A Novel Cl\(^{-}\) Inward-Rectifying Current in the Plasma Membrane of the Calcifying Marine Phytoplankton Coccolithus pelagicus\(^1\)

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We investigated the membrane properties and dominant ionic conductances in the plasma membrane of the calcifying marine phytoplankton Coccolithus pelagicus using the patch-clamp technique. Whole-cell recordings obtained from decalcified cells revealed a dominant anion conductance in response to membrane hyperpolarization. Ion substitution showed that the anion channels were selective for Cl\(^{-}\) and Br\(^{-}\) over other anions, and the sensitivity to the stilbene derivative 4,4'-disothiocyanostilbene-2,2'-disulfonic acid, ethacrynic acid, and Zn\(^{2+}\) revealed a pharmacological profile typical of many plant and animal anion channels. Voltage activation and kinetic characteristics of the C. pelagicus Cl\(^{-}\) channel are consistent with a novel function in plants as the inward rectifier that tightly regulates membrane potential. Membrane depolarization gave rise to nonselective cation currents and in some cases evoked action potential currents. We propose that these major ion conductances play an essential role in membrane voltage regulation that relates to the unique transport physiology of these calcifying phytoplankton.

Marine phytoplankton are key primary producers contributing as much as 40% of annual global carbon assimilation. Ion and nutrient transport across the plasma membrane of such unicellular marine algae is of central importance in maintaining cytoplasmic homeostasis and productivity in the marine environment. Despite their global importance, progress in understanding membrane transport mechanisms in marine phytoplankton has been slow.

The calcifying coccolithophorid phytoplankton such as Emiliania huxleyi and Coccolithus pelagicus often form massive monospecific blooms in oceanic waters that cover a total area of up to 1.4 million km\(^2\) annually (Brown and Yoder, 1994). They are responsible for forming extensive sedimentary beds of calcite and are considered to be the most significant producers of CaCO\(_3\) on Earth with a potential significant impact on global biogeochemical cycles and climate change (Riebesell et al., 2000; Zondervan et al., 2001) by contributing to carbon sequestration in ocean sediment and CO\(_2\) and dimethyl sulfide fluxes between the ocean and atmosphere. Although the ecophysiology of coccolithophores has been extensively studied, we know very little about the regulation of the underlying cellular processes during calcification.

Most calcifying plants and algae do so extracellularly, however coccolithophores are unique in that calcification occurs intracellularly. Plates or coccoliths are assembled in a specialized Golgi-derived coccolith vesicle and are secreted onto the cell surface where they interlock to form a shell or coccosphere (Fig. 1, A and B; for reviews, see Westbroek et al., 1984; Paasche, 2001). Coccogenesis is a highly regulated process and depends on a continuous flux of Ca\(^{2+}\) and dissolved inorganic carbon (Ci) most likely as HCO\(_3\)^\(^{-}\) (Buitenhuis et al., 1999; Berry et al., 2002) from the external medium into the coccolith vesicle. The molar fluxes of Ca\(^{2+}\) and Ci into the coccolith vesicle can equal the molar flux of photosynthetically fixed carbon (i.e. calcification/photosynthesis ratios of unity). Characterization of the ion transport mechanisms in the plasma membrane of coccolithophores is essential to understand the precise mechanisms and functional significance of calcification with respect to environmental physiology.

There is currently no information available concerning the electrical and ionic properties of the coccolithophore plasma membrane. To address this need, we have successfully applied the patch-clamp technique to investigate the primary membrane conductances in C. pelagicus cells. This provides a basis for understanding the membrane transport properties of these organisms and from which to identify pathways for and regulation of Ca\(^{2+}\) and Ci entry that is essential for calcification. Our results reveal...
a surprising regulation of membrane potential by a large Cl\(^-\)/H\(^+\) inward-rectifying conductance, which contrasts with the dominant K\(^+\)-rectifying properties reported for higher plant cells and marine diatoms and may reflect the unique transport requirements of this calcifying unicell.

**RESULTS**

**Cell Isolation**

The decalcification procedure produced intact cells with a clean plasma membrane on which high-resistance seals (1.34 G\(\Omega\) \(\pm\) 0.2, \(n = 216\)) could be obtained routinely with a patch pipette (Fig. 1). Decalcified cells remained viable, started to recalcify within hours, and after 2 to 3 d in culture, generated a complete layer of coccoliths (data not shown). Whole-cell recordings gave a mean cell capacitance of 7.6 pF (\(\pm\) 0.2, \(n = 174\)), which for an average cell diameter of 15 \(\mu\)m corresponds to a specific membrane capacitance of 1.07 \(\mu\)F cm\(^{-2}\).

