-channel level. The NNC’s sensitivity to Cl\textsuperscript{−} is mediated by a slowly gating Cl\textsuperscript{−}- dependent Cl\textsuperscript{−} channel with a mean conductance of 50 pS in the cell-attached configuration. Gating of the Cl\textsuperscript{−} channel is independent of voltage, and channel activity is independent of extra- and intracellular Ca\textsuperscript{2+}. Channel activity and the macroscopic current are reversibly blocked by bumetanide. In outside-out patches, changes of the extracellular Cl\textsuperscript{−} concentration do not affect channel activity, indicating that channel gating is not via direct interaction of extracellular Cl\textsuperscript{−} with the channel. As shown by recordings in the cell-attached configuration, the activity of the channels under the patch is instead governed by the Cl\textsuperscript{−} concentration sensed by the rest of the cell. We postulate a membrane-bound Cl\textsuperscript{−}-sensing receptor, which—on the increase of the extracellular Cl\textsuperscript{−} concentration—closes the Cl\textsuperscript{−} channel via a yet unidentified signaling pathway.

\section*{Introduction}

The maintenance of stable extracellular osmotic and ionic concentrations requires gathering and processing sensory information about these quantities (Wenning 1999). The discovery of mechanosensitive channels (Guharay and Sachs 1984) provides a mechanism for transducing changes in extracellular osmolality into electrical activity as shown for the hypothalamic osmoreceptors (Oliet and Bourque 1993). In comparison, the sensing of extracellular ion concentrations is as yet poorly understood. One example is the Ca\textsuperscript{2+} sensor of the parathyroid glands, which maintain the systemic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{e}) (Brown et al. 1993; Hebert et al. 1997). The molecular sensor, the Ca\textsuperscript{2+} sensing receptor (CaSR), belongs to the superfamily of G-protein-coupled receptors. The CaSRs also sense changes of other cations, e.g., La\textsuperscript{3+} and Mg\textsuperscript{2+}, as well as pH,ionic strength, and L-amino acids (Conigrave et al. 2000). Similarly, the aforementioned hypothalamic magnocellular neurosecretory neurons are also multimodal. In addition to sensing osmolality, they might also sense physiological variations of the extracellular Na\textsuperscript{+} concentration ([Na\textsuperscript{+}]\textsubscript{e}) (Voisin et al. 1999). Changes in [Na\textsuperscript{+}]\textsubscript{e} cause proportional changes in the relative permeability to Na\textsuperscript{+} of the mechanosensitive channels thereby modulating the osmoreceptive response. We report here on the transduction mechanism of a Cl\textsuperscript{−} receptor neuron, the nephridial nerve cell, which monitors the Cl\textsuperscript{−} concentration of leech blood.

The nephridial nerve cells (NNCs), 34 peripheral neurons, innervate the 34 nephridia, the leech excretory organs (Fig. 1, top left) (Wenning 1983). Due to a large fraction of divalent organic anions present in leech blood (~40 mM), its Cl\textsuperscript{−} concentration is rather low (36 ± 6 mM; mean ± SD) (Hoeger et al. 1989; Zerbst-Boroffka 1970). Feeding on mammalian blood causes the Cl\textsuperscript{−} concentration of leech blood to increase from 36 to 88 mM (±8) within 15 min, while the Na\textsuperscript{+} concentration increases by only 10 mM from 125 to 135 mM (Hildebrandt and Zerbst-Boroffka 1988; Wenning et al. 1980; Zerbst-Boroffka 1973). A Cl\textsuperscript{−} receptor is advantageous for an animal with a low blood Cl\textsuperscript{−} concentration and high Cl\textsuperscript{−} diet. Long-term extracellular recordings, such as shown in Fig. 1 (top right) demonstrated that the NNC’s electrical activity changes with, but does not adapt to, changes of the external Cl\textsuperscript{−} concentration ([Cl\textsuperscript{−}]\textsubscript{e}) and, by changing the composition of the bathing medium, that the NNCs are insensitive to changes of extracellular osmolality (Wenning 1989). More importantly, long-term recordings from semi-intact leeches before and after artificial meals confirm that the NNC’s activity correlates with the physiological changes of the blood Cl\textsuperscript{−} concentration (Wenning 1989). Intracellular recordings show that in normal, low [Cl\textsuperscript{−}]\textsubscript{e}, the NNC is depolarized and fires spontaneously while it hyperpolarizes and stops firing on the increase of [Cl\textsuperscript{−}]\textsubscript{e} (Wenning and Calabrese 1991). Voltage-clamp experiments (Wenning and Calabrese 1991) reveal that the NNC has a persistent high resting conductance for Cl\textsuperscript{−} that determines the membrane potential in normal, low [Cl\textsuperscript{−}]\textsubscript{e} (Fig. 1, model). In high [Cl\textsuperscript{−}]\textsubscript{e}, this inwardly directed, depolarizing Cl\textsuperscript{−} current is gated off. This “Cl\textsuperscript{−}-dependent Cl\textsuperscript{−} current” is...
halogen-specific, independent of voltage, and of extracellular Ca\(^{2+}\) and Na\(^+\) (Wenning and Calabrese 1991).

At 33 mM [Cl\(^-\)]\(_o\), the reversal potential of the Cl\(^-\)-dependent Cl\(^-\) current (\(E_{\text{Cl}}\)) was measured to be \(-16\) mV and the membrane potential (\(V_m\)) was between \(-30\) and \(-37\) mV (Wenning and Calabrese 1991). These observations indicate that the intracellular Cl\(^-\) concentration ([Cl\(^-\)]\(_i\)) is maintained well above the Cl\(^-\) equilibrium concentration (9.3 mM in low [Cl\(^-\)]\(_o\)) and is estimated to be around 17 mM (Fig. 1, model).

In the NNC, which receives no peripheral synaptic input, the depolarizing Cl\(^-\) current provides continuous excitation at the normal, low [Cl\(^-\)]\(_o\), keeping the NNC firing (Wenning and Calabrese 1991, 1995). The natural changes of the blood Cl\(^-\) concentration make the NNC of the leech a good model for studying the neural basis of ion homeostasis and the mechanism of ion sensing. To further characterize sensory transduction, we investigated the single-channel currents underlying the NNC’s sensitivity to Cl\(^-\). We describe a slowly gating anion
channel whose open probability decreases in high [Cl\(^{-}\)], and which has an indirect gating mechanism. Preliminary results appeared in abstract form (Erxleben et al. 1997; Wenning et al. 1996).

