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# Acetylcholine-activated $Cl^-$ Channel in the *Chara* Tonoplast

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**Abstract.** Acetylcholine has long been suggested to play a role in controlling physiological processes in plants, but no mechanism has been shown for its action. We show here that a chloride channel in the tonoplast (vacuolar membrane) of *Chara corallina* responds to acetylcholine. The channel has a conductance of 45 pS. The effect of acetylcholine is enhanced by nicotine, with the open probability increasing from 0.05 in the presence of 4 mM acetylcholine to 0.3 in the presence of 4 mM acetylcholine + 6 mM nicotine. Some effects of acetylcholine were seen at concentrations as low as 20 μM, with a maximum effect between 1 and 10 mM. In the intact cell, acetylcholine prolongs the depolarized phase of the action potential. We propose that this acetylcholine-gated channel has evolved separately from the mammalian acetylcholine-gated channel, and suggest that this represents a third form of acetylcholine signal transduction, after the nicotinic and muscarinic pathways in animal systems.

**Key words:** Acetylcholine — $Cl^-$ Channel — Tonoplast — *Chara* — Action potential — Signal transduction

## Introduction

Acetylcholine (ACh) is widely used as a transmitter among vertebrates and invertebrates. ACh is a simple molecule, synthesized in a single step from choline, a metabolite widely used in plants and animals in membrane phospholipids. One estimate is that its use evolved in animals at least 1 billion years ago, about the same time as the separation of plants and animals around 1.2 billion years ago (Walker, Brooks & Holden-Dye, 1996). In animals it is involved in two distinct pathways: the nicotinic receptor, in which the channel is activated directly by acetylcholine, and the muscarinic receptor, which requires an indirect G protein-based pathway (Sargent, 1993).

Evidence for a role for ACh in physiological processes in plants has been derived from several approaches. ACh has been shown to occur in plants, in some cases varying in concentration in response to physiologically relevant stimuli such as light (Fluck & Jaffe, 1974; Hartmann & Gupta, 1989; Tretyn, 1991). Enzymes involved in its synthesis and breakdown have been identified, including an acetylcholinesterase in *Nitella*, a Charophyte alga (Dettbarn, 1962; Hartmann & Gupta, 1989; Momonoki, 1992; Madhavan et al., 1995). Activity of plant cholinesterases has been shown to be selective for ACh over other choline esters, and sometimes showed sensitivity to inhibitors of mammalian ACh esterases, suggesting a common ancestor. Evidence for a receptor for ACh comes from binding studies (Hartmann & Gupta, 1989). There has also been a preliminary report of a receptor based on immunological cross-reactions with specific antibodies raised against the animal nicotinic ACh receptor (Madhavan & Pinkerton, 1997).

Exogenous application of ACh produces a number of physiological responses in plants, including mimicking or inhibiting alterations in membrane potential induced by red, far-red, or blue light (Fluck & Jaffe, 1974; Hartmann & Gupta, 1989; Tretyn, 1991). Such alterations in membrane potential frequently involve alteration of ion channel activity.

In plants, chloride channels play a number of important roles. They are components of signal transduction chains, including response to light (Schroeder, 1995; Cho & Spalding, 1996). In algae, chloride is often a major osmolyte in the vacuole, and hence regulation of chloride transport at the vacuolar membrane (tonoplast) is an important component of osmotic regulation (Gutknecht, Hastings & Bisson, 1978; Kirst, 1990; Bisson, Kiegle & Kiyosawa, 1992).

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In the alga *Chara*, the depolarization due to the action potential is largely due to activation of chloride channels in the plasma membrane (Shiina & Tazawa, 1987; Thiel, Homann & Plieth, 1997). There is also a component of the action potential that is due to channel activity at the tonoplast (Kikuyama & Shimmen, 1997). The chloride channel is therefore a good candidate to be involved in signal transduction chains as a ligand-gated channel. We report here that a chloride channel in the tonoplast of *Chara corallina* is activated by acetylcholine and nicotine.

