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Characterization of the High-Affinity Verapamil Binding Site in a Plant Plasma Membrane Ca^{2+}-selective Channel

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Abstract. Despite biochemical evidence for the existence of high-affinity phenylalkylamine receptors in higher plants, their effects on channel activity have only been demonstrated at relatively high concentrations. We have performed a quantitative single-channel analysis of the changes induced by extracellular verapamil in the *rca* channel [a wheat root plasma membrane Ca^{2+}-selective channel (Piñeros & Tester, 1995. *Planta* **195:**478–488)]. Concentrations as low as 0.5 μM verapamil induced a blockade of the inward current, with no evident reduction of the single-channel current amplitude. Blockade by verapamil was concentration and voltage dependent. Preliminary analysis suggested the blockade was due to a reduction in the maximum open state probability rather than a change in $V_{0.5}$. Further analysis of the association and dissociation rate constants revealed a binding site located 56 to 59% down the voltage drop from the extracellular face of the channel, with a $K_d(0)$ of 24 to 26 μM. This results in a K_d at −100 mV of 2 μM. Methoxyverapamil had qualitatively the same effects. This intra-pore binding site can be accessed directly from the extracellular side of the *rca* channel, but apparently not from the cytosolic side.

Key words: Calcium channel — Methoxyverapamil — Verapamil — Wheat roots

Introduction

Several types of organic compounds modulate the activity of Ca^{2+} channels from animal and plant cells. These compounds have been commonly classified according to the type of change induced in the channel gating: antagonists normally induce the closed state of the channel, in contrast to agonist, which increase the channel open state (Coronado & Affolter, 1986). Two groups of compounds have been the most widely employed: phenylalkylamines [such as methoxyverapamil (D600) and verapamil] and 1,4-dihydropyridines (DHPs: such as nifedipine, nitrendipine, 202-791 and Bay K8644). They have been used extensively to characterize Ca^{2+} channels in animal cells, providing a thorough understanding of their structure and function (Campbell , Leung & Sharp, 1988; Catterall, Seagar & Takahashi, 1988; Hosey & Lazdunski, 1988; Tsien, Ellinor & Horne 1991; Miller, 1992). Other groups of compounds such as benzothiazipines (diltiazem), bepridils and diphenylbutylpiperidines (DPBPs: pimozide) are also known to affect channel activity, although their effects have been less studied.

In contrast, much less is known about the structure of Ca^{2+} channels from plant cells (for an overview see Piñeros & Tester, 1997). Several of these compounds have been shown to disrupt normal plant functions [for a series of examples see Tester & MacRobbie, 1990; Hetherington et al., 1992]. In most cases the effects of these compounds have been assumed to be due to blockade of Ca^{2+} influx through putative Ca^{2+} channels, although these assumptions have been rarely tested. Phenylalkylamines have been used in affinity labelling and purification of putative Ca^{2+} channel proteins (Harvey, Venis & Trewavas, 1989; Thuleau et al., 1990), which have been shown to possess Ca^{2+} channel activity after reconstitution into artificial lipid bilayers (Tester & Harvey, 1989; Thuleau et al., 1993). Additionally, pharmacological studies on Ca^{2+} influx into protoplasts (Graziana et al., 1988; Rengel & Elliott, 1992; Schumaker & Gizinski, 1993), as well as electrophysiological measurements (Pantoja, Gelli & Blumwald, 1992; Gelli & Blumwald, 1993; Thuleau et al., 1993; Piñeros & Tester 1993, 1995,

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1997; Klüsener et al., 1995) have provided robust evidence for the existence of phenylalkylamine receptors in Ca^{2+} channels from higher plant cells. Nevertheless, the sensitivity of most known plant Ca^{2+} channels to the above compounds was found to be at relatively high concentrations, ranging from 10 to 100 μM, despite that fact that binding studies suggest an affinity of about 100 nM (Andrejauskas, Hertel & Marmé, 1985; Graziana et al., 1988; Harvey et al., 1989; Thuleau et al., 1990). Since a similar range of concentrations of some of these phenylalkylamines can block plant K^+ channels (Terry, Findlay & Tyerman, 1992; Thomine et al., 1994), the evidence for the existence of specific high affinity binding sites in plant Ca^{2+} channels is still incomplete.

