

The effect of external divalent cations on spontaneous non-selective cation channel currents in rabbit portal vein myocytes

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1. The effects of external divalent cations on spontaneous single non-selective cation channel currents were studied in outside-out patches from rabbit portal vein smooth muscle cells in K^+ -free conditions.
2. In an external medium containing 1.5 mM Ca^{2+} (Ca_o^{2+}) the majority of spontaneous channel currents had a unitary conductance of 23 pS, reversal potential (V_r) of +10 mV and a low open probability (P_o) at negative patch potentials. Some channels opened to a lower conductance state of about 13 pS suggesting that the cation channels have two conductance states. Open time and burst duration distributions could both be described by two exponentials with time constants of about of 1 ms and 7 ms for open times and 3 ms and 16 ms for burst durations.
3. In 0 Ca_o^{2+} the majority of spontaneous cation channels had a unitary conductance of 13 pS and V_r was shifted to +4 mV. Moreover the longer open time and longer burst duration time constants were both reduced to approximately half the values in 1.5 mM Ca_o^{2+} .
4. Compared to 0 Ca_o^{2+} the single channel currents in 3 μM and 100 μM Ca_o^{2+} had a 5- to 6-fold increase in P_o which was accompanied by increases in both open times and burst durations. In 3 μM and 100 μM Ca_o^{2+} the unitary conductance of the single channel currents was between 22 and 26 pS.
5. At positive membrane potentials the single channel currents had an increased P_o compared to negative potentials which was associated with increased open times and burst durations but these values were similar in 3 μM , 100 μM and 1.5 mM Ca_o^{2+} .
6. In 1.5 mM Sr_o^{2+} and 1.5 mM Ba_o^{2+} channels opened to the higher conductance state of about 22–25 pS and had a 3- to 7-fold greater P_o than in 0 Ca_o^{2+} .
7. In conclusion, external divalent cations have marked effects on the unitary conductance and kinetic behaviour of non-selective cation channels in rabbit portal vein smooth muscle cells.

In rabbit portal vein myocytes stimulation of α_1 -adrenoceptors by noradrenaline evokes a non-selective cation current (I_{cat}) in addition to Ca^{2+} -activated Cl^- and K^+ conductances (Byrne & Large, 1988). The proposed physiological role of I_{cat} is to produce membrane depolarisation with subsequent opening of voltage-dependent Ca^{2+} channels (VDCCs) to induce contraction of vascular smooth muscle. In addition, reversal potential measurements indicated that this conductance is highly permeable to divalent cations and it was proposed that Ca^{2+} may enter the cell through this channel to produce contraction independently of VDCCs (Byrne & Large, 1988; Wang & Large, 1991). Recent evidence suggests that a member of the transient receptor potential (TRP) family, TRPC6, is an essential component of I_{cat} (Inoue *et al.* 2001). This is an important observation because it

provides strong support for a physiological role for a TRP protein in native mammalian vascular smooth muscle cells. That is to say, the TRP channel is involved in the vasoconstrictor response to noradrenaline released from sympathetic neurons.

We have shown previously that external Ca^{2+} ions (Ca_o^{2+}) have both facilitatory and inhibitory actions on the amplitude of I_{cat} (Helliwell & Large, 1996). In 0 Ca_o^{2+} noradrenaline activated I_{cat} with a peak amplitude of approximately 25 pA showing that Ca_o^{2+} is not obligatory for stimulating I_{cat} . Increasing Ca_o^{2+} from 0 to micromolar concentrations increased the peak amplitude of I_{cat} by about 8-fold. However, increasing Ca_o^{2+} further ($> 200 \mu M$) decreased the amplitude of I_{cat} so that the amplitude of I_{cat} in 1.5 mM Ca_o^{2+} was similar to that in 0 Ca_o^{2+} . This produced a bell-shaped relationship between the amplitude

of I_{cat} and Ca_o^{2+} concentration with estimated equilibrium constants of about $6 \mu\text{M}$ and $400 \mu\text{M}$ Ca_o^{2+} for, respectively, the facilitatory and inhibitory effects (Helliwell & Large, 1996). The vastly different half-maximal values of Ca_o^{2+} concentration suggest that the facilitatory and inhibitory effects may be mediated by two distinct binding sites. In a fluctuation analysis study, the facilitatory effect of Ca_o^{2+} on I_{cat} was shown to be associated with changes in both the kinetics and amplitude of the unitary conductance underlying the whole-cell current (Helliwell & Large, 1998). In addition, a further study showed that external Sr^{2+} (Sr_o^{2+}) and Ba^{2+} (Ba_o^{2+}) also had facilitatory effects on the amplitude of I_{cat} when evoked in 0 Ca_o^{2+} , albeit with a potency and efficacy less than Ca_o^{2+} , which were associated with changes in the kinetics and single channel conductance of the cation channels (Aromolaran & Large, 1999). To provide a more detailed understanding of the facilitatory and inhibitory effects of Ca_o^{2+} , Sr_o^{2+} and Ba_o^{2+} on I_{cat} we have studied their effects at the single channel level.

A recent study from our laboratory showed that approximately 50% of outside-out patches contained spontaneous cation channel activity which had properties very similar to noradrenaline- and 1-oleoyl-2-acetyl-*sn*-glycerol-evoked single cation currents (Albert & Large, 2001), indicating that the spontaneous channel currents are I_{cat} . In the present study we describe the effects of external divalent cations on spontaneous single cation currents recorded in outside-out patches. A preliminary report has been given to the Physiological Society (Albert & Large, 2000).