**METHODS**

Leeches (Hirudo medicinalis L.) were obtained from a commercial supplier (Leeches USA, Westbury, NY) and kept in artificial pond water at 16°C. Experiments were carried out at room temperature (20–22°C).

Isolation of the nephridial nerve cell (NNC) is described in Wenning and Calabrese (1991). Briefly, a single nephridium and the dorsal part of its urinary bladder were dissected out and transferred to a silicone elastomer (Sylgard)-lined dish. The bladder wall was pinned inside out and a small cut through the bladder wall exposed the NNC. Dissection time per cell was 30 min.

The preparation was constantly superfused allowing complete changes of the bathing solution within 1–2 min. We used various Cl\(^{-}\) concentrations of the bathing medium (Table 1). “High [Cl\(^{-}\)],” refers to leech saline with Cl\(^{-}\) concentrations between 108 and 123 mM. “Low [Cl\(^{-}\)],” refers to artificial leech blood with Cl\(^{-}\) concentrations between 33 and 43 mM. Intermediate concentrations were obtained by mixing solutions with low and high Cl\(^{-}\) concentrations. Actual Cl\(^{-}\) concentrations are stated in the text and Figures. Dissections were done in high [Cl\(^{-}\)], saline.

**Intracellular recordings**

Intracellular recordings from the NNC began in leech saline. Glass microelectrodes were filled with a mixture of 4 M potassium acetate and 20 mM KCl and had resistances of 20–30 MΩ. To lower their capacitance, they were dipped into dimethyl-polysiloxane (Sigma, St. Louis, MO) prior to use. Measurements in discontinuous current clamp were made either with a NPI SEC-05 1 amplifier (NPI electronic GMBH, Tamm, Germany) using a switching frequency between 12 and 14 kHz or with an Axoclamp-2A (Axon Instruments, Foster City, CA) using a switching frequency of 2.5 kHz. Single-electrode voltage-clamp experiments were carried out in discontinuous mode (switch frequency of 2.5 kHz) with an Axoclamp-2A using a gain setting of ~0.8 nA/nV. We used 0 Ca\(^{2+}\)/5 mM Co\(^{2+}\) saline (Table 1) in voltage-clamp experiments to reduce spiking and to eliminate Ca\(^{2+}\)-mediated currents. To chelate intracellular Ca\(^{2+}\), glass microelectrodes were filled with 200 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA; Sigma) in 20 mM KCl. To increase the intracellular Cl\(^{-}\) concentration of the NNC, glass microelectrodes were filled with 3 M KCl (Schmidt and Calabrese 1992).

**Single-channel recordings**

Giga-ohm seals on the soma or distal neurites of the NNC (Fig. 1, top left) were obtained following 50–70 min of enzymatic digestion with a mixture of collagenase (5% wt/vol) Type IA (Sigma) and Trypsin (0.25%; Life Technologies, NY) at 22°C with agitation. Preparations were rinsed several times in leech saline and, if not immediately used, stored at 10°C for up to 3 h.

The pipette solutions for the single-channel recordings in the cell-attached configuration were either leech saline (high [Cl\(^{-}\)]) or artificial leech blood (low [Cl\(^{-}\)]) diluted by 10% with H\(_2\)O to make them hypo-osmotic with respect to the bathing solutions. A holding potential of nominally ~20 mV (see junction potential corrections) from the cell’s membrane potential was routinely used to increase the driving force on Cl\(^{-}\) and hence the amplitude of single-channel currents.

For the outside-out patches, we used two different Cl\(^{-}\) concentrations for the pipette solution: a Cl\(^{-}\)-free solution containing (in mM) 50 CsOH, 50 citric acid, 10 tetraethylammonium acetate (TEA), 10 HEPES, 5 EGTA, and 1 Mg-ATP or a 14 mM Cl\(^{-}\) solution containing (in mM) 50 CsOH, 50 citric acid, 10 tetraethylammonium chloride (TEA-Cl), 10 HEPES, 5 EGTA, 2 MgCl\(_2\), and 1 Na\(_2\)-ATP (all from Sigma). Pipette solutions were adjusted to pH 7.4.

Single-channel currents were recorded with an Axopatch 1 D or 200 (Axon Instruments) or an EPC9 (HEKA, Lambrecht, Germany). Inward currents are shown as downward deflections.

**Drugs**

Drugs were bath applied and were obtained from Sigma or Calbiochem (San Diego, CA). Channel blockers: DIDS (Sigma), SITS (Sigma), 5-nitro-2-(3-phenylpropyl-amino)benzoic acid (NPPB, Calbiochem), ZnCl\(_2\) (Sigma), Bumetanide (Sigma). Ca\(^{2+}\)-signaling: BAPTA, CdCl\(_2\) (Sigma). DIDS, SITS and NPPB were dissolved in DMSO (obtained as 1 ml aliquots from Sigma). DMSO caused slight depolarization and a drop in input resistance at concentrations 2 × 10\(^{-3}\) M (unpublished observations). Its final concentration was therefore kept ≤2 × 10\(^{-3}\) M.

**Junction potential corrections**

The junction potential at the reference electrode changes in response to changes in [Cl\(^{-}\)]. In intracellular recordings and voltage-clamp experiments, the two functions of the reference electrode (current return and stable reference potential) were assigned to separate electrodes using a bath probe thereby compensating for these potential changes (Wenning and Calabrese 1991). In addition, the bath probe potential provided a convenient way to indicate the actual time course of changes of [Cl\(^{-}\)].

For single-channel recordings, potential changes at the reference electrode were compensated by also using a bath probe (Axopatch 1D) or were minimized by using an agar bridge (Axopatch 200 and EPC9, both of which do not provide an input for a separate bath probe). The liquid junction potentials at the patch electrode and potential changes at the agar bridge were calculated using the “junction potential” module of AxoScope (Axon Instruments). The principal anions of leech blood, malate and succinate (Hoeger et al. 1989),

**TABLE 1. Solutions**

<table>
<thead>
<tr>
<th>NaCl, mM</th>
<th>KCl, mM</th>
<th>CaCl(_2), mM</th>
<th>Other Substances, mM</th>
<th>Cl(^{-}), mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low [Cl(^{-})],*</td>
<td>20–35</td>
<td>4</td>
<td>1.8</td>
<td>30–50 Na(_2)-malate, 0–10 Na(_2)-succinate, 10 HEPES</td>
</tr>
<tr>
<td>0 Ca(^{2+})/5 Co(^{2+})</td>
<td>30</td>
<td>4</td>
<td>0</td>
<td>5 CoCl(_2), 40 Na(_2)-malate, 10 Na(_2)-succinate, 10 HEPES</td>
</tr>
<tr>
<td>High [Cl(^{-})],†</td>
<td>100–120</td>
<td>4</td>
<td>1.8</td>
<td>10 glucose, 10 HEPES</td>
</tr>
<tr>
<td>0 Ca(^{2+})/5 Co(^{2+})</td>
<td>100</td>
<td>4</td>
<td>0</td>
<td>5 CoCl(_2), 10 glucose, 10 HEPES</td>
</tr>
</tbody>
</table>

