Completely functional double-barreled chloride channel expressed from a single *Torpedo* cDNA

(electric organ/*Xenopus* oocyte/patch clamp/voltage-gated channel/reconstitution)

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ABSTRACT We have performed an electrophysiological analysis of the recently cloned *Torpedo marmorata* Cl channel. Functional expression of Cl channels in oocytes of *Xenopus laevis* previously injected with cRNA yielded an outward-rectifying current activated by hyperpolarization. Replacement of Cl with other anions significantly reduced or inhibited the current. Single-channel recordings from cell-attached patches exhibited burst-like Cl channel activity with rapid fluctuations between three equally spaced current states (0 pS, 9 pS, and 18 pS). The properties of the cloned Cl channel were almost identical to those of the reconstituted native *T. californica* Cl channel and were in full agreement with the predictions of the double-barreled channel model [Hanke, W. & Miller, C. (1983) J. Gen. Physiol. 82, 25-45]. Our results imply that the cloned cDNA codes for the completely functional *Torpedo* electrophax Cl channel.

Plasma membrane Cl channels play an important role in various cellular functions such as regulation of cell volume, transepithelial transport, and control of excitability (1). Although dysfunction of Cl channels has been implicated in diseases such as cystic fibrosis and myotonia, a scarcity of information exists on their molecular structure. Recently the primary structure of the Cl channel from the electric organ of *Torpedo marmorata* was obtained by cloning its cDNA through an expression cloning approach (2). Extensive biophysical studies on the *T. californica* Cl channel, performed in the lipid bilayer system by Miller and colleagues (3-5), provided strong evidence for the existence of a functionally dimeric channel. Its single-channel properties are well described by the "double-barreled" channel model, although the assumption of equivalence and independence of two protochannels (barrels) forming an entire channel is undoubtedly a simplification (5, 6). The entire channel is slowly activated (within seconds) upon hyperpolarization (the cis side of the barrel corresponds to the intracellular side (2)). In the activated state, the channel exhibits bursts during which the two protochannels rapidly open and close independently of each other. The protochannel open-probability increases with depolarization, but with sustained depolarization the entire channel complex deactivates. Thus the channel has at least two gates with different kinetics and inverted voltage dependence (5).

Previous measurements of macroscopic currents in cRNA-injected oocytes were compatible with these complex gating processes (2). However, it was still an open question whether the protein encoded by the cDNA was sufficient to give rise to the complex behavior as observed in single-channel recordings of the native channel reconstituted into lipid bilayers; e.g., there might have been a missing subunit. The main purpose of the present study was to resolve this issue with patch clamp measurements in cRNA-injected oocytes.

Our experiments show that Cl channels expressed in *Xenopus laevis* oocytes previously injected with cRNA derived from *T. marmorata* Cl channel cDNA are indistinguishable from the native Cl channels of *T. californica* as regards ion selectivity, single-channel conductance, and voltage dependence. Thus a single polypeptide (either alone or in a homooligomeric complex) is sufficient to generate the complex apparent double-barreled structure of the *Torpedo* electrophax Cl channel.

MATERIALS AND METHODS

Oocyte Expression. Capped synthetic RNA was synthesized using T3 RNA polymerase (Stratagene capping kit) and plasmid 7134 (2) after linearization with Xba I and was taken up in diethyl pyrocarbonate-treated water. Oocytes from X. laevis were injected with about 50 nl of aqueous RNA solution by standard procedures (7). Between 4 and 200 ng of cRNA was injected in different experiments. Oocytes were kept at 18°C for 1-7 days before use in electrophysiological experiments.

Electrophysiological Measurements. Voltage clamp experiments. Standard two-microelectrode voltage clamp procedures were used to measure whole-cell membrane currents in oocytes. The oocytes were superfused with Ringer solution (116 mM NaCl/2 mM KCl/1.8 mM CaCl2/1 mM MgCl2/10 mM Hepes, pH 7.2) or a test solution (96 mM NaCl/2 mM KCl/1.8 mM CaCl2/1 mM MgCl2/5 mM Hepes, pH 7.6), which was modified by replacing part (50 mM) of the Cl- by either Br-, I-, SCN-, or cyclamate.