## Materials and Methods

*Chara corallina*, a freshwater alga, was grown in culture in the lab as described earlier (Bisson, 1984, 1985). From tonoplast-bound cytoplasmic droplets (Lühring, 1986) we formed outside (vacuole-side)-out patches with (in mM) 111 $Cl^-$, 100 $TEA^+$, 1 $Na^+$ and $10^{-4}$ $Ca^{2+}$ with 5 EGTA in the pipet (pH 7 with 5 HEPES) and 130 $Cl^-$, 100 $TEA^+$, 1 or 10 $Na^+$ and 10 $Ca^{2+}$ in the bath (pH 5 with 5 MES), unless otherwise stated. Patch pipets were pulled on a two-stage puller, coated with Sylgard, and fire-polished. We used a Dagan amplifier (Minneapolis, MN) to perform clamp experiments, and pClamp (Axon Instruments, Foster City, CA) to analyze the results. Membrane potentials are presented referenced to the cytoplasmic side, according to the convention proposed by Bertl et al. (1992). Probability of opening (*Po*) was calculated as the fraction of total recording time that the channel was open, if only one channel was apparent in the membrane. Often there were more than one channel open simultaneously, as shown by multiples of current levels. In this case, the maximum number of channels open simultaneously was taken as *N*, the total number of channels in the membrane. If the probability of opening appeared to follow a binomial distribution, the probability of opening was calculated appropriately, that is, the fraction of time that *N* channels were open was taken to be $Po^N$, the fraction of time that $N-1$ channels were open to be $Po^{N-1}(1-Po)$, etc., and the fraction of time that no channels were open was taken to be $(1-Po)^N$. If the open times did not follow a binomial distribution, the record was not used to calculate *Po*. This technique was preferred to the more common usage of calculated *NPo* as the total time that any number of channels were open, because we were comparing data from different patches, which might have captured different numbers of channels.

Conventional electrophysiology was performed as previously described (Stento et al., 2000). The cell was impaled with 2 microelectrodes, one measuring voltage and one injecting current. Action potentials were elicited by injecting depolarizing current. The time to the end of the fast phase of repolarization was determined as the intersection between the tangent lines to the steepest part of the repolarization and to the final slope (*see* Fig. 4). To obtain internodal cells with a membrane potential dominated by $K^+$ conductance, we incubated cells overnight in 0.1 mM $K_2SO_4$, 10 mM NaCl, 0 mM $Ca^{2+}$, 5 mM HEPES pH 7.0 (Hope & Walker, 1961).