**Membrane Potential Is Sensitive to Cl\(^-\) But Not K\(^+\)**

Zero current membrane potential (\(V_m\)) measurements were made in whole-cell current clamp mode under various internal and external ionic conditions (Table I). The K\(^+\) sensitivity of \(V_m\) was determined by perfusing 0.8 and 8 mM KCl-artificial seawater (ASW) over cells under current clamp with an intracellular solution containing 80 mM KCl. There was no significant change in \(V_m\) for this 10-fold change in external K\(^+\) concentration (Table I). However, \(V_m\) was highly sensitive to changes in external and internal Cl\(^-\) concentration, and \(V_m\) settled at or close to the calculated Cl\(^-\) equilibrium potential for each treatment (Table I).

**A Voltage-Dependent Cl\(^-\) Current Is Activated by Hyperpolarization**

Whole-cell voltage clamp recordings revealed a large conductance that was activated by hyperpolarization in every cell tested (\(n = 144\); Fig. 2A). The reversal potential of this current coincided precisely with the \(E_{Cl}\) when 8, 80, and 400 mM KCl were used in the recording pipette (Fig. 2B; Table I), showing that this current is an anion current. The reversal of the anion current at \(E_{Cl}\) was confirmed with tail current analysis (Fig. 2, C and D). The conductance of the anion current was strongly dependent on the internal Cl\(^-\) concentration (Fig. 2E), increasing with increased intracellular Cl\(^-\) concentration. Reversal of the anion current was insensitive to substitution of intracellular K\(^+\) for Cs\(^+\) (Table II).

Voltage-activation curves fitted to a Boltzmann function revealed shifts in the voltage activation of the Cl\(^-\) channel that closely followed \(E_{Cl}\). To assess the voltage dependence of gating, activation curves were derived from cells bathed in 2 mM SO\(_4\)\(^{2-}\)-ASW (to enable more accurate tail current resolution, see below). The half-activation voltage (\(V_{0.5}\)) was \(-4.4\) mV for a pipette solution containing 400 mM KCl and shifted negative to \(-30.1\) mV with 80 mM KCl in the
The activation kinetics of the Cl\textsuperscript{−} current were explored by fitting exponential curves to the initial non-steady-state currents stimulated by hyperpolarizing membrane pulses (Fig. 3). Activation was very rapid once the threshold voltage was reached with an average time constant of 2.5 ms (± 0.05, n = 8) for currents activated by a voltage step from −30 to −45 mV with 80 mM KCl in the pipette. The activation of the Cl\textsuperscript{−} current exhibited a voltage-dependent relationship whereby progressively more negative step voltages resulted in even faster current activation (Fig. 3B). The Cl\textsuperscript{−} current also exhibited rapid and voltage-dependent deactivation. Tail currents recorded in 2 mM SO\textsubscript{4}\textsuperscript{2−} ASW (Fig. 3C) deactivated more rapidly as the clamp command step became more positive (Fig. 3D).

The selectivity of the conductance to other anions was investigated by substituting intracellular Cl\textsuperscript{−} with Glu or nitrate (Table II). For each treatment, the inward current reversed at the equilibrium potential for Cl\textsuperscript{−}, showing that the anion current is highly selective for Cl\textsuperscript{−} over other larger anions. The relative permeability to Br\textsuperscript{−} was investigated in the same manner and revealed a reversal potential between E\textsubscript{Cl} and the calculated equilibrium potential for Br\textsuperscript{−} (Table II). The relative permeability sequence of the anion current was therefore Cl\textsuperscript{−} > Br\textsuperscript{−} > with no significant permeability to Glu NO\textsubscript{3}\textsuperscript{−}. However, the whole-cell conductance of the anion current when Br\textsuperscript{−} was the main permeating anion (i.e. pipette contained 400 KBr) was 1.39 ns/pF (± 0.38, n = 8), significantly lower than for the same concentration of KCl (6.65 ns/pF ±0.76, n = 8; see Fig. 2E).