Patch clamp experiments. Oocytes were mechanically skinned of their vitelline membranes in a hypotonic solution (8) and then placed into the recording dish containing either normal frog Ringer solution or K+ Ringer (Na+ replaced by K+). Patch pipettes (2.5 or 7 MΩ) were filled with the same solution as the bath solution.

All experiments were performed at room temperature. Stimulation and data acquisition were carried out with CED (Cambridge Electronic Design, Cambridge). Data were low-pass-filtered (1 kHz or 300 Hz) and sampled at 0.1 kHz or, for single-channel data, at 1 kHz. An EPC7 patch clamp amplifier (List Electronics, Darmstadt, F.R.G.) was used.

Double-Barreled Channel Model Parameters. The following equations, given by Hanke and Miller (3), were used to perform the statistical analysis. The reaction scheme

\[
\begin{align*}
S_0 & \xrightarrow{2\alpha} S_1 \xrightarrow{A} S_2 \\
S_1 & \xrightarrow{2\beta} S_2
\end{align*}
\]

[1]

gives the transitions between the three substates of the activated double-barreled Cl channel, S0 (nonconducting).

Abbreviation: RP, relative potential.
S1 (one subunit open), and S2 (both subunits open), governed by the opening rate constant λ and the closing rate constant μ.

The activation probability \( p \) of a single subunit of the channel (during bursting activity) is defined by the frequencies \( f \) of the substates S1 and S2:

\[
f_{S0} = (1 - p)^2; f_{S1} = 2p(1 - p); f_{S2} = p^2; \]

\[ p = (f_{S1} + 2f_{S2})/2. \]

The mean dwell time \( \tau \) in a substate depends on the rate constants:

\[
\tau_{S0} = 1/2\lambda; \tau_{S1} = 1/(\lambda + \mu); \tau_{S2} = 1/2\mu. \]

The relations of protochannel open probability, dwell times, and rate constants are given in the equations

\[
\rho(1 - p) = \lambda/\mu \]

\[ \lambda = 1/2\tau_{S0}; \mu = 1/2\tau_{S2} \]

\[ \lambda = p/\tau_{S1}; \mu = (1 - p)/\tau_{S1}. \]

The voltage dependence of \( p \) is described as

\[
p(V) = (1 + \exp[-zF(V - V_0)/RT])^{-1}, \]

where \( V_0 \) is the half-saturation voltage, and \( z \), the charge that moves across the membrane upon channel opening, was set equal to 1 (3).

**RESULTS**

**Ion Selectivity.** The *T. californica* Cl⁻ channel was found to be highly selective for Cl⁻ over other anions (9). Whereas only Br⁻ can pass the channel to an appreciable degree, I⁻ and SCN⁻ actually block it (10). We investigated whether the cloned *Torpedo* channel displays the same ion selectivity. Macroscopic current–voltage relationships of cRNA-injected oocytes were measured with the two-microelectrode voltage clamp technique. Since the channel is slowly activated by hyperpolarization (2), we first hyperpolarized the oocyte membrane to -120 mV for 10 s. The current response to a depolarizing voltage ramp was then used to determine current–voltage relationships both in uninjected controls (Fig. 1A) and in cRNA-injected oocytes (Fig. 1B). To test for qualitative ion selectivity, extracellular Cl⁻ was partially exchanged with 50 mM Br⁻, I⁻, SCN⁻, or cyclamate.

With control oocytes, there was nearly no effect of anion substitution at voltages more negative than -60 mV, indicating a small Cl⁻ conductance. With increasing depolarization, however, there was an increase in the slope of the current response, probably mediated by the activation of the endogenous Ca²⁺-dependent Cl⁻ channel (11–13). The apparent conductance sequence in native oocytes was SCN⁻ > I⁻ > Br⁻ > Cl⁻.

As reported previously (2), the current of cRNA-injected oocytes was outward-rectifying in the presence of Cl⁻. Partial substitution of Cl⁻ outside by other anions led to a decrease in overall current and to a shift of the reversal potential toward the more positive new Cl⁻ equilibrium potential. This indicates that Cl⁻ is the most permeant species. Using the substitution with presumably functionally inert cyclamate ions as a reference, larger currents were observed only with Cl⁻ and Br⁻. A sizable decrease in conductance was observed with I⁻ and SCN⁻, indicating that the latter ions block the channel. The block by I⁻ became more effective with depolarization, an effect compatible with I⁻ being driven into the channel pore by transmembrane voltage. Within the voltage range examined, the block by SCN⁻ was only slightly voltage-dependent, though this was somewhat obscured by the opposite effect of SCN⁻ on the endogenous Cl⁻ channel (Fig. 1A). Thus, the anion selectivity of the *Torpedo* Cl⁻ channel expressed in Xenopus oocytes agrees with that observed in reconstitution (4, 9).

