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Effect of Avermectins on Ca^{2+}-dependent Cl^- Currents in Plasmalemma of *Chara corallina* Cells

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Abstract. A natural complex of avermectins, aversectin C, and a component of this complex, avermectin A_1, were shown to change the conductivity of Ca^{2+}-dependent Cl^- channels of plasmalemma of *Chara corallina* cells by acting from the outer side of the cellular membrane. Low concentrations of aversectin C and avermectin A_1 increased the Cl^- current: $K_{1/2}$ = 35 ng/ml for the whole complex and $K_{1/2}$ = 21 pg/ml for A_1. Relatively high concentrations of the compounds suppressed the Cl^- current: $K_{1/2}$ = 2.2 μg/ml for aversectin C and $K_{1/2}$ = 4.2 ng/ml for A_1. The Hill coefficient for the interaction of avermectin A_1 with the corresponding targets was identical for stimulation and suppression of the Cl^- current: 2.8 and 2.5, respectively. Bicuculline, a nonspecific inhibitor of the $GABA_a$ receptors, did not influence stimulation of Cl^- currents caused by low concentrations of avermectins, but at the same time blocked suppression of the Cl^- currents by high concentrations of avermectins. Avermectins A_2, B_1, B_2, abamectin and 22,23-dihydroavermectin B_1 (ivermectin) did not affect the Cl^- currents of *Chara corallina* cells.

Key words: Ca^{2+}-dependent Cl^- channels — Voltage clamp — $GABA_a$ receptors — Aversectin C — Avermectin A_1 — *Chara Corallina*

Introduction

Avermectins are the eight macrocyclic lactones A_{ij} and B_{ij} (i = 1,2; j = a,b) produced by *Streptomyces avermitilis* (Burg et al., 1979). The natural complex and its component B_1 have high insecticidal, acaricidal and nematocidal activities against parasites of farm animals and plants and accordingly are used as active ingredient in antiparasitic formulations (Burg et al., 1979; Ostlind, Ciffli & Long, 1979; Drinyaev et al., 1999). The best known commercial preparations are based on abamectin, consisting of avermectins B_{1a} and B_{1b} in the ratio of 4:1, ivermectin, 22,23-dihydro-derivative of abamectin, and aversectin C, which is, in essence, a natural complex (Campbell, 1989; Drinyaev et al., 1999). Since avermectin A_1, A_2, B_2 turned out to be less active, as a rule, only the mechanisms of avermectins B_1 action have been studied.

Electrophysiological studies demonstrated that the effect of avermectin B_1 is, most likely, due to changes in Cl^- current in nerve and muscle cells of vertebrates and invertebrates (Friz, Wang & Gorio, 1979; Zufall, Franke & Hatt, 1989; Robertson, 1989; Schonrock & Borman, 1993). It is well established that the effect of avermectin B_1 on neurons of mammals is due to changes in Cl^- current through the $GABA_a$ receptor/Cl^- ionophore complex (Campbell, 1989; Eldefrawi & Eldefrawi, 1987; Fisher & Mrozik, 1992). In particular, it was shown (Huang & Casida, 1997) that in rat cerebellar granule neurons avermectin B_{1a} binds to two different sites on the $GABA_a$ receptor: the high affinity site with K_d = 5 nM and the low affinity site with K_d = 815 nM. 10–300 nM avermectin B_{1a} stimulates $GABA_a$-dependent Cl^- currents, but at concentrations of 1–1.5 μM it inhibits them.

Recently it was shown that avermectins have a cytostatic effect (Mosin et al., 1999; Kokoz et al., 1999; Korystov et al., 1999) and the avermectin A_1 act to a greater extent than the other avermectins. But there are almost no data regarding the electrophysiological mechanisms of avermectin A_1 effects and the differences between effects of avermectins A_1 and B_1. In this paper,

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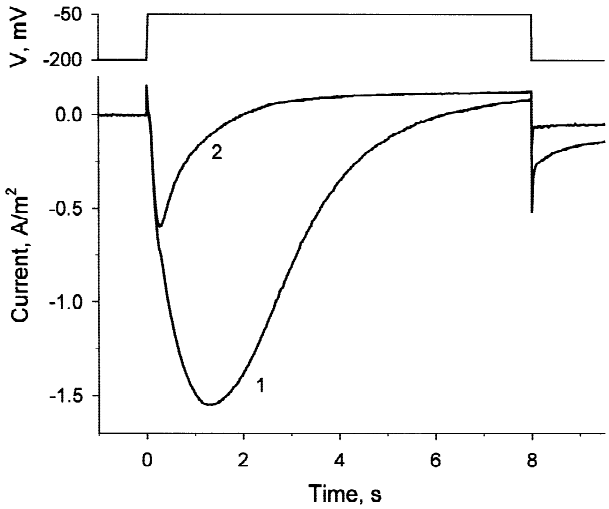

Fig. 1. Clamp potential, transient ion currents in control (curve 1) and after addition of 100 μM ethacrinic acid (curve 2) in plasmalemma of *Chara corallina* cells.

we demonstrate that avermectin A_1 (but not avermectins B_1, A_2, B_2) regulate the Ca^{2+}-dependent Cl^- currents. *Chara corallina* internodal cells were used as a convenient object for the study of transient excitable Cl^- currents (Lunevsky et al., 1983; Kataev, Zherelova & Berestovsky, 1984; Shiina & Tazawa, 1987). We show here that low concentrations of avermectin A_1 increase the Cl^- currents in cells of *Chara corallina*, while high concentrations of A_1 inhibit them, thus resembling the effect of avermectin B_1 on neurons of mammals.