### Table 1. Cl\textsuperscript{−} dependence of V\texttextsubscript{m} and anion current reversal potential

<table>
<thead>
<tr>
<th>[KCl] in Pipette</th>
<th>[KCl] in Bath</th>
<th>Measured V\textsubscript{m}</th>
<th>Calculated E\textsubscript{Cl}</th>
<th>Calculated E\textsubscript{K}</th>
<th>Reversal Potential of Inward Current</th>
</tr>
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<tbody>
<tr>
<td>80 mM KCl</td>
<td>80 mM KCl</td>
<td>0.85/543 mV</td>
<td>−29±1 mV</td>
<td>−38 mV</td>
<td>−120 mV</td>
</tr>
<tr>
<td>8 mM KCl</td>
<td>8 mM KCl</td>
<td>8/550 mV</td>
<td>−29±1 mV</td>
<td>−39 mV</td>
<td>−63 mV</td>
</tr>
<tr>
<td>8 mM KCl</td>
<td>8/550 mV</td>
<td>−58±2 mV</td>
<td>−58 mV</td>
<td>−9 mV</td>
<td>−62±4 mV</td>
</tr>
<tr>
<td>400 mM KCl</td>
<td>8/550 mV</td>
<td>−3±1 mV</td>
<td>−6 mV</td>
<td>−99 mV</td>
<td>−8±1 mV</td>
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<td></td>
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<td></td>
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<td>n</td>
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The Cl\textsuperscript{−} Current Rectifies in the Presence of External Sulfate

The presence of external SO\textsubscript{4}\textsuperscript{2−} had a dramatic influence on the rectifying properties of the Cl\textsuperscript{−} current. In the absence of SO\textsubscript{4}\textsuperscript{2−} in the external medium, the conductance, once activated, passed both outward and inward currents. This can be seen from the voltage-activated outward current and large persistent outward tail currents observed after current activation by a hyperpolarizing voltage pulse (Fig. 4A). Bath perfusion with 2 mM SO\textsubscript{4}\textsuperscript{2−} ASW caused a partial block of the voltage-activated outward current and corresponding tail currents (Fig. 4B) and partial rectification. Bath perfusion with 16 mM SO\textsubscript{4}\textsuperscript{2−} ASW abolished the voltage-activated outward current and outward tail currents resulting in full rectification (Fig. 4C). In contrast to the dramatic effects of external SO\textsubscript{4}\textsuperscript{2−} on the Cl\textsuperscript{−} current, substitution of MgCl\textsubscript{2} with MgSO\textsubscript{4} in the recording pipette had no significant effect (n = 5; data not shown).

Pharmacology and Regulation of the Cl\textsuperscript{−} Conductance

The voltage-dependent inward Cl\textsuperscript{−} current was reversibly blocked by external (200 µM, n = 9; Fig. 5A) but not internal Zn\textsuperscript{2+} (5 mM, n = 4). Full block was achieved by including 0.5 mM Zn\textsuperscript{2+} in the bath solution (n = 19). In SO\textsubscript{4}\textsuperscript{2−}-free media, Zn\textsuperscript{2+} ions blocked the outward voltage-activated and tail currents in addition to the inward component of the Cl\textsuperscript{−} current (Fig. 5B). The Cl\textsuperscript{−} current was blocked by both ethycrynic acid (n = 3) and the stilbene derivative 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (n = 5) in the external medium (Fig. 5, C and D). In all of the above treatments, the Cl\textsuperscript{−} current recovered on perfusion of fresh external media without inhibitor (data not shown).

Because algal and higher plant Cl\textsuperscript{−} channels are known to be sensitive to pH, ATP, and Ca\textsuperscript{2+} (Barbier-Brygoo et al., 2000), their effects were tested on the coccolithophore Cl\textsuperscript{−} current. The presence of up to 5 mM ATP in the pipette had no significant effect on
the Cl\(^{-}\) current (n = 5). Altering external pH from 8.0 to 5.5 had no significant effect (n = 3). Altering internal free Ca\(^{2+}\) from <10 nM to 1 \(\mu\)M had no effect on the Cl\(^{-}\) current (n = 7).

Depolarization-Activated Cation Currents

Depolarization-activated currents were investigated by selecting internal [Cl\(^{-}\)] such that Cl\(^{-}\) currents were not activated at the required holding voltage or by blocking the Cl\(^{-}\) current with Zn\(^{+}\). With 2 mM ZnCl\(_2\) in the bath, complete block of the Cl\(^{-}\) current was achieved (see Fig. 4B), and the cell could be clamped at voltages more negative than \(E_{Cl}\). On achieving block by Zn\(^{+}\) of the inward Cl\(^{-}\) current, membrane depolarization elicited a slowly activating outward current (Fig. 6A). With 400 mM KCl in the pipette and 2 mM Zn\(^{+}\)-ASW in the bath, the outward current activated from 0 mV and exhibited a \(V_{0.5}\) of +92 mV, voltage sensitivity of 20 mV for an e-fold change in current and gating charge of 1.3 (Fig. 6B). Tail currents reversed significantly more positive than \(E_K\) at −11 mV (±3, n = 3) and −17 mV (±3, n = 4) for pipette solutions containing 80 or 400 mM KCl, respectively (data not shown).