METHODS

Cell isolation

New Zealand White rabbits (2–3 kg) were killed by an i.v. injection of sodium pentobarbitone (120 mg kg^{-1}) and the portal vein was removed and placed in normal physiological salt solution (PSS). The tissue was dissected free of connective tissue and fat before being cut into strips and placed in 'Ca²⁺-free' PSS. The tissue was enzymatically dispersed in two sequential enzyme steps. First, the strips of tissue were incubated in 'Ca²⁺-free' PSS with 0.3 mg ml^{-1} protease type VIII (Sigma) for 5 min and then the strips were washed in 'Ca²⁺-free' PSS. In the second step the strips were incubated with 1 mg ml^{-1} collagenase type IV (Sigma) in $100 \mu\text{M}$ Ca²⁺-PSS for 10 min and were then washed in $100 \mu\text{M}$ Ca²⁺-PSS. All enzyme and wash procedures were carried out at 37°C . After the enzyme treatments the strips were incubated in $100 \mu\text{M}$ Ca²⁺-PSS at room temperature ($20\text{--}25^\circ\text{C}$) for 10 min before the cells were released into the solution by gentle mechanical agitation of the strips of tissue using a wide-bore Pasteur pipette. The suspension of cells was then centrifuged (1000 r.p.m.) to form a loose pellet which was resuspended in 0.75 mM Ca²⁺-PSS. The cells were then plated onto glass coverslips and stored at 4°C before use (1–6 h).

The normal PSS contained (mM): NaCl (126), KCl (6), CaCl_2 (1.5), MgCl_2 (1.2), glucose (10), and Hepes (11) and the pH was adjusted to 7.2 with 10 M NaOH. 'Ca²⁺-free' PSS, $100 \mu\text{M}$ Ca²⁺-PSS and 0.75 mM Ca²⁺-PSS had the same composition except that either Ca²⁺ was omitted or 1.5 mM CaCl_2 was replaced by $100 \mu\text{M}$ CaCl_2 and 0.75 mM CaCl_2 , respectively.

Electrophysiology

Single non-selective cation channel currents were recorded with a List L/M-PC patch clamp amplifier at room temperature using the standard isolated outside-out patch configuration (Hamill *et al.* 1981) and with recording techniques described previously (Albert & Large, 2001). Patch pipettes were manufactured from borosilicate glass and were routinely coated in Sylgard (Dow Corning, Germany) to reduce stray capacitance and fire polished to increase seal resistance giving pipette resistances between 6 and $10 \text{ M}\Omega$ when filled with the standard internal patch pipette solution. All experiments were carried out at a holding potential of -50 mV . To evaluate the unitary single cation channel current–voltage (I – V) characteristics, the membrane potential was manually stepped from -50 mV to between -90 mV and $+40 \text{ mV}$.

Single cation channel currents were initially recorded onto digital audiotape (DAT) using a CDATA digital tape-recorder (Cygnus Technology Inc., Delaware, PA, USA) at a bandwidth of 5 kHz (-3 dB). For off-line analysis single cation channel current records were filtered at 1 kHz (-3 dB , lowpass 8-pole Bessel filter, Frequency Devices, model LP02, Scensys Ltd, Aylesbury, UK) and acquired using a CED 1401 *plus* interface and CED Patch and Voltage Clamp Software (Version 6.0, Cambridge Electronic Design Ltd, Cambridge, UK) at a sampling rate of 10 kHz . Data were captured with a Pentium (P5-100) personal computer (Gateway, Ireland).

Single cation channel current amplitudes were calculated from idealised traces of at least 10 s duration using the half-amplitude crossing method. I – V relationships were calculated from single channel amplitude histograms in two ways; first, Gaussian curves were fitted to the histograms and the amplitudes of the major peaks, defined by the areas under the curves and reflecting the amplitude of the majority of events, were plotted and the unitary conductance was calculated from the slope of the relationship using linear regression (Origin software, Microcal, USA). The reversal potential (V_r) was calculated directly from the I – V relationship by interpolation. In some experiments, V_r was calculated by extrapolation and these values were similar to those obtained by interpolation. The unitary conductance and V_r from all the individual patches tested were then used to calculate mean conductance and mean V_r values. Secondly, in some experiments the amplitude of the majority of events from individual patches at different membrane potentials were pooled together and the unitary conductance and V_r were determined by linear regression. Lifetime distributions containing events of both the lower and higher conductance states were plotted using either a 0.5 ms or a 1 ms bin width and where appropriate were fitted with one or more exponential functions using the maximum likelihood method. Events which lasted for $< 0.664 \text{ ms}$ were excluded from analysis. P_o was calculated using the equation:

$$P_o = (\text{total open time/sample duration})/(\text{number of open levels}).$$

We have previously carried out burst duration analysis by determining a critical value (T_{crit}) from closed time distributions to distinguish between closures between bursts of channel activity and closures within a burst of activity (Albert & Large, 2001). In a method similar to Magleby & Pallotta (1983), T_{crit} was calculated as 3–4 times the fast closed time constant from closed time distributions with more than one time constant and where the fast time constant was at least 20 times smaller than any other time constant. Figure preparation was carried out using Origin software.

Solutions and drugs

The cells were perfused in a standard K⁺-free external solution containing (mM): NaCl (126), CaCl_2 (1.5), Hepes (11) and glucose (10), pH to 7.2 with 10 M NaOH. In 0 Ca_o^{2+} solution CaCl_2 was omitted and 1 mM BAPTA was added ($< 10 \text{ nM}$ free Ca_o^{2+} as calculated by EQCAL software, Biosoft, Feguson, MO, USA). In the $3 \mu\text{M}$ Ca_o^{2+} solution 0.934 mM CaCl_2 and 1 mM BAPTA were added (calculated using EQCAL

software, Biosoft, Ferguson, MO, USA) and in the 100 μM Ca_o^{2+} solution 100 μM CaCl_2 was added without added BAPTA. For the 1.5 mM Sr_o^{2+} and 1.5 mM Ba_o^{2+} solutions 1.5 mM CaCl_2 was replaced with 1.5 mM SrCl_2 or 1.5 mM BaCl_2 , respectively, and 1 mM BAPTA was added. The standard internal patch pipette solution contained (mM): CsCl (18), caesium aspartate (108), MgCl_2 (1.2), Hepes (10), glucose (11), BAPTA (10) and CaCl_2 (1) (free internal calcium concentration approximately 14 nM as calculated using EQCAL software); pH to 7.2 with Tris. Under these conditions voltage-gated Ca^{2+} currents, K^+ currents and Ca^{2+} -activated conductances are abolished at the holding potential of -50 mV and single non-selective cation channels could be recorded in isolation. Moreover, with the anion gradient the chloride equilibrium potential (E_{Cl}) is about -50 mV, i.e. the holding potential. All drugs were purchased from Sigma (UK). The values are the means of n cells \pm S.E.M. Statistical analysis was carried out using Student's t test with the level of significance set at $P < 0.05$.