In this paper, we describe a quantitative analysis of the changes induced by verapamil (a phenylalkylamine Ca^{2+} channel antagonist) on the *rca* channel [a wheat root plasma membrane Ca^{2+}-selective channel (Piñeros & Tester, 1995)].

Materials and Methods

Membrane Isolation, Planar Lipid Bilayers and Channel Reconstitution

Plasma membrane vesicles from wheat roots were isolated as described previously (Piñeros & Tester, 1995). Briefly, all the solutions employed in electrophysiological experiments were Millipore-filtered (diameter 0.2 μm: Millex-GS, Millipore). $CaCl_2$ solutions were unbuffered and the pH was adjusted to 5.5 by addition of small amount of HCl. Planar lipid bilayers were painted across a 0.3 mm hole in the wall of a styrene copolymer cup. The synthetic bilayer used contained (in mM): 8 PE, 3 PS, 1.5 PC and 12.5 cholesterol, dispersed in decane. The phospholipids were obtained from Avanti Polar Lipids (Alabama) and the cholesterol was from Sigma. The artificial bilayer separated a 500-μl volume in the styrene copolymer cup (*cis* side) from 1.5 ml in the outer Perspex chamber (*trans* side). Formation of the bilayer was monitored electrically by measuring the capacitance (with a final capacitance of 300–400 pF being commonly used for expeiments). Ionic conditions were established either by addition of stock solutions to either side of the bilayer or by perfusing the *cis* compartment with 50–60 chamber volumes of the desired solution. Bilayers were stable for up to several hours with holding potentials up to ±200 mV. Incorporation of channels was achieved by addition of vesicles to the stirred *cis* side and once channel activity was detected, further incorporation was avoided by perfusing away unfused vesicles with 50 volumes of the desired experimental solution. Considering the predominance of right-side-out vesicles obtained by the membrane isolation protocol, and the fact that membrane vesicles fuse into planar lipid bilayers in a defined manner (Cohen, 1986), channels fused into the bilayer had their extracellular face exposed to the *cis* chamber. All organic channel effectors were dissolved in ethanol at concentrations such that no more than 4% ethanol was present in the extracellular solution. This concentration of ethanol had no effect on channel activity. The concentrations of ethanol and verapamil used in the present study had no effect on the solution pH.

Single-Channel Recordings and Data Analysis

Single-channel currents were recorded under voltage clamp conditions using a Dagan 3900A amplifier (with 3910 expander; Dagan, Minneapolis, MN), connected to the bilayer chambers via 3 M KCl/1% agar salt bridges. Data were displayed on a digital storage oscilloscope (Gould 400, Gould Electronics, Hainhault, Essex, UK) and stored unfiltered on a digital audio tape recorder (DAT: Sony DTC-75ES), and simultaneously filtered at 100 Hz with an 8-pole low pass Bessel filter (Series 902, Frequency Devices, Haverhill, MA) and digitized at 2 kHz. Data acquisition and analysis was performed using PCLAMP 6.0 software (Axon Instruments, Foster City, CA). Membrane potentials were stepped from zero to the desired voltage.

Since the *trans* and *cis* sides are the cytosolic and extracellular sides respectively, voltages were measured in the outside chamber (*trans* side) with respect to the cup (*cis* side), following the same convention used for intact cells (i.e., cytosol with respect to the outside). Movement of Ca^{2+} from the *cis* to the *trans* chamber is indicated by a negative current and is shown as a downward deflection in the current traces (as for an inward current in intact cells). Single-channel dwell time distributions generated from recordings over a period of 5–20 min at each voltage (5-min records contained approximately 100 events) were fitted to Gaussian distributions. To avoid interference from capacitive currents, the initial 5 sec after applying the desired voltage were excluded from the analysis. Open-state probabilities were calculated from the ratio of the total open times to the total recording times derived from the curve fitting of the single-channel current-frequency distributions, as, for example, in Bertl and Slayman (1990). Gaussian and exponential distributions were fitted with the Simplex and Levenberg-Marquardt least squares method provided by PSTAT (PCLAMP 6.0) software. All other curve fittings such as Levenberg-Marquardt nonlinear regressions and Boltzmann equations were performed using DataFit 2.0c software (A Soft Answer, Macquarie Center, Australia, 1993). Liquid junction potentials were very small (±2 mV), thus they were not taken into account to correct membrane potentials.

