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Short communications

Calmodulin regulates the Ca^{2+}-dependent slow-vacuolar ion channel in the tonoplast of *Chenopodium rubrum* suspension cells

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Abstract. The patch-clamp technique was applied to vacuoles isolated from a photoautotrophic suspension cell culture of *Chenopodium rubrum* L. and vacuolar clamp currents, which are predominantly carried by the previously identified Ca^{2+}-dependent slow vacuolar (SV) ion channels, were recorded. These currents, which were activated by 1-s voltage pulses of −100 mV (vacuolar interior negative) in the presence of 100 μM Ca^{2+} (cytosolic side), could be blocked completely and reversibly by the calmodulin antagonist W–7 [N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide] and its chlorine-deficient analogue W–5; half-maximum inhibition was found at approx. 6 μM for W–7 and 70 μM for W–5. Inhibition was reversed by addition of 1 $\mu g \cdot ml^{-1}$ calmodulin purified from *Chenopodium* cell suspensions; reversal by bovine brain calmodulin was scarcely appreciable. We conclude that cytosolic calmodulin mediates the Ca^{2+} dependence of the SV-channel in the *Chenopodium* tonoplast.

Key words: Calmodulin – *Chenopodium* – Ion channel (slow vacuolar) – Suspension cell culture – Tonoplast

A prominent ion channel in the tonoplast of higher-plant cells is the slowly activated, vacuolar ion channel (SV-channel) which notably is permeable to univalent cations and some anions (Hedrich et al. 1986; Coyaud et al. 1987). This SV-channel is voltage-gated and activates at cytosolic Ca^{2+} activities ≥ 1 μM (Hedrich and Neher 1987). It exhibits a large conductance, i.e. about 70 pS (cf. Bentrup 1989). In recent patch-clamp studies on the tonoplast of vacuoles isolated from *Chenopodium rubrum* suspension cells we have shown that this channel is blocked by the acetylcholine antagonist (+)-tubocurarine (Weiser and Bentrup 1990), and by charybdotoxin which blocks a family of Ca^{2+}-activated large-conductance K^+ channels in animal tissues (Weiser and Bentrup 1991). We now present evidence that Ca^{2+} activation of the plant SV-channel appears to be mediated by cytosolic calmodulin.

The standard test solution contained 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 300 mM mannitol, and 5 mM Tris/2-(N-morpholino)ethanesulfonic acid (Mes) (pH 7.2). It was used both in the bath and in the patch-pipette. Test solutions were filtered (Schleicher und Schuell, Dassel, FRG; 0.2 μm) just before use. Calmodulin and calmodulin antagonists were added to the cytoplasmatic side of the vacuole by bath perfusion. All chemicals were of analytical grade.

Calmodulin was purified from *Chenopodium* suspension cells and maize coleoptiles by ammonium-sulfate precipitation and subsequent chromatography on diethylaminoethyl (DEAE) and phenylsepharose columns according to Lukas and Watterson (1988). Calmodulin concentrations were calculated as usual, i.e. by phosphodiesterase activation (Watterson et al. 1980) and protein was determined according to Bradford (1979). Spinach and bovine brain calmodulin were purchased from Sigma (Deisenhofen, FRG).

Vacuoles were prepared from protoplasts isolated from a heterotrophic suspension culture of *Chenopodium rubrum* L. as described previously (Bentrup et al. 1986). Patch-clamp measurements were performed according to Weiser and Bentrup (1990).