RESULTS

Properties of spontaneous single cation channel currents in 1.5 mM and 0 Ca_o^{2+}

We have shown previously in a fluctuation analysis study that an external solution containing 0, 3 μM or 100 μM Ca_o^{2+}

produced distinctive effects on the amplitude and kinetic behaviour of the unitary conductance underlying I_{cat} (Helliwell & Large, 1998). Therefore in the present study we have investigated the effects of these Ca_o^{2+} concentrations on the properties of spontaneous single non-selective cation channel currents. We initially recorded single cation channel currents in a 1.5 mM Ca_o^{2+} external solution and then exchanged the solution for one containing different divalent cation concentrations. This procedure created more stable outside-out patches with a better signal to noise ratio than recording directly with a lower Ca_o^{2+} solution.

In the first series of experiments, we compared the activity of single channel currents in 1.5 mM and 0 Ca_o^{2+} . Figure 1*A*(i) illustrates typical spontaneous single cation channel currents recorded in 1.5 mM Ca_o^{2+} from an isolated outside-out patch at a holding potential of -50 mV. The appearance of single channel currents was observed after the excision of the outside-out patch from the whole-cell configuration as described previously (Albert & Large, 2001). The majority of the single channel currents had

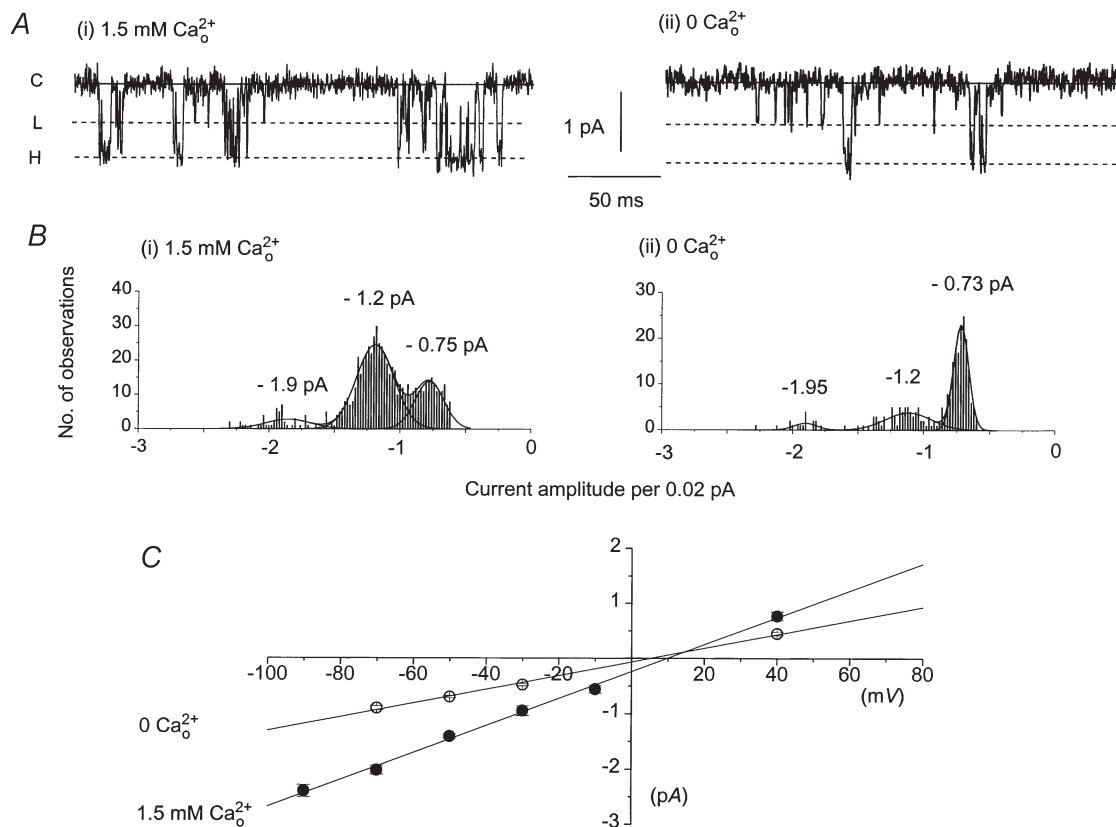


Figure 1. Effect of 0 Ca_o^{2+} on spontaneous single cation currents

A(i), spontaneous single inward cation currents recorded from an outside-out patch at -50 mV in 1.5 mM Ca_o^{2+} . The continuous line represents the closed level (C) and the dashed lines represent open levels. The dashed line marked 'L' represents the level of a lower conductance state and 'H' the higher conductance state. Note the flickering between closed and open levels denotes bursting behaviour; *A*(ii), effect of 0 Ca_o^{2+} on the single cation currents recorded from the same patch as shown in *A*(i). *B*(i), single cation channel current amplitude histogram for the events shown in *A*(i); *B*(ii), single channel current amplitude histogram for the channels shown in *A*(ii). Both histograms could be fitted by three Gaussian curves with different proportions of peaks at approximately -0.7 , -1.2 and -2 pA. *C*, current-voltage (I - V) relationships created from pooled main peak amplitude data for single cation currents recorded in 1.5 mM (●) and 0 (○) Ca_o^{2+} .