Solutions are pH 7.4 adjusted with NaOH or malic acid. For the pipette solution in cell-attached patches, solutions were diluted by 10%. * Based on Hoeger et al. (1989). † Based on Nicholls and Kuffler (1964); +10 mM NaOH for pH adjustment.
are not in the library of the junction Potential module. Therefore the liquid junction potential difference between high and low [Cl\textsuperscript{-2}]\textsubscript{i} was measured (Neher 1992). The potential difference was $-9.6 \pm 0.7$ mV ($n = 8$). Holding potentials (cell-attached patches) or absolute membrane potentials (excised patches) used for reversal potential measurements and current-voltage plots were corrected accordingly. The membrane potential of four enzyme-treated cells was measured in the whole cell configuration. It was $-48.6 \pm 7.5$ mV in high [Cl\textsuperscript{-2}]\textsubscript{o}, and $-32.5 \pm 11.5$ mV in low [Cl\textsuperscript{-2}]\textsubscript{o}, respectively. These values were used to estimate the NNC’s membrane potential in the cell-attached patches.

The Goldman-Hodgkin-Katz constant field equation (Hodgkin and Katz 1949) was used to describe rectification due to asymmetric [Cl\textsuperscript{-2}]\textsubscript{o}

$$I\textsubscript{Cl} = P_{Cl} \cdot V_{m}/RT ([Cl\textsuperscript{-2}]\textsubscript{o} - [Cl\textsuperscript{-2}]\textsubscript{i})/([Cl\textsuperscript{-2}]\textsubscript{o} - [Cl\textsuperscript{-2}]\textsubscript{i})$$

Here $I\textsubscript{Cl}$ (pA) is the single-channel current, $P_{Cl}$ (cm\textsuperscript{5}/s) the permeability, $V_{m}$ (V) the absolute membrane potential, and [Cl\textsuperscript{-2}]\textsubscript{o} and [Cl\textsuperscript{-2}]\textsubscript{i} (Mol/l), the intra and extracellular chloride concentration, respectively. The Faraday and Gas constant $F$ and $R$ as well as the absolute temperature $T$ have their usual values and dimensions.

**Data acquisition, storage, and analysis**

Data from intracellular recordings and voltage-clamp experiments were acquired at 2 kHz and stored using Clampex (Axon Instruments). Data reduction was necessary for display purposes of long-term recordings and spike amplitudes are therefore attenuated. Single-channel data were acquired at 1 or 2 kHz using a Digidata 1200 (Axon) or EPC9 (HEKA) and low-pass filtered at 200 Hz. Data was analyzed using the pClamp software suite (Axon) or the Pulse software (HEKA) in combination with a personal computer. The open probability (Po) of channels was determined by summing open times during 1–5 min of channel activity (depending on the frequency of openings) and dividing through the number of channels in the patch. To access the variability in Po, the activity for each experimental condition was divided in 10-s segments, which were then averaged. The standard deviation of this average is shown in the figures.

All values are expressed as means ± SD. Statistical significance was assessed by using a two-tailed Student’s t-test.

**RESULTS**

A Cl\textsuperscript{-2}-dependent, indirectly gated anion channel mediates the NNC’s sensitivity to the external Cl\textsuperscript{-2} concentration

The NNC’s tonic sensitivity to the extracellular Cl\textsuperscript{-2} concentration ([Cl\textsuperscript{-2}]\textsubscript{o}) relies on a high resting conductance for Cl\textsuperscript{-2} and the active maintenance of its nonequilibrium distribution. $E_{Cl}$ is estimated to be about $-21$ mV in low (38 mM) [Cl\textsuperscript{-2}]\textsubscript{o}, while the membrane potential is $-36.9 \pm 10$ mV under these conditions (Fig. 1; model). As previously shown in voltage-clamp measurements (Wenning and Calabrese 1991), the high resting conductance for Cl\textsuperscript{-2} is gated off by Cl\textsuperscript{-2}, it is inwardly directed and is carried by Cl\textsuperscript{-2} leaving the cell (Fig. 1; model). This current is therefore referred to as Cl\textsuperscript{-2}-dependent Cl\textsuperscript{-2} current. It was found to be independent of voltage and extracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) (Wenning and Calabrese 1991). These properties were used to identify the Cl\textsuperscript{-2}-dependent Cl\textsuperscript{-2} current on the single-channel level. By using bathing solutions of different Cl\textsuperscript{-2} concentrations (Table 1), we mimicked the physiological changes of the Cl\textsuperscript{-2} concentration that occur in leech blood after feeding on mammalian blood (Wenning et al. 1980; Zerbst-Boroffka 1973).

The simplest mechanism to account for the macroscopic observation of a Cl\textsuperscript{-2} current turned off in the presence of high [Cl\textsuperscript{-2}]\textsubscript{i} would be a block of a Cl\textsuperscript{-2} channel by extracellular Cl\textsuperscript{-2} itself. Consequently, channel activity in the cell-attached configuration should depend on the Cl\textsuperscript{-2} concentration of the pipette solution. We therefore used a pipette solution of either high or low Cl\textsuperscript{-2} concentration to study the activity of Cl\textsuperscript{-2} channels in the cell-attached configuration but did not see any consistent difference. A change of the Cl\textsuperscript{-2} concentration of the bathing medium, however, had pronounced effects on the open probability of a slowly gating channel (Figs. 2 and 3). Regardless of the Cl\textsuperscript{-2} concentration in the pipette, channel activity was low in high (123 mM) [Cl\textsuperscript{-2}]\textsubscript{o}, with brief and infrequent openings. When changing to low (43 mM) [Cl\textsuperscript{-2}]\textsubscript{o}, the open probability increased due to an increase of the burst duration. Channel activity subsided on changing the bathing solution back to high [Cl\textsuperscript{-2}]\textsubscript{o} (Fig. 2). In nine patches, which contained one or two active channels, judging from the maximum number of simultaneous openings at high activity, the mean activity (Po) in high (123–128 mM) [Cl\textsuperscript{-2}]\textsubscript{o}, was 0.027 (± 0.03) and increased to Po = 0.31 (± 0.34) on change to low (38–43 mM) [Cl\textsuperscript{-2}]\textsubscript{o}. This increase in Po was determined to be statistically significant using a paired t-test ($P < 0.013$).

