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Modulation of the ER Ca^{2+} channel BCC1 from tendrils of *Bryonia dioica* by divalent cations, protons and H_2O_2

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Abstract **Electrical properties of the ER Ca^{2+} channel BCC1 from tendrils of *Bryonia dioica* were analyzed after incorporation of BCC1 into black lipid bilayers. Single channel current fluctuations were modulated by divalent cations, protons and H_2O_2. Whereas the channel is permeable for Ca^{2+}, Ba^{2+} and Sr^{2+}, its conductance is strongly reduced in solutions containing $MgCl_2$. Cu^{2+} and Zn^{2+} are potent inhibitors of BCC1 in micromolar concentrations. The open channel conductance of BCC1 increases with acidification of the electrolyte solution. H_2O_2 shows strong inhibitory effects on BCC1. The channel is almost completely closed at submillimolar concentrations of H_2O_2. The effects of pH and H_2O_2 on channel properties are directional and affect BCC1 at the Ca exit side, but not on the entry site. Thus, cytosolic pH and H_2O_2 levels may play an important role in the modulation of the cytoplasmic free calcium concentration through BCC1.**

Key words: Calcium channel; Calcium signaling; Endoplasmic reticulum; Hydrogen peroxide; Tendril

1. Introduction

Calcium is a multifunctional signal transducer in plant cells [1,2]. The ion may enter the cell through influx from the apoplastic space or it may be released from internal stores. While it has been shown that a range of stimuli such as wind force, touch, cold shock or pathogen elicitation mobilize calcium from different cellular pools [3–5], the molecular mechanisms regulating calcium influx or calcium release are very poorly understood in plants. This is particularly true for the endoplasmic reticulum (ER). ER membranes harbor a primary active calcium ATPase (e.g. [6,7]) directed to pump calcium into the ER lumen. The ER thus has to be considered a calcium storage compartment. Klüsener et al. [8], who have recently identified the first plant ER ion channel, BCC1, from mechanosensitive tendrils of *Bryonia dioica*, have indeed shown it to be a calcium selective channel with clearly rectifying properties which serves to release calcium from the ER lumen into the cytoplasm. Triggered release of calcium from ER stores is probably of widespread importance for plants and may not only be relevant for the general mechanosensitivity of plant cells [2] but also for graviperception [9,10], for pathogen defenses (for review: [11]), for plant movements (e.g. [12]) and for morphogenetic processes, such as photomorphogenesis (e.g. [13]).

BCC1 offers the unique opportunity to study processes affecting ER calcium release at the molecular level, as this channel is readily prepared from *B. dioica* tendrils and can be incorporated in functional form into black planar lipid bilayers using a modified Mueller-Rudin technique [8,14]. Using this approach, we now present data showing that BCC1 activity is influenced by divalent cations, pH, and H_2O_2. BCC1 is consequently geared to integrate different cellular input signals and to transduce these into a calcium response. Our data suggest that regulated calcium release from the ER must be considered an important element of signal transduction in higher plants.

2. Materials and methods

Tendrils of *Bryonia dioica* (12–16 cm long [7]) were harvested and stored under liquid nitrogen until use. Microsomes and ribosome-free ER vesicles were prepared as described previously [7,8]. ER membrane fragments were resuspended in 250 mM sucrose, 6 mM $MgSO_4$, 25 mM HEPES-KOH pH 7.2 at a protein concentration of 0.5–1 mg/ml.

Planar lipid bilayers [8,14] were prepared from a solution of 80 parts (w/w) 1-palmitoyl-2-oleoyl-glycero-3-phosphatidylcholine and 20 parts (w/w) 1,2-dioleoyl-glycero-3-phosphatidylethanolamine (Avanti Polar Lipids Inc., Alabaster, AL) dissolved in *n*-decane (15 mg/ml). All reconstitution experiments [15,16] were carried out in perspex cuvettes, the hole on which the bilayer was painted being 0.1 or 0.2 mm in diameter. The lipid bilayer separates the cuvette into a *cis* and a *trans* compartment. We define the *trans* compartment to be at ground potential [8]. The value of the applied voltage is therefore that of the electrode potential in the *cis* compartment and a positive current (upward deflections) corresponds to a cation transfer from *cis* to *trans*.

