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Tonoplast Ion Channels from Sugar Beet Cell Suspensions[1]

Inhibition by Amiloride and Its Analogs

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ABSTRACT

The properties of the vacuolar membrane (tonoplast) ion channels of sugar beet (*Beta vulgaris*) cell cultures were studied using the patch-clamp technique. Tonoplast currents displayed inward rectification in the whole vacuole and isolated outside-out patch configurations and permeability ratios $P_{K+}/P_{Na+} = 1$ and $P_{K+}/P_{Cl-} = 5$. Amiloride and two of its analogs, 5-(*N*-methyl-*N*-isobutyl)-amiloride and benzamil, inhibitors of Na^+ channels in animal systems, blocked inward currents by reducing single-channel openings. Concentrations for 50% inhibition of vacuolar currents of 730 nanomolar, 130 nanomolar, and 1.5 micromolar for amiloride, benzamil, and 5-(*N*-methyl-*N*-isobutyl)-amiloride, respectively, were obtained from whole-vacuole recordings. The high inhibitory action (affinity) of amiloride and its analogs for the tonoplast cation channel suggests that these compounds could be used for the isolation and biochemical characterization of this protein.

Plant vacuoles may occupy up to 90% of the cell volume. The vacuole has major roles in pH and ionic regulation of the cytoplasm, turgor regulation of the cell, and the storage and retrieval of both organic and inorganic nutrients. The vacuolar membrane, the tonoplast, plays an important role in controlling the ionic concentrations in the cell, particularly for halophytes and salt-tolerant glycophytes that accumulate high concentrations of sodium chloride in their vacuoles.

Active (uphill) and passive (downhill) transport of sodium have been demonstrated in vacuoles and tonoplast vesicles isolated from beet and barley. Active sodium transport has been shown to be secondary, using the electrochemical potential difference for H^+ generated by H^+-transporting enzymes, ATPase (22), and pyrophosphatase (19), as the source of energy. A H^+-coupled Na^+ transport (Na^+/H^+ antiport) has been fairly well characterized in *Beta vulgaris* (3, 4) and in barley (8). Passive sodium transport via voltage-dependent ion channels in the tonoplast has also been demonstrated in *B. vulgaris* (5, 18). These ion channels are nonselective between Na^+ and K^+ and have a cation/anion selectivity of about 5:1. Moreover, tonoplast cation channels rectify, *i.e.* their conductance is much higher when the vacuolar membrane potential is negative with respect to the cytoplasm (18). In the physiological range of positive tonoplast potentials (about +20 mV), the conductance is very low. Such channel behavior seems to fit the role of halophyte vacuoles in relation to salinity. Halophytes, like *B. vulgaris*, must accumulate high concentrations of sodium, with a probable vacuole/cytoplasm concentration ratio of more than 10:1. Such a large vacuolar sodium concentration, together with the positive membrane potential, would result in a large outward (vacuole to cytoplasm) driving force of about 100 mV equivalent electrochemical potential difference. If the passive sodium conductance were appreciable, an electrochemical potential difference of 100 mV could not be maintained by the Na^+/H^+ antiport.

Despite increasing evidence concerning the presence of ion channels with a high selectivity for cations, and the operation of a Na^+/H^+ antiport in the tonoplast from different plant species, the biochemical characterization of these transport proteins has not been accomplished. Biochemical efforts aimed at the identification and purification of these proteins have been hampered by the lack of specific ligands suitable for labeling the proteins' subunit(s). Our previous studies have shown that the diuretic drug amiloride acts as a competitive inhibitor of the vacuolar Na^+/H^+ antiport, similar to its effects in animal cells (2). However, amiloride is not a specific inhibitor of the Na^+/H^+ antiport; it also inhibits several Na^+ transport systems including Na^+ channels, and Na^+-coupled transporters of glucose and amino acids (16). Studies of structure-activity relationships for amiloride and amiloride analogs on several ion transporters have demonstrated that appropriate modification of amiloride rendered compounds with increased affinity and specificity for a particular transport system. Introduction of hydrophobic substituents on the terminal nitrogen of the guanidino moiety, such as in benzamil, enhanced affinity for the Na^+ channel; whereas addition of alkyl groups on the 5-amino moiety, such as in MIA[2], enhanced affinity against the Na^+/H^+ antiport (16, and references therein).

We have previously shown that amiloride analogs bearing alkyl or alkenyl substituents on the nitrogen of the 5-amino moiety resulted in a 3- to 200-fold increase in potency of inhibition of the vacuolar Na^+/H^+ antiport of *B. vulgaris* cell suspensions (4). Recently, we (1) have used one of these radiolabeled analogs, [^{3}H]-MIA, to study its covalent incorporation to the tonoplast and to determine tonoplast proteins

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[2] Abbreviations: MIA, 5-(*N*-methyl-*N*-isobutyl)-amiloride; IC_{50}, concentration of inhibitor which reduced vacuolar currents by 50%.

containing specific binding sites for MIA. In this present paper, we report the use of the patch-clamp technique to study the effects of amiloride and two of its analogs, MIA and benzamil, on tonoplast channels, and we show that amiloride and benzamil inhibited the cation channel activity at submicromolar levels. The use of these compounds in combination with the photolabeling of tonoplast proteins (1) will provide a unique tool for the identification and purification of vacuolar sodium transport systems.