Results

In these experiments, the effect of extracellular (*cis*) verapamil on the inward and outward single channel currents carried by Ca^{2+} was examined. Methoxyverapamil (D600) had qualitatively the same effects as verapamil, but they were not analyzed quantitatively. Exposure of the extracellular side of the channel (*cis*) to concentrations as low as 0.5 μM verapamil induced a blockade of the inward current (*cis* to *trans*), with no evident reduction of the single-channel current amplitude (Fig. 1). At all extracellular concentrations tested, verapamil had no effect on the outward current (i.e., movement from *trans* to *cis: data not shown*). Figure 2 shows the open-state probability of the *rca* channel as a function of the membrane potential at different concentrations of verapamil. The curve fittings suggest that the blockade is due to a reduction in the maximum open state probability, rather than a change in $V_{0.5}$ (i.e., the voltage at which the open state probability is 0.5). The open state probability of the channel at positive membrane potentials remained unchanged at all concentrations of verapamil tested (*data not shown*), suggesting that the binding of the blocker is reversed by positive membrane potentials. Verapamil blockade was also reversed upon perfusion of the *cis* chamber with $CaCl_2$.

Since the transition rates of the blockade reaction induced by verapamil were slow enough to be resolved at

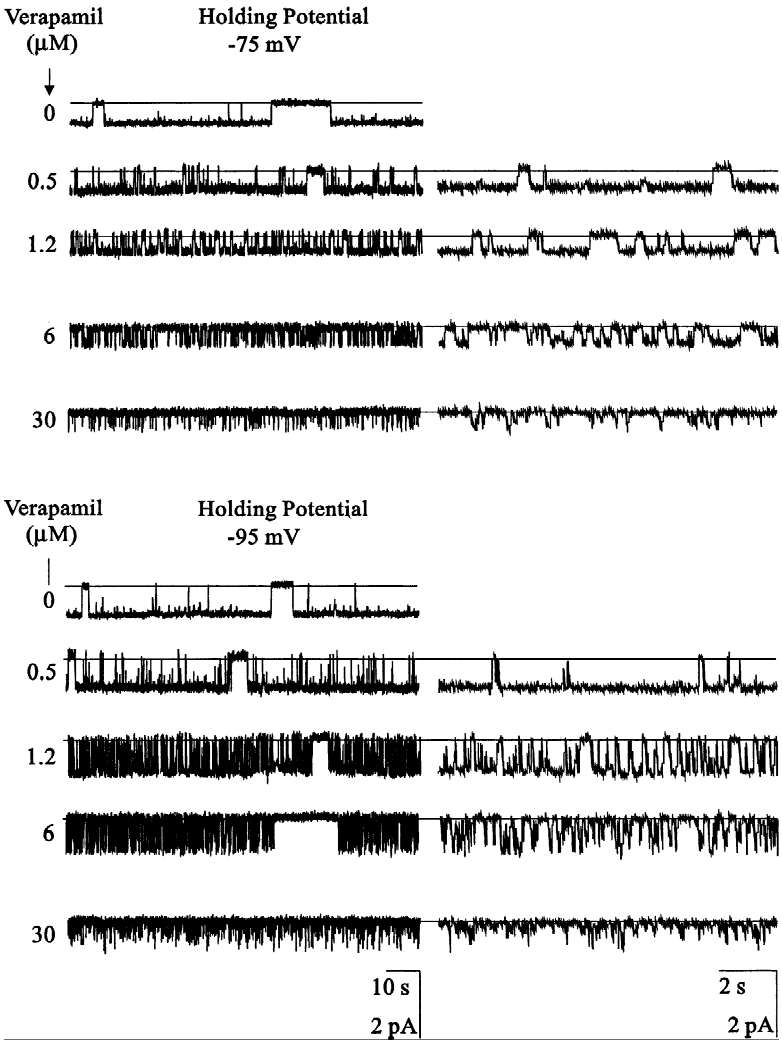

Fig. 1. Effect of different concentrations of verapamil on single channel activity. The channel was exposed to symmetrical 1 mM $CaCl_2$ (pH 5.5). Verapamil was added from a stock solution to the *cis* (extracellular) chamber at the concentrations indicated on the left margin of the left set of traces. Membrane potentials were stepped from zero to the voltage indicated at the top of each set of traces. The horizontal lines represent the closed state. The set of traces on the right are an enlargement of last 10 sec of their corresponding trace on the left and their time scale is shown at the bottom. All traces from the same bilayer. Similar observations were recorded in a total of six bilayers.