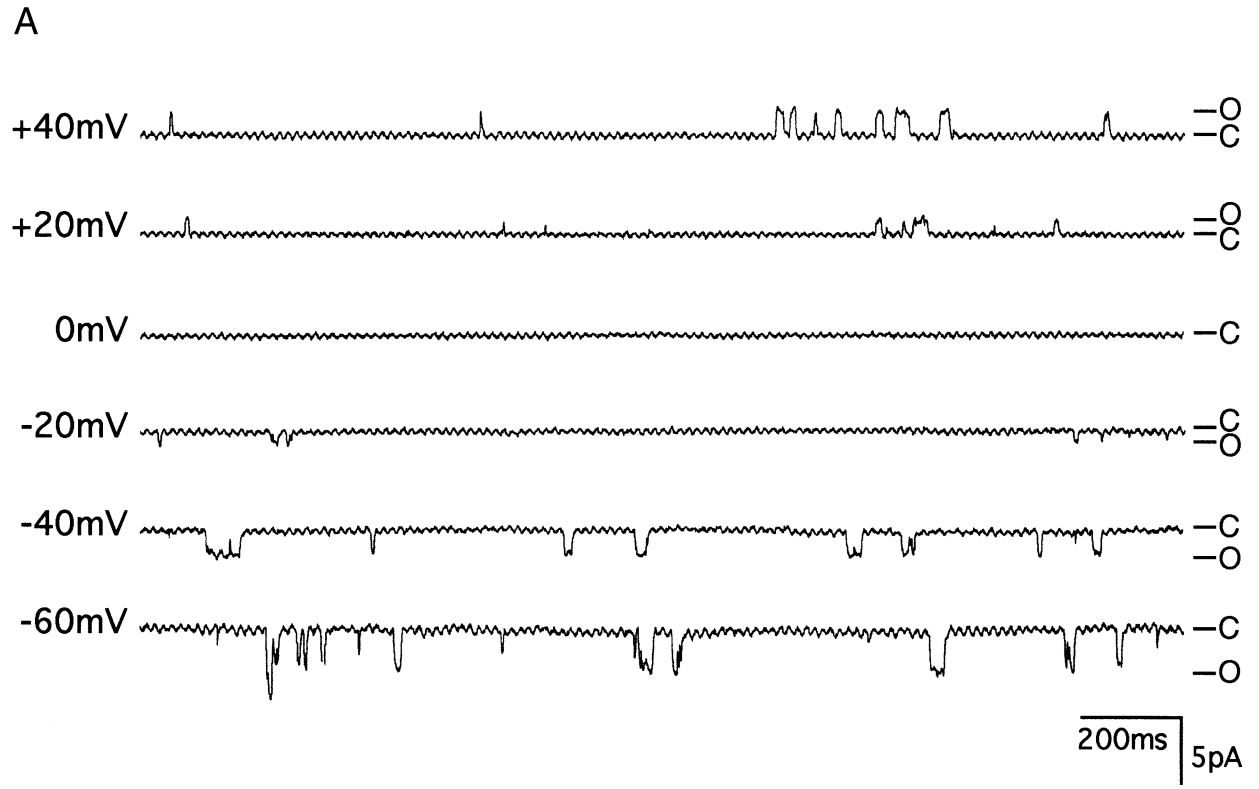
## Results

In $K^+$-free solutions, the $K^+$ channel that normally dominates the patch records (Laver & Walker, 1987; Lühring, 1986) is not seen. However, in $K^+$-free solutions, we occasionally (in less than 10% of patches) saw a channel that usually ran down rapidly. Increasing $Ca^{2+}$ activity up to 10 mM in the pipet increased the occurrence of these channels and decreased run-down, but not sufficiently to enable us to easily study the channel. Addition of nucleotides such as ATP, GTP, cAMP, etc., to the pipet solution did not prevent rundown. Addition of acetylcholine (ACh: 1–10 mM) to the outside (vacuole side) could occasionally prevent or reverse rundown. Current traces from such a channel with 10 mM ACh in the bath are shown in Fig. 1*A*, with current voltage relations in Fig. 1*B*. The conductance is 43 pS and the reversal potential is −2.2 mV.

Addition of acetylcholine to the cytoplasmic side, however, gave a consistently higher likelihood of seeing a channel that did not run down (in 10–20% of patches). Nicotine, but not muscarine, enhanced the open probability (Fig. 2). The open probability as a function of acetylcholine concentration is shown in Fig. 3.

In all, 15 I-V curves were measured, two without ACh, 6 with ACh in the bath, 2 with ACh in the pipet, and 5 with ACh and nicotine in the pipet. The average intercept was 0.421 ± 2.58 mV (SE) and the mean conductance was 44.5 ± 3.8 pS. There were no consistent differences due to the ACh treatment. There was also no difference when $Na^+$ concentrations were the same on both sides or when there was a 1:10 gradient. Based on these data, and on comparison with anion channels reported in the literature (*see* Discussion) we concluded that this channel is an anion channel.

If acetylcholine has this effect on the tonoplast chloride channel, what physiological effect might we expect? We examined the effect of ACh on the action potential (Fig. 4). While the depolarizing phase was not affected, there was a significant slowing of the repolarizing phase. The rate of repolarization immediately after the peak potential (arrows labeled "1" in Fig. 4) was significantly ($p = 0.000112$) less in the presence of 4–6 mM ACh, and the time to the end of the major repolarization phase (arrows labeled "2" in Fig. 4) was significantly longer ($p = 0.0464$) (*see* Table 1). This is consistent with an increase in $Cl^-$ conductance at the tonoplast (*see* Discussion). Such a result could also be produced if the $K^+$ channels at the plasma membrane were inhibited. We therefore examined the effect of ACh on $K^+$ conductance at the plasma membrane. We did this by inducing the cells to be in the "K state" (Bisson & Walker, 1982), which has the electrogenic pump off and the membrane dominated by $K^+$ conductance. We tested the dependence of the membrane potential on $K^+$, and showed that ACh had no effect on the nature of the dependence (Fig. 5). This shows that in the presence of ACh, $K^+$ conductance still dominates the membrane potential. If $K^+$ conductance was partially inhibited,