Table 1. Characteristics of spontaneous single cation channel currents in different Ca_o^{2+} concentrations at a holding potential of -50 mV

$[\text{Ca}_o^{2+}]_o$	γ (pS)	V_r (mV)	P_o	O_{r1} (ms)	O_{r2} (ms)	B_{r1} (ms)	B_{r2} (ms)	Frequency (bursts s^{-1})	n
0	$12 \pm 2^{1**}$	$+4 \pm 2^*$	0.02 ± 0.01	$0.7 \pm 0.1^*$	$3.9 \pm 0.4^*$	2.9 ± 1.4	$5.8 \pm 1.1^*$	12 ± 3	6
$3 \mu\text{M}$	22 ± 2	$+10 \pm 2$	$0.11 \pm 0.03^{**}$	1.3 ± 0.3	$17 \pm 2.3^{**}$	2.8 ± 0.4	$43 \pm 4^{**}$	$17 \pm 4^*$	7
$100 \mu\text{M}$	26 ± 2	$+12 \pm 2$	$0.15 \pm 0.03^{**}$	1.1 ± 0.2	6.2 ± 1.1	3.9 ± 0.7	$42 \pm 9^*$	$45 \pm 8^{**}$	6
1.5 mM	23 ± 1	$+10 \pm 1$	0.05 ± 0.02	1.2 ± 0.3	7.4 ± 0.2	3.1 ± 0.3	16 ± 1.4	14 ± 1	19

γ is the single channel conductance. * $P < 0.05$, ** $P < 0.01$ compared with values in 1.5 mM Ca_o^{2+} . ¹ Lower conductance state. The kinetic parameters were determined using openings to both the lower and higher conductance states.

peak amplitudes of approximately -1 to -2 pA at -50 mV although Fig. 1A(i) also shows that there were events which had lower amplitudes. To measure the amplitudes of the channel currents we fitted Gaussian curves to single channel current amplitude histograms. Figure 1B(i) shows that the channel currents from the patch shown in Fig. 1A(i) could be fitted with three Gaussian curves with peaks at -0.75 , -1.2 and -1.9 pA. The areas under the curves consisted of 195, 441 and 52 events, respectively, for the -0.75 , -1.2 and -1.9 pA peaks indicating that the peak at -1.2 pA is the main open state of the channel currents in 1.5 mM Ca_o^{2+} . The smaller currents had similar amplitudes when the single channel currents were lowpass filtered at 2 kHz (-3 dB) indicating their amplitudes were not truncated by over-filtering of brief events. The observation of two amplitude events suggests a possible equilibrium between two conductance states of the cation channel. Figure 1B(i) also shows there were a few openings with a larger amplitude (-1.9 pA) which probably represented multiple openings of both the lower and higher conductance states, indicating there was more than one cation channel in the patch. The higher amplitude events (-1.2 pA) had either brief discrete openings or longer openings containing flickerings between the open and closed states indicating a bursting behaviour (see Colquhoun & Hawkes, 1995). The lower amplitude events (-0.75 pA) opened for brief periods of time with less obvious ‘flickery’ behaviour. In addition, the single channel currents had a low activity at -50 mV ($P_o < 0.01$, see Table 1).

Figure 1A(ii) shows the effect of 0 Ca_o^{2+} on the channel currents from the same patch as shown in Fig. 1A(i). Changing from 1.5 mM to 0 Ca_o^{2+} altered both the amplitude distribution and kinetic behaviour of the single channel currents. In 0 Ca_o^{2+} the most noticeable feature is that whereas a few channel currents reach the higher open state most of the channel currents have a smaller amplitude. This is shown in the amplitude histogram which could be fitted with three Gaussian curves with peaks of -0.73 , -1.2 and -1.95 pA (Fig. 1B(ii)). The areas under the curves consisted of 163, 80 and 5 events respectively for the -0.73 , -1.2 and -1.95 pA peaks indicating that the -0.73 pA peak is the main open state in 0 Ca_o^{2+} . These results suggest that the channel exists in

two states and that Ca_o^{2+} alters the equilibrium between the two states with high Ca_o^{2+} favouring the higher conductance state. The small number of larger openings (-1.95 pA, Fig. 1B(ii)) probably represent multiple openings as in Fig. 1B(i).

Figure 1C shows the I - V relationships of the single channel currents. For 1.5 mM Ca_o^{2+} the relationship of the larger conductance channel current was plotted since this represented the major event. In 0 Ca_o^{2+} the lower conductance channel current was measured as this was the predominant event. In 1.5 mM Ca_o^{2+} the I - V relationship behaved ohmically between -90 mV and $+40$ mV, with a unitary conductance of 23 pS and V_r of $+10$ mV. In 0 Ca_o^{2+} the I - V relationship had an unitary conductance of 13 pS and V_r of $+4$ mV (Fig. 1C). Table 1 shows that the mean V_r in 0 Ca_o^{2+} was significantly different to the mean V_r of the single channel currents in 1.5 mM Ca_o^{2+} .

Table 1 shows the mean values of unitary conductance and V_r of single cation currents recorded from individual patches in 1.5 mM or 0 Ca_o^{2+} . The mean data were not different from the pooled values. Table 1 shows that, compared to 1.5 mM Ca_o^{2+} , in 0 Ca_o^{2+} there was no significant difference in P_o but there were marked changes in other kinetic parameters of channel activity which are described below.