the single channel level, the kinetics of the open and closed times were analysed to elucidate further the mechanism by which verapamil blocks the channel. The blocking reaction was treated as a one-to-one interaction between verapamil and the channel pore, resulting in a discrete slow blockade reaction scheme as described by Moczydlowski (1992):

$$C \underset{K_\beta}{\overset{K_\alpha}{\rightleftharpoons}} O + B \underset{K_{on}(V)}{\overset{K_{off}(V)}{\rightleftharpoons}} OB \qquad (1)$$

The measurable rate constants $K_{on}(V)$ and $K_{off}(V)$ describe the blocking ($O + B \rightarrow OB$) and unblocking ($OB \rightarrow O + B$) reactions respectively. The ratio of these constants represents $K_d(V)$, the apparent equilibrium dissociation constant for the binding of verapamil to a site in the unblocked channel O at the membrane potential (V), to give the blocked state of the channel, OB. For the purposes of the current analysis, the blocker-independent channel-gating reaction was simplified to a reversible one-step process, closed-open ($C \rightleftharpoons O$).

The time constants for the blockade reactions were obtained by exponential fitting of the dwell time distributions of the open and closed states (Fig. 3). Since the reaction scheme used in this work has only one open state, a single exponential was used to fit the open dwell time distributions to give the unblocked time constant, τ_U. However, as the reaction scheme contains two ''closed'' states, C (closed) and OB (blocked), the dwell time distribution for the closed state is predicted to be the sum of two exponentials. Theoretically, since these two closed states are not in direct communication, the two observed closing time constants could be independently identified—in the absence of verapamil the channel would exhibit the time constant associated with the channel opening, and upon exposure to verapamil the dwell time distribution would include a new time constant, τ_B, equal to the mean dwell time of the blocker. Unfortunately, due to the small number of transitions (i.e., openings and closures) in the absence of verapamil at less negative membrane potentials (see control traces in Fig. 1; also see traces in Piñeros and Tester 1995, 1997), it