**Activation and Deactivation of Expressed Cl⁻ Current.** The resting membrane potential of injected oocytes was usually between -30 and -20 mV. From a holding potential of -30 mV whole-cell current responses were elicited by two subsequent hyperpolarizing pulses. The newly expressed Cl⁻ current was activated by the first hyperpolarizing pulse (Fig. 2A). During the interstimulus interval of 5 s only part of the Cl⁻ conductance was deactivated. This accounts for the initial large Cl⁻ current at the beginning of the second pulse. The subsequent small current decrease during the hyperpolarization most probably represents the transition of activated channels from higher conductance levels at depolarizing potentials to lower conductance levels at more negative membrane potentials (4).

A similar pulse protocol was used to search for expressed Cl⁻ channels in cell-attached patches. In most patches no channel openings were observed except those of stretch-activated channels, which exhibited more or less spontaneous activity (14). In a few patches, extremely high "leakage" current was present, which changed direction close to a relative potential (RP) of 0 mV—i.e., near the oocyte resting membrane potential. The amplitude of this current slowly decreased with sustained depolarization and drastically increased upon the hyperpolarizing test pulses (Fig. 2B). The time course of activation and deactivation of this current was similar to that of the whole-cell current (Fig. 2A). This
current could also be recorded with K+ Ringer in the bath and pipette, suggesting that the hyperpolarization-activated inward current was carried by an efflux of Cl− from the cytoplasm through newly expressed channels. In K+ Ringer the change of current direction was shifted to slightly hyperpolarizing potentials, which is consistent with a depolarization of the oocyte membrane.

The fact that either no specific Cl− currents or, in <5% of the patches, predominantly very large currents (ranging from several tens to several hundreds of pA) were recorded suggested that the newly expressed Cl− channels were incorporated into the oocyte plasma membrane in clusters.

Single-Channel Recordings. In <1% of the cell-attached patches, activity of single newly expressed Cl− channels was recorded that could easily be identified and distinguished from native channels by its activation behavior and single-channel characteristics. At RP = 0 mV, no channel openings were observed. Upon slow hyperpolarization the channel was activated between −50 mV and −70 mV RP. Fig. 3 shows bursts of single-channel activity at RP = −70 mV. The duration of bursts varied between 1 and 30 s and tended to decrease with prolonged hyperpolarization. Characteristically the channel rapidly fluctuated between three conductance levels during the burst, the lowest being equal to the closed state (0 pS).

According to the double-barreled channel model, the three conductance levels during a burst were defined as substates SO (nonconducting), S1 (one channel subunit open), and S2 (both channel subunits open). The frequency of the channel being in a certain substate was voltage-dependent (Fig. 4). At RP = −30 mV, the channel was predominantly in the S2 substate, whereas substate SO was more frequent with stronger hyperpolarizations. This is consistent with the macroscopic outward-rectifying properties of the Torpedo Cl− channel. In Fig. 4, only hyperpolarizing potentials are shown because, in that experiment, depolarization caused the channel to stop bursting within seconds or several tens of seconds. Even at RP = −30 mV, the channel normally deactivated within <1 min. We did not further investigate these slow voltage-dependent activation and deactivation channel processes.

Histograms of the single-channel current amplitude during bursts revealed that the substates are equally spaced. Fig. 5A shows an amplitude histogram of currents measured at RP = −90 mV with a superimposed fit of three Gaussian equations. Fig. 5B demonstrates the linear relationship between the mean current amplitude in the substates S1 and S2 and the RP. Linear regression analysis indicated chord conductances of 8.95 pS and 17.88 pS for the S1 and the S2 substate, respectively. For both substates, the extrapolated reversal potential was close to RP = 0 mV. Provided that the single-channel current was exclusively carried by Cl−, this indicates that the membrane potential of the oocyte was nearly identical to the Cl− equilibrium potential, as would be

Fig. 3. Occurrence of bursts of single-channel activity recorded in the cell-attached mode after prolonged (>10 min) hyperpolarization to RP = −70 mV. During a burst, the channel rapidly fluctuates between three substates. External and pipette solution, Ringer.

