Antibodies to the CFTR modulate the turgor pressure of guard cell protoplasts via slow anion channels

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Received 16 January 2001; revised 20 February 2001; accepted 22 February 2001

First published online 19 March 2001

Edited by Maurice Montal

Abstract The plasma membrane guard cell slow anion channel is a key element at the basis of water loss control in plants allowing prolonged osmolite efflux necessary for stomatal closure. This channel has been extensively studied by electrophysiological approaches but its molecular identification is still lacking. Recently, we described that this channel was sharing some similarities with the mammalian ATP-binding cassette protein, cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel [Leonhardt, N. et al. (1999) Plant Cell 11, 1141–1151]. Here, using the patch-clamp technique and a bioassay, consisting in the observation of the change in guard cell protoplasts volume, we demonstrated that a functional antibody raised against the mammalian CFTR prevented ABA-induced guard cell protoplasts shrinking and partially inhibited the slow anion current. Moreover, this antibody immunoprecipitated a polypeptide from guard cell protein extracts and immunolabeled stomata in *Vicia faba* leaf sections. These results indicate that the guard cell slow anion channel is, or is closely controlled by a polypeptide, exhibiting one epitope shared with the mammalian CFTR.

Key words: Guard cell; Anion channel; Cystic fibrosis transmembrane conductance regulator; ATP-binding cassette protein; *Vicia faba*

1. Introduction

Guard cells are located at the end of the transpiration stream within the plant. Their control of stomatal aperture is crucial to minimize water loss from leaf tissues while balancing the requirement of CO_2 exchange for photosynthesis. Stomatal closing is mediated by anion and K^+ efflux from guard cells. Essentially, two types of anion channels have been identified at the guard cell plasma membrane using the patch-clamp technique [2,3]. However, the distinction between these two proteins is not ascertained [4]. Rapid anion channels activate and deactivate rapidly, giving rise to transient depolarization [5]. Slow anion channels activate slowly and allow sustained anion efflux suggesting their role in ion efflux during stomatal closure [5]. Besides the electrophysiological characterization obtained for these ionic channels, the molecular identification of anion channels implicated in stomatal movement regulation is still lacking. Alternative approaches to identify guard cell anion channels have already been tempted using high affinity ligands [6] but to our knowledge, antibodies raised against ionic channels have never been used in plants.

In plant cells, we recently obtained physiological evidence for the intervention of ATP-binding cassette (ABC) proteins in the modulation of stomatal movements and the control of ion channel activities [1,7]. The ABC superfamily is probably the largest and most diverse family of proteins that mediate ATP-dependent transfer of solutes across membranes [8]. Among ABC transporters identified in animal cells, the cystic fibrosis transmembrane conductance regulator (CFTR) constitutes a chloride channel, which is regulated by binding and hydrolysis of cytoplasmic ATP. Furthermore, CFTR is a receptor for the sulfonylurea glibenclamide [9] and is inhibited by this compound [10]. The clinical importance of mutations affecting the CFTR has contributed to the expansion of the research in this field, including the development of inhibitors and immunological tools. Based on our biophysical and pharmacological results we have proposed that the guard cell slow anion channel could be a CFTR homolog, or tightly controlled by such an ABC protein [1]. In order to investigate further this hypothesis, monoclonal antibodies raised against the mammalian CFTR were used to characterize an ABC protein previously identified in guard cells.

2. Materials and methods

2.1. Plant material, guard cell protoplasts (GCPs) isolation and patch-clamp recordings

Plants of *Vicia faba* were grown as described previously [7]. GCPs were isolated from 3–4-week-old plant leaves of *V. faba* by enzymatic digestion [7] and were subsequently used for swelling measurements or patch-clamp experiments. The whole-cell patch-clamp technique was performed to record anion currents specific from slow anion channels in *V. faba* GCPs exactly as described previously [1].