In addition to the slow outward currents, fast transient voltage-activated inward current spikes with a threshold between −50 and +10 mV were observed to a varying degree depending on the batch of cells. No action potential currents were detected in some batches, whereas in others, up to 70% of the cells exhibited this excitability (Fig. 6C). Under conditions of free-running membrane potential, the consequence of activating the action current would lead to a regenerative action potential. Action potential currents were observed in experiments where the recording pipette contained KCl, K-Glu, or CsCl. In every cell exhibiting excitability, the evoked currents...
the activation and the wide range of anions transported by this channel are regarded as iso-osmotic with the seawater membrane barrier for turgor generation, and coccolithophores cite plates does not present a significant mechanical resistance to osmotic regulation. Unlike most algal and higher plant cell walls, the coccolithophorid coccosphere consisting of interlocking calcite plates does not present a significant mechanical barrier for turgor generation, and coccolithophores are regarded as iso-osmotic with the seawater medium. Moreover, in the stable oceanic ionic environment (Kennish, 2001), coccolithophores are not normally subjected to large rapid fluctuations in salinity. It is unlikely therefore that the primary role of the Cl− channel described here is to mediate large osmolyte fluxes in response to external osmotic changes. Rather, the rapid kinetics of the C. pelagicus Cl− channel indicates a role in membrane potential regulation (see below).

A second characteristic of the C. pelagicus Cl− current that differs from higher plant anion channels is the insensitivity to modulatory factors. For example, guard cell plasma membrane anion channels are activated in a Ca2+- and ATP-dependent manner and are sensitive to intra- and extracellular pH (Schulz-Lessdorf et al., 1996), whereas the C. pelagicus Cl− channel was insensitive to these factors. Furthermore, guard cell anion channels are also modulated by organic acids (Hedrich et al., 1994) and the hormone abscisic acid, demonstrating the essential role these channels play in integrating responses to metabolic state and hormonal signaling with plasma membrane solute fluxes. Metabolic regulation of ion channels has also been demonstrated in Chara spp., where pH modulation of anion fluxes play a key role in cytosolic pH regulation (Johannes et al., 1998). The insensitivity of the Cl− channel in C. pelagicus to ATP, intracellular Ca2+, and extracellular pH indicates that this channel is primarily regulated by membrane voltage and the Cl− gradient across the plasma membrane although there may be other, as yet uncharacterized regulatory mechanisms.

The third contrasting characteristic of C. pelagicus anion channels is their high selectivity for Cl− over larger anions. Anion channels in broad bean (Vicia faba) guard cells (Dietrich and Hedrich, 1998) and Arabidopsis hypocotyl cells (Thomine et al., 1997; Frachisse et al., 2000) are significantly permeant to NO3−. Hypocotyl cells from Arabidopsis are also permeant to SO42−, supporting a role in the transport of mineral nutrients (Frachisse et al., 1999). In contrast, the fast inward rectifier Cl− channels of C. pelagicus are permeant to other halides but impermeant to NO3− and SO42−. It is clear therefore that the Cl− channel in C. pelagicus cannot be involved in uptake or release of these mineral nutrients. This is

### Table II. Effect of anion substitution on Cl− current

<table>
<thead>
<tr>
<th>Primary Salt in Pipette</th>
<th>Reversal Potential of Anion Current</th>
<th>n</th>
<th>Calculated $E_{Cl}$</th>
</tr>
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<tbody>
<tr>
<td>80 mM CsCl</td>
<td>−41 ± 2</td>
<td>30</td>
<td>−40</td>
</tr>
<tr>
<td>80 mM Cs-Glu</td>
<td>−70 ± 2</td>
<td>28</td>
<td>−68</td>
</tr>
<tr>
<td>80 mM CsNO3</td>
<td>−61 ± 2</td>
<td>11</td>
<td>−60</td>
</tr>
<tr>
<td>400 mM KCl</td>
<td>−8 ± 1</td>
<td>20</td>
<td>−6</td>
</tr>
<tr>
<td>400 mM KBr</td>
<td>−3 ± 3</td>
<td>9</td>
<td>−95</td>
</tr>
<tr>
<td>80 mM K-Glu</td>
<td>−75 ± 3</td>
<td>10</td>
<td>−69</td>
</tr>
</tbody>
</table>

DISCUSSION

The voltage and kinetic characteristics of the Cl− current of C. pelagicus show unique properties that distinguish it from anion currents of higher plants and other algae in several ways. First, it is activated by negative voltages, has a very steep voltage dependence and fast activation and deactivation kinetics, and rectifies strongly in the presence of normal seawater SO42− concentrations. In contrast, with few exceptions higher plant plasma membrane anion channels are activated by depolarization and can mediate anion influx at voltages more positive than the anion equilibrium potential (Barbier-Brygoo et al., 2000). Although anion channels activated by hyperpolarizing membrane potential have been described (Terry et al., 1991; Barbara et al., 1994; Elzenga and VanVolkenburgh, 1997), full functional characterization remains limited.