Materials and Methods

Plant Material

Cells of *Chara corallina* were grown in "artificial pond water" (APW) containing (in mM) 0.1 KCl, 1.0 NaCl, 0.1 $CaCl_2$. Temperature was maintained at 18–20°C.

Electrical Recordings

A detailed procedure of current measurements was described elsewhere (Lunevsky et al., 1983; Kataev et al., 1984). Transient Cl^- currents were studied in intact and perfused cells. Voltage was clamped and currents were measured on the area of the cell with a length of 2 mm (isolation was achieved by air bridges). Microelectrodes were made out of pyrex glass. The cell was placed in a fluoroplastic chamber filled with solution containing (in mM) 0.1 KCl, 0.5 $CaCl_2$, 1.0 NaCl, 1.0 HEPES/NaOH, pH 7.2. A Dagan 8500 amplifier was used for voltage clamping. A computer IBM/AT 486 equipped with an AD/DA board PCL-718 and «Bio Quest» software was used.

Perfusion Technique

In perfusion experiments, cells were dried up with a filter paper and then positioned in the teflon chamber and cut off from both sides. One side section was filled with solution containing (in mM) 3 EGTA, 10 KCl, 20 HEPES/(KOH), 280 sucrose, pH 7.4. In the opposite section, where the other end of the cut-off cell was directed, a potential electrode was inserted and was filled with a solution containing (in mM) 0.5 $CaCl_2$, 0.05 EGTA, 10 KCl, 20 HEPES/(KOH), 280 sucrose, pH 7.4. The solution was poured in such a way that initially there was a weak flow with the rate of not more than 5–6 μm/sec directed from the first section to the second. The central section was filled with solution containing (in mM) 0.1 KCl, 1.0 NaCl, 0.5 $CaCl_2$, 1.0 HEPES/(NaOH), 200 sucrose, pH 7.4. After 10–15 min incubation, the destruction of tonoplast occurred and, after brief perfusion, there was only plasmalemma left with the associated layer of cytogel (Tazawa, Kikuyama & Shimmen, 1976; Smith & Walker, 1981). Thereafter the direction of perfusion was reversed and solution was replaced for the solution with EGTA and Ca^{2+} (Kataev et al., 1984; Shiina & Tazawa, 1987).

Preparation of Avermectins

Aversectin C was isolated and characterized as described in detail elsewhere (Drinyaev et al., 1999; Korystov et al., 1999). Aversectin C was purified by a multi-step chromatographic procedure from an ethanolic extract of the *S. avermitilis* mycelia. The composition of the product was checked by HPLC, UV spectroscopy, NMR and mass-spectrometry. Avermectin content (active ingredient) of aversectin C was about 86–90% and impurities consisted of: avermectin-related compounds (5–9%); neutral lipids (4–7%); nitrogen-bearing components (<1%); salts (<1%). HPLC chromatograms of aversectin C demonstrated the following avermectin profile: A_{1a}, 9%; A_{1b}, 4%; B_{1a}, 36%; B_{1b}, 6%; A_{2a}, 19%; A_{2b}, 4%; B_{2a}, 21%; B_{2b}, 1%. Individual avermectins were chromatographically isolated from aversectin C. Abamectin and ivermectin were supplied by Merck Co. (Darmstadt, Germany). Aversectin C, isolated individual avermectin fractions, abamectin and ivermectin were dissolved in 96% ethanol (1 mg/ml) and then diluted as required. The final concentration of the alcohol did not affect Cl^- currents.

Data Analysis

The values characterizing the action of the drugs on Cl^- currents were determined from the average dose-effect curve that was normalized to the control value by iterative nonlinear least-squares regression by the Sigma Plot program (Jandel Scientific).

Results

Is there a $GABA_A$-dependent Regulation of the Cl^- Current in *C. corallina* Cells?