Ca^{2+} channels were inserted into the bilayer by adding ER vesicles (0.5–1 μg of protein) to the *cis* aqueous solution. All experiments were carried out at 18°C. Single channel recordings were performed under voltage clamp conditions using a BLM-120 (Biologic, Echirolles, France) current amplifier. The amplifier signal was filtered with a corner frequency of 1 kHz and recorded continuously on a DAT recorder (DTR-1204, Biologic). Current tracks were digitized with a sampling frequency of 10 kHz using the computer (Power Mac 7100/66) controlled EPC-9 patch-clamp amplifier [17] and Pulse software [18] from Heka (Lambrecht/Pfalz, Germany). Single channel current amplitudes and open state probabilities were determined with the help of the single channel analysis program Mac-TAC (Instrutech Inc., New York). Mac-TAC uses the 50%-threshold-crossing method [19] for the detection of signals.

3. Results and discussion

3.1. Channel activity of BCC1 is modulated by divalent cations

Following incorporation into black lipid membranes, the current-voltage relationship of the ER calcium channel was determined for various cations using symmetrical 50 mM divalent cation chloride solutions (Fig. 1a). The open channel exhibited ohmic I/V relationships, with single channel conductances declining in the order $Ca^{2+} \approx Ba^{2+} > Sr^{2+} > Mg^{2+}$ (Fig. 1). Whereas the channel shows a good permeability for Ca^{2+}, Ba^{2+} and Sr^{2+}, its permeability is strongly reduced in electro-

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lytes containing $MgCl_2$, most probably because Mg^{2+} possesses a large hydration shell, which dehydrates very slowly [20]. In solutions containing $CaCl_2$ and $MgCl_2$, a slight anomalous molar fraction effect [21] could be observed (Fig. 1b). For this experiment, the electrolyte solutions contained a constant total amount of 50 mM divalent cation chloride, but different molar fractions of $CaCl_2$ and $MgCl_2$. It is obvious that the single channel conductance of BCC1 is reduced with declining Ca^{2+} and rising Mg^{2+} concentrations. Whereas the dotted line is the theoretically expected curve, if permeation of Ca^{2+} and Mg^{2+} ions were independent of each other, the experimentally determined relationship is non-linear, and the conductances are smaller than the theoretical ones. The anomalous molar fraction effect is often interpreted in such a way that the ion channel either contains more than one binding site, or that several ions may bind to the same site [22].