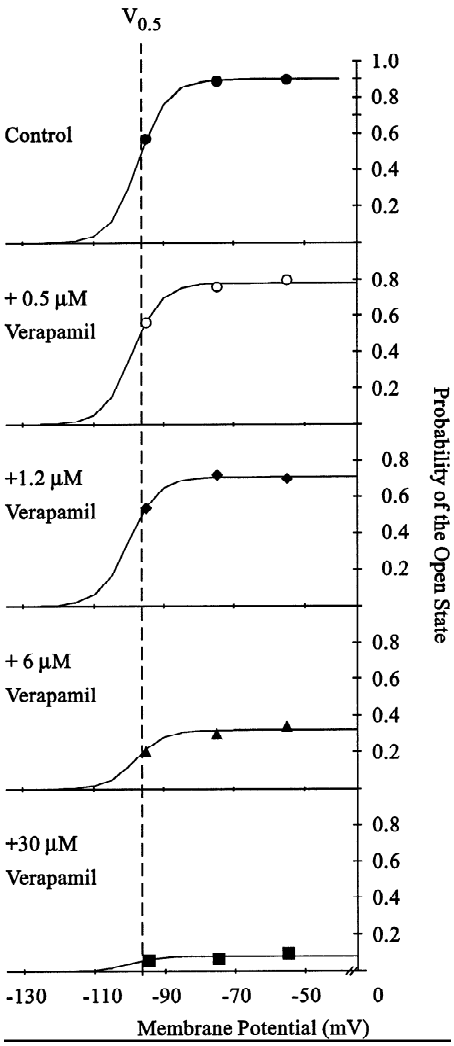

Fig. 2. Changes in open probability at steady state as a function of the membrane potential at different extracellular verapamil concentrations, as indicated in the left margin of each panel. $P_{(open)}$ was calculated as described in Materials and Methods. The smooth curves were drawn according to the Boltzmann equation, $P_{(open)} = P_{max}/(1 + \exp(V_{0.5} - V)/N)$, where P_{max} is the maximum observed open state probability, $V_{0.5}$ is the voltage where $P_{(open)} = 0.5$ and N is the steepness factor of 6 mV equivalent to RT/Fz, R, T, and F having their usual meaning and z representing the gating charge of 4.2 as estimated in Piñeros and Tester (1995, 1997). Best fits were obtained by varying $V_{0.5}$ to −97, −99, −100, −98, and −100 mV and P_{max} to 0.90, 0.78, 0.71, 0.32 and 0.08 for the 0, 0.5, 1.2, 6 and 30 μM verapamil concentrations respectively.

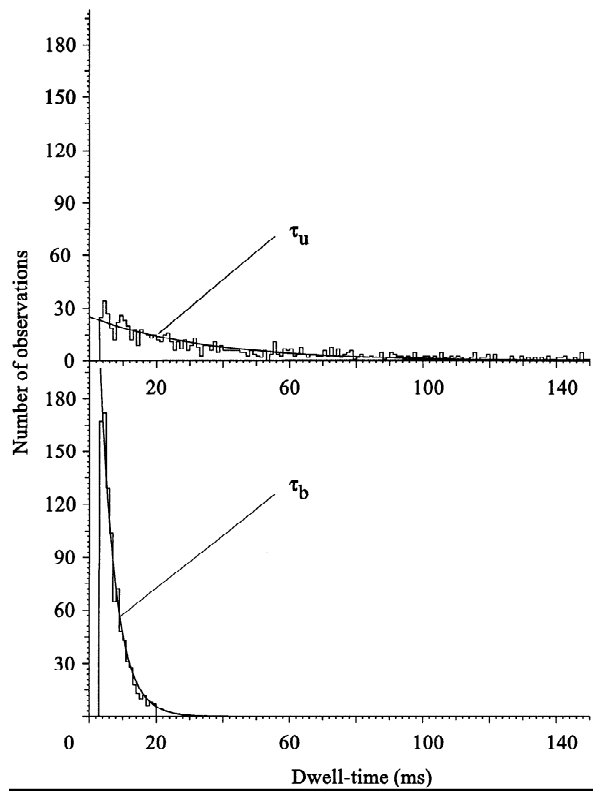

Fig. 3. Estimation of time and rate constants for the blocking and unblocking reactions in the presence of verapamil. Example of dwell time histograms for the unblocked (top) and blocked (bottom) states of the channel, generated from single channel recordings such as those shown in Fig. 1. The single exponential curves fitted for estimates of unblocked (τ_U) and blocked (τ_B) time constants were calculated as described in the text. This particular example is taken from a record at −75 mV with 1.2 μM verapamil, similar to that shown in Fig. 1.

was not possible to accumulate a large number of events to generate a representative dwell-time histogram, and so determination of the time constant resulting from spontaneous channel activity was not feasible. In the presence of verapamil, the number of events in the dwell-time distributions of the closed state attributable to the transitions between *O* (open) and *C* (closed) states at the less negative membrane potentials were likely to be insignificant compared to the large number of events between *O* + *B* (unblocked) and *OB* (blocked) states. Thus, the mean dwell times of these distributions are likely to describe almost entirely the *O* + *B* (unblocked) to *OB* (blocked) transitions. At more negative membrane potentials [i.e., near $V_{0.5}$, where a higher number of transitions between *O* (open) and *C* (closed) states occurs] the mean dwell time of these distributions is only dominated by the *O* + *B* (unblocked) to *OB* (blocked) transitions at high verapamil concentrations. Given these assumptions, the dwell time distribution of the closed state for each of the different membrane potentials and concentrations of verapamil tested were fitted with a single exponential.