MATERIALS AND METHODS

Plant Material

Cell suspension cultures of sugar beet (*Beta vulgaris*) were grown as previously described (3).

Isolation of Vacuoles

Vacuoles were isolated by osmotic shock of protoplasts as described before (18).

Electrical Measurements

Ionic currents across the tonoplast were measured using conventional patch-clamp techniques (9). The seal between the patch pipette and the vacuole was attained by slightly pressing the pipette against the vacuole followed by the application of gentle suction to the pipette. Patch pipettes were made from borosilicate glass (Rochester Sci. Co, Rochester, NY), pulled in two stages with a pipette puller (Narishige Co, Japan) and fire polished. The establishment of the electrical seal was monitored by recording the current response to a 10 ms voltage pulse of 10 mV. Seal resistances of the order of 3 to 10 GΩ were obtained almost immediately after the application of suction to the patch pipette, thus obtaining the vacuole-attached configuration (9). The whole-vacuole configuration (11), obtained from the vacuole-attached mode after breaking the small area of tonoplast within the pipette by applying a 30 ms voltage pulse of 1 V, was used to measure the currents across the intact tonoplast. Single channel currents were recorded from isolated outside-out patches (vacuolar side exposed to the pipette solution), obtained from the whole vacuole configuration by pulling the patch pipette away from the vacuole (9).

Vacuolar current-voltage relations were derived from steady-state currents elicited by 5 s voltage pulses. The membrane potential was held at 0 mV and alternated pulses of ± 10 mV were applied every 10 s; this protocol was repeated with increments of ±10 mV up to ±100 mV. The reversal potential was derived using a double-pulse protocol (11, 15). Leakage current was not subtracted from the current voltage relations.

Single channel recordings were obtained by continuously polarizing isolated outside-out patches of tonoplast to levels of potential between ±80 mV. Current-voltage relationships for single channels were obtained by plotting the single channel current against the corresponding level of patch potential.

Whole-vacuole and single channel currents were recorded at 22°C with a 3900 integrating patch clamp system (Dagan Corporation, Minneapolis, MN). During the whole-vacuole recordings, the 3900 patch clamp system voltage-clamped the vacuole at the desired level of potential, measured the vacuole capacitance, and corrected for the series resistance established between the pipette and the vacuole.

Single channel recordings were low pass filtered at 500 Hz with a four pole Bessel filter contained in the patch clamp amplifier. The signal from the patch-clamp system was digitized at 44 kHz by a pulse code modulator and stored on videotape (DAS 900, Dagan, Minneapolis, MN). For subsequent analysis, data were digitized and processed with the PAT V 6.0 and VCAN programs developed by J. Dempster (University of Strathclyde, Glasgow, U.K.) in a PCII-386 computer.

Solutions

The composition of the pipette filling solution was: 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM Tris/Mes (pH 7.5), 100 mM KCl or NaCl, adjusted to 550 mOsmol with D-mannitol. The bathing solution was composed of 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM Tris/Mes (pH 7.5), 100 mM KCl or NaCl, and osmolarity adjusted to 550 mOsmol with D-mannitol.

Chemicals

Amiloride was obtained from Sigma Chemical Co. Amiloride analogs were obtained from E. J. Cragoe, Jr. (Landsdale, PA). Amiloride was dissolved in double-distilled water, MIA and benzamil were dissolved in 30% ethanol.

RESULTS

Whole Vacuole Currents

In the whole-vacuole configuration, with 100 mM NaCl in the pipette solution and 100 mM KCl in the bath, voltage pulses were applied to the tonoplast from a holding potential (V_h) of 0 mV to ±80 mV in ±20 mV steps (Fig. 1A). The negative voltage pulses elicited an instantaneous inward current of −10 to −50 pA, followed by a time-dependent inward current that increased gradually during the first second and remained constant for the duration of the 5 s pulse (Fig. 1B). The slow increase in the whole-vacuole inward current indicates a gradual activation of the tonoplast channels. On the other hand, positive voltage pulses did not elicit a measurable outward current (Fig. 1). Similar results to those shown in Figure 1 were also obtained with KCl in the pipette (not shown).

The voltage dependance of the vacuolar currents indicates that they are carried by channels in the tonoplast. This view is supported by our previous report (18) in which we showed the presence of inward rectifying cation channels in the tonoplast of sugar beet cell cultures (also see below).