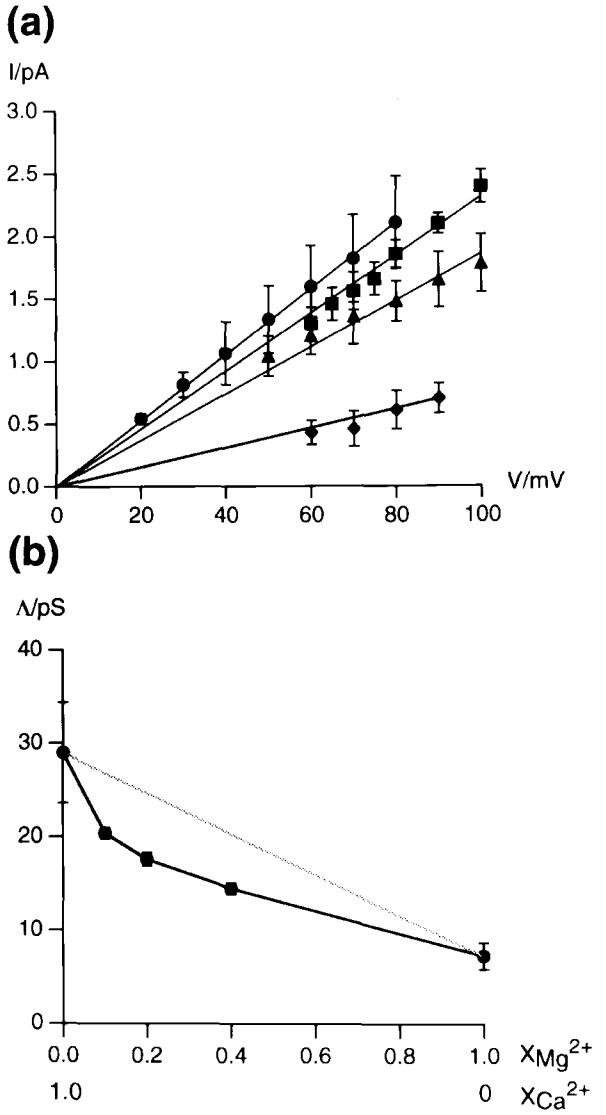

Fig. 1. Activity of BCC1 in the presence of different divalent cations. Current-voltage relationships for the ER Ca^{2+} channel in symmetrical 50 mM $CaCl_2$ (a) (●, $n=6$, $\Lambda=29$ pS), $BaCl_2$ (■, $n=9$, $\Lambda=26$ pS), $SrCl_2$ (▲, $n=6$, $\Lambda=18$ pS), $MgCl_2$ (◆, $n=5$, $\Lambda=8$ pS). Anomalous molar fraction effect of Ca^{2+}/Mg^{2+} mixtures (b). The solid line shows the experimentally determined single channel conductances of BCC1 in electrolytes with a total concentration of 50 mM divalent cations, as a function of different molar fractions of $CaCl_2$ and $MgCl_2$. The dotted line is the theoretically expected curve, if permeation of Ca^{2+} and Mg^{2+} ions were independent of each other.