With low (39 mM) [Cl\textsuperscript{-2}] in the recording pipette, currents of this slowly gating Cl\textsuperscript{-2}-dependent channel were inward with amplitudes of about 1–2 pA at the cell’s membrane potential (Fig. 4, A and C). The current amplitude increased with hyperpolarization and decreased with depolarization as expected for an anion channel. The channel is subsequently referred to as Cl\textsuperscript{-2}-dependent Cl\textsuperscript{-2} channel because Cl\textsuperscript{-2} is the permeant anion under our experimental conditions. Two other classes of anion channels were occasionally observed (for example the channels marked with asterisks in Fig. 3). They had conductances of 5–10 and 90 pS, respectively, but their activity was Cl\textsuperscript{-2} independent, and these channels were therefore not further characterized.

A total of 140 patches were examined. In 43 of these we recorded Cl\textsuperscript{-2} channels. In 24 of these 43, we also recorded spikes (Fig. 5). The activity of the Cl\textsuperscript{-2}-dependent Cl\textsuperscript{-2} channel preceded the characteristic bursts of action potentials of the NNC. Indicating that opening of these channels initiate, rather than follow, the depolarization caused by turning on the Cl\textsuperscript{-2}-dependent Cl\textsuperscript{-2} current in low [Cl\textsuperscript{-2}]\textsubscript{o}. The activity of the channels under the patch is governed by the Cl\textsuperscript{-2} concentration of the bathing medium, which suggests that gating is not by direct interaction of Cl\textsuperscript{-2} with the channel but rather via an intracellular signaling pathway.

Since the macroscopic Cl\textsuperscript{-2}-dependent Cl\textsuperscript{-2} current is voltage independent (Wenning and Calabrese 1991), the underlying Cl\textsuperscript{-2}-dependent Cl\textsuperscript{-2} channel should also be voltage insensitive. The voltage dependence of the Cl\textsuperscript{-2}-dependent Cl\textsuperscript{-2} channel was examined using two different approaches. First, we measured the open probability (Po) during steady polarization at different membrane potentials (Fig. 4, B and C). In terms of a Boltzmann fit, the voltage sensitivity of the example shown in Fig. 4 and a second patch was such that 200 mV were needed for an e-fold change in Po of the channel, indicating that Po was largely voltage independent. Second, we examined channel activity during voltage ramps. Channels opened at random with no preference at de- or hyperpolarized potentials (Fig. J Neurophysiol • VOL 86 • OCTOBER 2001 • WWW.JN.org
FIG. 2. A change from high (gray shading; 123 mM) to low (no shading; 43 mM) \([\text{Cl}^2 -]\) led to the opening of a Cl\(^{-}\) channel in the NNC in cell-attached patches. Continuous recording for 12 min with low (39 mM) \([\text{Cl}^2 -]\) in the pipette. Cl\(^{-}\) channels, which were initially closed (traces 1 and 2), started to open after changing from high to low \([\text{Cl}^2 -]\) (trace 3), and channel activity subsided on returning to high \([\text{Cl}^2 -]\) (traces 5 and 6). Once the channel opened, the holding potential was changed as indicated (†, †, traces 3 and 4), to demonstrate that channel openings do not depend on the membrane potential. Note the long duration of channel openings. (- - -, closed states.) The transition from high to low \([\text{Cl}^2 -]\) causes a slant in the holding current. Right: the cartoons illustrate the magnitudes of the expected Cl\(^{-}\) movement across the patch in high \([\text{Cl}^2 -]\) and low \([\text{Cl}^2 -]\). \(V_m\) was estimated using whole cell recordings from four enzyme-treated preparations (see METHODS). \([\text{Cl}^2 -]\) is assumed to stay constant in high and low \([\text{Cl}^2 -]\). Note that while \(E_{\text{Cl}}\) over the patch remains constant when changing \([\text{Cl}^2 -]\), the driving force—and hence channel current amplitude—change due to the change of \(V_m\) and \(V_{\text{patch}}\), respectively.

FIG. 3. A change from high (gray shading; 123 mM) to low (no shading; 43 mM) \([\text{Cl}^2 -]\) led to the opening of a Cl\(^{-}\) channel in the NNC in cell-attached patches, regardless of the Cl\(^{-}\) concentration in the pipette. Continuous recording for 8 min with high (110 mM) \([\text{Cl}^2 -]\) in the pipette. The patch was initially held at \(-20\) mV from the membrane potential of the cell and changed to \(-40\) mV (end of trace 3; †) in low \([\text{Cl}^2 -]\). In high \([\text{Cl}^2 -]\), channel activity is low (3 openings in traces 1 and 2). After the change from high to low \([\text{Cl}^2 -]\), channel activity increased (trace 3). In the recording shown here, a 2nd anion channel was present (*) but its activity was Cl\(^{-}\)-independent. Symbols and cartoons as in Fig. 2.
Characterization of the channels in excised patches

We further characterized the Cl$^-$-dependent Cl$^-$ channel in excised patches. If gating is indeed indirect—as suggested by the experiments in the cell-attached configuration—changes of [Cl$^-$]o should not affect channel activity when recorded in (outside-out) excised patches, while the open probability would change with changes of [Cl$^-$]o if gating were direct. In outside-out patches, we identified a slowly gating Cl$^-$ channel with $\sim 1 \text{pA}$ outward current at 0-mV holding potential. Current amplitudes increased with depolarization and decreased on hyperpolarization (Fig. 7A). With 0 mM Cl$^-\text{in}$ the pipette solution, we did not observe inward currents. The nonreversal of currents confirms that the channel is Cl$^-$ selective and impermeable to citrate, the organic anion present in the pipette solution (METHODS).

Gating of the Cl$^-$ channel in the outside-out configuration was not affected by changes of the extracellular Cl$^-$ concentration (Fig. 8). The average ratio of the open probability in low (43 mM) versus high (128 mM) [Cl$^-\text{in}$] (Po low/Po high [Cl$^-\text{in}$]) was near 1 (0.93 ± 0.36; n = 6). The slope conductance was determined from outward currents 0 mV. As expected for a Cl$^-$ channel, the slope conductance of outward currents in the outside-out patches increased with increasing [Cl$^-\text{in}$] (Fig. 7A). On average, the slope conductance was $25.5 \pm 8 \text{ pS}$ in low versus $37 \pm 12 \text{ pS}$ in high [Cl$^-\text{in}$] (n = 5). With Cl$^-\text{free}$ solution in the recording pipette and low outside [Cl$^-$], the single-channel conductance in the outside-out patches was on average half that of the Cl$^-$ channel identified in the cell-attached configuration (compare Figs. 4A and 7A). This difference in slope conductance raises the possibility that we are looking at different channels in these two configurations.