It was apparent that Ca_o^{2+} has an effect on open time distributions. Figure 2A illustrates open time histograms created using both the lower and higher conductance states of the single channel currents shown in Fig. 1A. In 1.5 mM Ca_o^{2+} the open time distribution was fitted by two exponentials with time constants of 1.4 ms (O_{r1}) and 7.2 ms (O_{r2}), i.e. similar to our previous work (Albert & Large, 2001). In 0 Ca_o^{2+} the majority of events were open for briefer durations (< 2 ms) and the open lifetime distributions could also be fitted with two time constants of 0.7 ms (O_{r1}) and 4.3 ms (O_{r2} , Fig. 2A). The mean data for six patches are shown in Table 1. The open time distributions suggest that the cation channels have at least two open states which are both significantly reduced on removing Ca_o^{2+} .

Previously, we have shown that these cation channel currents exhibited bursting behaviour and the burst

duration histograms were fitted by two exponentials (Albert & Large, 2001). Closures between bursts of single cation channel activity were distinguished from closures within a burst of activity by calculating a critical value (T_{crit}) from closed time distributions as shown previously (see Albert & Large, 2001). Figure 2*B* shows the closed time distributions created from the single channel currents shown in Fig. 1*A*. In both 1.5 Ca_o^{2+} and 0 Ca_o^{2+} the closed time distributions could be fitted by the sum of two exponentials with markedly different time constants of approximately 2 ms ($C_{\tau 1}$) and 200 ms ($C_{\tau 2}$), suggesting $C_{\tau 1}$ represents brief closures within a burst of channel activity and $C_{\tau 2}$ represents long closures between bursts. The marked differences between $C_{\tau 1}$ and $C_{\tau 2}$ make it unlikely that the calculated T_{crit} values are seriously affected by underestimation of the closed states due to more than one channel in the patch. Figure 2*C* shows burst duration histograms created from the single channel currents shown in Fig. 1*A*. In 1.5 mM Ca_o^{2+} the

burst duration histogram was composed of brief events (< 5 ms) and a similar number of bursts with durations of between 5 ms and 90 ms (Fig. 2*C*). The histogram could be fitted by two exponentials with time constants of 2.8 ms ($B_{\tau 1}$) and 16 ms ($B_{\tau 2}$). The mean data from 19 patches in 1.5 mM Ca_o^{2+} are shown in Table 1 and these values are similar to those reported previously (Albert & Large, 2001).

In Figure 1*A*(ii) it is not immediately obvious that the lower conductance cation channel currents in 0 Ca_o^{2+} exhibit bursting behaviour compared to the channel currents in 1.5 mM Ca_o^{2+} . However in 0 Ca_o^{2+} the closed time distributions could also be fitted by the sum of two exponentials with mean time constants of 2.6 ± 0.3 ms ($C_{\tau 1}$) and 188 ± 48 ms ($C_{\tau 2}$, $n = 6$, Fig. 2*B*). These two vastly different time constants indicate that $C_{\tau 1}$ represents closures within a burst of channel activity and $C_{\tau 2}$ represents closures between bursts of activity and

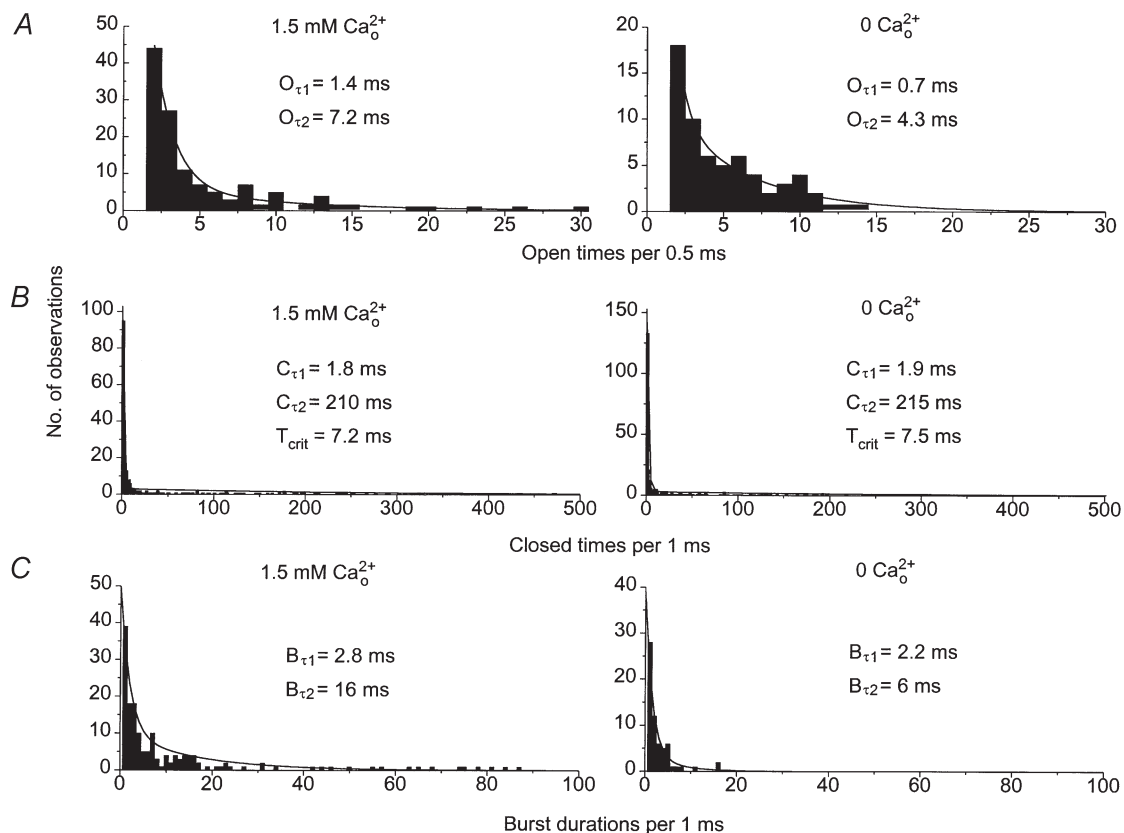


Figure 2. The effect of 0 Ca_o^{2+} on open time and burst duration distributions