2.2. Monoclonal antibodies

Mouse monoclonal anti-CFTR antibodies, kindly provided by Dr. G. Banting [11], contained 10 μg proteins ml^{-1} and were diluted to a

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Abbreviations: ABC, ATP-binding cassette; BSA, bovine serum albumin; CFTR, cystic fibrosis transmembrane conductance regulator; GCPs, guard cell protoplasts; PVDF, polyvinylidene difluoride; SUR, sulfonylurea receptor

0014-5793/01/$20.00
PII: S0014-5793(01)02289-X

final concentration of 0.1 μg proteins ml^{-1}. Antibodies mAbCF4 and mABCF8 were raised against the CFTR peptide 881–896 and 1035–1050, respectively. They were purified from the neat tissue culture supernatant by means of protein A beads and immunodetected using antibodies raised against mouse IgG coupled to alkaline phosphatase [17]. Two rabbit anti-rat monoclonal antibodies raised against the C-terminal 21 amino acids of the sulfonylurea receptor SUR1 and SUR2A were kindly provided by Dr. S. Seino [18].

2.3. GCPs swelling measurements

Swelling of *V. faba* GCPs was followed by incubation for 4 h under light (300 μmol m^{-2} s^{-1} PAR) in a buffer containing 500 μM $MgCl_2$, 500 μM $CaCl_2$, 30 mM KCl, 5 mM MES (pH 5.5 with KOH), 450 mM D-sorbitol. During the experiment, protoplasts were gently shaken (shaking speed 80 min^{-1}) and kept at 21 ± 2°C. For each experimental condition, 600 GCPs diameters were measured in a hemocytometer by the same method as used for the measurement of stomatal aperture [7]. In order to take into account the time necessary at the end of the experiment for counting 600 protoplasts per condition, pharmacological treatments were shifted in time to keep a 4-h incubation duration for each sample.

2.4. Protein extraction, Western blots and immunoprecipitation

Proteins from *V. faba* GCPs were extracted and immunoprecipitations were conducted as described in Cotelle et al. [19]. Briefly, aliquots of proteins extracted from *V. faba* GCPs were added to 500 μl of washing medium (50 mM Tris–HCl, pH 8, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100 (v/v)) and incubated for 2 h at room temperature in the presence of a saturating amount of mAbCF8. Protein A Sepharose beads rehydrated overnight in 0.1 M Tris–HCl, pH 8, were added to samples (2% w/v) and similarly incubated. Beads were separated by centrifugation at 12 000 × *g* for 1 min and washed three times with the washing medium. The pellet was resuspended in SDS sample buffer, loaded onto 7.5% acrylamide gels for SDS–PAGE and immunoblotted using mAbCF8 (0.2 μg ml^{-1}). Antibodies mAbCF8 were immunodetected using anti-mouse IgG antibodies coupled to alkaline phosphatase.

2.5. Immunolocalization

Immunolocalizations were conducted in 3–4-week-old plant leaves of *V. faba*. Tissues were fixed, dehydrated, embedded, sliced, dewaxed and rehydrated as described by Weiss et al. [20]. Immunohistochemistry was performed on leaf sections using 0.2 μg ml^{-1} mAbCF8 as previously described for Western blots. Negative controls include sections incubated in the absence of primary antibody.

3. Results

3.1. Anti-CFTR antibodies modulate the turgor pressure of GCPs

In order to compare slow anion channels and the chloride channel CFTR, the antibodies mAbCF4 and mAbCF8 raised against the human CFTR [11] were used in *V. faba* GCP swelling experiments. The sulfonylurea glibenclamide and its antagonist, the K^+ channel opener cromakalim, were also applied since we previously demonstrated that they block or activate guard cell slow anion currents respectively [1].

The diameter of GCPs incubated under light was increased in the presence of 10 μM glibenclamide whereas addition of 100 nM ABA or 20 μM cromakalim induced GCPs shrinking. In the same conditions, the turgor of GCPs was not affected by the addition of anti-CFTR antibodies such as mAbCF8 reported for its functional efficiency against the CFTR in animal cells [12] (Fig. 1A, $n = 4$). However, the simultaneous application of ABA and mAbCF8 (0.1 μg ml^{-1}) led to the complete abolishment of the ABA-induced GCPs shrinking (Fig. 1B, $n = 8$). This inhibition of the ABA-induced GCPs shrinking was observed whatever the incubation temperature conditions, 22 or 4°C, attesting that mAbCF8 was active from