To resolve this issue, we first identified the Cl$^-$ channel based on its Cl$^-$ sensitivity in the cell-attached mode and then excised the patch into the inside-out configuration measuring the conductance in both configuration. Under these conditions, the single-channel conductance for outward currents carried by a high Cl$^-$ concentration in the pipette did not change (Fig. 6, B and C). Next we examined whether the difference in the Cl$^-$ concentration on the intracellular side of the membrane, that is, the bathing medium in the inside-out configuration and the pipette solution in the inside-out configuration, respectively, affected single-channel conductance. When the pipette solution for the outside-out patches contained 14 mM Cl$^-$ (METHODS), the average conductance for outward currents was $69 \pm 3 \text{ pS}$ (n = 3), which is similar to the single-channel conductance measured in the cell-attached and inside-out configuration (compare Figs. 4A and 6, B and C, to 6D).

To facilitate a comparison of the single-channel conductance measured under different conditions, we fitted the currents of Fig. 6 with the Goldman-Hodgkin-Katz constant field equation (Hodgkin and Katz 1949) that describes the rectification due to asymmetric ion distribution. A fit to the currents in the cell-
attached configuration gives a permeability $P_{Cl}$ of $2.2 \times 10^{-13}$ cm$^3$/s and an internal chloride concentration $[Cl^-]_i$ of 18 mM, which is in good agreement with previous estimates from voltage-clamp investigations of the NNC’s macroscopic chloride currents (Fig. 1, model) (Wenning and Calabrese 1991). Since the resting potential for cell-attached patches was estimated and absolute membrane potentials were subject to errors despite numerical corrections of junction potentials, a correction factor was used to shift the fitted curve along the voltage axis. Corrections were $-8$ mV in $B$, $4.4$ and $18$ mV for $C$ for high and low $[Cl^-]_o$, respectively, and $18$ mV for $D$. On excision of the patch into a saline with a $[Cl^-]_i$ close to physiological concentration (33 mM $[Cl^-]_i$, Fig. 6C), the I-V relationship for both outward as well as inward currents is well described by the GHK equation with the same $P_{Cl}$ of $2.2 \times 10^{-13}$ cm$^3$/s and 33 mM $Cl^-$ in the bathing solution. With a high (nonphysiological) $[Cl^-]_i$ of 123 mM; however, the I-V relationship in the range of inward currents markedly deviates from the GHK prediction (123 mM $[Cl^-]_i$, Fig. 6C). With $[Cl^-]_i$ in the physiological range (14 mM, Fig. 6D), the I-V relationship and permeability for outside-out patches is also correctly predicted by the GHK equation.

By fitting a Boltzmann curve to the data, the voltage sensitivity of the $Cl^-$ channel’s open probability in the outside-out configuration was assessed. Of the five patches analyzed, the example shown in Fig. 7B had the highest voltage sensitivity ($+54$ mV in 128 mM $[Cl^-]_o$ and $+101$ mV in 43 mM $[Cl^-]_o$ for an e-fold change in Po). Of the other four patches, only one showed comparable voltage sensitivity ($+121$ mV for an e-fold change in Po). From the remaining three patches, two had a positive and one a negative voltage dependence (>200-mV polarization for an e-fold change in Po) similar to that found in the cell-attached patches. We consider this low voltage sensitivity insignificant for the physiological response.

Another characteristic of the $Cl^-$-dependent $Cl^-$ current is its independence of $[Ca^{2+}]$, suggesting that no $Ca^{2+}$ entry is required for the response to changes of $[Cl^-]_o$, (Wenning and Calabrese 1991). To test whether the macroscopic response of the NNC depends on the intracellular $Ca^{2+}$ concentration ([Ca$^{2+}]_i$), we used electrodes filled with BAPTA to chelate [Ca$^{2+}$], and, in addition, CdCl$_2$ (10$^{-4}$ M) in the extracellular solution to block influx of Ca$^{2+}$ through Ca$^{2+}$ channels. The sensitivity of the NNC to $[Cl^-]_o$ was not diminished—if anything, the depolarization on change from high to low $[Cl^-]_o$ was even stronger (Fig. 9). Gating of the $Cl^-$ channel in the outside-out patches was found to be independent of the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}]_i$) since channel activity persisted in Ca$^{2+}$-free, EGTA-buffered pipette solution.

Gating of some $Cl^-$ channels depends on the intracellular $Cl^-$ concentration ([Cl$^-]_i) (reviewed in Foskett 1998). We tested whether manipulating $[Cl^-]_i$ of the NNC would affect the macroscopic response to $[Cl^-]_o$. Leech neurons can be loaded conveniently with $Cl^-$ by using microelectrodes filled with 3 M KCl (Schmidt and Calabrese 1992). When loading the NNC with $Cl^-$, $V_m$ settled at $-45.4 \pm 11.5$ mV ($n = 11$) in 123 mM $[Cl^-]_o$, which is somewhat depolarized from the control value of $-58.7 \pm 8.8$ mV ($n = 49$; $P < 0.001$ (unpaired t-test)) (Wenning and Calabrese 1991). On the change to 38 mM $[Cl^-]_o$, the cells depolarized further to $-18.7 \pm 15.3$ mV ($n = 11$) as compared with $-35.8 \pm 12.3$ mV ($n = 11$; $P < 0.01$ (unpaired t-test)) (Wenning and Calabrese 1991), and more importantly, showed a threefold increase of the input conductance. Thus an increase of $[Cl^-]_i$, shifts $V_m$ but does not change the $Cl^-$ sensitivity of the NNC.

The macroscopic $Cl^-$-dependent $Cl^-$ current and the $Cl^-$ channel in the outside-out patches are independent of $[Ca^{2+}]_i$, and largely voltage-insensitive, suggesting that the $Cl^-$ channel mediates the NNC’s sensitivity to $[Cl^-]_o$. The $Cl^-$ channel
in the outside-out patches shares its gating characteristics—slow gating—with the Cl−-dependent Cl− channel identified in the cell-attached configuration. The Cl−-dependent Cl− channel in the cell-attached configuration responds to changes of the Cl− concentration of the bathing medium rather than to changes of the pipette solution, indicating indirect gating. Consistent with indirect gating is that the Cl− channels in the outside-out patches are insensitive to changes in [Cl−]o (Figs. 7 and 8).