A, open time distributions of the single channel currents shown in Fig. 1*A*. In 1.5 mM and 0 Ca_o^{2+} the distributions could be fitted by the sum of two exponentials with time constants $O_{\tau 1}$ and $O_{\tau 2}$. Changing from 1.5 mM to 0 Ca_o^{2+} reduced $O_{\tau 1}$ from 1.4 ms to 0.7 ms and $O_{\tau 2}$ from 7 ms to 4 ms. *B*, closed time distributions of the single channel currents shown in Fig. 1*A* could be described by two exponentials with markedly different time constants of approximately 2 ms and 200 ms. The faster time constant ($C_{\tau 1}$) was used to calculate T_{crit} (see Methods). Changing from 1.5 mM to 0 Ca_o^{2+} did not significantly alter any of these values. *C*, burst duration distributions of the single channel currents shown in Fig. 1*A* could both be fitted by the sum of two exponentials with time constants $B_{\tau 1}$ and $B_{\tau 2}$. Changing from 1.5 mM to 0 Ca_o^{2+} did not alter $B_{\tau 1}$ whereas $B_{\tau 2}$ was reduced from 16 ms to 6 ms.

therefore in 0 Ca_o^{2+} the cation channels do exhibit bursting behaviour. Figure 2C shows that in 0 Ca_o^{2+} the majority of the bursts had brief durations (< 5 ms) with only a few events having burst durations longer than 10 ms and that the distribution could be fitted by the sum of two exponentials with time constants of 2.2 ms (B_{r1}) and 6 ms (B_{r2} , Fig. 2B). The mean data from six patches are shown in Table 1 and it is evident that reducing Ca_o^{2+} from 1.5 mM to a Ca^{2+} -free solution significantly decreased the longer burst duration but did not affect the faster burst period. Therefore it can be concluded that there are marked differences in single channel conductance, open times and burst durations of these single channel currents in 0 Ca_o^{2+} and 1.5 mM Ca_o^{2+} .

Properties of single cation channel currents recorded in 3 μM and 100 μM Ca_o^{2+}

In the present study, we investigated the effects of 3 μM Ca_o^{2+} since this concentration is close to the half-maximal facilitatory effect on the amplitude of noradrenaline-

evoked I_{cat} recorded with whole-cell recording with little apparent inhibitory effect. We also studied the effect of 100 μM Ca_o^{2+} since at this concentration the facilitatory effects of Ca_o^{2+} on I_{cat} is near maximal (Helliwell & Large, 1998). In addition, using 3 μM and 100 μM Ca_o^{2+} allowed us to compare the properties of the single cation channels with the kinetic and single channel conductance values determined with fluctuation analysis of the whole-cell current (Helliwell & Large, 1998).

Figure 3A shows typical effects of reducing Ca_o^{2+} from 1.5 mM to 3 μM on single channel activity in the same patch at -50 mV. In 3 μM Ca_o^{2+} the main peak amplitudes of the single channel currents, between 1 and 2 pA at -50 mV, were similar to the amplitudes in 1.5 mM Ca_o^{2+} and the unitary conductance was 22 pS (Table 1). Figure 3A also shows that reducing Ca_o^{2+} from 1.5 mM to 3 μM increased the time for which the channel currents were in the open state which was manifest as an increase in P_o from 0.05 to 0.15 (see Table 1 for mean values). It can