The selectivity of the tonoplast channels responsible for the time-dependent current was determined using a double pulse protocol (Fig. 2). From a V_h = 0 mV, the tonoplast was clamped at −100 mV in order to open the channels. From the −100 mV level, the tonoplast potential was clamped to less negative potentials at which the deactivation of the inward currents (reversal potential) could be recorded (Fig. 2A). Under asymmetrical solutions ($[NaCl]_{vac}$ = 10 mM; $[NaCl]_{cyt}$

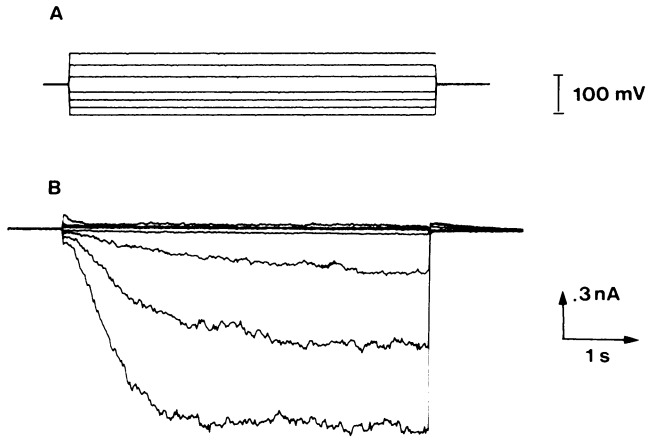

Figure 1. Recording of inward-rectifying currents in a whole-vacuole configuration in response to applied voltages. The interior of the vacuole was loaded with 100 mM NaCl; the bathing solution contained 100 mM KCl. Negative currents indicate a current flux into the vacuole, whereas positive currents indicate currents from the vacuole to the bathing solution. Superposition of multiple recordings of currents (B) across the tonoplast elicited by consecutive voltage pulses (A). From a holding potential of 0 mV the tonoplast was first polarized to −10 mV for 5 s and then returned to 0 mV; a pulse of 10 mV of the same duration was applied 5 s later, again returning to 0 mV. This cycle was repeated 10 times with increments of ±10 mV, each time returning to 0 mV. Negative potentials elicited large inward currents (downward deflection), while positive potentials induced only small outward currents (upward deflection). Only few recordings are shown for clarity.

= 100 mM), deactivation of the inward currents gave a reversal potential of 40 mV (Fig. 2B), indicating that the tonoplast channels are more selective for Na^+ than for Cl^-, with a permeability ratio, $P_{>Na^+}/P_{>Cl^-}$, equal to 4.5, as calculated from the Goldman equation. The permeability ratio $P_{>K^+}/P_{>Na^+}$ was obtained from the reversal potential with 100 mM KCl in the bathing solution. A reversal potential of 0 mV (Fig. 2B) suggests that the tonoplast of sugar beet vacuoles are unselective as between Na^+ and K^+.

The diuretic drug amiloride and several of its derivatives have been shown to inhibit Na^+ transport systems in various animal cell membranes (16). In the present work we have investigated the effect of amiloride and two of its analogs on vacuolar currents in *Beta vulgaris* cell suspensions. MIA was obtained by substitution of the protons of the nitrogen atom in the 5-amino terminal moiety of amiloride by methyl and iso-butyl groups. Benzamil was obtained by substitution of one of the protons on the terminal nitrogen atom of the guanidino moiety by a benzyl group (16).

Vacuolar inward currents were inhibited by the addition of amiloride to the bathing solution (Fig. 3). At 0.5 μM, amiloride reduced the inward current by 40% (expressed as a percentage of the current observed at −60 mV in the absence of amiloride). Increasing concentrations of amiloride induced greater inhibitions such that at 400 μM, an inhibition of 98% was observed (Fig. 3). An IC_{50} for amiloride of 730 nM was calculated from a plot of the percentage current inhibition at −60 mV *versus* the amiloride concentration (Fig. 3, inset).