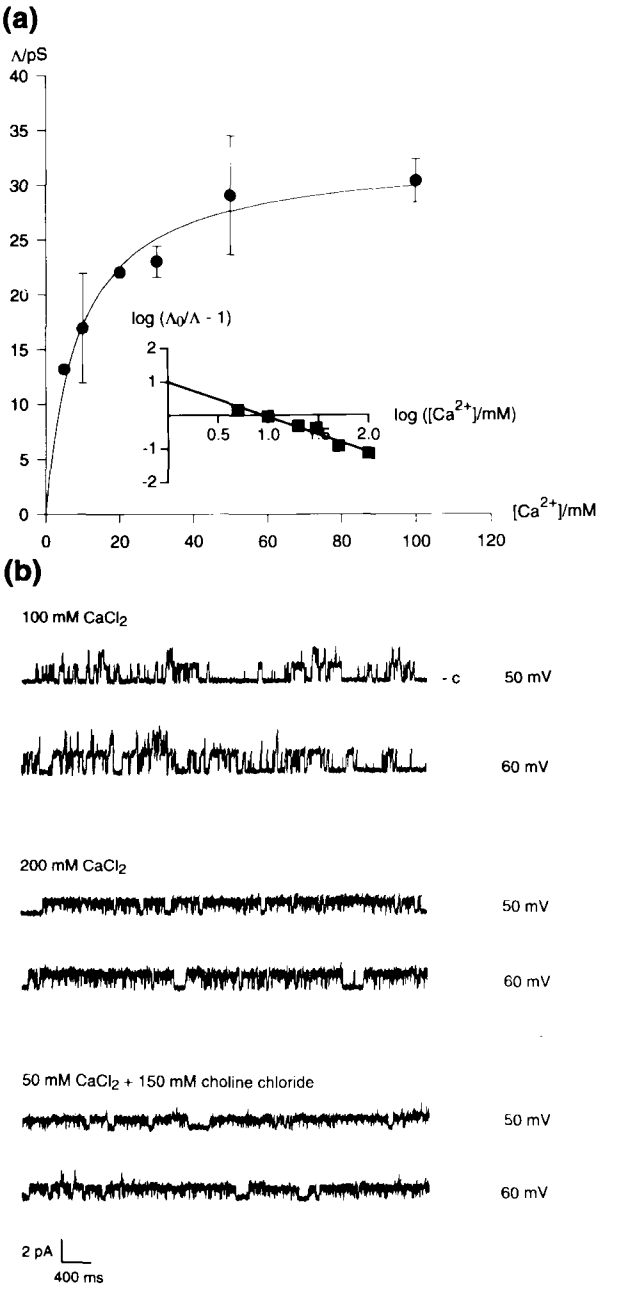

Fig. 2. Calcium dependence of BCC1 activity. Ca^{2+} concentration dependence of BCC1 open channel conductance (a). Data from at least three bilayer recordings were averaged and fitted to a Hill function with exponent $\nu=1$. Current fluctuation traces of BCC1 in electrolytes with different Ca^{2+} concentrations (b). Shown are typical recordings at 50 and 60 mV.

In Fig. 2a, the single channel conductance of BCC1 is plotted against the calcium concentration. Data points ($n=3$) were fitted to a Hill-function of the following equation: $\Lambda=\Lambda_0\,[1/(1+(K_m/[Ca^{2+}])^{\nu})]$, where Λ is single channel conductance, Λ_0 maximum conductance ($\Lambda_0=32.45$ pS), K_m 8.80 mM, ν Hill coefficient. For the fitting of the data points, an exponent of $\nu=1$ was used. Half-maximal conductance is achieved at a Ca^{2+} concentration of $K_m=8.80$ mM. The inset

shows a double-logarithmic plot of the data points. A linear relationship with a slope of 1 could be determined.

Raising the Ca^{2+} concentration to 200 mM resulted in a reduction of single channel conductance ($\Lambda = 21.6$ pS; $n = 3$). The recorded fluctuation traces show very strong flickering gating properties (Fig. 2b). Conductance values are reduced, because flicker frequency is too high to be resolved by the electronic measuring device. An averaged value is adopted, which is determined by the ratio of open to closed times of flicker events. The same effect was observed when the ionic strength of the electrolyte solution was raised by adding 150 mM choline chloride to 50 mM $CaCl_2$ (cf. Fig. 2b). Single channel conductance was reduced to 18 pS, compared to 29 pS in solutions without choline chloride. The flickering closures of the open state [23] therefore do not represent a blocking effect of high concentrations of Ca^{2+} ions, but a more general effect of high ionic strength.

A number of divalent cations block calcium passage through the ER calcium channel in low concentrations: Cu^{2+} ($I_{50} = 1.2$ μM, $n = 3$), Zn^{2+} ($I_{50} = 3.4$ μM, $n = 3$) and Cd^{2+} ($I_{50} = 100$ μM, $n = 3$). The dose-response curves for the channel blockade by Cu^{2+} and Zn^{2+} are shown in Fig. 3. All tested divalent cations only blocked the channel when they were added to the Ca^{2+} entry side (*cis* compartment), while having no effect when added to the exit (*trans*) side (data not shown). It is therefore likely that these ions bind to the Ca^{2+} binding sites, thus blocking the channel sterically [24].