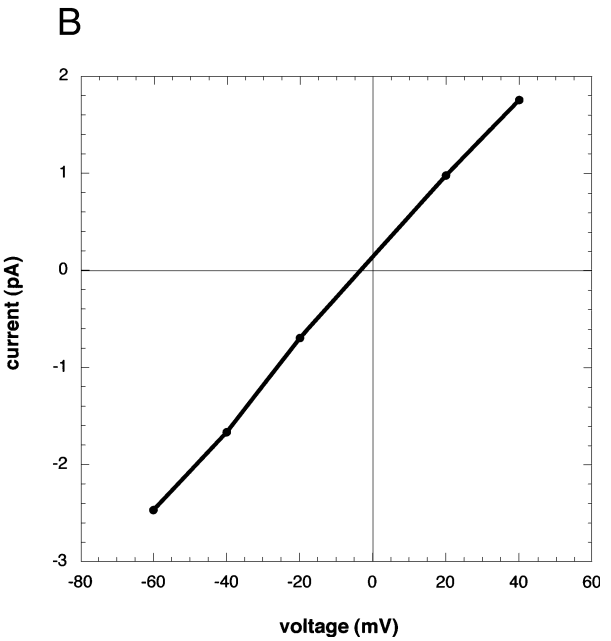




**Fig. 1.** (*A*) Patch-clamp record of single-channel currents seen in $K^+$-Free solutions. Bath (mM): 130 $Cl^-$, 100 $TEA^+$, 10 $Na^+$ and 10 $Ca^{2+}$; pipet: 111 $Cl^-$, 100 $TEA^+$, 1 $Na^+$ and $10^{-4}$ $Ca^{2+}$. (*B*) Current-voltage curve. Conductance is 43 pS; intercept is −2.2 mV.

but still substantially greater than any other conductance, the same result would be seen. In this case, one would predict that ACh would cause a decrease in conductance. However, this was not the case: the average control conductance was 1.9 ± 0.90 $Sm^{-2}$ (mean ± SE, $n$ = 5) and increased by 0.57 ± 0.36 $Sm^{-2}$ on addition of 4 mM ACh. Therefore, the alteration in the shape of the action potential is unlikely to be due to an increase in $K^+$ conductance at the plasma membrane, and is most likely due to an increase in $Cl^-$ conductance at the tonoplast.

## Discussion

The tonoplast of *Chara corallina* is dominated by $K^+$ channel activity, with anion channels reported only rarely. Previous reports noted a calcium-dependence

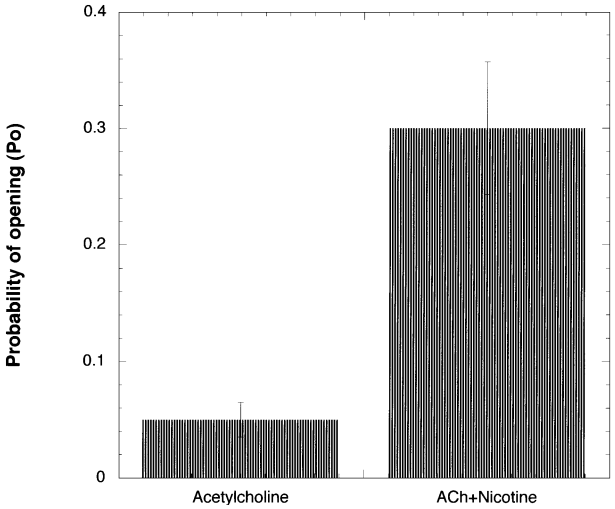



**Fig. 2.** Effect of the addition of 6 mM nicotine on the ACh-induced open probability. Acetylcholine column: treated with 4 mM ACh only. Ach + nicotine: treated with 4 mM ACh and 6 mM nicotine. Experiments performed at 60 mV. Error bars shown are standard error. For ACh, n = 9; for Ach + nicotine, $n = 5$.

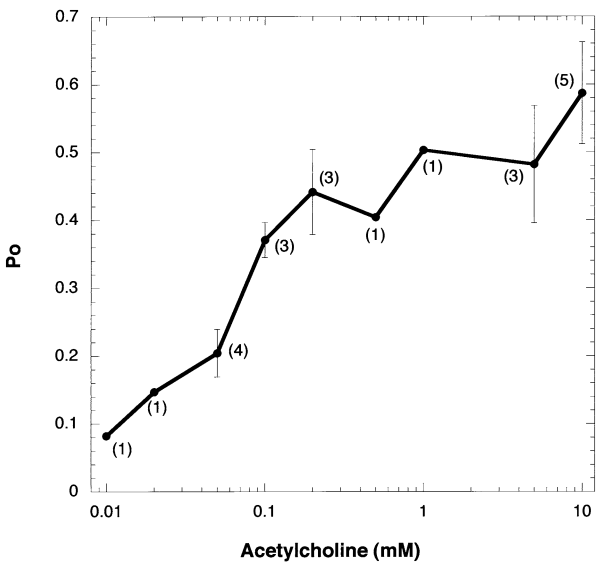



**Fig. 3.** Concentration dependence of the open probability (*Po*) on ACh concentration in the pipet. All pipet solutions also contained 6 mM nicotine. Error bars represent standard deviation. *n* for each point is indicated in parentheses.