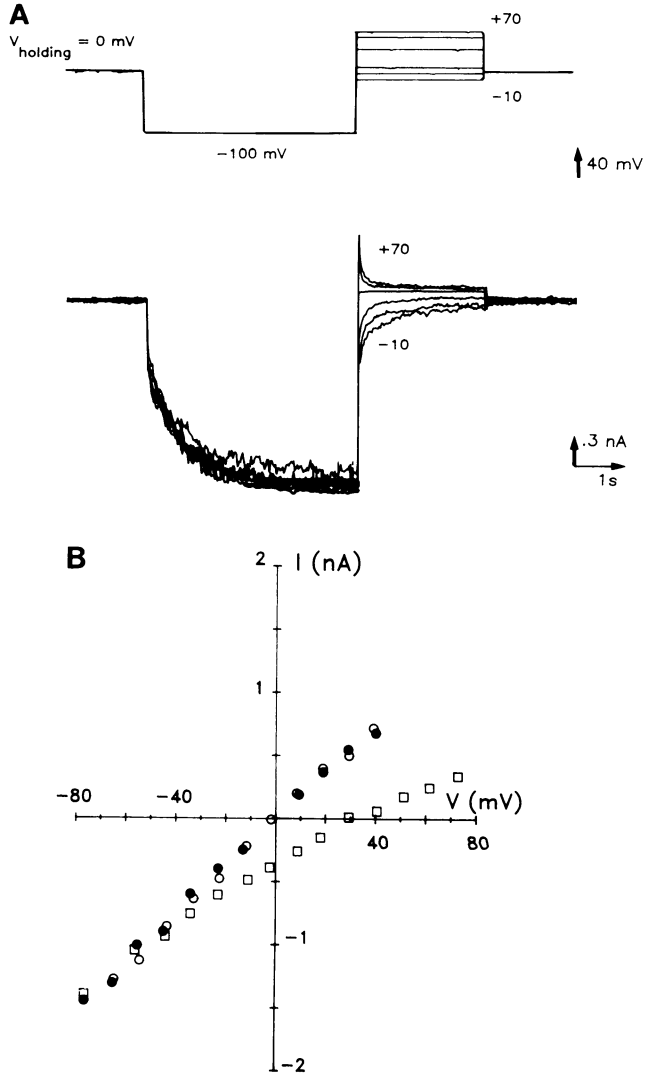

Figure 2. Selectivity of the tonoplast inward currents to cations. A, The selectivity of the tonoplast inward currents was obtained by employing a tail-current protocol with 10 mM NaCl in the vacuole and 100 mM NaCl in the bathing solution. From a V_h = 0 mV, the tonoplast was clamped at −100 mV to open the channels. Next, the tonoplast was changed to a second potential and the current was measured at the beginning of the second pulse. The deactivating currents change direction (reversal potential) at 40 mV indicating that Na^+ is 5 times more permeant than Cl^-. B, The deactivating (tail) currents are plotted against the tonoplast potential for three different conditions: (□), 10 mM NaCl in the vacuole and 100 mM in the bathing solution; (○), 100 mM NaCl at both sides of the vacuole; and (●), 100 mM NaCl in the pipette and 100 mM KCl in the bath. A reversal potential of zero mV with 100 mM KCl in the bath indicates that the tonoplast is as permeable to Na^+ as to K^+.

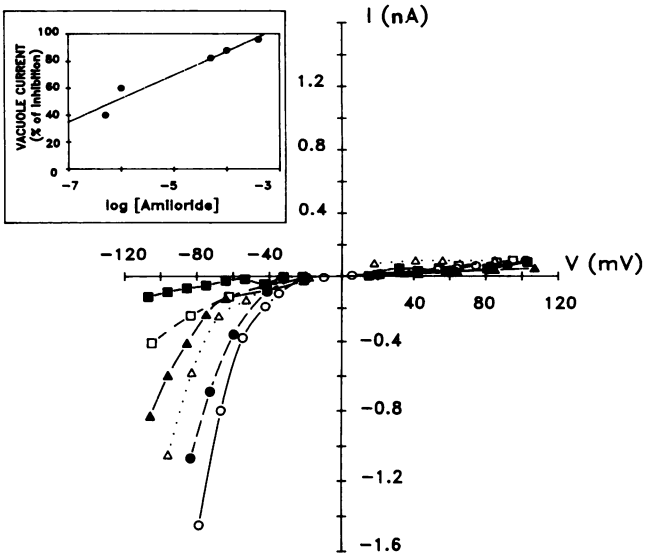

Figure 3. Effect of increasing concentrations of amiloride on the whole vacuole currents with 100 mM NaCl in the vacuole and 100 mM KCl in the bath. Conditions as described in "Materials and Methods." Inset, Plot of the percentage current inhibition at −60 mV *versus* the logarithm of amiloride concentration. An IC_{50} = 730 nm for amiloride was calculated. The line is that of best fit by the least square method. (○), Control; (●), 0.5 μM; (△), 1 μM; (▲), 50 μM; (□), 100 μM; (■), 400 μM.

Figures 4 and 5 show the effect of MIA and benzamil, respectively, on the vacuolar inward currents. MIA inhibited inward currents by 40% at 1 μM and by as much as 92% at 10 μM (Fig. 4). An IC_{50} for MIA of 1.5 μM was calculated from a plot of percentage current inhibition at −60 mV *versus* the MIA concentration (Fig. 4, inset). At 0.1 μM, extravacuolar benzamil reduced the inward currents by 45% (Fig. 5). Higher concentrations of benzamil further inhibited the inward currents, with a 95% inhibition obtained at 12.5 μM benzamil. An IC_{50} for benzamil of 130 nM was calculated (Fig. 5, inset). The inhibitory effects of amiloride and its analogs were reversible, substitution of the extravacuolar medium for an inhibitor-free solution restored the channel currents (results not shown).