Pharmacological characterization of the Cl−-dependent Cl− current and the Cl−-dependent Cl− channel

We next compared pharmacological characteristics of the Cl−-dependent Cl− current and the Cl− channel identified in
the outside-out patches to provide further independent evidence for their identity. We tested known Cl\textsuperscript{2-} channel blockers for their ability to reduce both channel activity in the outside-out patches as well as the macroscopic Cl\textsuperscript{2-}-dependent Cl\textsuperscript{2-} current.

To characterize the macroscopic Cl\textsuperscript{2-}-dependent Cl\textsuperscript{2-} response, we used voltage- or current-clamp experiments to screen for pharmacological agents. Recordings started in high [Cl\textsuperscript{2-}]\textsubscript{o} (108–128 mM). Under these conditions, the input resistance is high and recordings stabilize more rapidly than in low (38–43 mM) [Cl\textsuperscript{2-}]\textsubscript{o} (Wenning and Calabrese 1991). The NNC was voltage-clamped at −60 mV, its membrane potential in high [Cl\textsuperscript{2-}]\textsubscript{o} (Fig. 1, model). On the change to normal, low [Cl\textsuperscript{2-}]\textsubscript{o} (not drawn to scale), the open probability of the Cl\textsuperscript{2-} channel (mean ± SD) was largely independent of membrane voltage (B). For an e-fold change in Po, a +54 mV change is required in high [Cl\textsuperscript{2-}]\textsubscript{o} (r = 0.96) and +101 mV (r = 0.6) in low [Cl\textsuperscript{2-}]\textsubscript{o}, respectively.

![Graphs showing Cl\textsuperscript{2-} channel conductance and voltage dependence](image)

**FIG. 7.** Slope conductance (A) and voltage dependence (B) of the Cl\textsuperscript{2-} channel in high (128 mM) and low (43 mM) [Cl\textsuperscript{2-}]\textsubscript{o}, from a single patch in the outside-out configuration with Cl\textsuperscript{2-}-free solution in the pipette. The slope conductance (A) was calculated from the current at membrane potentials ≥0 mV. The lines through the points are a polynomial fit. The open probability of the Cl\textsuperscript{2-} channel (mean ± SD) was largely independent of membrane voltage (B). For an e-fold change in Po, a +54 mV change is required in high [Cl\textsuperscript{2-}]\textsubscript{o} (r = 0.96) and +101 mV (r = 0.6) in low [Cl\textsuperscript{2-}]\textsubscript{o}, respectively.

![Graphs showing Cl\textsuperscript{2-} channel conductance and voltage dependence](image)

**FIG. 8.** Sequential recordings from a single patch to show that the open probability (Po) of the Cl\textsuperscript{2-} channel was independent of [Cl\textsuperscript{2-}]\textsubscript{o} in the outside-out configuration. The change of single-channel amplitude showed the expected decrease with the decrease of [Cl\textsuperscript{2-}]\textsubscript{o} from 128 to 43 mM. The difference in the holding potential is due to changes of the junction potentials (see METHODS).
(38–43 mM) [Cl\(^-\)]\(_o\), the NNC develops an inward current of \(~4–5\) nA (Wenning and Calabrese 1991). Since these large inward currents tended to compromise voltage control, we used intermediate Cl\(^-\) concentration between 60 and 85 mM to decrease the inward currents associated with the decrease in [Cl\(^-\)]\(_o\).

Five known Cl\(^-\) channel blockers were screened for their effectiveness on the macroscopic current: the two stilbenes DIDS and SIDS, bumetanide, and Zn\(^{2+}\). Of those, only bumetanide reliably blocked both the macroscopic Cl\(^-\)-dependent Cl\(^-\) response and single-channel Cl\(^-\) currents.

Bumetanide, best known to block the Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransporter present in epithelial and nonepithelial cells, has been shown to also block the CFTR-Cl\(^-\) channel (cystic fibrosis transmembrane conductance regulator) (Reddy and Quinton 1999) at higher concentrations. Strong and reversible inhibition of the NNC’s response to change of [Cl\(^-\)]\(_o\) was observed at \(10^{-3}\) M (\(n = 7\)). At \(10^{-4}\) M, the inhibitory effect of bumetanide was less pronounced (\(n = 4\)). In three preparations, we tested the effect of bumetanide at \(10^{-3}\) M under voltage clamp, asking whether it is able to abolish an existing, or prevent the onset of a developing, inward current and whether it changes the input conductance of the NNC. In the example shown in Fig. 10, the response to changes of [Cl\(^-\)]\(_o\), was first tested in bumetanide-free solution. On the change from high (114 mM) to low (70 mM) [Cl\(^-\)]\(_o\) (A, ↑), the NNC turned on an inward current (\(−2.3 \) nA) with a concomitant three- to fourfold increase in input conductance. The time course of the changes in [Cl\(^-\)]\(_o\), and hence fluid exchange rate, are indicated by the bath probe potential. On the height of the response to low [Cl\(^-\)]\(_o\), bumetanide was added to the superfusate (A, bar). Bumetanide reversibly decreased the inward current to 25% (\(≈0.6\) nA) and the input conductance by 50%. Washout (B) restored the inward current and the high-input conductance characteristic for low [Cl\(^-\)]\(_o\). The change to high [Cl\(^-\)]\(_o\) (B, ↓) decreased the inward current to almost 0 nA with a concomitant decrease of the input conductance. When returning to low [Cl\(^-\)]\(_o\), now with bumetanide present (C, bar and ↑), the NNC developed a much smaller inward current (\(−0.6\) nA) with a less pronounced increase of the input conductance. Washout yielded the full response to low [Cl\(^-\)]\(_o\), namely the large inward current (3 nA) and a further increase of the input conductance (D). With the change back to high [Cl\(^-\)]\(_o\), (D, ↓), the inward current was turned off, the input conductance decreased and the NNC returned to its initial 0-nA holding current and small input conductance (D, end). The swift and rather dramatic changes of both the inward current and input conductance due to the change of [Cl\(^-\)]\(_o\) and its block by bumetanide are emphasized in the top panel. In four preparations, the effect of bumetanide was tested in discontinuous current clamp (data not shown). As expected, application of bumetanide (\(10^{-5}\) M) in low [Cl\(^-\)]\(_o\) led to hyperpolarization and an increase in the input resistance. Again, the response was reversible. When tested at the same concentration (\(10^{-5}\) M) on the Cl\(^-\) channels in the outside-out configuration (\(n = 6\)), with low or high [Cl\(^-\)]\(_o\), bumetanide completely and reversibly blocked channel openings (Fig. 11).