Typical Cl^- currents through the plasma membrane are shown in Fig. 1. Addition of 0.1 mM ethacrynic acid, a classical blocker of Cl^- channels, inhibited these currents. According to Lunevsky et al. (1983), the major portion of this current is the Ca^{2+}-dependent Cl^- current. Figure 2 shows that neither GABA nor nembutal, the

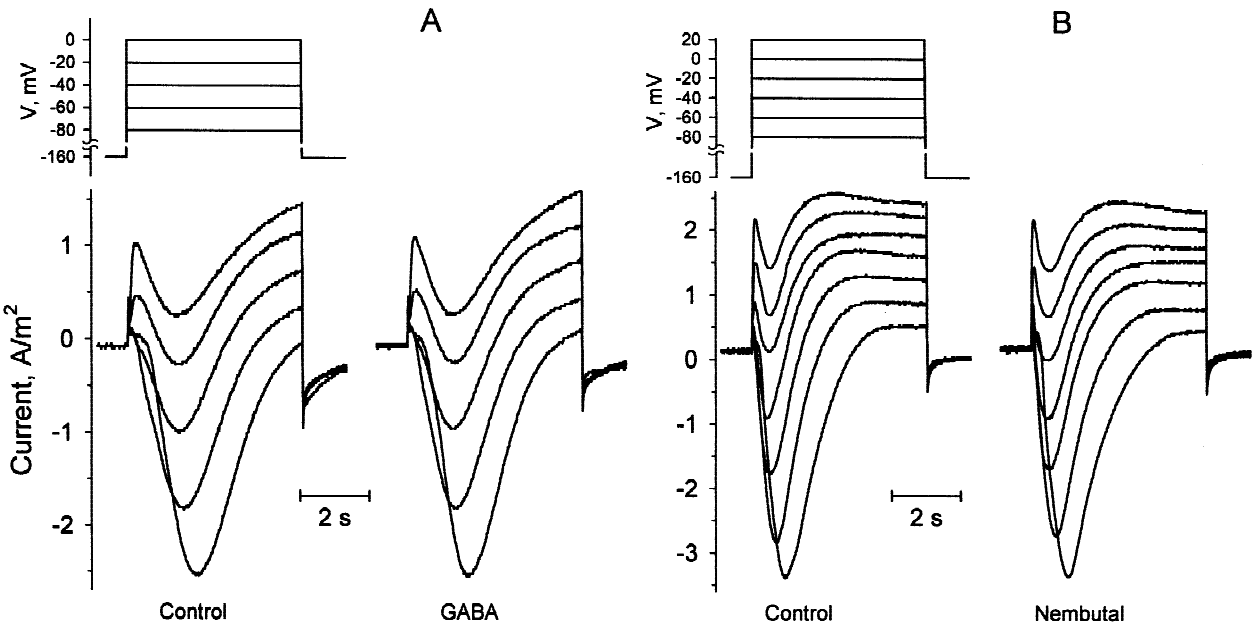

Fig. 2. Ca^{2+}-dependent Cl^- currents. (*A*) Control and after 40 min addition of 5 μM GABA. (*B*) Washout and 40 min exposure to 160 μM nembutal. (At top are shown the respective stimulation protocols).

most effective agonist of the $GABA_a$ receptors, exerted a noticeable effect on the amplitude and kinetic characteristics of the Ca^{2+}-dependent Cl^- current. In all experiments, before and after addition of these substances, currents were visibly the same. This allowed to conclude that, in *C. corallina* cells, the $GABA_a$-dependent regulation of Cl^- current is absent.

Effect of Aversectin C on Cl^- Current

The effect of aversectin C on Cl^- currents ($n = 11$) is presented in Fig. 3*A*. Aversectin C, in a concentration-dependent manner, induced both the increase and subsequent suppression in the Cl^- current. Time-course of the effect of aversectin C is shown in Fig. 3*B*. It is noteworthy that the effect of aversectin C developed slowly during several tens of minutes. The average dose-effect curve for aversectin C normalized to the control value is presented in Fig. 3*C*. The normalized dose-effect curve may be described as a product of two Hills functions.

$$\frac{I_{ex}}{I_{control}} = \left(1 + \frac{A_1(x/k_1)^{\eta 1}}{1 + (x/k_1)^{\eta 1}}\right)\left(1 - \frac{A_2(x/k_2)^{\eta 2}}{1 + (x/k_2)^{\eta 2}}\right)$$

(where x is concentration, k_1, k_2 are the dissociation constants, η_1, η_2 are the Hill coefficients characterizing cooperativity of interaction, A_1, A_2 are the amplitudes of current activation and suppression) (Fig. 3*D*). Half-maximal current activation was found at 35 ng/ml and half-maximal current inhibition was seen at 2.2 μg/ml. The other parameters are: $\eta_1 = 1.4$; $A_1 = 0.4$ (for activation) and $\eta_2 = 1.1$; $A_2 = 1.0$ (for inhibition).

