A9C sensitive Cl\textsuperscript{-} accumulation in A. thaliana root cells during salt stress is controlled by internal and external calcium

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The involvement of chloride in salt stress symptoms and salt tolerance mechanisms in plants has been less investigated in the past. Therefore, we studied the salt-induced chloride influx in Arabidopsis expressing the GFP-based anion indicator Clomeleon. High salt concentrations induce two phases of chloride influx. The fast kinetic phase is likely caused by membrane depolarization, and is assumed to be mediated by channels. This is followed by a slower “saturation” phase, where chloride is accumulated in the cytoplasm. Both phases of chloride uptake are dependent on the presence of external calcium. In general: with high [Ca\textsuperscript{2+}] less chloride is accumulated in the cytoplasm. Surprisingly, also the internal calcium availability has an impact on chloride transport.

A complete block of the second phase of chloride influx is achieved by the anion channel blocker A9C and trivalent cations (La\textsuperscript{3+}, Gd\textsuperscript{3+} and Al\textsuperscript{3+}). Other channel blockers and diuretics were found to inhibit the process partially. The results suggest that several transporter species are involved here, including electroneutral cation-chloride-cotransporters, and a part of chloride possibly enters the cells through cation channels after salt application.

Introduction

Many cellular factors have been found to influence the plants ability to withstand excessive salt in the soil.\textsuperscript{15} Focusing on the ionic aspects of high salinity, sodium (Na\textsuperscript{+}) and chloride (Cl\textsuperscript{-}) are the most abundant ions found on arable land affected by salinity worldwide. Until now salt stress research has mainly paid attention to Na\textsuperscript{+}-transport and its cellular regulation. Genetic manipulations of the involved Na\textsuperscript{+}-transport mechanisms already led to improvements of salt tolerance in model organisms and certain crop species.\textsuperscript{6-9} However, the transport of the concomitant anion (i.e., chloride) and the regulatory mechanisms are less investigated. Chloride has long been considered an inert anion being tolerated in a wide concentration range and as counter-ion to K\textsuperscript{+} responsible for cell turgor regulation.\textsuperscript{10} Nevertheless, many plant species are highly sensitive to high [Cl\textsuperscript{-}]\textsuperscript{11-15} and the term ‘chloride toxicity’ is well established,\textsuperscript{14} although the effects of chloride toxicity show a broad variability, even within species.\textsuperscript{15}

The principal driving force for Cl\textsuperscript{-} flux across the plasma membrane is its electrochemical gradient. Under normal conditions (i.e., [Cl\textsuperscript{-}]\textsubscript{out} < [Cl\textsuperscript{-}]\textsubscript{cyt}) there is a pH-dependent chloride uptake via a ΔpH-driven symport of Cl\textsuperscript{-} and nH\textsuperscript{+}.\textsuperscript{16-19} Here, ATP is consumed by H\textsuperscript{+}-pumps, and the term to be referred to, is secondary active transport.

Under salt stress conditions, however, Cl\textsuperscript{-} ions move along an “energy-independent” way driven by the electrochemical gradient.\textsuperscript{20} Thus, it is proposed, that the influx of chloride across the plasma membrane is mostly passive.\textsuperscript{14} Anyway, passive influx is possible only, when the membrane potential is more positive than its equilibrium potential. This situation can occur when the plasma membrane is depolarized. There is evidence that depolarization due to sodium influx under salt stress conditions (i.e., 100 mM NaCl and more) does not yield values more positive than -50 mV.\textsuperscript{21,22} According to the Nernst equation such a membrane potential at 100 mM [Cl\textsuperscript{-}]\textsuperscript{out} would yield no more than 14 mM [Cl\textsuperscript{-}]\textsubscript{cyt}.

In a previous study\textsuperscript{23} higher cytoplasmic chloride concentrations were reported. A cytoplasmic chloride concentration of approximately 75 mM was observed after several hours of salt application (100 mM NaCl). This seemingly contrasting finding triggered investigations reported in this study.