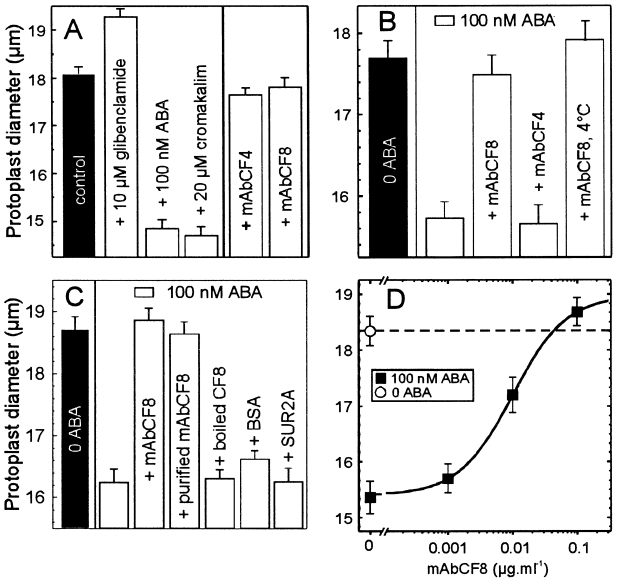

Fig. 1. Mammalian anti-CFTR antibodies modulate the turgor pressure of *V. faba* GCPs. A: Swelling of *V. faba* GCPs was induced under light (solid bar), promoted by glibenclamide or reduced by ABA or cromakalim. The presence of anti-CFTR antibodies (mAbCF4 or mAbCF8, 0.1 μg ml^{-1}) did not significantly affect protoplast diameters. B: ABA-induced GCPs shrinking was abolished by mAbCF8 antibodies raised against mammalian CFTR. Swelling of *V. faba* GCPs was induced under light (solid bar) while shrinking was obtained by application of 100 nM ABA (open bar). Simultaneous addition of ABA with 0.1 μg ml^{-1} mAbCF8 suppressed the GCPs shrinking at room temperature as well as at 4°C. Conversely, the same concentration of mAbCF4 did not affect the response to ABA. C: In similar experiments, a simultaneous application of ABA with 0.1 μg ml^{-1} mAbCF8, purified or not on protein A beads, completely reversed the effect of ABA. When mAbCF8 was previously boiled or substituted by 10 mg ml^{-1} BSA, ABA-induced shrinking was maintained. When anti-SUR2A antibodies replaced anti-CFTR antibodies, the shrinking effect of ABA was restored. D: Dose-dependent increase in GCPs diameter triggered by increasing concentrations of mAbCF8 in the presence of 100 nM ABA (solid square). A–D: 600 GCP diameters (mean ± S.E.M.) were measured for each bar or data point.

the external side of the cell and not via a mechanism needing first its endocytosis. The presence of mAbCF4 did not affect the response to ABA, in accordance with the fact that this antibody was never described in animal cells for its ability to modify the CFTR activity (Fig. 1B). Use of mAbCF8 coming either from the hybridoma tissue culture supernatant or the monoclonal antibody after purification on protein A beads completely suppressed the ABA-induced loss in guard cell turgor (Fig. 1C, $n = 7$). When this antibody was previously boiled or substituted by bovine serum albumin (BSA), the shrinking effect of ABA was preserved. Furthermore, antibodies raised against other ABC protein channel regulators such as the SUR (anti-SUR1 antibody, data not shown; anti-SUR2A antibody, Fig. 1C) did not exhibit a significant effect on GCP swelling. In order to test the specificity of mAbCF8, experiments using different concentrations of the antibody were performed. The inhibitory effect of mAbCF8 on guard cell ABA-induced shrinking was dose-dependent, saturable, irreversible and obtained at a half-maximal concentration of 0.01 μg ml^{-1} (Fig. 1D).

3.2. The monoclonal antibody mAbCF8 interferes with slow anion channels

GCP slow anion currents were recorded in whole-cell patch-clamp experiments before and after external perfusion with 0.01 μg ml^{-1} mAbCF8 on the same protoplast. Slow anion currents were partially but irreversibly inhibited within 10 min when mAbCF8 was added in the bath medium. However, these currents were neither affected by the perfusion of boiled mAbCF8 (Fig. 2) nor by the addition of mAbCF8 in the pipet solution (data not shown). When applied in the pipet, the absence of mAbCF8 effect could be related either to the absence of this epitope at the cytosolic side or to the high polypeptide content in the pipet resulting in a diffusion problem. Based on 11 independent GCPs, the mean (±S.E.M.) slow anion channel current inhibition by mAbCF8 was 23±11%. Regarding the control of stomatal movements, this inhibition by mAbCF8 is significant. Indeed, it has been shown that the same concentration of a classical anion channel inhibitor (9-AC, 100 μM) was able to induce stomatal opening in darkness equivalent to the control under light whereas it only induced a 50% inhibition of the slow anion current [13,14].