Higher plant anion channels activated by depolarization can be broadly divided into those that exhibit either fast (millisecond) or slow (second) activation kinetics. The plasma membrane anion channels of the guard cell have been studied in detail (Keller et al., 1989; Schroeder and Keller, 1992; Hedrich, 1994; Dietrich and Hedrich, 1998). The fast activation and rapid inactivation of R-type guard cell anion channels reflect a likely role in transient stabilization of $V_{m}$, whereas the slow kinetics of activation and inactivation and the wide range of anions transported by the $S$-type channel are properties suited to longer term solute loss during turgor regulation. Unlike most algal and higher plant cell walls, the coccolithophorid coccosphere consisting of interlocking calcite plates does not present a significant mechanical barrier for turgor generation, and coccolithophores are regarded as iso-osmotic with the seawater medium.
not unexpected because these algal unicells cells bloom in ocean waters that typically contain $<30 \mu M$ NO$_3^-$ and 2 $\mu M$ PO$_4^{3-}$, conditions, which impose the requirement of strict conservation of cellular nutrient pools acquired actively against such a large gradient.

The \textit{C. pelagicus} Cl\textsuperscript{-} current unusually displays features that closely resemble the classic K\textsuperscript{+} inward rectifier (Hille, 2001). These are: (a) opening at negative voltages with a steep voltage dependence, (b) gating and conductance dependent on the concentration of permeant ion, shifting toward the new equilibrium, and (c) fast activation kinetics. These characteristics are also common to animal ClC0 and ClC1-type Cl\textsuperscript{-} channels, which play a key role in stabilization of membrane potential (Jentsch et al., 2002, and refs. therein). The properties of the \textit{C. pelagicus} Cl\textsuperscript{-} current together with the observations that the current was highly selective, did not exhibit rundown, and was present in every cell, lead us to conclude that it functions as the primary plasma membrane inward rectifier, playing a fundamental role in the regulation of membrane potential and membrane excitability in this planktonic alga. Moreover, the dependence of voltage activation on intracellular Cl\textsuperscript{-} shows that the gating is coupled to the Cl\textsuperscript{-} electrochemical gradient, supporting the contention that $E_{Cl}$ dominates the membrane potential in \textit{C. pelagicus}. This is in marked contrast to the marine diatom \textit{Cosinodiscus wailesii}, where both current clamp and voltage clamp recordings indicate that the major inward-rectifying conductance is K\textsuperscript{+} selective and membrane potential is dominated by $E_K$ (Gradmann and Boyd, 1999). Interestingly, a single-channel study of \textit{Valonia utricularis} protoplasts has recently revealed a Cl\textsuperscript{-} channel with remarkably similar properties to the Cl\textsuperscript{-} conductance described here for \textit{C. pelagicus} (Heidecker et al., 1999), raising the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Activation and deactivation kinetics of the \textit{C. pelagicus} Cl\textsuperscript{-} current. A, Inward Cl\textsuperscript{-} currents activated by step hyperpolarization from a holding potential of $-30 \text{ mV}$. Activation kinetics were obtained by fitting a single exponential curve to each trace (solid lines). Pipette solution contained 80 mM KCl, and bath solution contained 16 mM SO$_4^{2-}$ ASW. B, Graph of activation time constant and hyperpolarizing test voltage for eight cells under the same recording conditions as illustrated by A. C, Example of deactivating tail currents from which deactivation time constants were derived. Cl\textsuperscript{-} currents were activated by hyperpolarizing the membrane to $-110 \text{ mV}$ followed by a sequence of deactivation voltage steps. Pipette solution contained 80 mM KCl and bath consisted of 2 mM SO$_4^{2-}$ ASW to resolve tail currents and to enable curve fitting of a single exponential to the deactivating current. D, Graph to illustrate deactivation time constant versus repolarization tail voltage. Deactivation time constants were derived from four cells under the same conditions illustrated in C. SEs are indicated.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Effect of SO$_4^{2-}$ on Cl\textsuperscript{-} current. A, Current traces (left) and corresponding steady-state current-voltage curve (right) for a family of hyperpolarizing voltage pulses in SO$_4^{2-}$-free ASW. The intracellular solution contained 40 mM KCl. Cell capacitance was 6 pF, and seal resistance was 2 G\textOmega. Note the outward Cl\textsuperscript{-} current at potentials positive of $E_{Cl}$ and the prolonged tail current. B, The same cell as in A except in the presence of 2 mM SO$_4^{2-}$ ASW. C, The same cell as in A except in the presence of normal 16 mM SO$_4^{2-}$ ASW. Note the disappearance of outward Cl\textsuperscript{-} current and tail current.}
\end{figure}
possibility that a dominant Cl$^{-}$/H$^{+}$ inward rectifier could be present in a range of marine algae.