Single Channel Currents

To obtain more information on the mode of action of the different inhibitors, the effects of amiloride, MIA, and benzamil on the single channel currents were studied on isolated outside-out patches of tonoplast. Original records of single channel currents at −60 mV, with 100 mM NaCl in the pipette solution and 100 mM KCl in the bath, are shown in Figures 6A, 7A, and 8A. Isolated patches of tonoplast generally presented no less than three channels, as suggested by the different levels of current observed in the single channel recordings. In the absence of inhibitors (Figs. 6A, 7A, and 8A), a pipette potential of −60 mV induced a step-wise response in the current, representing the opening (O) and closing (C) of single channels. The presence of more than one channel in the patch is indicated by the different levels in the current (O_1, O_2, . . . , O_n). The distribution of amplitudes of single channel currents (Figs. 6C, 7C, and 8C), clearly showed that the actual number of channels present in a particular patch was more than one. In addition to the peak corresponding to the zero current level (closed state), two or more peaks are observed, with amplitudes being multiples of the first current level. Gaussian fits to the different peaks from the amplitude distribution showed that the mean single channel currents were −4.0 ± 0.1 pA (Fig. 6C), −3.8 ± 0.1 pA (Fig. 7C), and −3.9 ± 0.3 pA (Fig. 8C).

Figure 6B shows the single channel activity after addition of 100 μM amiloride to the bath. It is clearly observed that upon addition of amiloride, the openings are less frequent, and in contrast to Figure 6A, few simultaneous openings occurred. The distribution of amplitudes of single channel currents (Fig. 6D), showed that the channels were closed most of the time. This is indicated by the increase in the relative frequency of the zero current level (closed state) and a corresponding decrease in the frequency of the single channel currents. Single channel currents from isolated outside-out patches were recorded continuously for up to 1 h without an apparent decrease in activity (results not shown). This suggests that the observed effect of amiloride is not a consequence of inactivation of the channels.

Upon addition of 5 μM MIA to the bath, the single channel activity in the patch was diminished (Fig. 7B), although some openings were still recorded. The amplitude distribution (Fig. 7D) showed that the relative frequency of the zero current level increased in the presence of MIA, indicating a reduction on the opening of the channels. The spread of the Gaussian fitted to the zero current level distribution (Fig. 7D), may be

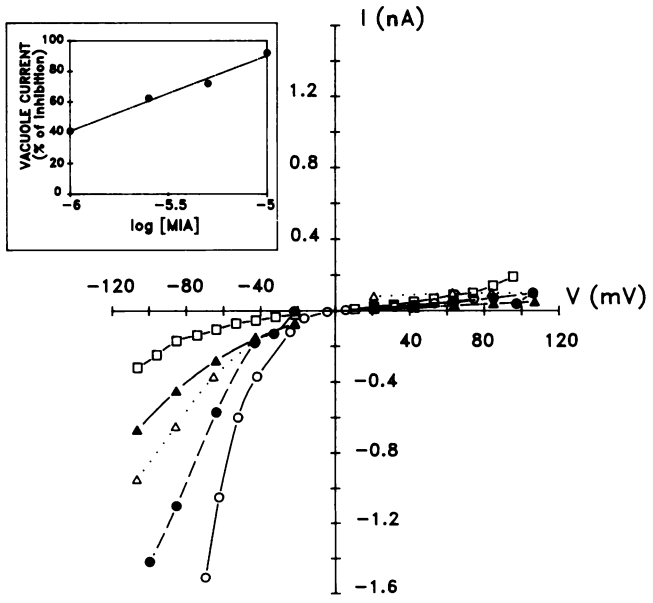

Figure 4. Effect of increasing concentrations of MIA on the whole vacuole currents. Conditions as in Figure 3. Inset, Plot of the percentage current inhibition at −60 mV *versus* the logarithm of MIA concentration. An IC_{50} = 1.5 μM for MIA was calculated. The line is that of best fit by the least square method. (○), Control; (●), 1 μM; (△), 2.5 μM; (▲), 5 μM; (□), 10 μM.

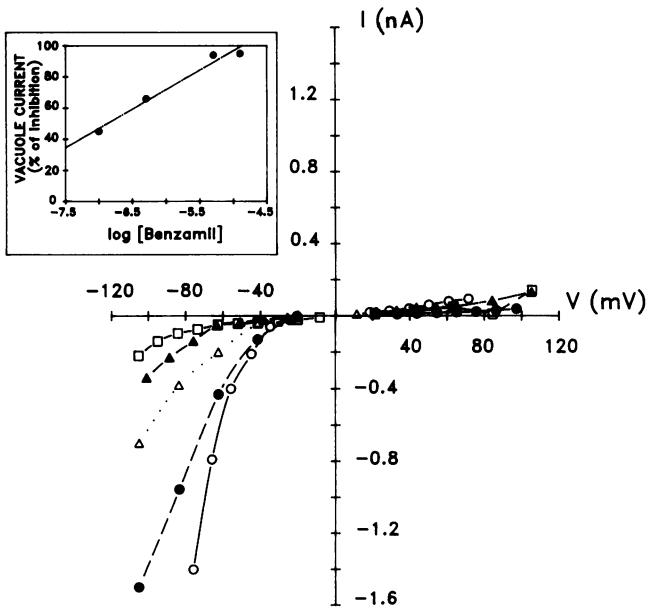

Figure 5. Effect of increasing concentrations of benzamil on the whole vacuole currents. Conditions as in Figure 3. Inset, Plot of the percentage current inhibition at −60 mV *versus* the logarithm of benzamil concentration. An IC_{50} = 130 nM for benzamil was calculated. The line is that of best fit by the least square method. (○), Control; (●), 0.1 μM; (△), 0.5 μM; (▲), 5 μM; (□), 12.5 μM.

due to the few single channel openings recorded in the presence of MIA (*cf.* Fig. 7, B and D).