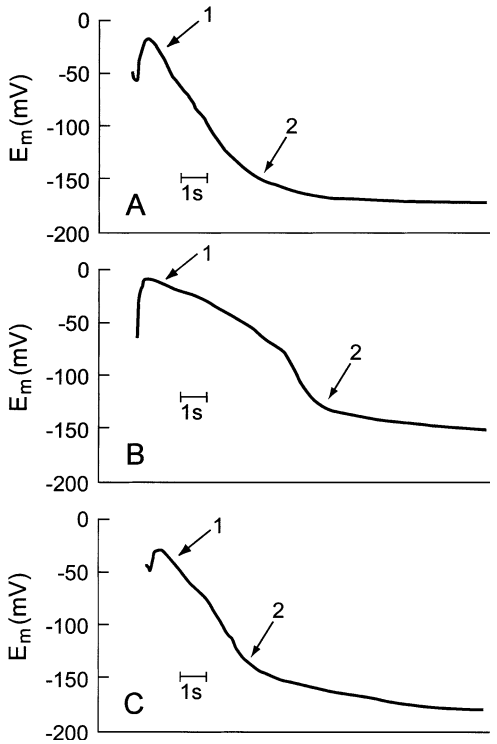



**Fig. 4.** Action potentials recorded with the two-electrode method in intact internodal cell. (*A*) Before adding ACh. (*B*) After adding 2 mM ACh into the incubation solution. (*C*) 30 min after the ACh was washed out. Quantitative measurements of the repolarizing phase were made at the arrow marked "1" (initial rate of repolarization) and "2" (end of fast repolarization phase).

of the anion channel. Tyerman & Findlay (1989) reported that the $Cl^-$ channel is frequently observed in attached patches, but seldom in detached patches, unless low $Ca^{2+}$ concentrations were present at the cytoplasmic face. Then they noted brief appearances of the channel, consistent with our experience of rundown of the channel. They found that the channel had a conductance of 21 pS in attached patches with 130 mM $Cl^-$ in the bath. In detached patches, the conductance was 59 pS with 130 mM $Cl^-$. The current did not rectify with symmetrical $Cl^-$ concentrations.

Berecki et al. (1999, 2001) found a similar channel, non-rectifying, with a conductance in detached patches of 37 pS or 48 pS at 190 mM $Cl^-$, and lower in attached patches, similar to what was seen by Tyerman and Findlay. However, they found the opposite $Ca^{2+}$ sensitivity, with almost no activity in attached patches or when $Ca^{2+}$ was low on the cytoplasmic side, but with increasing *Po* with increasing cytoplasmic concentration. They showed a maximum *Po* at about 0.8 mM and a half-maximal around 0.1 mM. These concentrations are significantly higher than normal cytoplasmic $Ca^{2+}$ concentrations (Williamson, 1984). However, a significant increase in activity is seen at $10^{-6}$ M, a concentration that may be achieved during excitation, e.g., by an action potential (Williamson & Ashley, 1982). Moreover, $Ca^{2+}$ sensitivity is modulated by pH; lowering the cytoplasmic pH from 7.2 to 6.0 lowered the half-maximal calcium concentration from 100 μM to 5 μM (Berecki et al., 2001). Since the $Cl^-$ channel is sensitive to physiologically relevant changes in cytoplasmic conditions, it may play a role in signal transduction events.

The channel we describe here is more similar to that of Berecki et al. in that its activity is increased by an increase in cytoplasmic $Ca^{2+}$. Its conductance was 44 pS. In most cases, no rectification was seen but in 3

**Table 1.** Effect of acetylcholine on the depolarizing phase of the action potential.

| | Control | + Acetylcholine (4–6 mM) |
|---|---|---|
| Initial rate of repolarization (mV/sec) | 35 ± 1.27 (7) | 19 ± 0.89 (9) |
| Time to end of fast repolarizing phase (sec) | 4.63 ± 0.2 (7) | 5.31 ± 0.24 (9) |

See text for details. Mean ± SE (*n*).