The effect of bumetanide on the Cl\(^-\) channels was tested in excised patches (o/o) with one or two channels present. The macroscopic response was tested on the whole cell with the peripheral arborizations of the NNC in the nephridium intact (Fig. 1, top left). Penetration of the drug in the periphery might be slower than onto the exposed cell body, which might explain the incomplete block of the macroscopic current.

The macroscopic current and the Cl\(^-\) channel identified in outside-out patches were reliably blocked by bumetanide. We therefore conclude that the Cl\(^-\) channel of the outside-out patches are the same as the Cl\(^-\)-dependent Cl\(^-\) channels identified in cell-attached patches and are indeed those that mediate the NNC’s sensitivity to [Cl\(^-\)]\(_o\).

DISCUSSION

The NNCs of the leech, peripheral neurons close to the nephridium (Fig. 1, top left), monitor blood Cl\(^-\) concentration by a novel slowly gating Cl\(^-\) channel whose activity is inversely correlated to the external Cl\(^-\) concentration. In principle, a Cl\(^-\) channel with positive voltage dependence could explain the observed depolarization on the change from high to low [Cl\(^-\)]\(_o\), simply due to a more depolarized E\(_{Cl}\). A key feature of the macroscopic Cl\(^-\)-dependent Cl\(^-\) current, however, characterized previously in voltage-clamp experiments (Wenning and Calabrese 1991) is its voltage independence. To account for the threefold increase in Cl\(^-\) conductance that accompanies the 23-mV depolarization on the change from high (133 mM) to low (38 mM) [Cl\(^-\)]\(_o\) (Fig. 1, model), voltage sensitivity of a Cl\(^-\) channel would have to be >23 mV for an e-fold change in membrane potential. This is far more than we ever observed in cell-attached or excised patches (Figs. 4B, 6A, and 7B).

Alternatively, the shift in E\(_{Cl}\), and the concomitant depolarization, could initiate a Ca\(^{2+}\) influx. Increase in [Ca\(^{2+}\)], could in turn activate a Cl\(^-\) current and lead to further depolarization. The Cl\(^-\)-dependent Cl\(^-\) current of the NNC is, however, independent of extracellular [Ca\(^{2+}\)] (Wenning and Calabrese 1991), and, as shown here, the macroscopic response to changes of the extracellular Cl\(^-\) concentration persisted when [Ca\(^{2+}\)] was chelated with BAPTA (Fig. 9). Consistent with the macroscopic current being independent of [Ca\(^{2+}\)], gating of the Cl\(^-\) channel identified in the outside-out configuration is also independent of [Ca\(^{2+}\)], because the pipette solution was Ca-free and contained EGTA as a Ca\(^{2+}\)-chelator.

The Cl\(^-\)-dependent Cl\(^-\) channel identified in the cell-attached patches shares both the inverse correlation of channel activity with [Cl\(^-\)]\(_o\), and the voltage independence with the macroscopic Cl\(^-\)-dependent Cl\(^-\) current (Figs. 4 and 6) (Wenning and Calabrese 1991). We therefore conclude that the Cl\(^-\)-dependent Cl\(^-\) channel identified in cell-attached patches accounts for the Cl\(^-\) sensitivity of the NNC. The Cl\(^-\) channel identified in the outside-out configuration shares the independence of voltage and [Ca\(^{2+}\)], with the macroscopic Cl\(^-\)-dependent Cl\(^-\) current. To further confirm that the Cl\(^-\) channels identified in the outside-out patches—which are insensitive to changes of [Cl\(^-\)]\(_o\) (Fig. 8), see following text—underlie the macroscopic current, we used pharmacological tools.

Bumetanide provided an efficient tool to block both the macroscopic Cl\(^-\) current and the Cl\(^-\) channels identified in the outside-out configuration (Figs. 10 and 11). Bumetanide is structurally related to NPPB, a widely used Cl\(^-\) channel blocker. Bumetanide is best known to block the Na\(^+\)/K\(^+\)/2Cl\(^-\) co-transporter in epithelial cells. The Na\(^+\)/K\(^+\)/2Cl\(^-\) co-transporter elevates [Cl\(^-\)]\(_o\), above thermodynamic equilibrium and thereby yields Cl\(^-\) outflow through the appropriate channels on
FIG. 10. Bumetanide (10^{-3} M) reversibly blocked the macroscopic response of the NNC to [Cl^{-}]_o. The whole experiment is shown on top emphasizing the changes in the inward current and input conductance. The bath probe potential indicates changes of [Cl^{-}]_o and hence of the bath exchange rate. The experiment is shown in detail in four consecutive sets of expanded traces (A–D): voltage trace (top), current trace (middle), and the bath probe potential (bottom). A: recordings began in high (114 mM) [Cl^{-}]_o, and the NNC was held at -60 mV. The input conductance was assessed by administering steps of -10 mV (1 s). On the change to low (70 mM) [Cl^{-}]_o (arrow), the NNC turned on the Cl^{-}-dependent inward current. In the presence of bumetanide (bar), the inward current turned off and the input conductance decreased. Washout of bumetanide (B) restored the inward current and the high-input conductance. Note that the NNC was still in low [Cl^{-}]_o. The change back to high [Cl^{-}]_o (↓) turned off the Cl^{-}-dependent Cl^{-} current (end of B). The change to low [Cl^{-}]_o, now in the presence of bumetanide (C), gave rise to a small inward current and an increase in input conductance. Washout of bumetanide with low [Cl^{-}]_o gave rise to the full Cl^{-}-specific response (D). When the bathing medium was finally changed back to high [Cl^{-}]_o, (↓), the holding current returned to 0 nA and the input conductance decreased to its initial low value. Bathing media contained 5 mM Co^{2+} and 0 Ca^{2+} (Table 1). The bath probe potential and current traces are not in the same vertical register in the expanded traces (A–D).
ever, as shown in native sweat ducts, bumetanide also blocks the

patches underlie the NNC's response to \([\text{Cl}^{2-}]_o\) (data not shown), and blocked the Cl\(^{2-}\) channels identified in the outside-out configuration (Fig. 11). The similar pharmacology indicates that the Cl\(^{2-}\) channels identified in the outside-out patches underlie the NNC's response to \([\text{Cl}^{2-}]_o\) and are the same as the Cl\(^{2-}\)-dependent Cl\(^{2-}\) channels identified in the cell-attached configuration.