Benzamil markedly inhibited single channel activity (Fig. 8). In the presence of 200 nM benzamil in the bath few channel openings were observed (Fig. 8B). The decrease in the opening of the channels is correlated with an increase in the relative frequency of the zero channel level of the amplitude distribution, where a narrow peak at the zero current level is observed (Fig. 8D).

Similar to the results obtained in the whole-vacuole configuration studies, substitution of the extravacuolar medium for an inhibitor-free medium restored single channel activities at control levels (results not shown).

The current-voltage relation of single channel currents is shown in Figure 9. In agreement with the pattern observed in whole-vacuole recordings, single channel currents showed inward rectification with a conductance of 65 pS at negative potentials, and less than 10 pS at positive potentials (Fig. 9). Addition of 100 μM amiloride to the bathing solution of an isolated outside-out patch of tonoplast did not affect the single channel conductance in the range ±80 mV (Fig. 9). MIA at 5 μM and benzamil at 200 nM, had similar effects on the single channel activity; namely, they did not affect the single channel conductance (Fig. 9).

DISCUSSION

Voltage-dependent ion channels have been described in plasma membranes of animal (14) and plant cells (13, 21). In plant cells, voltage and time-dependent inward-rectifying channels have also been found in the tonoplast (11, 18). The voltage dependence of the inward currents and the permeability ratios, $P_{>K^+}/P_{>Na^+}$ = 1 and $P_{>K^+}/P_{>Cl^-}$ = 5, obtained from whole-vacuole configuration (Fig. 2), can be correlated with the inward rectifying channels recorded from isolated patches of tonoplast from sugar beet cell cultures (Fig. 9) and Pantoja *et al.* (18). Similar channels have been observed in the vacuoles of several plant species (13).

The inward, time-dependent currents in sugar beet vacuoles were inhibited by amiloride, MIA and benzamil (Figs. 3, 4, and 5). Benzamil has the highest ability to inhibit the inward currents; it has an IC_{50} = 130 nM, and thus is six times more inhibitory than amiloride (IC_{50} = 730 nM) and twelve times more inhibitory than MIA (IC_{50} = 1.5 μM). Amiloride and benzamil have been shown to inhibit Na^+ channels in animal systems at submicromolar concentrations (6, 20). In epithelial Na^+ channels, IC_{50} values of 100 and 300 nM for amiloride (6, 20), and 10 nM for benzamil have been reported (6). Our results are in contrast to those described by Hedrich and Kurkdjian (12), who reported a 20% inhibition of the vacuolar currents of sugar beet vacuoles by 2 mM amiloride.

The pattern of inhibition obtained in beet vacuoles (benzamil > amiloride > MIA) was similar to that reported for the inhibition of Na^+ channels in animal cells (6, 16, 20).

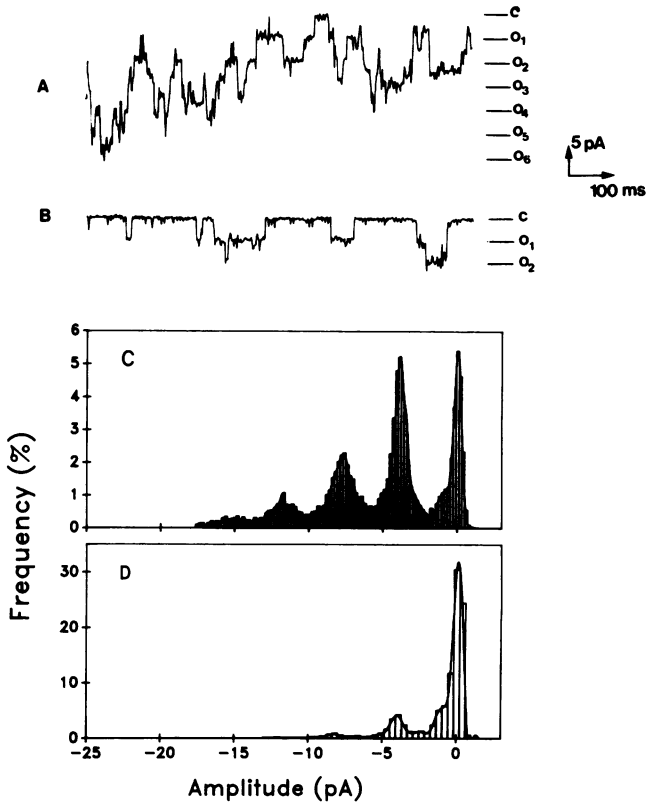

Figure 6. Effect of 100 μM amiloride on single channel currents of an isolated outside-out tonoplast patch. Conditions as described in "Materials and Methods," with 100 mM NaCl in the pipette and 100 mM KCl in the bath. A and B, Single channel currents induced by a pipette potential of −60 mV in the absence, and in the presence of 100 μM amiloride, respectively; C and D, amplitude distributions and Gaussian fit for the recordings shown in A and B, respectively.