Effects of external SO$_4^{2-}$/H$^{+}$ ions on the rectifying behavior of the Cl$^{-}$ current imply that the presence of SO$_4^{2-}$/H$^{+}$, a major conservative ionic component of seawater (28–30 m$^3$; Kennish, 2001) confers specific functional properties on the channel. The lack of effect of cytosolic SO$_4^{2-}$/H$^{+}$ shows that it influences channel behavior by binding to an external site. However, external SO$_4^{2-}$/H$^{+}$ does not affect Cl$^{-}$ efflux, therefore the SO$_4^{2-}$/H$^{+}$-binding site must be distinct from the channel pore or permeation pathway. In the absence of SO$_4^{2-}$/H$^{+}$, the Cl$^{-}$/H$^{+}$ channel, once activated, can pass Cl$^{-}$ ions both into and out of the cell. External SO$_4^{2-}$/H$^{+}$ blocks Cl$^{-}$ influx, probably by accelerating deactivation of the current such that tail currents are extremely brief. The presence of SO$_4^{2-}$/H$^{+}$ in seawater therefore enhances the rectification of the Cl$^{-}$ current by preventing any significant Cl$^{-}$ influx when the channel is activated. A further key role for the Cl$^{-}$/H$^{+}$ channel in C. pelagicus most likely lies in charge balance for other transport processes. Of particular significance is the likely balance of charge necessary during Ci uptake for calcification. Several reports suggest HCO$_3^{-}$/H$^{+}$ may be used as an external Ci substrate for calcification (Buitenhuis et al., 1999; Berry et al., 2002) and that coccolithophores can maintain Ci fluxes into calcite at rates similar to that of photosynthetic carbon uptake (Buitenhuis et al., 1999; Paasche, 2001). The necessary efflux of ions to balance Ci uptake could readily be met by the Cl$^{-}$/H$^{+}$ inward rectifier. Cl$^{-}$ efflux via the inward rectifier may also act to balance efflux of cations during the operation of active transport that is likely to occur to generate a coupling gradient for high-affinity nutrient acquisition. Information on the primary chemiosmotic pumps in the plasma membrane of halotolerant algae and unicellular marine phytoplankton is unfortunately very limited. The utilization of H$^{+}$/ATPases to generate electrochemical gradients is unlikely in ma-

Figure 5. Cl$^{-}$ channel pharmacology. A, Block of inward and tail Cl$^{-}$ current by perfusion of 200 $\mu$M ZnCl$_2$ in the external ASW medium. B, Complete block of Cl$^{-}$ current by 2 mM ZnCl$_2$ in the ASW medium. The pipette solution contained 400 mM KCl, and the external solution was SO$_4^{2-}$/H$^{+}$-free ASW. Note the tail current is also completely abolished by Zn$^{2+}$. C, The Cl$^{-}$ current is blocked by 0.5 mM ethacrynic acid (EA). The pipette solution contained 80 mM CsCl and the external solution was 16 mM SO$_4^{2-}$/H$^{+}$ ASW. D, Cl$^{-}$ current block by 300 $\mu$M 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid. The pipette contained 80 mM Cs-Glu solution, and the external medium was 16 mM SO$_4^{2-}$/H$^{+}$ ASW.

Figure 6. C. pelagicus plasma membrane currents activated by depolarization. A, Depolarizing voltages activate time-dependent outward currents with rapid deactivation. The pipette contained 400 mM KCl intracellular solution. B, Voltage activation characteristics of the outward cation current with pipette containing 400 mM KCl ($n = 3$). The activation curve is fitted with a Boltzmann function (see “Materials and Methods”) showing a $V_{0.5}$ of +90 mV. C, An "action potential"-like current is stimulated by depolarization. A non-leak subtracted current responses to a family of depolarizing voltage pulses from −74 mV to +126 mV. Seal resistance was 0.4 GOhm, and cell capacitance 7.5 pF. Pipette solution was 80 mM K-Glu. Examples of the evoked inward current spikes are marked with arrows. D, Expanded leak subtracted traces from A showing the voltage-dependent delay in activation of the action potential spike and detail of the time course of the action potential current.
rine algae at seawater pH of 8.0. Several studies have suggested that marine algae use a Na\textsuperscript{-}/H\textsuperscript{+}-based economy at the plasma membrane (Shono et al., 1996; Hildebrand et al., 1997; Popova et al., 1998; Gimmeler, 2000). Moreover, a Na\textsuperscript{-}-ATPase has been cloned from *Heterosigma akashiwo* (Shono et al., 2001). It is now essential to address the question of primary transport mechanisms in calcifying unicellular algae to fully understand how these marine algal cells integrate plasma membrane ion transport during calcification, nutrient acquisition, cell signaling, and cellular ionic homeostasis.