Fig. 4. Fluctuations of the newly expressed Cl− channel between the substates S0, S1, and S2 during bursts recorded (cell-attached patch) at different relative potentials as indicated at the end of each current trace. With less-hyperpolarizing potentials, the channel is more frequently in the S2 substate. Stars indicate spontaneous openings of stretch-activated channels; the current trace is cut off at these positions. External solution, Ringer.
and activity data is histograms for the substates. 

\[ \text{Lines} \] \text{ductance of experiments (each expected gating protochannels predicts different concentrations of Cl}^\text{-}(200 \text{mM double-barreled channel indirect evidence for but }} \]

\( \text{to channel expressed applied T.} \)

\[ \text{quencies (0)} \] \text{required by model.} \)

Substate the \( \text{distribution of substate calculated by dividing the time-averaged mean \text{value between the bursts and substate frequency (given by Eq. 8).} \)

\( \text{Bauer Neurobiology: } \)

\[ \text{amplitudes shown in Table 1; } \]

\( \text{in the reconstitution two substate amplitudes during the half-saturation values VO of } -58 \text{mV and } -105 \text{mV RP} \) differed greatly in these experiments. A possible explanation could be a variability of intracellular pH values in these experiments. A change of pH on the cis side by 1 unit has been found to shift the \( V_O \) value by >60 mV in the bilayer experiments (3). In addition, membrane injury induces considerable local intracellular acidification in oocytes (15). This might also occur upon membrane stretch produced by the cell-attached patch electrode.

A further test of the model was the comparison of the mean dwell times \( \tau \) in the three substates during a burst. \( \text{RP} = -90 \text{ mV was chosen to increase the frequency of substate S0. The values for } \tau \text{ shown in Table 1 are of the same order of magnitude as the data given by Hanke and Miller (3) for a membrane potential of } -100 \text{ mV (low-pass filtering 0.5–1 kHz). Since the resting membrane potential of injected oocytes measured in Ringer solution was nearly always between } -30 \text{ and } -20 \text{ mV, a relative potential of } -90 \text{ mV might correspond to an absolute potential of about } -115 \text{ mV. This estimation allows a comparison with data obtained in the bilayer system. On the basis of the measured mean dwell times, the rate constants } \lambda \text{ and } \mu \text{ were calculated independently in two different ways according to Eqs. 6 and 7. As required by the double-barreled model, the two methods gave similar values for the rate constants (Table 1). In addition, these values are in good agreement with the bilayer data given for membrane potentials of } -100 \text{ mV and } -120 \text{ mV (3).} \)

**DISCUSSION**

Our results show that the properties of the newly expressed T. marmorata Cl\(^{-}\) channel are almost identical to those

**Fig. 5.** (A) Distribution of current amplitude during bursts of channel activity at RP = -90 mV. Fit of three Gaussian curves to the data is superimposed. Mean current amplitude of the channel in substate S0 was equal to the mean current value between the bursts and was set equal to zero. The mean of the Gaussian fit to the current amplitude of S2 (-1.62 pA) is exactly twice the mean amplitude of S1 (-0.81 pA). This evaluation is from the experiment shown in Fig. 4. (B) Mean current amplitudes as determined by fits to amplitude histograms for the substates S1 and S2 in dependence of the relative potential. Lines are linear regressions to the data points and denote the chord conductance for the substates S1 (8.95 ± 0.26 pS, slope ± SE; \( r^2 = 0.998 \)) and S2 (17.88 ± 0.37 pS; \( r^2 = 0.999 \)).

expected with a high acquired Cl\(^{-}\) conductance. The conductance of the subunits of the expressed Cl\(^{-}\) channel (each about 9 pS) was slightly smaller than that found in bilayer experiments (each about 10 pS), which might be due to the different concentrations of Cl\(^{-}\) in the reconstitution experiments (200 mM Cl\(^{-}\) on both sides) and in the oocytes.