The NNC's resting conductance is three times larger in low than in high \([\text{Cl}^{2-}]_o\) (90 vs. 30 nS) (Wenning and Calabrese 1991), indicating that the depolarizing Cl\(^{2-}\)-dependent Cl\(^{2-}\) current dominates the membrane conductance under normal conditions (Figs. 1, model; 10, change to low \([\text{Cl}^{2-}]_o\) in the 1st trace). However, from 140 patches in the cell-attached configuration that lasted long enough to test channel activity at different \([\text{Cl}^{2-}]_o\) (\(\approx 5\) min), only 43 contained the Cl\(^{2-}\)-dependent Cl\(^{2-}\) channel. This suggests that the channel density might be lower in the cell body than in the sensory projections themselves. This suggestion is corroborated by earlier findings that the Cl\(^{2-}\)-sensitivity of the NNC is abolished when the peripheral projections are cut near the cell body (Wenning 1989).

Gating of the Cl\(^{2-}\)-dependent Cl\(^{2-}\) channel is not through interaction of Cl\(^{2-}\) with the channel itself. In cell-attached recordings, its activity increases markedly on change from high to low Cl\(^{2-}\) in the bathing medium rather than to changes of the pipette solution (Figs. 2, 3, and 5), suggesting indirect gating. Consistent with an intracellular signaling pathway is the fact that the Cl\(^{2-}\) channel identified in the outside-out configuration is insensitive to changes in \([\text{Cl}^{2-}]_o\) (Fig. 8).

The properties of the leech Cl\(^{2-}\)-dependent Cl\(^{2-}\) channel differ fundamentally from other known Cl\(^{-}\) channels. One feature of the leech Cl\(^{2-}\)-dependent Cl\(^{2-}\) channel, a dependence of the open probability on the permeating ion itself, has been described for CIC-type Cl\(^{-}\) channels of Torpedo electroplaque. However, the open probability of CIC-type Cl\(^{-}\) channels increases with increasing \([\text{Cl}^{2-}]_o\) (Chen and Miller 1996; Pusch et al. 1995; Richard and Miller 1990), while it decreases in the leech Cl\(^{2-}\)-dependent Cl\(^{2-}\) channel. Moreover, gating of the CIC-type channels is direct while it is indirect in the Cl\(^{2-}\)-dependent Cl\(^{2-}\) channel of the leech NNC.

The Cl\(^{2-}\)-dependent Cl\(^{2-}\) channel is modulated by the NNC's endogenous peptide, FMRF-NH\(_2\) (Wenning et al. 1993b). Voltage-clamp experiments showed that FMRF-NH\(_2\) turns off the Cl\(^{2-}\)-dependent Cl\(^{-}\) current (Wenning and Calabrese 1995), suggesting a second, probably also indirect, gating mechanism. The observation that the conductance of the Cl\(^{2-}\)-dependent Cl\(^{2-}\) channel is only 50% when Cl\(^{2-}\) is absent on the cytoplasmic side compared with the case when Cl\(^{2-}\) is present (compare Figs. 6 and 8) might indicate that the channel is "locked" into a subconductance state with no cytoplasmic Cl\(^{2-}\) (outside-out patches). Subconductance states were frequently observed in cell-attached recordings (Fig. 6A, asterisk) and in outside-out patches with Cl\(^{2-}\) present on the cytoplasmic side but never in outside-out patches with no cytoplasmic Cl\(^{2-}\). A dependence of channel gating on intracellular Cl\(^{2-}\) has been reported for other Cl\(^{-}\) channels (see Foskett 1998) and for SLO-2 K\(^{+}\) channels from Caenorhabditis elegans (Yuan et al. 2000). In the intact NNC, however, we expect only small changes in \([\text{Cl}^{2-}]_i\), and the reduced conductance of the channels seen in the absence of

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**FIG. 11.** Bumetanide (at \(10^{-3}\) M) completely and reversibly blocked the Cl\(^{-}\) channels identified in the outside-out configuration. Single-channel currents were recorded during voltage ramps (+50 mV, 2 s) with 14 mM [Cl\(^{-}\)] in the pipette and high (107 mM) [Cl\(^{-}\)] in the bathing medium. Shown are 10 consecutive traces each; before, in the presence of bumetanide, and 3 min after washout. Average leak and capacitive currents were obtained from records with no openings and are subtracted from the traces.
[Cl\(^-\)]\(_m\) seems thus irrelevant under physiological conditions. Also, loading of the cell with Cl\(^-\) shifted \(V_m\) but did not abolish the macroscopic response.

The NNC and, as shown more recently, a growing number of vertebrate neurons (Ben-Ari et al. 1997; Enz et al. 1999; Reuter et al. 1998; Rohrbough and Spitzer 1996), belong to the type of neurons that maintain \(E_{Cl}\) well above \(V_m\) so that activation of any Cl\(^-\) currents will lead to depolarization. In the NNC, the Cl\(^-\)-dependent Cl\(^-\) current dominates \(V_m\) in the normal ionic environment (36–42 mM [Cl\(^-\)]\(_m\)) (Wenning et al. 1980; Zerbst-Boroffka 1970) providing continuous excitation and keeping the cell firing bursts of action potentials (Fig. 1, top right) (Wenning 1989; Wenning and Calabrese 1991). The combination of an \(E_{Cl}\) above \(V_m\) and second-messenger-gated Cl channels drives fluid transport across many vertebrate epithelia. Neurons that maintain \(E_{Cl}\) above \(V_m\) and hence have depolarizing Cl\(^-\) currents, serve a variety of purposes. A depolarizing Ca\(^{2+}\)-dependent Cl\(^-\) current was found to contribute to chemo-electrical transduction in olfactory sensory neurons (Reuter et al. 1998). In immature vertebrate neurons, a nonpassive distribution of Cl\(^-\) with \(E_{Cl}\) positive from the membrane potential provides excitation in response to GABAergic innervation (Ben-Ari et al. 1997; Rohrbough and Spitzer 1996). The leech NNC’s ability to monitor extracellular Cl\(^-\) concentration, without adapting to it (Fig. 1, top right), relies also on the active maintenance of its nonequilibrium distribution and a high resting conductance for Cl\(^-\). This Cl\(^-\) conductance is turned off when the Cl\(^-\) concentration in leech blood increases as it does, for example, after a blood meal. We show here that the underlying Cl\(^-\) channel is a novel, slowly gating, voltage- and Ca\(^{2+}\)-independent Cl\(^-\) channel that is indirectly gated and whose activity is inversely correlated to the external Cl\(^-\) concentration. Future experiments will focus on the signaling mechanism of the Cl\(^-\)-dependent Cl\(^-\) channel.

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