For maintaining the charge balance in a salt stressed cell, it is crucial, that uptake of chloride is counterbalanced either by the uptake of a cation or by loss of another anion. It is known that the presence of nitrate in soils can ameliorate the toxic effect of excess Cl\textsuperscript{-},\textsuperscript{24} seemingly due to the competitive effect of NO\textsubscript{3}\textsuperscript{-} on Cl\textsuperscript{-} influx.\textsuperscript{25-29} However, an opposite effect has also been described.\textsuperscript{30,31} Na\textsuperscript{+} ions are in surplus during salt stress. If NO\textsubscript{3}\textsuperscript{-} is transported out and Na\textsuperscript{+} and Cl\textsuperscript{-} into the cell then, the charge...
A hypersaline environment results in a disturbance of the ionic homeostasis not only for Cl\textsuperscript{−} and K\textsuperscript{+}, but also for Ca\textsuperscript{2+}.\textsuperscript{2,21} NaCl treatment causes cytosolic increase of Ca\textsuperscript{2+},\textsuperscript{2,21} which is hypothesized to work as a messenger triggering specific stress responses (e.g., SOS1 transporter).\textsuperscript{38,39} Calcium is also known to meliorate the growth of plants on saline soils.\textsuperscript{36,40-42}

It was previously shown that NaCl-induced chloride transport is restricted by external divalent cations like Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (10 mM) and completely blocked by low concentrations of lanthanum ([La\textsuperscript{3+}] < 2 mM).\textsuperscript{23} Yet, no plasma membrane localized chloride transporter is known, which can be inhibited by calcium. Thus, there was also need to characterize this feature of NaCl-induced chloride entry in more detail. Hence, we have studied salt stress-induced chloride influx into Arabidopsis root cells expressing Clomeleon under influence of different anion transport inhibitors, and diuretics; as well as varying calcium concentrations.

**Results**

Chloride influx into arabidopsis root cells exhibits two distinct phases- the fast “depolarization phase” and the slow “saturation phase.” After the application of high external concentrations of NaCl to Arabidopsis roots expressing Clomeleon,\textsuperscript{23} two distinct phases of chloride influx kinetics can be observed in epidermal roots cells (Fig. 1).

The rapid part of the kinetic lasts just a few minutes (full red line) and is dependent on the applied salt concentration. Here, it is named the “depolarization phase,” because passive chloride influx due to sodium triggered membrane depolarization is assumed.\textsuperscript{37,21,22,32,33,43,44} After approximately 8 min the chloride influx slows down and a second phase follows: the “saturation phase” (dotted red line). The amplitudes of both phases depend not only on the applied salt concentration (Fig. 1), but also on the extracellular pH (Fig. S1) and on the kind of corresponding cations.\textsuperscript{36}

External and internal calcium determine the chloride influx kinetics during both phases. External calcium is able to inhibit the salt-induced chloride influx.\textsuperscript{23} This has been also described earlier\textsuperscript{44} and is studied here in more detail. The inhibition of Cl\textsuperscript{−}-influx is more pronounced with increasing concentrations of external calcium (Fig. 2). Omitting Ca\textsuperscript{2+} during the entire experiment, leads to a maximum chloride influx during the “depolarization phase” (Fig. 2, blue curve). Superfusion with 10 mM CaCl\textsubscript{2} inhibits this phase significantly. During the “saturation phase” a dependency on external [Ca\textsuperscript{2+}] becomes obvious too. Ten mM CaCl\textsubscript{2} are able to block the chloride influx completely during this phase. Generally, the higher the externally applied [Ca\textsuperscript{2+}], the less pronounced is the chloride influx during both phases.

Similar results can be obtained when internally available calcium is varied, i.e., when plants are grown on media with varying Ca\textsuperscript{2+} concentrations (Fig. 3). This is a novel finding. Here,
increased concentrations of calcium in the growth medium lead to a less pronounced chloride uptake. Calcium deficient plants (grown on a medium lacking calcium) show the strongest response to salt application and also to the hypotonic shock caused by withdrawal of salt (Fig. 3, red curve). A possible effect of an adaptation to higher chloride concentration of plants grown on media containing more CaCl₂ (Fig. S2B) on the chloride influx during salt stress can be ruled out. Since 50 mM [Cl⁻] in the growth medium have only a marginal effect on the chloride influx (Fig. S3), consequently, the effect of 10 mM [Cl⁻] as shown in Figure 3 (black curve) has to be much less pronounced.

In order to determine, how calcium can influence the chloride transport during the slow phase, a calcium channel blocker—nifedipine—was added (Fig. 4). Nifedipine, a dihydropyridine, blocks Ca²⁺ entry by binding to the α₁ subunit of voltage-gated cation channels. Here, it was applied approximately 5 min after onset of salt stress, i.e., during the “depolarization phase.” No significant change in the first phase, when compared with the control, occurred. However, during the “saturation phase” the addition of nifedipine led to an enhanced chloride influx.