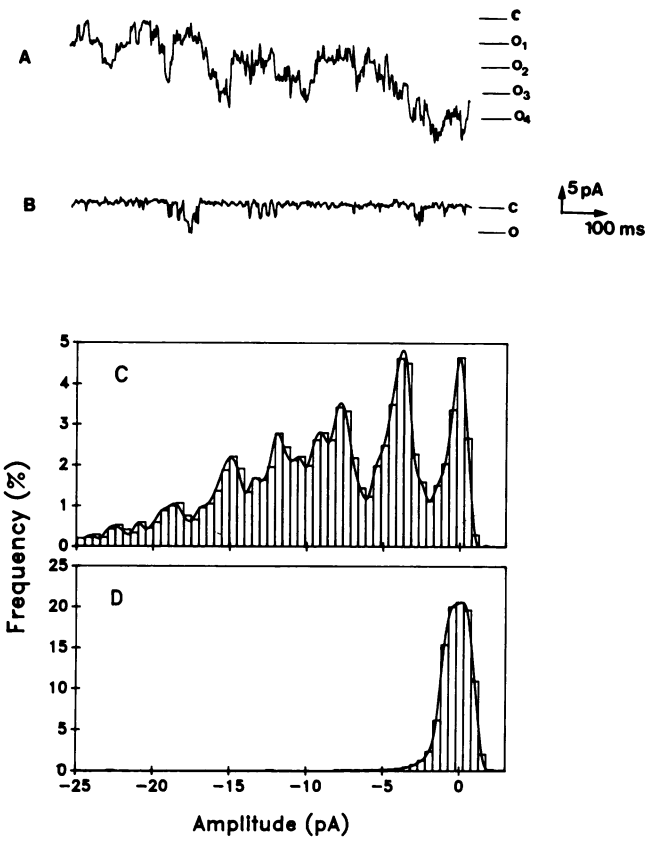

Figure 7. Effect of 5 μM MIA on single channel currents of an isolated outside-out tonoplast patch. Conditions as described in Figure 6. A and B, Single channel currents induced by a pipette potential of −60 mV in the absence and presence of 5 μM MIA, respectively; C and D, amplitude distributions and Gaussian fit for the recordings shown in A and B, respectively.

Substitution on the terminal nitrogen of the guanidino moiety of amiloride by a hydrophobic group (*e.g.* a benzyl group in benzamil) enhanced the ability of the compound to inhibit Na^+ channels (6, 7, 20). In contrast, hydrophobic substitutions on the 5 amino-group of amiloride (*e.g.* methyl and isobutyl groups in MIA) reduced the inhibitory potency of the compound on Na^+ channels (16).

Amiloride and its analogs inhibited sugar beet vacuolar currents (Figs. 3, 4, and 5), decreased the single channel activities (Figs. 6, 7, and 8) but had no effect on single channel currents (Fig. 9). The ability of amiloride and its analogs to block vacuolar currents is similar to that described by Hamilton and Eaton (10), and Palmer and Frindt (17) for Na^+ channels of epithelial kidney cells from *Xenopus laevis* and rats, respectively.

The apparent increase in inhibition by amiloride and its analogs at more negative potentials (Figs. 3, 4, and 5), suggests that the inhibition may be voltage-dependent. The voltage-dependence of the amiloride effect has been reported previously for Na^+ channels of animal tissues (10, 20). The voltage-dependent inhibitory effect may be of experimental significance. In electrophysiological experiments, the inhibition of cation channels by amiloride and/or its analogs should be tested at more negative intravacuolar membrane potentials. In uptake experiments, changes of vacuolar Na^+ concentrations which alter tonoplast membrane potential could significantly change the blocking efficiency of amiloride. Such an alteration could incorrectly indicate a competitive effect of amiloride with respect to Na^+ concentrations. In the absence of membrane potential, the affinity of the inhibitors for the channel will be decreased. Thus excess of amiloride and its analogs will be needed in binding experiments.