Effect of Avermectins A_1, A_2, B_1, B_2 on Cl^- Current

Because aversectin C contains several avermectin, individual avermectins were tested. Data presented in Fig. 4 show that avermectin A_2, B_1, B_2 did not change the Ca^{2+}-dependent Cl^- current in *C. corallina* cells, even if applied for half an hour ($n = 6$). Fig. 5*A* shows typical changes in the amplitude Cl^- currents induced by different concentrations of avermectin A_1. Under low concentrations of 10 to 100 pg/ml, the amplitude of Cl^- current increased more than two-fold. Increase of the concentration decreased the amplitude (40 min exposure to 10 ng/ml A_1 caused a several-fold decrease of Cl^- current amplitude). The typical time course of changes in Cl^- current is shown in Fig. 5*B*. The average dose-effect curve normalized to the control value for avermectin A_1 is presented in Fig. 5*C* ($n = 7$). Separation of the normalized dose-effect curve into two components (Fig. 5*D*) allowed to calculate concentrations of avermectin A_1 causing half-maximal activation ($k_1 = 21$ pg/ml) and inhibition ($k_2 = 4.2$ ng/ml). Other parameters of the

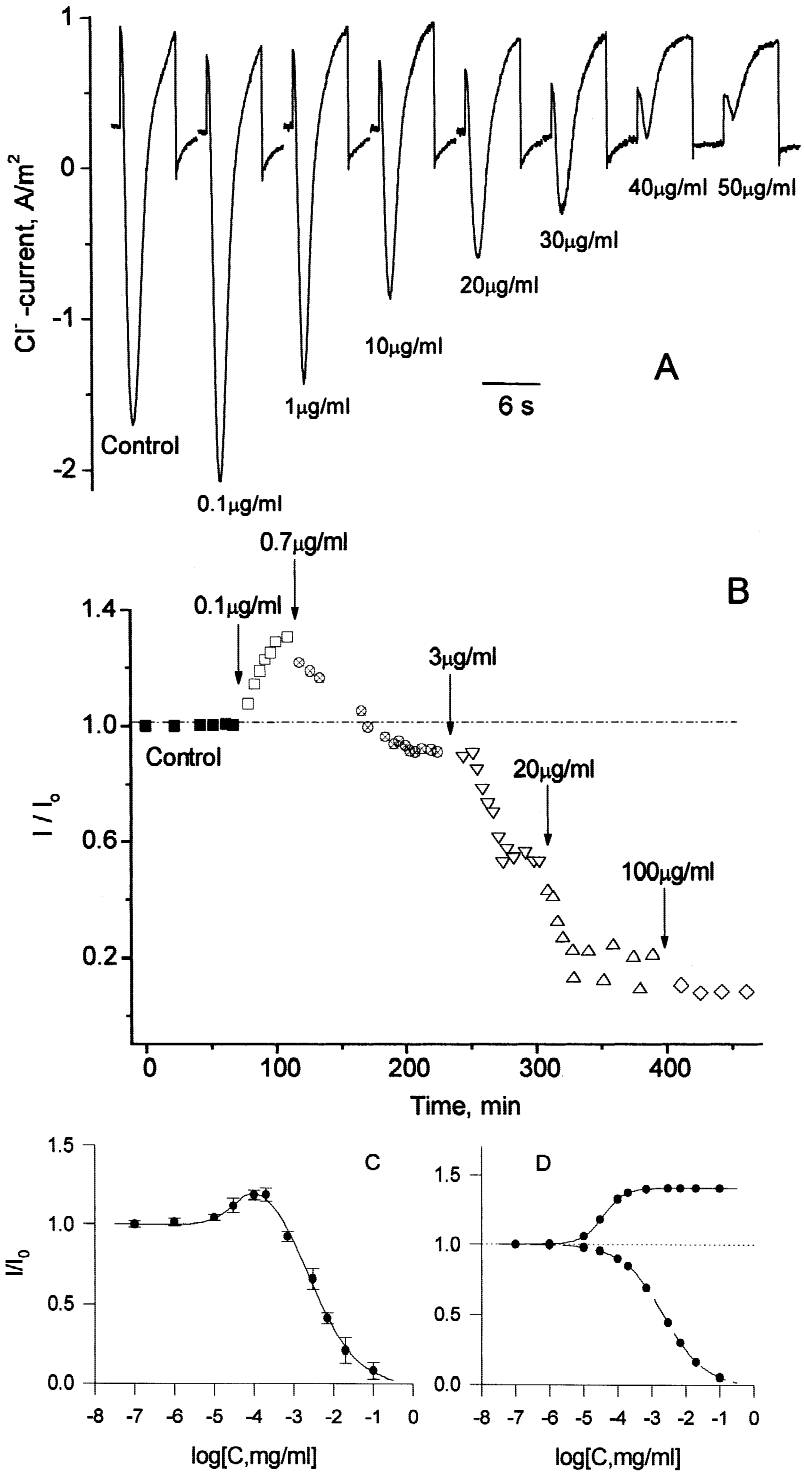

Fig. 3. Effect of the natural complex of avermectins on Ca^{2+}-dependent Cl^- currents in *C. corallina* cells. (*A*) Currents after 15 min exposure to different concentrations of natural complex. (*B*) Time course of the change in current amplitude. (*C*) Averaged dose-effect curve ($n = 6$). (*D*) Normalized curves of increase and inhibition of currents under the action of different concentrations of the natural complex obtained by separation, and normalization of the dose-effect curve. Here and below, bars at experimental points correspond to dispersion of the results of measurements.

approximation are: $\eta_1 = 2.8$; $A_1 = 1.31$ (for activation) and $\eta_2 = 2.5$; $A_2 = 0.86$ (for inhibition).