3.3. Human anti-CFTR antibodies detect immunologically related polypeptides in guard cells

The efficiency of the mAbCF8 in swelling and patch-clamp experiments led us to investigate the presence of a polypeptide immunologically related to the CFTR in total protein extracts of *V. faba* GCPs (Fig. 3). Identification of *V. faba* guard cell polypeptides corresponding to a CFTR homolog was assayed using mAbCF8 in Western blot (Fig. 3A, lane Gcp) or immunoprecipitation followed by Western blot (Fig. 3A, lane Ip). In the latter case, a single 74-kDa polypeptide was clearly identified. In contrast, when the monoclonal antibody anti-SUR2A, inefficient during swelling experiments, was used in Western blots no polypeptide was detected (data not shown).

Anti-CFTR antibodies were used during preliminary CFTR immunolocalization studies on human tissue sections [11]. In leaf tissue, mAbCF8 labeled *V. faba* guard cells (Fig. 3C). The staining level observed in guard cells compared to mesophyll cells demonstrates that the 74-kDa polypeptide is preferentially located in guard cells. These results confirm both patch-clamp and bioassay experiments and postulate a role of this polypeptide in the control of stomatal movement.

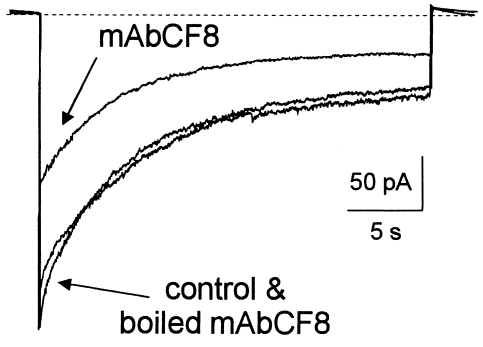

Fig. 2. Mammalian anti-CFTR antibodies reduce *V. faba* GCPs slow anion current. Superimposed whole-cell patch-clamp recordings showing a slow and incomplete relaxation of anion currents induced by hyperpolarization of the membrane potential from 0 to −120 mV in fava bean GCPs. Slow anion currents were recorded before (control), and after perfusion with 0.1 μg ml^{-1} boiled mAbCF8 and finally in the presence of 0.1 μg ml^{-1} mAbCF8. Whole-cell capacitance was 4.9 pF. Seal resistance was 2 GΩ. Dashed line refers to zero current.

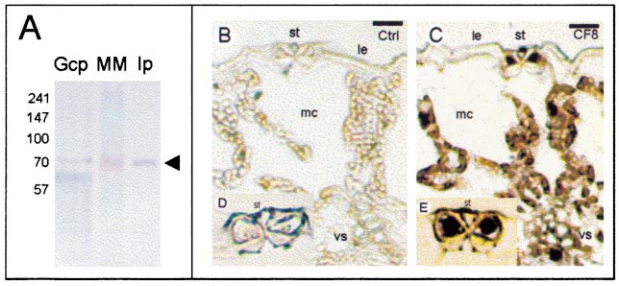

Fig. 3. Immunoprecipitation of a *V. faba* guard cell polypeptide with a monoclonal antibody raised against the human CFTR and immunolocalization of CFTR epitopes in *V. faba* leaf sections. A: Lane Gcp; immunodetection of polypeptides from *V. faba* GCPs in Western blots using mAbCF8. Lane Ip: After immunoprecipitation and immunodetection by mAbCF8 of 50 μg proteins from *V. faba* GCPs, a 74-kDa polypeptide was detected (right arrow). MM: molecular mass markers. B, C: Alkaline phosphatase immunolabeling in *V. faba* leaf sections incubated in the absence of the primary antibody (negative control, B) or in the presence of 0.2 μg ml^{-1} mAbCF8 (C). Scale bar: 30 μm. le: lower epidermis, st: stomatal complex, mc: mesophyll cell, vs: vessels. D, E: Magnifications of (B) and (C) to compare the negative control (D) to the reactivity obtained in guard cells using mAbCF8 (E).