Involvement of calmodulin in controlling a given Ca^{2+}-dependent cellular process may be demonstrated by the reversible inhibition of calmodulin binding by two naphthalenesulfonamide derivatives, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W–7) and N-(6-aminohexyl)-1-naphthalenesulfonamide (W–5) (Hidaka et al. 1981). Figure 1 demonstrates that W–5 markedly inhibits the whole-vacuole current in *Chenopodium*. More succinct evidence for the notion that calmodulin indeed mediates the Ca^{2+} dependence comes from the observation in Fig. 1 that addition of *Chenopodium* calmodulin counteracts the inhibition by W–5. Bovine brain calmodulin, on the other hand, had no appreciable affect on the inhibitory action of W–5. While in these and all subsequently shown experiments 100 μM Ca^{2+} was used to maximize the response, qualitatively similar results

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Abbreviations: SV-channel = slowly activated, vacuolar ion channel; W–5 = N-(6-aminohexyl)-1-naphthalenesulfonamide; W–7 = N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide

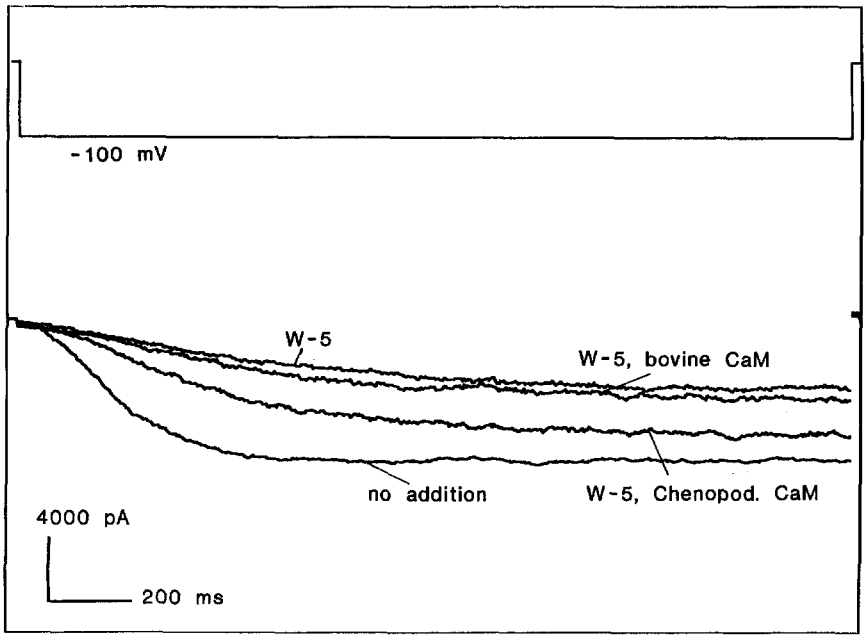

Fig. 1. Whole-vacuolar electric clamp currents from a *Chenopodium* vacuole subjected to a 1-s voltage pulse of −100 mV (inside negative). The SV-currents were recorded upon addition to the cytoplasmic side of 50 μM of the calmodulin antagonist W–5, and upon addition of W–5 plus 1 μg · ml^{-1} calmodulin *(CaM)* from *Chenopodium* and bovine brain, as indicated

have been obtained with 1 μM Ca^{2+}, i.e. the presumed cytosolic Ca^{2+} concentration.

It was also found that calmodulin from spinach and maize was nearly as effective as *Chenopodium* calmodulin (data not shown). Clearly, homologous, i.e. plant, calmodulin is required to activate this tonoplast channel. A comparable ion-channel activation by calmodulin and competitive inhibition by the antagonist W–7 has been shown for a Ca^{2+}-dependent potassium channel in neurones of the snail *Euhadra* by Onozuka et al. (1987).

We tested the inhibitory action of W–7, and its chlorine-deficient analogue W–5, over a reasonable concentration range. Figure 2 illustrates that both calmodulin antagonists can suppress the vacuolar clamp current almost completely, W–7 clearly being the more effective analogue. Half-maximum inhibition (IC_{50}) occurred at about 6 μM for W–7 compared to approx. 70 μM for W–5. These concentrations and the differential activity are in accord with previous studies, i.e. W–7 clearly being more effective than its chlorine-deficient analogue W–5; Hidaka et al. (1981) showed that cell proliferation is half-inhibited when 32 μM W–7 or 200 μM W–5 is injected into Chinese hamster ovary cells.