The time constants obtained from the exponential fits of the open and closed dwell time distributions are related to the rate constants of the blocking and unblocking reactions by the following equations (Moczydlowski 1992):

$$K_{off}(V) = 1/\tau_B \quad (2)$$

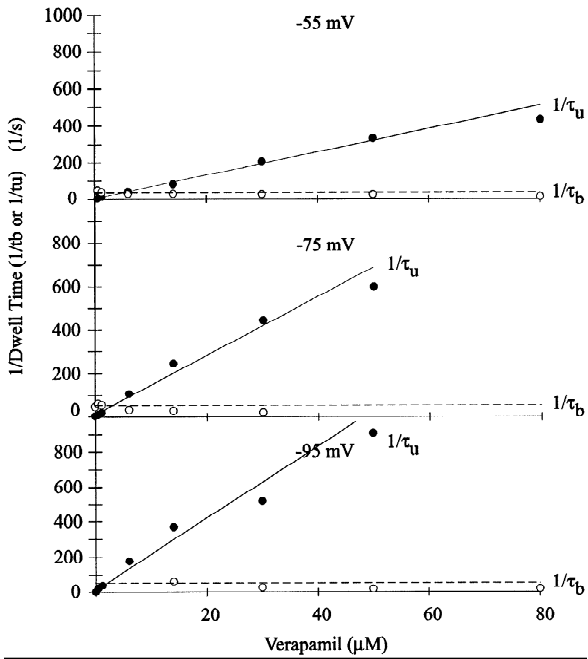

Fig. 4. Dependence of the reciprocal time constants of the blocked ($1/\tau_B$) and unblocked ($1/\tau_U$) states on the extracellular (*vis*) concentration of verapamil at three different membrane potentials. Values were estimated as described in the text. The slope of the linear relationship between $1/\tau_U$ and the verapamil concentration corresponds to the rate constant (K_{on}) and the ordinate intercept corresponds to α, as described by Eq. 3. K_{off} equals $1/\tau_B$ (Eq. 2). Estimated values for these constants are summarized in Table 1.

$$K_{on}(V) \cdot [B] + K_{\alpha} = 1/\tau_U \quad (3)$$

where [*B*] is the concentration of channel blocker. Plots of the reciprocal time constants against verapamil concentration confirm that the reciprocal time constant for the blocked state ($1/\tau_B$) was relatively independent of the verapamil concentration, whereas there was a clear increase of the reciprocal time constant of the unblocking reaction ($1/\tau_U$) with increasing concentration of verapamil (Fig. 4). Table 1 summarises the resulting rate constants K_{α}, $K_{on}(V)$ and $K_{off}(V)$ estimated at three membrane potentials.

The voltage dependence of the rate constants for the blocking and unblocking reactions (Fig. 5) is such that K_{on} values increased and K_{off} values remained relatively constant as the membrane potential became more negative, suggesting that a negative cytosolic voltage attracts verapamil towards the cytosolic side of the channel, thus speeding its binding to the channel.

These data can be expressed quantitatively by the following Boltzmann distributions:

$$K_{off}(V) = K_{off}(0)\exp(-0.05 \cdot FV/RT) \quad (4)$$

$$K_{on}(V) = K_{on}(0)\exp(-0.61 \cdot FV/RT) \quad (5)$$

Table 1. Values for the rate constant K_{α} in the reaction describing closure of the Ca^{2+} channel and the rate constants of its blocking (K_{on}) and unblocking (K_{off}) by verapamil (*see* Fig. 4 and text for details). r^2 for K_{on} linear regressions were between 0.977 and 0.981 Errors represent the standard error of the slope (K_{on} values) or y-intercept (K_{off} values).