Chloride flux is sensitive to A9C and to other inhibitors. Considering electrophysiological aspects we assumed that during salt application, chloride cannot enter the cell simply “downhill.” A transporter family taken into account is the group of cation-chloride-cotransporters. Until now, only one putative cation-chloride-cotransporter (AtCCC) in Arabidopsis thaliana has been reported. In Xenopus oocytes, this transporter was characterized as a bumetanide-sensitive Na⁺-K⁺-2Cl⁻-cotransporters (NKCC).

Here, the effects of different diuretics (e.g., bumetanide), channel blockers and inhibitors were studied in more detail, in order to test whether or not a cation-chloride-cotransporter is involved in the NaCl-induced chloride entry. The experiments with Arabidopsis thaliana plants expressing Clomeleon were aimed at the “saturation phase” only, since active chloride influx is proposed.

The results (Figs. 5 and 6) of the experiments show, that anthracene-9-carboxylic acid (A9C, 100 μM, see also Table S1), a potent anion channel inhibitor, is capable to inhibit the chloride influx completely. The inhibition by indanyloxyacetic acid 94 (IAA-94, 100 μM), a known anion channel blocker (Fig. 6, Table S1) is around 40% lower than that of A9C and comparable to the effect of 4,4'-diisothiocyanatoestilbene-2,2'-disulfonic acid (DIDS, 5 μM). Diuretics (Fig. 6, Table S1) such as bumetanide (BUME, 100 μM), furosemide (FURO, 100 μM) and torasemide (TORA, 100 μM), capable to inhibit NKCCs in mammalian cells also have capabilities to partially inhibit chloride entry into plant cells. However, their inhibitory effect is less than that observed with IAA-94 and DIDS (Fig. 6, Table S1).

It was previously shown that the trivalent cation La³⁺ is a non-selective anion channel blocker. Along with lanthanum, other trivalent cations such as gadolinium and aluminum, are known to inhibit the activity of calcium channels. La³⁺ was already shown to be able to block salt stress-induced chloride flux into Arabidopsis root cells. Here, besides La³⁺ we also tested the effect of the other two trivalent cations Gd³⁺ and Al³⁺ (Fig. 7) and could show that both are potent inhibitors of chloride transport as well. The removal of the trivalent cations from the perfusion solution shows that in case of Al³⁺ and La³⁺ (red and green trace) the block is irreversible, whereas Gd³⁺ (black trace) can be partially washed out to resume Cl⁻-entry. Another difference becomes obvious, when inspecting the kinetics directly after addition of the respective trivalent cation. While with La³⁺ and Gd³⁺ the inhibition occurs some minutes after addition, with Al³⁺ the response is immediate.
shows that also internally available calcium has an influence on both phases of chloride influx. From the electrophysiological point of view it seems unlikely that the import of chloride during the second phase is passive through channels. The influence of external calcium during this second was already reported earlier. Our results are in agreement with this finding, showing that the second phase can be blocked by calcium (Fig. 2).

Figure 6. Inhibition rate of different effectors on salt induced chloride influx. The inhibition rate was calculated as a percentage decrease in the slope of the emission ratio of control experiments (without inhibitor) for the time interval 45 min ≤ t ≤ 60 min. Data obtained from 5 experiments each, as shown in Figure 5. The CCC inhibitors are given as gray columns.

Discussion

The use of “self-reporting” plants expressing genetically encoded indicators for anion monitoring can provide a general view on the transport of specific ions. The capability of monitoring ion flux in whole and intact plants represents an approach toward a better comparability of results of lab experiments to the conditions plants experience in the field. In the case of Clomeleon, evidence can be provided about the amount of chloride in the cytoplasm of plant cells in a living object during different treatments. Nevertheless, Clomeleon has cross-sensitivities (pH, redox). Hence, all results have to be interpreted carefully, because salt stress may also impact on these “cross-parameters.”

NaCl-induced chloride influx into Arabidopsis root cells exhibits two distinct phases possibly reflecting two different transport mechanisms. During salt treatment passive import of chloride into the cytoplasm was postulated. However, this can occur only, when the plasma membrane becomes depolarized. Depolarization has been frequently observed during increased NaCl uptake. In particular, Felle showed in Sinapis alba a membrane depolarization due to chloride treatment. He observed two different kinetic phases in membrane potential changes after increasing the external [Cl]. The initial depolarization of the plasma membrane occurred not as a consequence of Na+ or K+ flux and was followed by a slow hyperpolarization.