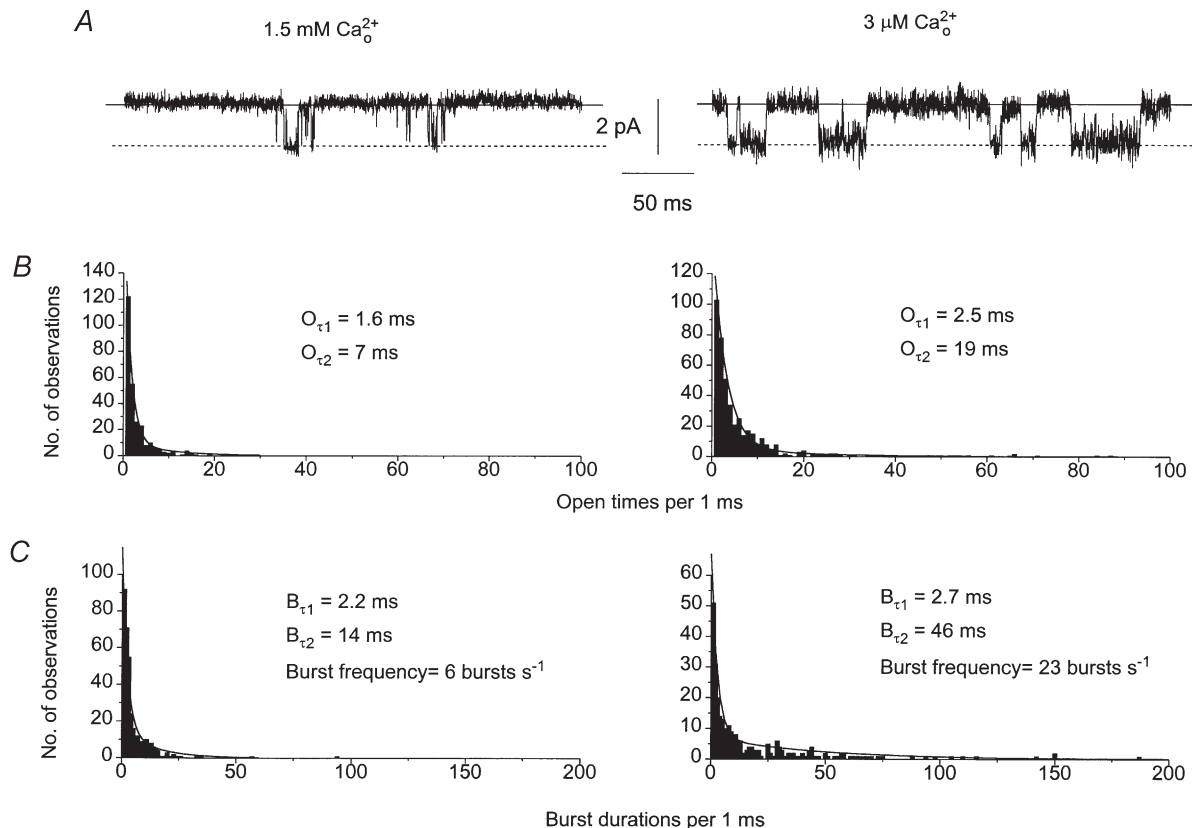


Figure 3. Effect of reducing Ca_o^{2+} concentration from 1.5 mM to 3 μM on single cation channel current activity

A, effect of reducing Ca_o^{2+} from 1.5 mM to 3 μM on single channel currents recorded from the same patch. Note the increased channel activity and longer openings in 3 μM Ca_o^{2+} . *B*, effect of changing Ca_o^{2+} from 1.5 mM to 3 μM on the open time distribution of the single cation channels shown in Fig. 3A. Both open time distributions could be described by the sum of two time constants, $O_{\tau 1}$ and $O_{\tau 2}$. Note that reducing Ca_o^{2+} to 3 μM did not alter $O_{\tau 1}$ but it did increase $O_{\tau 2}$ from 7 ms to 19 ms. *C*, effect of reducing Ca_o^{2+} from 1.5 mM to 3 μM on the burst duration distributions of the single channel currents shown in Fig. 3A. The burst duration distributions could be described by two time constants, $B_{\tau 1}$ and $B_{\tau 2}$. In 3 μM Ca_o^{2+} $B_{\tau 1}$ was not changed whereas $B_{\tau 2}$ was increased from 14 ms to 46 ms.

be seen in Fig. 3A that the open time of the channel appears to be longer in Ca_o^{2+} which is confirmed in Fig. 3B where it can be seen that the longer open time (O_{r2}) increases from 7 ms to 19 ms on changing from 1.5 mM to 3 μM Ca_o^{2+} but there is little change in the faster open time constant (O_{r1}). Also in 3 μM Ca_o^{2+} there was an increase in the longer burst duration time constant (B_{r2} , 46 ms compared to 14 ms in 1.5 mM Ca_o^{2+}), with no change in the faster burst time constant (B_{r1}). Also burst frequency was increased from 6 bursts s^{-1} in 1.5 mM Ca_o^{2+} to 23 bursts s^{-1} in 3 μM Ca_o^{2+} (Fig. 3C and see Table 1).

Changing from 1.5 mM to 100 μM Ca_o^{2+} significantly increased channel activity (Fig. 4A) with an increased P_o (from 0.06 in 1.5 mM Ca_o^{2+} to 0.22 in 100 μM Ca_o^{2+} in this patch, also see Table 1). This increased activity was not due to a change in open times (O_{r1} and O_{r2} , Fig. 4B, Table 1) but to significant increases in the longer burst duration time constant (Fig. 4C, B_{r2} , from 16 ms in 1.5 mM Ca_o^{2+} to 45 ms in 100 μM Ca_o^{2+} , Table 1) and the burst frequency (from 9 bursts s^{-1} to 34 bursts s^{-1} , Fig. 4C, Table 1).

The effects of Ca_o^{2+} on the kinetic behaviour of single cation channel currents are voltage dependent

In a recent study we have shown that the spontaneous single cation currents have a higher P_o and increased open time and burst duration at +40 mV compared to -50 mV (Albert & Large, 2001) and therefore we have compared the effects of different Ca_o^{2+} concentrations on single cation channel activity at -50 mV and +40 mV. Figure 5A, B and C shows the effect of voltage on single cation channel currents recorded from three different patches in different Ca_o^{2+} concentrations. Increasing Ca_o^{2+} from 3 μM to 1.5 mM had a dramatic effect on the kinetic behaviour of cation channel activity at -50 mV as described previously (Figs 3 and 4, Table 1). However, at +40 mV increasing Ca_o^{2+} from 3 μM to 1.5 mM had no effect on single cation channel activity (Fig. 5A, B and C) with the single channel currents having similar P_o , open and closed times and burst durations (Table 2). Therefore the effect of Ca_o^{2+} on the channel current kinetics was voltage dependent and only observed at negative potentials.