Voltage	K_{α}	K_{on}	K_{off}
(mV)	(s^{-1})	($s^{-1}M^{-1}$)	(s^{-1})
−55	$1.25 \cdot 10^1$	$5.66 \cdot 10^6 \pm 3.52 \cdot 10^5$	$3.99 \cdot 10^1 \pm 2.9$
−75	$2.65 \cdot 10^1$	$1.23 \cdot 10^7 \pm 8.50 \cdot 10^5$	$5.23 \cdot 10^1 \pm 4.0$
−95	$3.86 \cdot 10^1$	$1.74 \cdot 10^7 \pm 1.10 \cdot 10^6$	$5.67 \cdot 10^1 \pm 17$

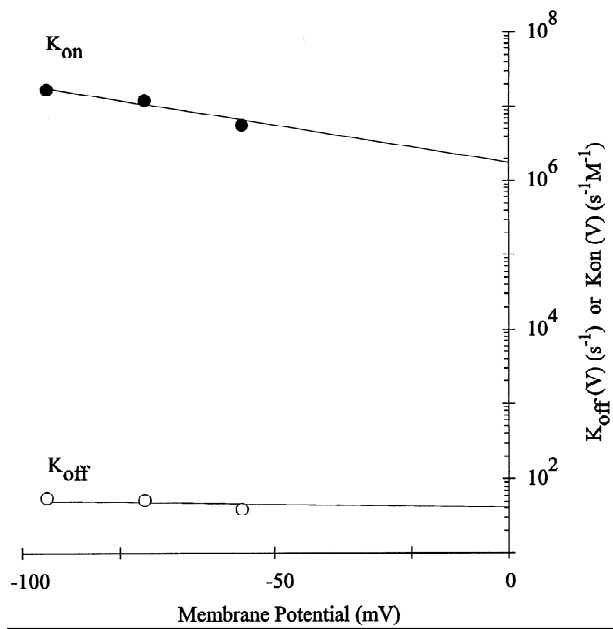

Fig. 5. Voltage dependence of the dissociation and association rate constants. Semilogarithmic plot of $K_{off}(V)$ and $K_{on}(V)$ as a function of membrane potential. Lines were drawn according to Eq. 4 and 5.

where K_{on} (0), the value of $K_{on}(V)$ at 0 mV is equal to $1.78 \cdot 10^6$ s^{-1} M^{-1} and $K_{off}(0)$, the value of $K_{off}(V)$ at 0 mV is equal to $4.20 \cdot 10^1$ s^{-1}. *F*, *R*, *T* have their usual meanings and −0.05 and 0.61 correspond to $z\delta_2$ and $z\delta_1$ respectively. The sum of the equivalent valences ($Z^1 = z\delta_1 + z\delta_2$) quantifies the effects of the electric field on the rates (Moczydlowski 1992), and equals 0.56. The ratio $K_{off}(0)/K_{on}(0)$ [i.e., $K_d(0)$] is $2.4 \cdot 10^{-5}$ M verapamil.

The apparent dissociation constants $K_d(V)$ [i.e., $K_{off}(V)/K_{on}(V)$] may also be calculated using a model where verapamil traverses the membrane field (Woodhull, 1977). $K_d(V)$ values against voltage were fitted to the following Boltzmann distribution:

$$K_d(V) = K_d(0)\exp(z^1 \cdot FV/RT) \quad (6)$$

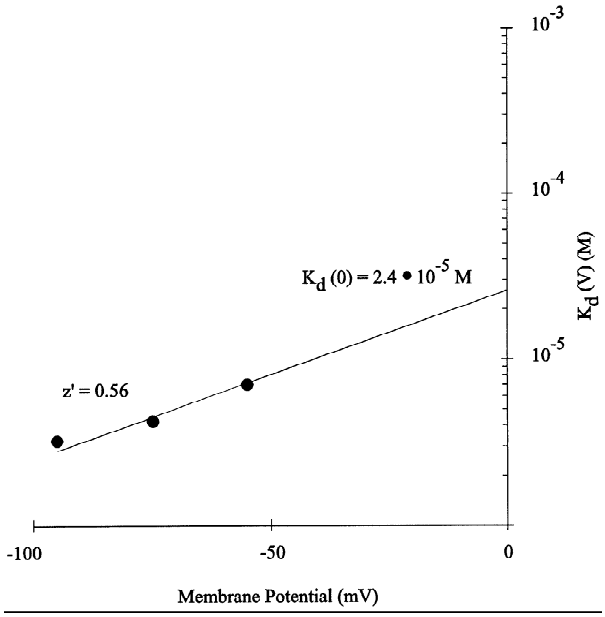

Fig. 6. Voltage dependence of the $K_d(V)$ values [given by the ratio of $K_{off}(V)$ and $K_{on}(V)$] values. The line was drawn according to Eq. 6.