In agreement with Felle and with Shabala et al. our results show, that the chloride influx triggered by NaCl, as shown in Figure 1 also exhibits two distinct phases. They are named here accordingly to their putative characteristics: “depolarization” and “saturation” phase. The salt-induced membrane depolarization recorded in Arabidopsis by Shabala et al. was dependent on externally added calcium. Calcium on its own produced a less negative membrane potential. Consequently, the subsequent salt-induced depolarization had lower amplitude. The recorded influence of calcium on the depolarization was explained by the action of calcium on K+ efflux. Other studies propose that the ameliorative effect of calcium lies in its effect on Na+-permeable non-selective cation channels (reviewed in refs. 51 and 52).

We assume that the rapid “depolarization” phase of chloride influx (Figs. 1–4) is triggered by changes in membrane potential. Thus, the amplitude of chloride influx in this phase should be strongly dependent on the depolarization caused by salt treatment. As long as the membrane potential is depolarizing a passive influx of chloride is possible. Furthermore, we propose that external calcium ameliorates the salt-induced chloride influx in the rapid phase (Fig. 2) by reducing the depolarization amplitude. It remains to be shown that this is due to a block of cation channels.

From the electrophysiological point of view it seems unlikely that the import of chloride during the second phase is passive through channels. The influence of external calcium during this second was already reported earlier. Our results are in agreement with this finding, showing that the second phase can be blocked by calcium (Fig. 2).

Figure 3 shows that also internally available calcium has an influence on both phases of chloride influx. Plants grown on media containing different Ca concentrations were used here to study the effect of internal Ca+ availability. Salt-induced chloride influx is maximal when calcium is low on both sides of the plasma membrane. Increasing Ca+ supply in the growth medium and thus increasing internal Ca+ availability (Fig. S2A) reduces the observed chloride influx (Fig. 3). This finding is in line with studies which demonstrate the amelioration of salt stress symptoms when calcium is added to the growth substrate. Internal Ca+ availability leads to an increased Ca+ efflux rate and may
thus increase the calcium concentration in the cell wall and in the vicinity of the root.37 Internal calcium stores are mobilized due to salt treatment and lead to an abrupt but only short-lasting increase in the cytoplasmic calcium content.37 The timing of this increase corresponds to the here observed rapid phase of the chloride influx. Taken together, calcium acts as a mediator of the salt-induced chloride influx during both phases irrespective of its localization.

Chloride entry depends on salt sensitivity and salt pre-treatment of the plant. Hajibagheri et al.38 used X-ray microanalysis and present data which nicely give numbers between 100 and 1000 μM chloride per gram dry weight in Zea mays. Since dry weight is about 5% of fresh weight these numbers correspond to 5 to 50 mM Cl− related to fresh weight. This is in good agreement with the data presented here (Figs. 1–3) when it is taken into account that the experiments presented here are within a few hours time frame and that Hagibagheri et al.38 monitored ion concentrations over 2 wk.

Characteristics of the chloride transport during the slow 'saturation'- phase - a pharmacological approach. The uptake of chloride into the plant cells and especially the kind of transporters involved, have not been characterized yet. As already mentioned, we propose for the rapid phase of chloride influx a passive import mechanism. For the slow “saturation” phase an electroneutral transport of chloride via some kind of cotransporter is likely from the electrophysiological point of view.

In animal systems four different groups of electroneutral cation-chloride cotransporter (CCC) are distinguished due to their sensitivity to various inhibitors.86,89 Here, they are able to inhibit the chloride flux by 30% to 50% (Fig. 6). Other cation-chloride-cotransport inhibitors, aiming specifically at NCCs and KCCs, show different results (Fig. S4) and led to acidification of the cytoplasm as revealed by control experiments (Fig. S5). Due to the pH cross-sensitivity of Clomeleon, data obtained in presence of these inhibitors cannot solely be interpreted in terms of chloride concentration changes. This result suggest, that NKCCs are involved, but do not cover the entire NaCl-induced chloride transport observed here. On the one hand, this may be explained by the properties of AtCCC1. Colmenero-Flores et al.86 have shown that AtCCC1 is dependent on the coexistence of all three ions (Na+, K+ and Cl−). But, during salt stress only Na+ and Cl− are present in excess. Thus, the performance of AtCCC1 would depend mainly on the availability of K+. On the other hand, we cannot omit the possibility, that at least a part of the chloride transport during this slow phase is mediated by channels. There are several anion channels in the plasma membrane of plant cells and seven chloride channels (ClCs) characterized in Arabidopsis thaliana so far.68,69 None of these characterized ClCs is localized in the plasma membrane68,69 and the majority of them is responsible for nitrate rather than chloride homeostasis in the cell, due to a characteristic amino acid motif in the selectivity filter of these transporters.65-69 From anion channels localized in the plasma membrane none is known to be inhibited by calcium. This is in contrast to the results shown here (Figs. 2 and 3). In addition, a channel driven transport of chloride would be electronegic (i.e., non-electroneutral) and a hyperpolarization as observed by Felle37 would not allow the entry of negative charged ions unless there is a co-transport of positive ions (Na+ or H+) or an antiport of negative ions (e.g., nitrate).