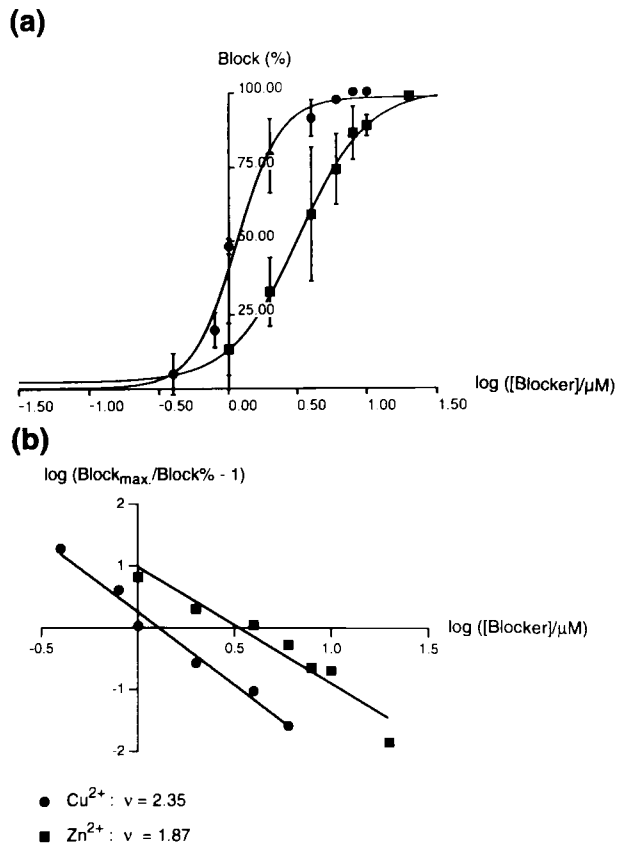

Fig. 3. Blockade of BCC1 by divalent cations. Dose-response curve ($n = 3$) for channel blockade by Cu^{2+} (●) and Zn^{2+} (■) (a). Block (%) is defined as 100. ($1-P_{oa}/P_{ob}$), with P_{oa} and P_{ob} being single channel open state probabilities after and before the addition of Cu^{2+} or Zn^{2+}. Double-logarithmic plot for blockade of BCC1 by Cu^{2+} (●) and Zn^{2+} (■) (b). The curve was fitted by the following equation: $\log(\text{Block}_{max}/\text{Block}\% - 1) = \nu \cdot \log K_m - \nu \cdot \log([\text{Blocker}]/\mu M)$, with Block_{max} being 100%.