Although amiloride and its analogs inhibited the tonoplast cation channel in a reversible manner, their high inhibitory potency suggests that these compounds would be useful for the design of affinity labels for the biochemical identification of the channel of sugar beet tonoplast. Recently, Barkla *et al.* (1) have used the differential affinity of MIA and amiloride for the identification of polypeptides associated with the Na^+/H^+ antiport of these membranes. Photoirradiation of tonoplast in the presence of [^{3}H]MIA resulted in label incorporation by several polypeptides (1). For the identification of specifically labeled polypeptides, amiloride was used to protect against [^{3}H]MIA labeling. Their studies showed the presence of two groups of polypeptides, each group differing in their affinity for amiloride and [^{3}H]MIA. Barkla *et al.* (1) concluded that a group of polypeptides of molecular masses

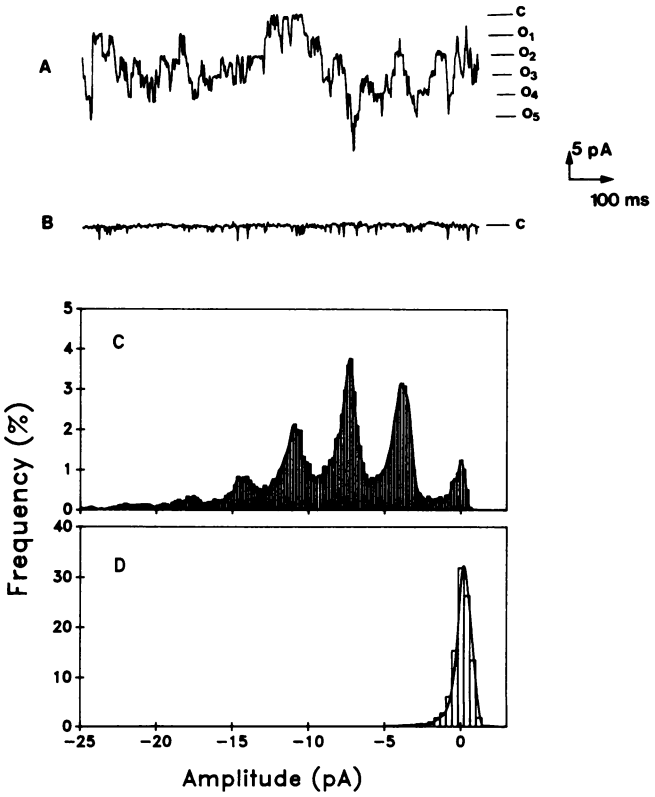

Figure 8. Effect of 200 nM benzamil on the single channel currents of an isolated outside-out tonoplast patch. Conditions as described in Figure 6. A and B, Single channel currents induced by a pipette potential of −60 mV in the absence and presence of 200 nM benzamil, respectively; C and D, amplitude distributions and Gaussian fit for the recordings shown in A and B, respectively.

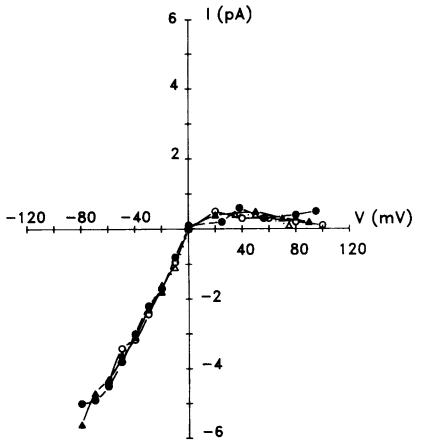

Figure 9. Effects of amiloride, MIA, and benzamil on the single channel currents of sugar beet tonoplast. Inward rectifying currents with a conductance of 65 pS were recorded with 100 mM NaCl in the pipette and 100 mM KCl in the bath. Pipette and bathing solution always contained 2 mM $MgCl_2$, 0.1 $CaCl_2$, 5 mM Tris-Mes buffer (pH 7.5), and adjusted to an osmolarity of 550 mOsmol with D-mannitol. (○), Control; (●), 100 μM amiloride; (▲), 5 μM MIA; (△), 200 nM benzamil.

174, 38, and 35 kD, with a high affinity for MIA could be associated with the tonoplast Na^+/H^+ antiport. Their results also showed that a second group of polypeptides of molecular masses 223, 123, and 32 kD displayed a higher affinity to amiloride (amiloride completely protected against labeling by [^{3}H]MIA). These results, together with the differential affinities of amiloride for the tonoplast cation channel (730 nM, Fig. 3) *versus* the Na^+/H^+ antiport (140 μM) (4), would suggest the possible association of the second group of polypeptides with the putative cation channel of sugar beet tonoplast.

Preliminary studies have shown that benzamil is a relatively weak inhibitor of the tonoplast Na^+/H^+ antiport from sugar beet cell suspensions; up to 5 μM benzamil inhibited H^+-dependent Na^+ fluxes by only 10% (BJ Barkla, E Blumwald, unpublished data). Thus the high affinity of benzamil for the tonoplast cation channel (130 nM, Fig. 5) suggests that benzamil, and amiloride, could be used as high affinity probes for the biochemical identification of the tonoplast cation channel polypeptide(s). Currently these probes are being used to protect differentially against photolabeling of tonoplast proteins by [^{3}H]MIA.

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