An outward-rectifying current is likely to regulate the *C. pelagicus* membrane potential in response to events that cause depolarization. Unlike the *C. pelagicus* Cl\textsuperscript{-} current, the outward cation current exhibits voltage activation and kinetic characteristics that are similar to those of higher plants (White, 1997). Because nonselectivity is a common feature of many plant cation channels (Pineros and Tester, 1997), we are currently investigating the selectivity and regulation of this current to determine whether it can mediate the sustained Ca\textsuperscript{2+} influx required for intracellular calcification.

Although the occurrence of action potentials and associated currents is widely distributed among algae and higher plants, the excitable property of the *C. pelagicus* membrane is thus far unique. With the exception of the *Chlamydomonas* sp. photoreceptor current, the kinetics of algal and plant action potentials and membrane potential transients studied to date are slow (Harz et al., 1992; Miedema and Prins, 1993; Schonknecht et al., 1998), the time course of the characean action potential being 3 to 5 s. In contrast, the kinetics of the *C. pelagicus* currents that underlie action potentials are very rapid, with the current response complete within 70 ms. Furthermore, in algae and higher plants, the *E*\textsubscript{Cl} is usually far more positive than *V*\textsubscript{m} and anion channels underlie electrical depolarization during regenerative action potentials and oscillations. For example, in *Chara* spp., plasma membrane Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels underlie action potentials that are initiated by electrical depolarization or mechanical stimuli (Shimmen, 1997; Thiel et al., 1997; Biskup et al., 1999). A similar mechanism for membrane excitability involving transient increase in Cl\textsuperscript{-} conductance is present in the marine diatom *C. valesii* where they are proposed to be involved in buoyancy regulation (Gradmann and Boyd, 2000). The action potential currents in *C. pelagicus* differ fundamentally in that the inward-depolarizing current underlying this excitability is not carried by Cl\textsuperscript{-}, because activation occurs at voltages positive of *E*\textsubscript{Cl} and *E*\textsubscript{K}, implying voltage activation of Ca\textsuperscript{2+} and/or Na\textsuperscript{+} channels during the depolarizing phase. The function of the rapid action potential current activated by moderate membrane depolarization in *C. pelagicus* is not clear, but it likely plays a role in environmental signaling possibly via transient Ca\textsuperscript{2+} elevation.

In summary, using the whole-cell patch-clamp technique, we have characterized the major Cl\textsuperscript{-} inward rectifier of the calcifying phytoplankton *C. pelagicus*. To our knowledge, this represents the first successful patch-clamp study of any marine phytoplankton cell providing detailed information on the properties and conductances of the plasma membrane. The results show that, unlike marine diatoms, a novel Cl\textsuperscript{-} inward rectifier tightly regulates the coccolithophore membrane potential, modulates membrane excitability, and may act as an electrical shunt for essential nutrient and Cl transporters. The *C. pelagicus* plasma membrane also exhibits a nonselective, outward-rectifying cation current that may also act as a Ca\textsuperscript{2+} influx pathway. A major goal is to understand how these predominant conductances in the membrane are coordinated to regulate Ca\textsuperscript{2+} and Cl uptake during calcification.

**MATERIALS AND METHODS**

**Growth of Cell Culture**

*Coccolithus pelagicus* (PLY 182G) cultures were obtained from the Plymouth Culture Collection and maintained as batch cultures in ASW consisting of 450 mM NaCl, 30 mM MgCl\textsubscript{2}, 16 mM MgSO\textsubscript{4}, 8 mM KCl, 10 mM CaCl\textsubscript{2}, and 2 mM NaHCO\textsubscript{3}. The ASW was supplemented with 500 μM NaNO\textsubscript{3}, 32 μM K\textsubscript{2}HPO\textsubscript{4}, 1 μM Fe-EDTA, and trace metals (Guillard and Ryther, 1962). Cultures were maintained in 250-mL polycarbonate flasks at 15°C under 150 μmol m\textsuperscript{-2} s\textsuperscript{-1} light from cold fluorescent tubes. Cultures typically had a 4- to 6-d lag period when seeding cultures with a starting cell density of 300 to 500 cm\textsuperscript{-3}. The exponential growth phase occurred between d 6 and 24, after which the culture entered a stationary phase. A maximum cell density of approximately 10,000 cells cm\textsuperscript{-3} was observed. (See Table 1 for details on cell density and growth conditions.)