Thus, it can be suggested that binding of avermectin A_1 to two sites on the plasma membrane is responsible for changes in Ca^{2+}-dependent Cl^- currents in *C. corallina* cells.

Protective Action of Bicuculline

Figure 6*A* shows the Cl^- currents ($n = 4$) before and after action of 10 μM and 100 μM bicuculline, a nonselective blocker of $GABA_a$-receptors, on two different cells. As can be seen there is no direct effect of bicu-

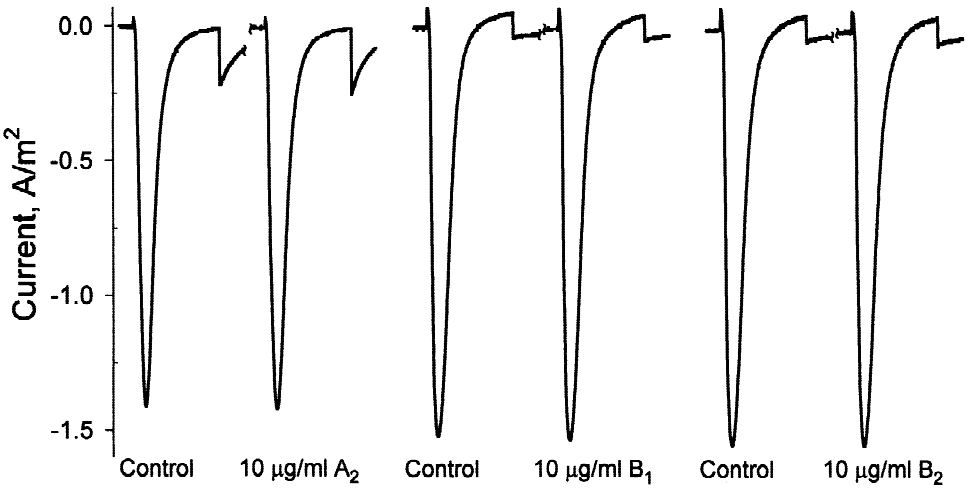

Fig. 4. Ca^{2+}-dependent Cl^- currents in *C. corallina* cells in control and after 30 min exposure to 10 μg/ml avermectins A_2, B_1, B_2.

culline on the Cl^- current. Bicuculline did not affect Cl^- currents at low concentrations of A_1 (Fig. 6*B*), but suppressed the inhibitory effect of 10 ng/ml avermectin A_1 (Fig. 6*C*). A dashed line in Fig. 6*C* shows the time course of suppression of the current by 10 ng/ml avermectin A_1 without bicuculline.

Actions of Avermectins from the Inner Side of Plasma Membrane of *C. corallina* Cells

A typical picture of development of the Cl^- current under the intracellular perfusion in control and in the presence of aversectin (a natural complex of avermectins) is presented in Fig. 7 ($n = 16$). Responses were registered when EGTA solution was substituted for a solution containing Ca^{2+} (times of substitution are indicated by arrows) and the voltage was clamped at −100 mV. Avermectins applied from the inside of the cell did not change the amplitude and kinetics of Cl^- currents. Since the Cl^- currents, registered upon addition of Ca^{2+} ions to the inside, were identical in all parameters to the Ca^{2+}-dependent Cl^- currents (Lunevsky et al., 1983) described earlier, it is possible to conclude that, in *C. corallina* cells, avermectins act only from the outer surface of the plasma membrane.

Effects of Avermectin A_1 on Characteristic Time of the Transition of Ca^{2+}-dependent Cl^- Channels from Inactivated State to Closed State

Figure 8*A* demonstrates changes in the Ca^{2+}-dependent Cl^- currents induced by 10 ng/ml avermectin A_1. It is noteworthy that at the same frequency of stimulation but at lower concentration of the A_1, the amplitude of the current did not change (*see* Fig. 5*B*). Moreover, increase in the pause between the two consecutive pulses of measurements of the Cl^- currents from 4 to 8 min also resulted in stabilization of the amplitude and frequency characteristics of the current (Fig. 8*B*). These data suggest a simple interpretation of changes in the current amplitude that is shown in Fig. 8*A*: avermectins of the A_1 group increase, in a concentration-dependent way, the characteristic time of the transition of Ca^{2+}-regulated Cl^- channels in *C. corallina* from the inactivated state into the closed state.