The effect of channel inhibitors like A9C on different plant anion channels was described earlier by others.70 The data reported here show that anion channel inhibitors are able to reversibly inhibit the second phase of salt-induced chloride influx (Figs. 5 and 6). This is in contrast to the assumption of an electroneutral CCC. But since the effects of such inhibitors are not clearly correlated with just one transporter species, this statement is rather speculative.

La3+ and Gd3+ are usually used to block calcium permeable channels. However, Lewis et al.48 showed that La3+ causes non-selective effects on many different transport mechanisms, also inhibiting anion channels. In addition, lanthanides influence many other cellular functions (e.g., Kawano et al.71) and can even alter gene expression.72,73 Al3+ as well is known to block calcium permeable transporters.74 The data given here (Fig. 7) suggest that Gd3+, La3+ and Al3+ inhibit channels (most probably calcium permeable channels).75,76 responsible for the salt-triggered changes of membrane potential mentioned above, thus having an indirect effect on the chloride transport. The differences between the onsets of inhibition after inhibitor application in Figure 7 can be explained by the target location. Al3+ shows an immediate inhibition (Fig. 7, red curve) and thus very likely binds irreversibly to a target on the exterior surface of the plasma membrane,74 whereas La3+ and Gd3+ are able to enter the cytoplasm77 and possibly act on several and different cellular targets and hence present a biphasic action giving rise to a transient signal peak.

The calcium channel blocker nifedipine seemingly acts in a different way. We could show an opposite effect, i.e., an acceleration of the chloride influx during the second phase (Fig. 4).
The magnitude of this kinetic corresponds to that obtained with calcium-deficient plants (Fig. 3, black curve). Nifedipine has no influence on the “depolarization phase” suggesting that calcium acts differentially on both phases of chloride influx.

One of the possibilities explaining those partially inconsistent results would be an electroneutral Cl⁻/H⁺ symport as described in Sinapis alba or in Chara.⁵,⁶ The intruding protons should normally lead to an acidification of the cytoplasm. As this is obviously not the case (Fig. S1), the protons are certainly pumped out immediately. This, in consequence, would lead to a hyperpolarization of the cell as described by Felle.⁷

Cation channels could provide another route through which chloride ions invade the cells during the slow phase. Some properties of the hyperpolarization-activated calcium permeable channel found in Arabidopsis root hairs described by Véry et al.⁷ fit well with the findings presented here. This channel is selective for Ca²⁺ over K⁺ and Cl⁻ (PCa/PK = 15; PCa/PCl = 25) and is fully blocked by < 100 μM trivalent cations (La³⁺, Al³⁺, Gd³⁺). The proposed inhibition mechanism of trivalent cations is a direct blocking of the channel pore.⁴⁵ On the other hand, nifedipine, which enhances the chloride influx (Fig. 4), is supposed to bind directly to the calcium ion within the channel pore and thereby stabilizes long lasting channel openings.⁴⁶ This could support chloride influx as seen in Figure 4.