Specimens of *C. pelagicus* were prepared for scanning electron microscopy by filtering cells onto 13-mm polycarbonate filters, drying for 24 h at 40°C, and mounting the dried filter on an aluminum stub. Stubs were gold-coated gold before examination with a microscope (JSM-35C, JEOL, Tokyo).

**Decalcification and Protoplast Isolation**

Samples of cells (5 cm\textsuperscript{3}) were allowed to settle passively before removing the culture solution to produce a concentrated cell sample in approximately 0.2 cm\textsuperscript{3}. Five cubic centimeters of 25 mM EGTA in Ca\textsuperscript{2+}-free ASW was then added to the cell concentrate, and the cells were mixed gently with a plastic transfer pipette. The cells were allowed to settle for a further 15 min before removing the EGTA-ASW media and adding a further 5 cm\textsuperscript{3} of fresh EGTA-ASW. After a further 10 min, the cells were mechanically agitated by a series of rapid aspirations and expulsions with a plastic transfer pipette before transferring to a recording chamber with a coverslip base. The chamber was secured onto a cooled microscope stage (Research Instruments, Penzryn, UK) mounted on an inverted microscope (Axiovert, Zeiss, Welwyn Garden City, UK) and maintained at 15°C. To establish that the cells were undamaged by the isolation procedure, samples of decalcified cells were maintained as above, and recalcification was monitored by inspection on the inverted microscope over 4 d.

**Patch-Clamp Recording and Analysis**

Patch electrodes were fabricated from GC150F glass capillaries (Clark Electromedical, Pangbourne, UK) using a pipette puller (P-833, Narashige, Japan) and filled with an internal solution containing (mM): 140 KCl, 2.5 MgCl\textsubscript{2}, 5 HEPES, pH 7.2. The patch-pipette resistances varied between 1 and 5 MΩ, and the bridge balance was adjusted to maintain the internal and external conductances equal. Impalements were made with a glass micropipette, filled with an internal solution containing (mM): 140 KCl, 2.5 MgCl\textsubscript{2}, 5 HEPES, pH 7.2. The patch-pipette resistances varied between 1 and 5 MΩ, and the bridge balance was adjusted to maintain the internal and external conductances equal.
Phytoplankton Whole-Cell Currents

Tokyo. Unpolished pipettes were filled with 0.22 μm of filtered internal recording solution (Millipore, Watford, UK) consisting of 5 mM MgCl₂, 5 mM HEPES, and 2 mM EGTA, pH 7.2. K⁺ and Ca²⁺ salts were added as described in “Results” and figure legends. The osmolarity was brought to between 1,000 and 1,200 mosmol L⁻¹ by adding sorbitol. Pipettes were connected to the head stage of an amplifier (Axopatch 200B, Axon Instruments, Foster City, CA) mounted on a micromanipulator (Research Instruments, Pencryn) connected to a PC running PCLAMP acquisition and analysis software (Axon Instruments).

The recording chamber volume was 1.5 cm³, and external solutions were exchanged using gravity-fed input and suction output at a rate of 5 cm³ min⁻¹. Patch pipettes varied in resistance from 3 to 15 MΩ depending on the filling solution. The tips of the electrodes were coated in beeswax to minimize stray capacitance but were not fire-polished. Whole-cell recordings were obtained by applying combinations of gentle suction, negative pipette potential, and application of a transient current pulse. The whole-cell recording typically stabilized within 60 s, and a further 3 to 5 min was allowed for the pipette contents to fully equilibrate with the cell before voltage-clamp recordings were made. Corrections were made for liquid junction potentials as described previously (Taylor et al., 1996). Leak subtraction was either achieved on-line using the acquisition software (eight pre-pulses) or offline using the input resistance measured just before a family of voltage-clamp pulses. Cell capacitance was estimated using the compensation circuitry of the amplifier, and steady-state currents were corrected for the pipette series resistance offline.

Voltage activation curves were acquired by measuring the peak tail current activated by a series of voltage pulses. The tail currents were normalized to the maximum peak current and plotted against activation voltage before fitting with a Boltzmann function using the CLAMPAPEX (axon instruments) software as follows:

\[ I = I_{\text{max}}/1 + \exp((V_{1/2} - V)/S)/S \]

where \( I_{\text{max}} \) is the normalized maximum peak tail current (i.e., 1), \( V_{1/2} \) is the test voltage, and \( S \) is the slope of voltage activation given by \( S = RT/\Delta F \) where \( R \), \( T \), and \( F \) have their usual thermodynamic meaning and \( S \) is the gating charge.

Received July 25, 2002; returned for revision August 18, 2002; accepted October 20, 2002.

LITERATURE CITED