Discussion

Since avermectins are widely used in agriculture and many preparations utilized in plant growing are based on these compounds, the methods for determining the traces of avermectins in plants have recently been given much attention. However, the direct effect of avermectins on plants, especially on their ion-transporting systems, has not yet been studied. It is evident from this study that the natural complex of avermectins (aversectin C) and avermectin A_1 at very low concentrations block Ca^{2+}-dependent Cl^- current: the half-maximum effect ($K_{1/2}$) is 2.2 μg/ml for aversectin C and 4.2 ng/ml for avermectin A_1. The analysis of the dose-effect curves (Figs. 3 and 5) and the estimation of the molecular weights show that the Ca^{2+}-dependent Cl^- current in our experiments was almost completely blocked at aversectin concentration of about 10 μM and avermectin A_1 concentration of 10 nM. Recall for comparison that, according to published data, the concentrations of known and widely used blockers of Ca^{2+}-dependent Cl^- current in plants such as A-9-C, ethacrynic acid, ABA, etc., necessary for complete blockage, is no less than 0.1 mM (Shiina & Tazawa, 1988; Lunevsky et al., 1983; Tyerman, Findley & Paterson, 1986; Schauf & Wilson, 1987).

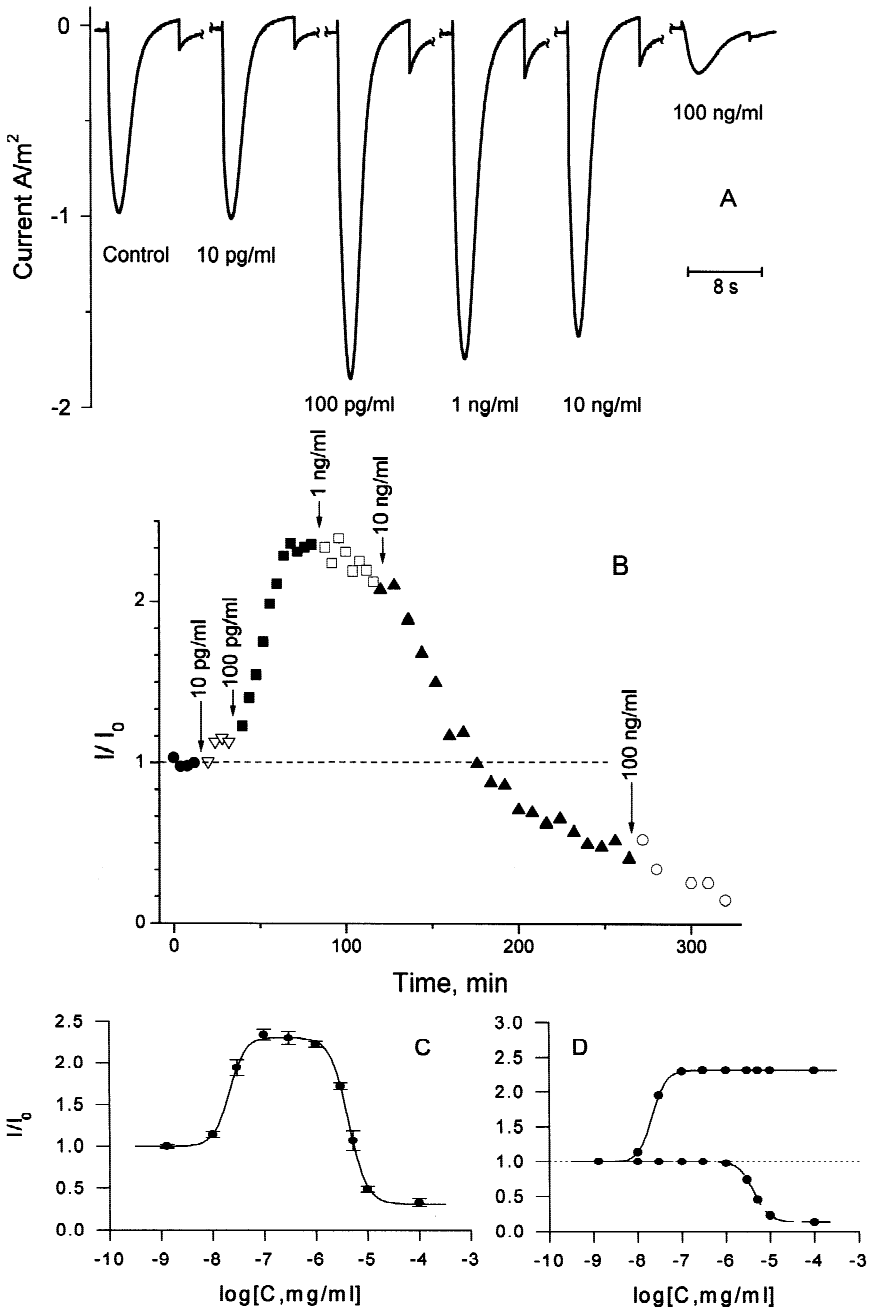

Fig. 5. Effect of avermectin A_1 on Ca^{2+}-dependent Cl^- currents. (*A*) Recording of currents after 15 min exposure to different concentrations of A_1. (*B*) Time-course of A_1-induced changes in the amplitude of Cl^- currents. (*C*) Averaged dose-effect curve ($n = 7$). (*D*) Normalized curves of increase and suppression of currents by different concentrations of avermectin A_1 obtained by separation and normalization of the dose-effect curve.