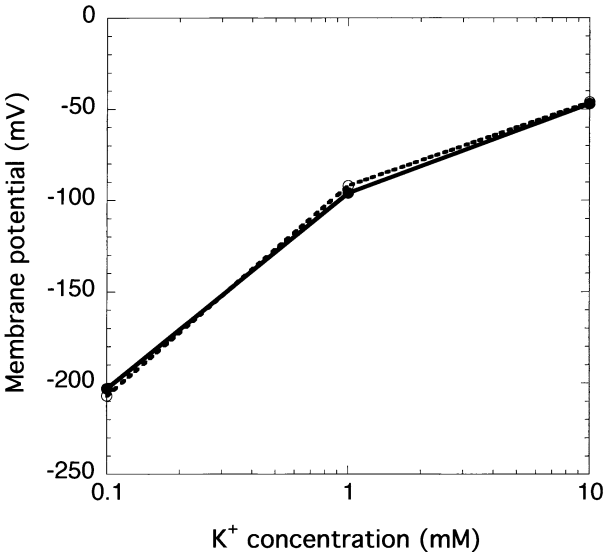



**Fig. 5.** The dependence of the membrane potential of cells in the K state on external $K^+$ concentration in the presence (*open circles*, *dashed lines*) and absence (*filled circles*, *solid line*) of Ach.

out of 15 measurements, no positive currents were observed. There is a possibility that this is a nonselective cation channel, but this seems unlikely. Such a nonselective cation channel has not been reported in the *C. corallina* tonoplast. The $K^+$ channel reported here is highly selective for $K^+$ over $Na^+$ ($P_{Na}/P_K$ = 0.01 (Lühring, 1986) or 0.02 (Laver & Walker, 1991)). We tried a number of other cation substitutions, including choline. In these cases, the channel characteristics were similar, although the patches were unstable and therefore the results difficult to analyze. We attempted measurement with bi-ionic gradients or anion substitutions, including other halide ions, but these also resulted in unstable patches. Because of the similarity to previously reported $Cl^-$ channels, the best explanation is that this is a $Cl^-$ channel. We report here a unique characteristic of this channel, that it is activated by acetylcholine and nicotine.

Is this activation of physiological significance? We show that the addition of ACh to the intact cell prolongs the depolarized phase of the action potential (Fig. 4). Is this likely due to the same channel? Increasing the open probability of the tonoplast channel would tend to maintain the cell more depolarized. With a cytoplasmic concentration of about 15 mM (Hope & Walker, 1975; Tyerman & Findlay, 1989) and a vacuolar concentration of 106 mM (Bisson & Bartholomew, 1984), the equilibrium potential for $Cl^-$, $E_{Cl}$, is about 49 mV, vacuole positive to the cytoplasm. Thus, increasing $Cl^-$ conductance would make the cell more positive, as measured with an electrode in the vacuole. If the channel is a nonselective cation channel it would have less effect, since the concentration of ($K^+$ + $Na^+$) is about the same in the cytoplasm (125 mM + 9 mM; Hope & Walker, 1975) as in the vacuole (80 mM + 54 mM; Bisson & Bartholomew,1984). Therefore, increasing cation permeability would drive the vacuolar potential towards zero, which would not be able to counteract a negative potential at the plasma membrane.

In the intact cell, ACh has its effect without the addition of nicotine. In detached patches, ACh alone has some effect, although nicotine greatly potentiates this effect (Fig. 3). In vivo, either ACh alone can have a significant effect because of the additive effect of a great number of channels, or there is an endogenous activator that plays the role of nicotine. To activate a tonoplast channel, ACh must cross the plasma membrane to enter the cytoplasm. The fact that in detached patches ACh can sometimes have an effect when added to the vacuolar side of the membrane indicates that it can cross the tonoplast membrane, and it is likely to be able to cross the plasma membrane as well. We generally waited an hour after application of ACh to provide sufficient time for it to penetrate the membrane, although we did not do a systematic study of the length of time needed for maximal effect.

We cannot rule out the possibility that in the intact cell ACh is having its physiological effect on a plasma membrane channel. We showed that ACh does not inhibit the $K^+$ channel, which is responsible for generating the membrane potential when the channel is in the "K state" (Bisson & Walker, 1982), dominated by $K^+$ conductance. It is possible that the $K^+$ channel that is activated in the repolarizing phase of the action potential is a different channel that is inhibited by ACh. Alternatively, inactivation of the $Cl^-$ channel responsible for depolarization could be inhibited by ACh. To test these hypotheses, experiments on plasma membrane patches are needed. In any event, a clear physiological effect of ACh on electrophysiology of the intact *Chara* cell is seen.