where, as above, z^1 is the equivalent valence defined as δz, where δ and z are the fraction of the voltage across the channel sensed by verapamil and the charge on the verapamil molecule respectively (Fig. 6). The best fitting suggests a z^1 of 0.59 and a $K_d(0)$ of $2.6 \cdot 10^{-5}$ M verapamil, which is similar to those estimated above, suggesting that both approaches provide a accurate estimate of $K_d(0)$.

Discussion

Blockade of the *rca* channel by low micromolar concentrations of verapamil was consistent with many previous observations made on animal and plant cells. Calcium influx into protoplasts from *Physcomitrella patens* (Schumaker & Gizinski 1993), *Daucus carota* (Graziana et al., 1988) and *Amaranthus* (Rengel & Elliott 1992) was reduced upon exposure to verapamil and verapamil derivatives such as D600 and D888 (desoxyverapamil), although up to 100 μM verapamil did not block the calcium influx into plasma membrane vesicles from *Triticum aestivum* (Huang et al., 1994) nor *Zea mays* (Marshall et al., 1994). Nevertheless, interpretation of these pharmacological results is difficult as the effect of verapamil on other experimental variables (e.g., membrane potential) is unknown.

The decrease in channel open times induced by verapamil observed in the *rca* channel has also been reported for single Ca^{2+} channels in the tonoplast (Pantoja et al., 1992; Gelli & Blumwald, 1993) and endoplasmic reticulum (Klüsener et al., 1995), and in the plasma membrane of both higher plants (Thuleau et al., 1993) and algae (Aleksandrov, Aleksandrova & Berestovsky, 1990). Similar pharmacological responses to phenylalkylamines have been reported in electrophysiological studies in animal cells. For example, as in the present work, the frequency and duration of the open state of single channel recordings from skeletal muscle transverse tubules was decreased upon exposure to D600 (Coronado & Affolter, 1986).

The results in the present work suggest a high affinity binding site for verapamil [as judged from the low $K_d(0)$] located 56 to 59% of the voltage drop from the extracellular face of the channel. Comparative studies between D600 and D890 (a quaternary derivative) have suggested that the neutral form of the phenylalkylamine compound can permeate the lipid membrane in the uncharged form, dissociate, and then bind to the receptor accessed from the cytosolic face of the channel (Hescheler et al., 1982). Although our results agree with the existence of a binding site located closer to the cytosolic side of the channel than to the extracellular one, they suggest an exclusive extracellular access of verapamil to this site (Piñeros & Tester, 1995).

Our estimated $K_d(0)$ of 24 to 26 μM for verapamil from these single-channel study is higher than the apparent dissociation constants of 130, 72, 85 nM for verapamil and verapamil derivatives binding to crude membrane preparations of *Cucurbita pepo* L. (Andrejauskas et al., 1986), *Zea mays* (Harvey et al., 1989) and *Daucus carota* (Graziana et al., 1988) respectively. The difference between these estimates and that obtained in the present work may be due to different ionic or lipid environments, or a consequence of the experimental approaches used. When the K_d for verapamil is estimated at a more physiological membrane potential [i.e., −100 mV, where the channel spends half of the time in the open state (Fig. 2)], an affinity for binding of 2 μM is found. This value is closer to those obtained by biochemical techniques from the $K_d(0)$. Whether such voltages are present in the membrane preparations used by these earlier workers is however, unknown.

Our results provide clear evidence for the existence of a specific intra-pore phenylalkylamine receptors in Ca^{2+}-selective channels from higher plant cells. Although the channel is sensitive to submicromolar concentrations of verapamil, its affinity is in the low micromolar range. However, the precise value for the affinity of verapamil binding depends on membrane potential, complicating interpretation of results on the affinity of plant Ca^{2+} and K^+ channels to verapamil and similar compounds. Nevertheless, the use of similar pharmacological approaches is allowing a better understanding of the structure and function of plant channels, as well as

their differences and similarities with other well known animal channels.

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