**Test of Double-Barreled Channel Model.** The model of a double-barreled channel composed of two independently gating protochannels predicts a binomial distribution for the substate frequencies (4). This is indeed fulfilled for the reconstituted T. californica channel (3, 4), providing strong, but indirect evidence for the double-barreled channel model. We applied the same kind of analysis to the T. marmorata channel expressed from cRNA, using the equations given in Materials and Methods.

Substate frequencies \( f \) and activation probability \( p \) during bursts were evaluated in three different experiments. The distribution of substate frequencies during a burst was always found to be nearly identical to the binomial distribution required by the model. In Fig. 6A, measured substate frequencies (○) are compared with theoretical values (bars) calculated from Eq. 2. Protochannel activation probability \( p \) was determined by dividing the time-averaged current by twice the mean protochannel current. The activation probability \( p \) increased with less-hyperpolarizing potentials. Fig.

**Fig. 6.** Comparison of measured data with values predicted by the requirements of the double-barreled model. (A) Histogram of relative frequencies \( f \) of the three substates during bursts at the indicated potentials. Filled circles denote the experimental data, and hatched bars denote theoretical values according to Eq. 2. The activation probability \( p \) was directly determined as the time-averaged current in a burst divided by the mean current amplitude of the substate S2. (B) Dependence of the activation probability \( p \) on relative potential. ●, Data for the same experiment evaluated in A; ■, data of another experiment, where the voltage dependence of \( p \) was shifted to the right. The continuous curves are fits (least squares) to the two sets of data according to Eq. 8. \( V_O = -105.47 \text{ mV (●)} \) or \( -57.65 \text{ mV (●).} \)

6B shows the voltage dependence of \( p \) from two different experiments. Each set of measured values for \( p \) could be described by a Boltzmann distribution (Eq. 8). The voltage dependence (given by the half-saturation values \( V_O \) of -58 mV and -105 mV RP) differed greatly in these experiments. A possible explanation could be a variability of intracellular pH values in these experiments. A change of pH on the cis side by 1 unit has been found to shift the \( V_O \) value by >60 mV in the bilayer experiments (3). In addition, membrane injury induces considerable local intracellular acidification in oocytes (15). This might also occur upon membrane stretch produced by the cell-attached patch electrode.
Table 1. Measured and calculated values of several kinetic
parameters predicted by the double-barreled channel model

<table>
<thead>
<tr>
<th>Parameter(s)</th>
<th>Value(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean $T_{90}$, $T_{95}$, $T_{98}$</td>
<td>11.7, 16.4, 25.1 ms</td>
</tr>
<tr>
<td>$f_{50}, f_{51}, f_{52}$</td>
<td>Measured</td>
</tr>
<tr>
<td></td>
<td>0.09, 0.43, 0.48</td>
</tr>
<tr>
<td></td>
<td>Calculated (Eq. 2)</td>
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<tr>
<td>$p$ (calculated, Eq. 3)</td>
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</tr>
<tr>
<td>$\lambda$ (calculated, Eqs. 6/7)</td>
<td>42.7/42.5 s$^{-1}$</td>
</tr>
<tr>
<td>$\mu$ (calculated, Eqs. 6/7)</td>
<td>19.9/18.4 s$^{-1}$</td>
</tr>
</tbody>
</table>

Mean $\tau$, mean dwell time for the indicated substrate; $f$, substrate frequency; $p$, channel subunit activation probability; $\lambda$, opening rate constant; $\mu$, closing rate constant.

Similar clustering of newly expressed channels has been observed for other cloned channels (e.g., K$^+$ channels; ref. 22). It is unclear whether this represents an artifact of the oocyte expression system, or whether clustering of channels also occurs in the electric organ, where single-channel data are not available.

A dimeric or a more general, multimeric channel structure may be of general importance since two other double-barreled Cl$^-$ channels, identified in the distal rabbit neuron (18) and in Aplysia neurons (19, 20), and a triple-barreled cardiac inward-rectifying K$^+$ channel (21) have been described. Our data show that the single polypeptide encoded by the cDNA is able to generate the completely functional double-barreled Torpedo Cl$^-$ channel. This heterologous expression of a channel with a multibarreled structure offers many possibilities for study of the relationship of structure and function in this novel class of channels.

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