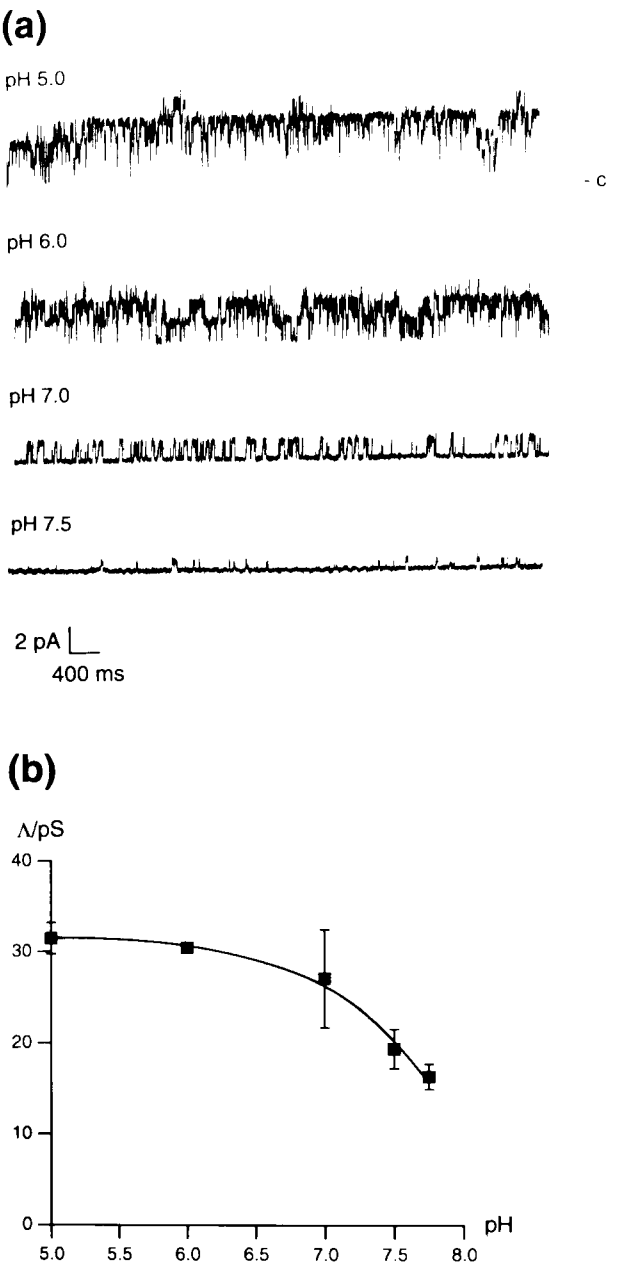

Fig. 4. Dependence of BCC1 on pH. Current fluctuations of the ER Ca^{2+} channel at different pH (a). Shown are typical recordings from four different bilayers in symmetrical 50 mM $CaCl_2$ at an applied voltage of 50 mV. Dependence of open channel conductance on pH ($n = 3$) (b). Single channel conductance of BCC1 was measured in symmetrical 50 mM $CaCl_2$ solutions.

In Fig. 3b, the blockade of BCC1 by Cu^{2+} and Zn^{2+} is shown as a function of blocker concentration on a double-logarithmic plot. The slopes of the regression lines are $\nu = 2.35$ for Cu^{2+} and $\nu = 1.87$ for Zn^{2+}, suggesting that more than one Cu^{2+} or Zn^{2+} ion is necessary to block the channel.

3.2. BCC1 is regulated by pH

The process of insertion of the ER calcium channel into the bilayer and the open channel conductance of BCC1 are pH-dependent. Decreasing the pH value increased the number of

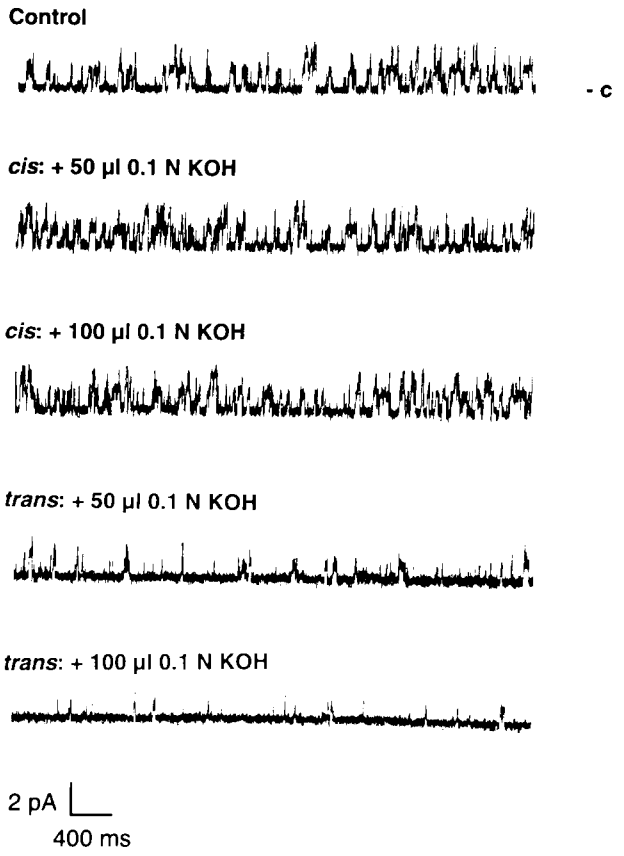

Fig. 5. Sidedness of the effect of pH on BCC1 activity. KOH was first added to the *cis* and then to the *trans* compartment. Single channel fluctuations were recorded at an applied voltage of 50 mV in 50 mM $CaCl_2$, and taken from the same bilayer.

reconstituted ion channels (Fig. 4a) and their mean conductance value (Fig. 4b). In Fig. 4a current fluctuation traces of BCC1 are shown at pH values between pH 5.0 and pH 7.5. The recordings were done under symmetrical ionic conditions using different bilayers for each pH value. However, the traces shown represent the typical fluctuation patterns at the respective pH. A similar pH effect on the insertion of pore-building proteins into black lipid bilayers was observed for the tetanus [25] and diphtheria toxins [26] as well as for the phalloporin from *Amanita phalloides* [27]. Protonation of the channel protein obviously turns it into a conformation in which insertion into the bilayer is energetically favored. This would be the case for example, if the channel protein is converted into a more hydrophobic state by protonation of negatively charged amino acid residues.