The effect produced by avermectin A_1 on Ca^{2+}-dependent Cl^- current in *C. corallina* cells is qualitatively similar to the effect of avermectin B_{1a} on $GABA_a$-dependent Cl^- currents in rat cerebellar granule neurons: low concentrations activate Cl^- currents, and relatively high concentrations block them, the K_d (activation)/K_d (inhibition) ratio in rat cerebellar ganule neurons and *C. corallina* cells being of the same order of magnitude. This fact can be interpreted as being in favor of the hypothesis that the sites of binding of Cl^- channels to avermectins are universal.

It has been shown earlier (Pouliot et al., 1997) that avermectins of the B_1 group are inhibitors of P glycoprotein. The main function of P glycoprotein is the ATP-dependent transport of foreign substances out of the cell, which, to a large extent, determines the multidrug resis-

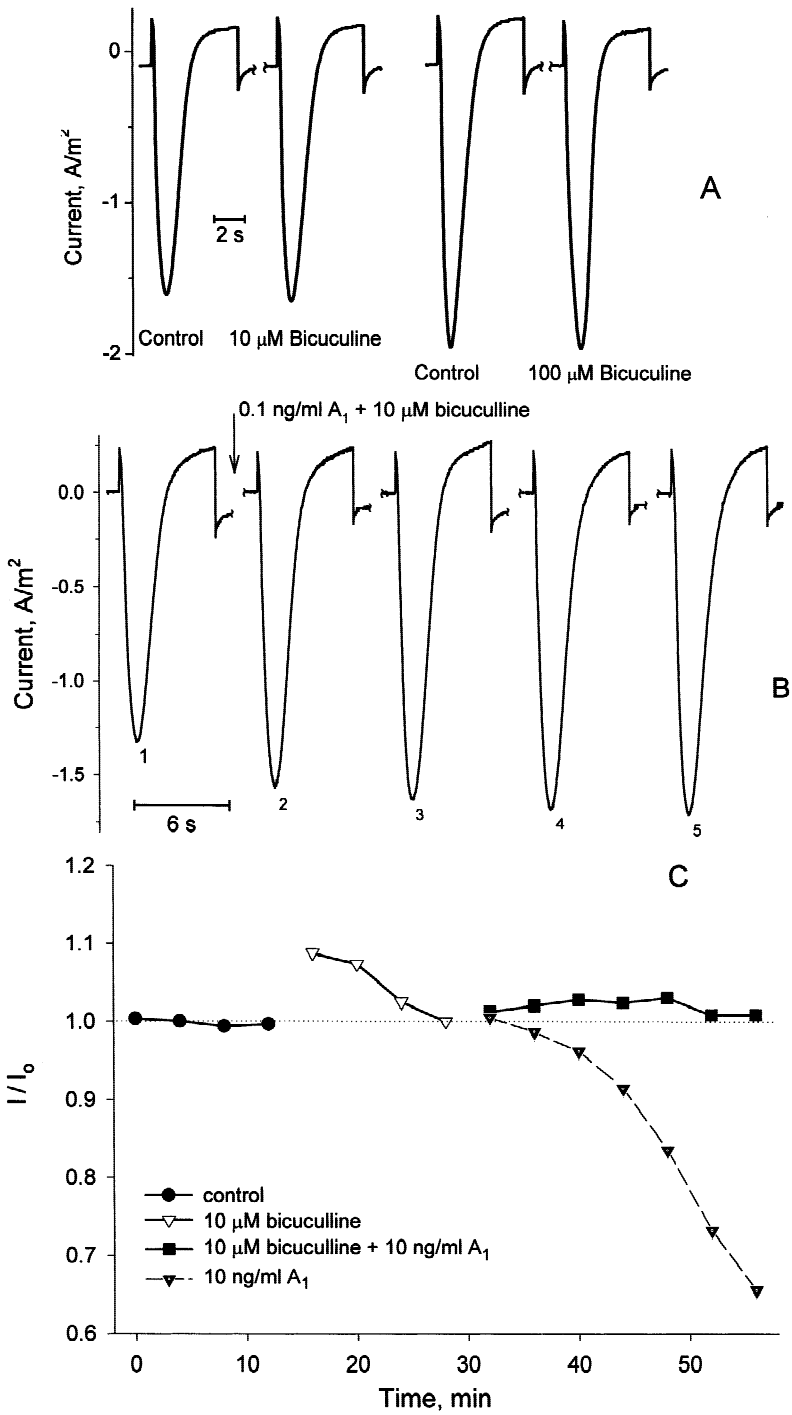

Fig. 6. Protective action of bicuculline. (*A*) Currents in control and after 20 min exposure to 10 and 100 μM bicuculline. (*B*) Effect of 0.1 ng/ml avermectin A_1, which was added after exposure to 10 μM bicuculline. Time internal between consecutive recordings was 4 min. (*C*) Effect of 10 ng/ml avermectin A_1, which was added after exposure to 10 μM bicuculline. Dashed line shows the change in amplitude induced by 10 ng/ml avermectin A_1 in the absence of bicuculline.

tance of tumor cells to chemotherapy (Lautier et al., 1996; Van der Heyden et al., 1995). There is evidence that P glycoprotein itself is a chloride channel or a component thereof (Gill et al., 1992). Starting from the above hypothesis and our results, it may be supposed that avermectins of group A_1 have the same effect on P glycoprotein-associated multidrug resistance as avermectins of the B_1 group.