Material and Methods

In vivo monitoring of chloride influx. Arabidopsis thaliana plants expressing the ratiometric GFP-based anion indicator Clomeleon were obtained from The European Arabidopsis Stock Centre; NASC stock #N9404 to #N9415) were used to quantify changes in cytoplasmic chloride concentration in cells of the root hair zone near the hypocotyl during perfusion with different solutions. Whole plantlets were fixed in a self-made perfusion chamber (flux rate ca. 2.5 ml/min; chamber volume ca. 1.6 ml) and cytoplasmic chloride [Cl]cyt was recorded by fluorescence imaging (Fₑ₄ = 436 nm; Fₑ₅ = 480 and Fₑ₆ = 535 nm). Fluorescence images were taken every 12 sec with a ratio imaging system (F₄/₅ = 436 nm/F₅/₆ = 480 and F₆/₅ = 535 nm). Fluorescence emission spectra (Fig. 4) were obtained under different NaCl-concentrations in potassium phosphate buffer at pH 7.4 with a fluorescence spectrophotometer (Varian Cary Eclipse, Agilent Technologies). Fluorescence emission spectra R = Fₑ₄/Fₑ₅ were plotted over Cl⁻-concentrations and a sigmoidal Boltzman curve was fitted to the data (Fig. S6) using Origin 7.0 (OriginLab Corp.) and normalized to [Cl⁻] = 13 mM (value obtained by IC measurements). Normalized in vivo fluorescence imaging data obtained with the TILL system were converted into absolute chloride concentrations using the normalized in vitro calibration curve.

Table 1. Parameters for sample preparation and the used wavelengths

| Parameters | Na | K | Ca |
|------------|----|---|----|
| Wavelength (nm) | 598 | 589 | 422.8 |
| Releasing agent | KCl | CsCl₂ | LaCl₃ |
| Dilution | 1:50 (1:100) | 1:50 (1:100) | 1:20 |

The disclosure of a potential conflict of interest is not applicable for this work.

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No potential conflicts of interest were disclosed.

Plant treatment. Transgenic plants were grown on sterile vertical agar as described in Plieth et al. (2002) at a light regime 50 μE, dark/light cycle 16/8 h. Six to 14 d-old plants were used for experiments. The concentrations of Murashige & Skoog (MS, #M0222) used for culture medium are indicated in the figures or figure legends. For various calcium concentrations in growth medium, macro nutrient salts were prepared individually according to the composition of ready-to-use MS medium recipe. CaCl₂ was added in concentrations given in figure legends. MS micro salts (#M0301) and MS vitamin solution (#M0409) were filter-sterilized and added after autoclaving.

Two different basic buffer systems were used for perfusion experiments. MES buffer system composed of 5 mM MES/KOH (pH 5.8). SM buffer system consisted of 5 mM MES/KOH (pH 5.8) supplemented with salts (KCl, CaCl₂ and MgCl₂; 0.1 mM each).

Cation content measured by atomic absorption spectroscopy (AAS). Total Ca, K and Na contents of the plant material specified above were measured by means of AAS (AA S-series; Thermo Scientific Inc.). Plant material was prepared by dry ashing. Before measuring, sample dilutions were prepared by adding releasing agent as indicated in Table 1.

Anion content measured by ion chromatography (IC). Inorganic anions were eluted from ground plant material by hot-water extraction. The aqueous phase was recovered by centrifugation and subsequent chloroform precipitation. A reversed-phase Strata C18-E column was used to separate the polar components according to manufacturers’ instructions. 200 μl of the recovered sample were used for analysis by ion chromatography (ICS-2500; Dionex) using an IonPac AS11 Hydroxide-Selective Anion-Exchange Column (Dionex). The ions were eluted with 50 mM NaOH. Calculation and analysis of the chromatograms were done using the Chromeleon 6.6 software (Dionex).

Calibration and data processing. Clomeleon with N-terminal 6x His-Tag and C-terminal Strep-Tag was expressed in E. coli (SG13009), subsequently purified via Strep-Tactin® column (#2-1001; IBA) and Ni²⁺-NTA agarose (30210; Qiagen), and reduced in 50 mM DTT (#6908) at 4°C over night. Fluorescence emission spectra (Fig. S6) were obtained under different NaCl-concentrations in potassium phosphate buffer at pH 7.4 with a fluorescence spectrophotometer (Varian Cary Eclipse, Agilent Technologies). Fluorescence emission ratios R = Fₑ₄/Fₑ₅ were plotted over Cl⁻-concentrations and a sigmoidal Boltzman curve was fitted to the data (Fig. S6) using Origin 7.0 (OriginLab Corp.) and normalized to [Cl⁻] = 13 mM (value obtained by IC measurements). Normalized in vivo fluorescence imaging data obtained with the TILL system were converted into absolute chloride concentrations using the normalized in vitro calibration curve.

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Supplemental Materials
Supplemental materials may be found here: www.landesbioscience.com/journals/psb/article/24259

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