Are ACh and nicotine the endogenous activators of this channel? ACh is easily synthesized from

common components of the cell, acetate and choline. Many studies have shown that plants contain ACh (Fluck & Jaffe, 1974; Hartmann & Gupta, 1989; Tretyn, 1991), and an acetylcholinesterase has been shown to exist in the Charophyte *Nitella* (Dettbarn, 1962). Activation of this channel may be one of its physiological functions. Nicotine, on the other hand, occurs only in certain plants, and is much more complicated to synthesize. There may be another endogenous ligand that plays the same role of enhancing ACh activation.

Is this channel related to the nicotinic ACh receptor (nAChR) found in animals? There is no obvious candidate for this receptor in the *Arabidopsis* database. Moreover, the channel shows many significant differences from the animal receptor. The permeant ion is an anion, rather than a cation. However, a small alteration in sequence can convert the neuronal nicotinic ACh receptor from cation to anion selective (Galzi & Changeux, 1995). Other differences are of greater concern. In the animal nAChR, nicotine and acetylcholine appear to bind at the same site, while in this channel, since their actions are synergistic, they appear to bind at different sites. Another difference is topological. In the animal nAChR, the binding site for ACh is on the external side. The equivalent binding site at the tonoplast would be at the vacuolar side. Instead, the response to ACh appears to be on the cytoplasmic side.

We therefore suggest that this channel is unrelated to the nAChR. Since ACh is such a common and simple molecule, it is not surprising that different pathways have evolved to utilize it as a signaling molecule. In animals, two separate evolutionary events gave rise to the muscarinic and nicotinic receptors. In plants, another separate event could have given rise to this channel.

These findings call into question the single report of heterologous gene expression in this important model plant system. Injection of mRNA coding for nAChR into the cytoplasm of *Chara* was reported to result in expression of ACh-induced channel activity (Lühring & Witzemann, 1995). Based on our experiments, it remains a question as to whether this was true heterologous expression or a measurement of the endogenous ACh-sensitive channel.

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## References

Berecki, G., Varga, Z., Van Iren, R, Van Duiyn, B. 1999. Anion channels in *Chara corallina* tonoplast membrane: Calcium dependence and rectification. *J. Membrane Biol.* **172:**159–164

Berecki, G., Eijken, M.., Van Iren, F., Van Duiyn, B. 2001. Tonoplast anion channel activity modulation in *Chara corallina*. *J. Membrane Biol.* **184:**131–141

Bertl, A., Blumwald, E., Coronado, R., Eisenberg, R., Findlay, G., Gradmann, D., Hille, B., Kohler, K., Kolb, H.-A., MacRobbie, E., Meissner, G., Miller, C., Neher, E., Palade, P., Pantoja, O., Sanders, D., Schroeder, J., Slayman, C., Spanswick, R., Williams, A. 1992. Electrical measurements on endomembranes. *Science* **258:**873–874

Bisson, M., Bartholomew, D. 1984. Osmoregulation or turgor regulation in *Chara? Plant Physiol.* **74:**252–255

Bisson, M.A. 1984. Calcium effects on electrogenic pump and passive permeability of the plasma membrane of *Chara corallina*. *J. Membrane Biol.* **81:**59–67

Bisson, M.A. 1985. The effect of darkness on active and passive transport in *Chara corallina*. *J. Expt. Bot.* **37:**8–21

Bisson, M.A., Kiegle, E., Kiyosawa, K. 1992. Turgor regulation of solutes in Charophyte algae. *Curr. Topics Plant Biochem. Physiol.* **11:**178–191

Bisson, M.A., Walker, N. 1982. Transitions between modes of behaviour (states) of the Charophyte plasmalemma. *In:* Plasmalemma and Tonoplast: Their Functions in the Plant Cell. E. Marré and R. Hertel, editors, pp. 35–40. Elsevier Biomedical Press

Cho, M.H., Spalding, E.P. 1996. An anion channel in *Arabidopsis* hypocotyls activated by blue light. *Proc. Nat. Acad. Sci. USA* **93:**8134–8138

Dettbarn, W.D. 1962. Acetylcholinesterase activity in *Nitetta*. *Nature* **194:**1175–1176

Fluck, R.A., Jaffe, M.J. 1974. The acetylcholine system in plants. *Curr. Adv. Plant Sci.* **11:**1–22

Galzi, J.-L., Changeux, J.-P. 1995. Neuronal nicotinic receptors: Molecular organization and regulations. *Neuropharmocology* **32:**563–582

Gutknecht, J., Hastings, D., Bisson, M. 1978. Ion transport and turgor pressure regulation in giant algal cells. *In:* Membrane Transport in Biology. Vol. III. Transport Across Multimembrane Systems. G. Giebisch, D. Tosteson, and G. Ussing, editors, pp. 125–174. Springer, Berlin

Hartmann, E., Gupta, R. 1989. Acetylcholine as a signaling system in plants. *In:* Second Messengers in Plant Growth and Development, pp. 257–287. Alan R. Liss, Inc.