Finally, Fig. 3 shows the kinetics of vacuolar-clamp-current inhibition by the antagonists and its reactivation by calmodulin. In the given experimental set-up (e.g. given chamber perfusion rate) half-inhibition by W–5 and W–7 requires approx. 40 s and 110 s, respectively, whereas addition of calmodulin to vacuoles pretreated with W–5, reactivates the current more quickly, i.e. to 50% within 20 s. We further note that upon withdrawal of the inhibitory substances from the perfusion fluid the block of vacuolar current is released, even in the absence of added calmodulin. Secondly, withdrawal of calmodulin while W–5 or W–7 is still present again blocks the vacuolar current (data not shown).

In conclusion, our data support the idea that plant

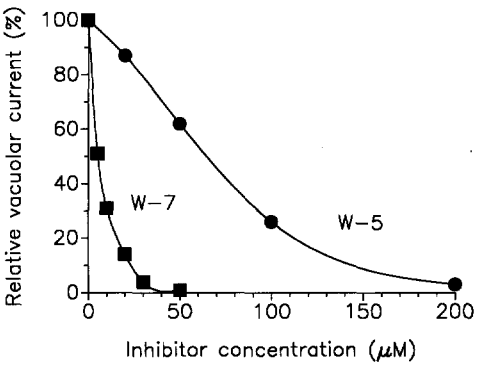

Fig. 2. Relative vacuolar currents as a function of the concentration of the calmodulin antagonists W–5 and W–7. Values (means, $n=3$) have been normalized to steady-state control currents (see Fig. 1). Half-inhibition is obtained with approx. 70 μM W–5 or 6 μM W–7

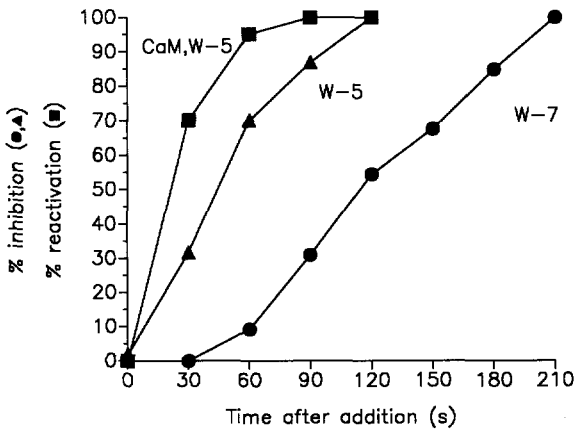

Fig. 3. Kinetics of the vacuolar clamp currents: inhibition by 50 μM W–5 or 10 μM W–7, respectively, added at t=0, and reactivation by 1 μg · ml^{-1} *Chenopodium* calmodulin *(CaM)* added at t=0 to a vacuole in the presence of 50 μM W–5. For 100% values see steady-state currents in Fig. 1

calmodulin and the calmodulin-antagonists W–5 and W–7 compete for a putative calmodulin binding site on the SV-channel in the *Chenopodium* tonoplast. Consequently we suggest that this cation channel is regulated by cytosolic or membrane-associated calmodulin as a function of the cytosolic Ca^{2+} activity. Presumably calmodulin binding to the SV-channel is sufficiently firm to withstand dissociation during vacuole preparation, because Ca^{2+}-dependent current activation is commonly observed without addition of calmodulin. So far, calmodulin stimulation of membrane-associated processes has been reported for a Ca^{2+}/Mg^{2+}-ATPase identified in plasmalemma fractions isolated from maize leaves (Robinson et al. 1988) and coleoptiles (Briars et al. 1988). A calmodulin-stimulated dissipation of the pH gradient generated in tonoplast vesicles described by Ladror and Zielinski (1990) was not very pronounced, required ATP, and was brought about by bovine brain calmodulin.

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