The relationship between open channel conductance and pH value is shown in Fig. 4b. Whereas the mean open channel conductance decreases only slightly from $\Lambda = 31.5$ pS ($n = 5$) at pH 5.0 to $\Lambda = 29$ pS ($n = 38$) at pH 7.0, a strong reduction by 13 pS is observed in the range between pH 7.0 and pH 7.75 ($\Lambda = 16.3$ pS, $n = 3$). At pH 8.0, current fluctuations were no longer visible, even if a calcium channel, which was active at pH 7.0, was titrated in a continuous experiment to pH 8.0. All above mentioned experiments were carried out under symmetrical pH conditions, in order to avoid asymmetrical changes in surface potential. However, to test the sidedness of the pH effect on single channel conductance, it was necessary to change the pH of the electrolyte solution asymmetrically in some measurements. The results of these experiments are summarized in Fig. 5. Whereas the upper (control) trace illustrates current fluctuations of BCC1 under symmetrical neutral pH conditions, the lower traces show the respective current fluctuations after adding 50 and 100 µl KOH first to the *cis* and

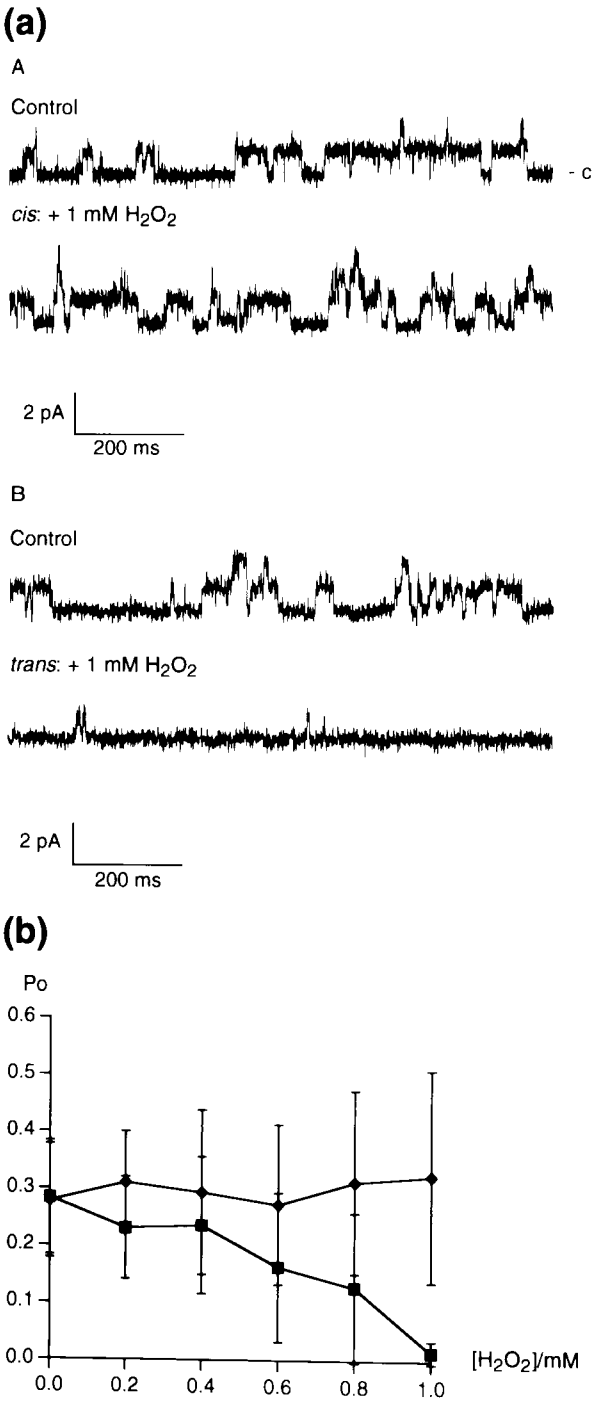

Fig. 6. Effect of H_2O_2 on single channel properties of BCC1. Sidedness of the H_2O_2 effect (a). Shown are current recordings of BCC1 in the absence or presence of 1 mM H_2O_2. Electrolyte: 50 mM $CaCl_2$, 10 mM HEPES, pH 7.0. Membrane voltage: 50 mV. A: H_2O_2 was added first to the *cis* compartment. B: H_2O_2 was then added to the *trans* compartment. Influence of H_2O_2 on the open state probability P_o of the ER Ca^{2+} channel (b). The same experimental conditions as in (a) were used. ◆: H_2O_2 was added to the *cis* compartment; ■: H_2O_2 was added to the *trans* compartment.

then to the *trans* compartment. Only addition of KOH to the *trans* compartment (cytoplasmic side of the channel) modified current fluctuation traces.

3.3. H_2O_2 affects BCC1 channel activity

Reactive oxygen species (ROS) in plants are released during defense reactions (for review: [28]), and there is now evidence that an oxidative burst can also be induced mechanically [29]. Moreover, it has been shown that hydrogen peroxide influences calcium signaling in *Arabidopsis thaliana* [30]. It was therefore tested whether the properties of the ER calcium channel are modified by hydrogen peroxide. The results of these experiments are summarized in Fig. 6. H_2O_2 had a clear inhibitory effect on BCC1 when it was added to the *trans* compartment (cytoplasmic side of the channel), while having no effect when added to the *cis* compartment. The channel is nearly completely inhibited at 1 mM H_2O_2 (Fig. 6a) with the beginning of P_o reduction seen around 0.1 mM. The