In order to obtain a partial sequence of the 74-kDa polypeptide by micro-sequencing, several experiments were conducted to pool and purify a sufficient amount of the protein. Proteins from GCP were immunoprecipitated by mAbCF8, controlled on gel and blotted on a polyvinylidene difluoride (PVDF) membrane. The N-terminal part of the sample corresponding to the 74-kDa polypeptide was analyzed using Edman sequencing. Unfortunately, despite several trials, we never obtained enough protein to read a partial sequence.

4. Discussion

Plasma membrane guard cell anion channels were previously suspected as possible CFTR homologs [15,16]. We recently showed that these anion channels were glibenclamide-sensitive [1], as already demonstrated for the CFTR in mammalian cells [10]. In order to approach the molecular identity of the guard cell slow anion channels, we took advantage of functional antibodies raised against the human CFTR.

This study demonstrates that:

1. GCPs obtained after enzymatic digestion are functional and able to modulate their turgor pressure in response to biotic (ABA, light) and abiotic modulators (glibenclamide, cromakalim or anti-CFTR antibodies). The simple measurement of the GCP diameter constitutes a functional bioassay to evaluate the effect of large compounds or peptides (herein antibodies) classically stopped by the cell wall.
2. A 'CFTR-like' epitope is present at the guard cell plasma membrane and accessible from the external side of the cell. Indeed, among the two anti-CFTR and the two anti-SUR antibodies tested, only one, mAbCF8, antagonized the GCPs shrinking induced by 100 nM ABA (Fig. 1). Our experiments show that the 'CFTR-like' epitope is located at the external side of the cell for the following reasons. First, slow anion currents were inhibited by external perfusion of mAbCF8 (Fig. 2) but not when added in the

pipet. Second, the inhibition of ABA-induced GCP shrinking was observed at 4°C discarding a mechanism needing first the endocytosis of mAbCF8. The decrease of the slow anion current by mAbCF8 demonstrates an interaction between the antibody and the anion channel but does not allow concluding for a direct interaction between both partners. In animal cells, the functional activity of mAbCF8 but not mAbCF4 has already been reported since mAbCF8 inhibits the Cl^- pathway and detects the CFTR protein in rat kidney brush-border membranes [12].

3. The interaction between mAbCF8 and its 'receptor' is specific since in one hand it is dose-dependent and saturable (Fig. 1D) and in the other hand it can not be mimicked by other unrelated peptides (BSA) or antibodies raised against related ABC protein such as anti-SUR antibodies (Fig. 1C).
4. The 'receptor', which binds mAbCF8 and finally controls the turgor pressure of GCPs, could be the 74-kDa polypeptide present in guard cells. Indeed, in *V. faba* leaf sections, mAbCF8 strongly labeled guard cells compared to mesophyll cells (Fig. 3C) and immunoprecipitated only this 74-kDa polypeptide (Fig. 3A). In this case, the 74-kDa polypeptide could be either a regulator of the channel or the channel itself. However, it can not be excluded that another polypeptide, which was not detected during immunoprecipitation because of its low expression level, might be the receptor of mAbCF8. In both cases, these experiments support our previous data showing that CFTR modulators control slow anion currents and regulate stomatal movements [1].

A similar approach using a high affinity ligand has already been conducted in order to identify the R-type anion channel [6]. However, the authors never described any element dealing with the sequence of the channel they identified. It could be assumed that, like in our case, these authors were also limited by the quantity of the protein available. Interestingly our study demonstrates for the first time that a functional antibody could be used to modify the activity of a plant anion channel. Taken together, these results support the hypothesis that the slow anion channel could be a plant CFTR homolog or controlled by a polypeptide exhibiting such a CFTR epitope. We hope that the antibodies described in this study might be used in the near future for the screening of a guard cell expression library in order to identify the molecular nature of the slow anion channel.

Acknowledgements: The authors wish to thank Dr. G. Banting (Department of Biochemistry, University of Bristol, UK) and Dr. S. Seino (Center for Biomedical Science, Chiba, Japan) for the generous gift of antibodies raised against CFTR and SUR, respectively. Dr. J Vidal (IBP, Orsay, France) is acknowledged for technical advice regarding purification of antibodies. We also thank Mathilde Louwagie and Jérome Garin (CEA Grenoble, France) for their help in the microsequencing experiments. We thank Dr. Markus Klein (Neuchâtel, Switzerland) for critical reading of the manuscript. This work was supported by Commissariat à l'Energie Atomique.

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