Hope, A.B., Walker, N.A. 1961. Ionic relations of cells of *Chara australis* R. Br. IV. Membrane potential differences and resistances. *Austr. J. Biol Sci.* **14:**26–44

Hope, A.B., Walker, N.A. 1975. The Physiology of Giant Algal Cells. Cambridge University Press, London

Kikuyama, M, Shimmen, T. 1997. Role of $Ca^{2+}$ in triggering tonoplast action potential in intact *Nitella flexilis*. *Plant Cell Physiol* **38:**941–944

Kirst, G.O. 1990. Salinity tolerance of eukaryotic marine algae. *Annu. Rev. Plant Physiol. Plant Molec. Biol.* **41:**21–53

Laver, D.R., Walker, N.A. 1987. Steady-state voltage-dependent gating and conduction kinetics of single $K^+$ channels in the membrane of cytoplasmic drops of *Chara australis*. *J. Membrane Biol.* **100:**31–42

Laver, D.R., Walker, N.A. 1991. Activation by $Ca^{2+}$ and block by divalent ions of the $K^+$ channel in the membrane of cytoplasmic drops from *Chara australis*. *J. Membrane Biol.* **120:**131–139

Lühring, H. 1986. Recording of single $K^+$ channels in the membrane of cytoplasmic drop of *Chara australis*. *Protoplasma* **133:**19–27

Lühring, H., Witzemann, V. 1995. Internodal cells of the giant green alga *Chara* as an expression system for ion channels. *FEBS Lett* **361:**65–69

Madhavan, S., Pinkerton, S.G. 1997. Do plant cells have the receptor for acetylcholine? Immunodetection of acetylcholinesterase and acetylcholine receptor in leaf cells. *Plant Physiol.* **114S:**820

Madhavan, S., Sarath, G., Lee, B.H., Pegden, R.S. 1995. Guard cell protoplasts contain acetylcholinesterase activity. *Plant Sci.* **109:**119–127

Momonoki, Y.S. 1992. Occurrence of acetylcholine-hydrolyzing activity at the stele-cortex interface. *Plant Physiol.* **99:**130–133

Sargent, P.B. 1993. The diversity of neuronal nicotinic acetylcholine receptors. *Ann. Rev. Neurosci.* **16:**403–443

Schroeder, J.I. 1995. Anion channels as central mechanisms for signal transduction in guard cells and putative functions in roots for plant-soil interactions. *Plant Mol. Biol.* **28:**353–361

Shiina, T., Tazawa, M. 1987. $Ca^{2+}$-activated $Cl^-$ channel in plasmalemma of *Nitellopsis obtusa*. *J. Membrane Biol.* **99:**137–146

Stento, N.A., Ryba, N.G., Kiegle, E.A., Bisson, M.A. 2000. Turgor regulation in the salt-tolerant alga *Chara longifolia*. *Plant Cell Env.* **23:**629–637

Thiel, G., Homann, U., Plieth, C. 1997. Ion channel activity during the action potential in *Chara*: New insights with new techniques. *J. Exp. Bot.* **48:**609–622

Tretyn, A. 1991. Acetylcholine in plants: Presence, metabolism and mechanism of action. *Bot. Rev.* **57:**34–73

Tyerman, S.D., Findlay, G.P. 1989. Current-voltage curves of single $Cl^-$ channels which coexist with two types of $K^+$ channel in the tonoplast of *Chara corallina*. *J. Expt. Bot.* **40:**105–117

Walker, R.J., Brooks, H.L., Holden-Dye, L. 1996. Evolution and overview of classical transmitter molecules and their receptors. *Parasitology* S3–S33

Williamson, R.E. 1984. Calcium and the plant cytoskeleton. *Plant Cell Env.* **7:**431–440

Williamson, R.E., Ashley, C.C. 1982. Free $Ca^{2+}$ and cytoplasmic streaming in the alga *Chara*. *Nature* **296:**647–651