reduction of open state probability by increasing concentrations of H_2O_2 and the clear sidedness of this effect are also shown in Fig. 6b. There is little information about the total amount of ROS released during the so-called oxidative burst. In cultured soybean (*Glycine max*) cells, medium levels of H_2O_2 rise to 1.2 mM during the course of the defense response [31]. It has been proposed that ROS lead to lipid peroxidation [11,32,33] and therefore may function as substrates for lipoxygenase-mediated production of octadecanoid precursors from linolenic acid [11]. It is suggested that H_2O_2 does not only function on the cell surface, but that a certain amount of it penetrates into the cells, modifying gene expression [34] and Ca^{2+} signalling [30]. It was shown recently [30] that H_2O_2 applied exogenously to tobacco seedlings induces a rise in intracellular Ca^{2+} concentration. However, it remains to be elucidated if Ca^{2+} was mobilized from some intracellular or extracellular sources in these experiments. In contrast to the results of Knight, we could not observe an increased Ca^{2+} release from the ER after H_2O_2 treatment in our experiments, but, on the contrary, we found an inhibition of BCC1. This inhibition is most probably caused by a loss of channel function due to oxidation of amino acid residues. It may well be that H_2O_2 affects different calcium channels differently, as Knight et al. [3,4,30] have presented evidence that different stimuli mobilize different cellular calcium pools. Thus, H_2O_2 could be involved in the process of differential release of calcium from cellular pools by inhibiting certain channels while activating others. However, intracellular levels of H_2O_2 are unknown at present, and further work will be necessary to show whether they may reach or exceed the threshold level (0.1 mM) required to affect BCC1. Since mechanisms exist that protect the cell from H_2O_2 damage (e.g. catalase), average intracellular levels of H_2O_2 should normally be very low. However, it is conceivable that under conditions of challenge and/or locally, they may fluctuate considerably. This remains to be demonstrated.

4. Conclusion

Immediate responses of cells to a variety of stimuli include changes in calcium, proton and H_2O_2 levels. Little is known so far about the cellular mechanisms of signal integration in situations where several of these factors would come into play. Our data have identified a cellular target, BCC1, that strongly responds to transmembrane Ca^{2+} and cytosolic H^+ as well as H_2O_2 concentrations and that may serve to integrate these cellular signals into a unified output response.

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