The data presented here (low effective concentrations, a high efficacy observed only after the application of avermectins from the outer side of plasmalemma, etc.) suggest that avermectin A_1 has its own receptor coupled with the Ca^{2+}-sensitive chloride ionophore complex. Then it can be concluded that the natural complex contains at least two pairs of avermectins (A_1 and B_1) capable of blocking both types of Cl^- channels, $GABA_a$

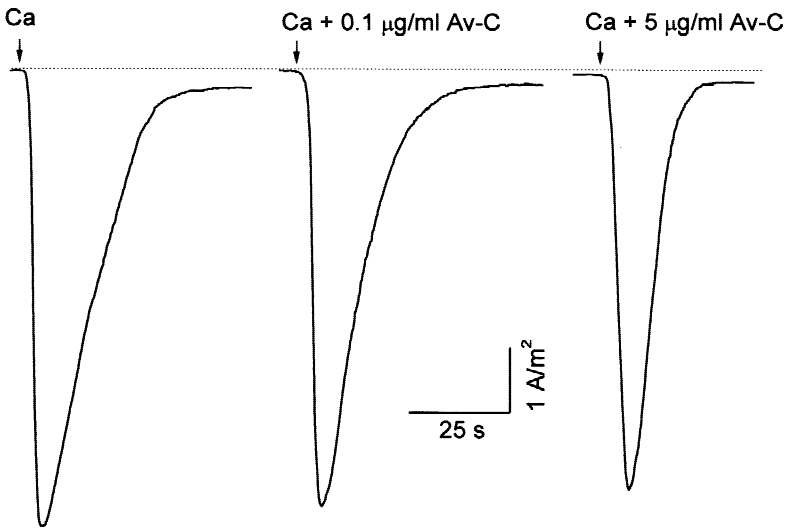

Fig. 7. Effect of the natural complex of avermectins from the inner side of plasmalemma. Currents were recorded under cell perfusion by replacement of solution containing EGTA with solution containing Ca^{2+}.

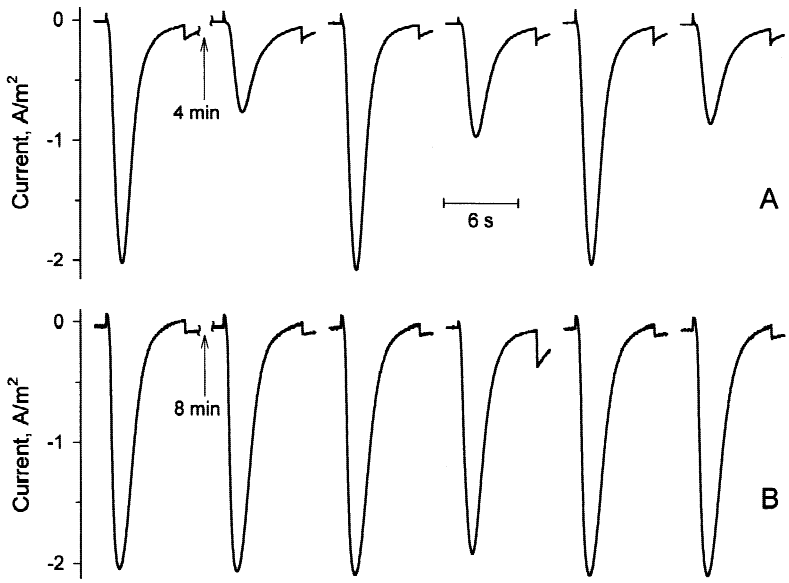

Fig. 8. Effect of avermectin A_1 on the characteristic time of transition of Ca^{2+}-regulated Cl^- channels from the inactivated state to the closed state. Cl^- currents in the presence of 10 ng/ml avermectin A_1 recorded at different frequency of stimulation: (*A*) 4 mHz, (*B*) 2 mHz.

and Ca^{2+}-sensitive. Therefore, the effect of the natural complex of avermectins can be stronger than that of individual components.

The data of this study show that $GABA_a$ does not activate Cl^- currents in *C. corallina*. It is also known that the selectivity and pharmacology of Ca^{2+}-dependent Cl^- channels in *Chara* cells are similar to those of such channels in mammalian neurons (Hedrich & Jeromin, 1992). These facts suggest that the cytostatic effect of avermectins in vitro and the neurotoxic effect in vivo (Lankas, Cartwright & Umbehauer, 1997) produced by avermectins in animal organism are, at least partially, related to changes in Ca^{2+}-dependent Cl^- currents. In addition, it can be concluded that avermectins may affect not only the pest but host plants, too.

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