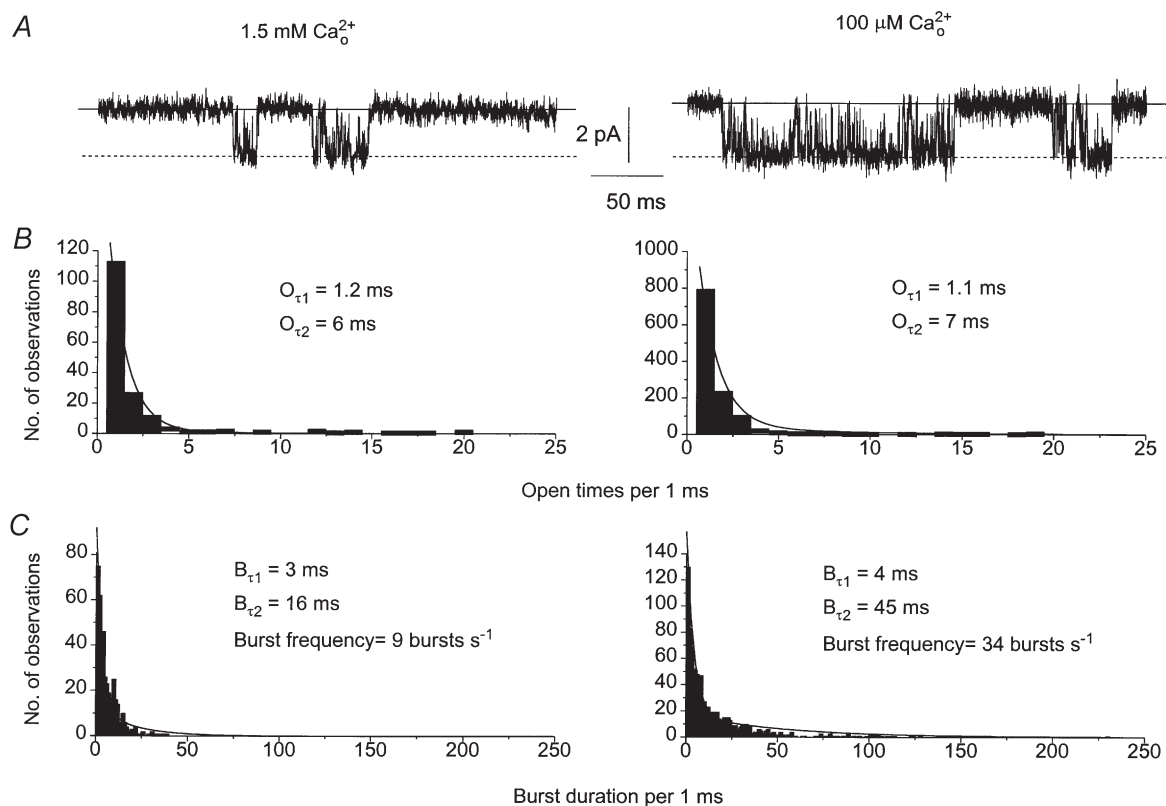


Figure 4. Single cation channel activity in 100 μM Ca_o^{2+}

A, effect of reducing Ca_o^{2+} from 1.5 mM to 100 μM on channel currents recorded from the same patch. In 100 μM Ca_o^{2+} the channel currents showed significantly longer bursts of channel activity. B, effect of reducing Ca_o^{2+} from 1.5 mM to 100 μM on the open time distributions of the single channel currents shown in Fig. 4A. Changing from 1.5 mM to 100 μM Ca_o^{2+} did not alter O_{r1} or O_{r2} . C, burst duration distributions of the single channel currents shown in Fig. 4A. Reducing Ca_o^{2+} from 1.5 mM to 100 μM did not change B_{r1} but increased the B_{r2} from 16 ms to 52 ms.

Table 2. The effects of different Ca_o^{2+} concentrations on spontaneous single cation channel currents at a holding potential of +40 mV

$[\text{Ca}_o^{2+}]$	P_o	O_{r1} (ms)	O_{r2} (ms)	B_{r1} (ms)	B_{r2} (ms)	n
3 μM	0.51 ± 0.14	1.4 ± 0.3	37 ± 4	3.2 ± 0.4	128 ± 22	4
100 μM	0.47 ± 0.11	1.7 ± 0.3	43 ± 2	2.9 ± 0.2	112 ± 15	4
1.5 mM	0.62 ± 0.15	1.5 ± 0.2	40 ± 8	3.8 ± 0.5	104 ± 19	6

The effects of Sr_o^{2+} and Ba_o^{2+} on spontaneous single cation channel currents

We have shown previously that 1.5 mM Sr_o^{2+} and 1.5 mM Ba_o^{2+} increased the amplitude of I_{cat} by approximately 8- and 3-fold, respectively, when I_{cat} was evoked in 0 Ca_o^{2+} (Aromolaran & Large, 1999); i.e. Sr_o^{2+} and Ba_o^{2+} also have facilitatory effects on I_{cat} . Therefore we investigated the effect of Sr_o^{2+} and Ba_o^{2+} on spontaneous single channel currents. Figure 6A shows a typical response of single channel currents produced by the substitution of 1.5 mM Ca_o^{2+} with 1.5 mM Sr_o^{2+} at -50 mV. In 1.5 mM Sr_o^{2+} the single cation channels spent more time in the open state reflecting a significant increase in P_o from 0.03 in 1.5 mM Ca_o^{2+} to 0.12 in 1.5 mM Sr_o^{2+} (see also Table 3). Figure 6B shows the I - V relationship of the cation channel currents recorded in 1.5 mM Sr_o^{2+} shown in Fig. 6A. The relationship was linear between -90 and -30 mV with a slope conductance of 25 pS and an extrapolated V_r of $+9$ mV. Figure 6C shows the open time histogram of the

single cation currents shown in Fig. 6A. In 1.5 mM Sr_o^{2+} the open time distribution could be described by the sum of two exponentials with time constants of 1.2 ms (O_{r1}) and 7 ms (O_{r2}), i.e. similar to the values in 1.5 mM Ca_o^{2+} . Figure 6D illustrates the burst durations distribution of the single channel currents shown in Fig. 6A. The burst duration histogram could be described by the sum of two exponentials with time constants of 2.5 ms (B_{r1}) and 31 ms (B_{r2}) indicating that the longer burst duration (B_{r2}) was significantly increased on changing from 1.5 mM Ca_o^{2+} to 1.5 mM Sr_o^{2+} . Table 3 shows the mean values of unitary conductance, P_o , mean open and closed times and burst durations in 1.5 mM Sr_o^{2+} .

Figure 7A illustrates a typical response of spontaneous single channel currents in 1.5 mM Ba_o^{2+} . Changing from 1.5 mM Ca_o^{2+} to 1.5 mM Ba_o^{2+} increased P_o of the single channel currents from 0.05 to 0.10 but although an increase in P_o was observed in all patches tested the increase was not statistically significant. Figure 7B shows

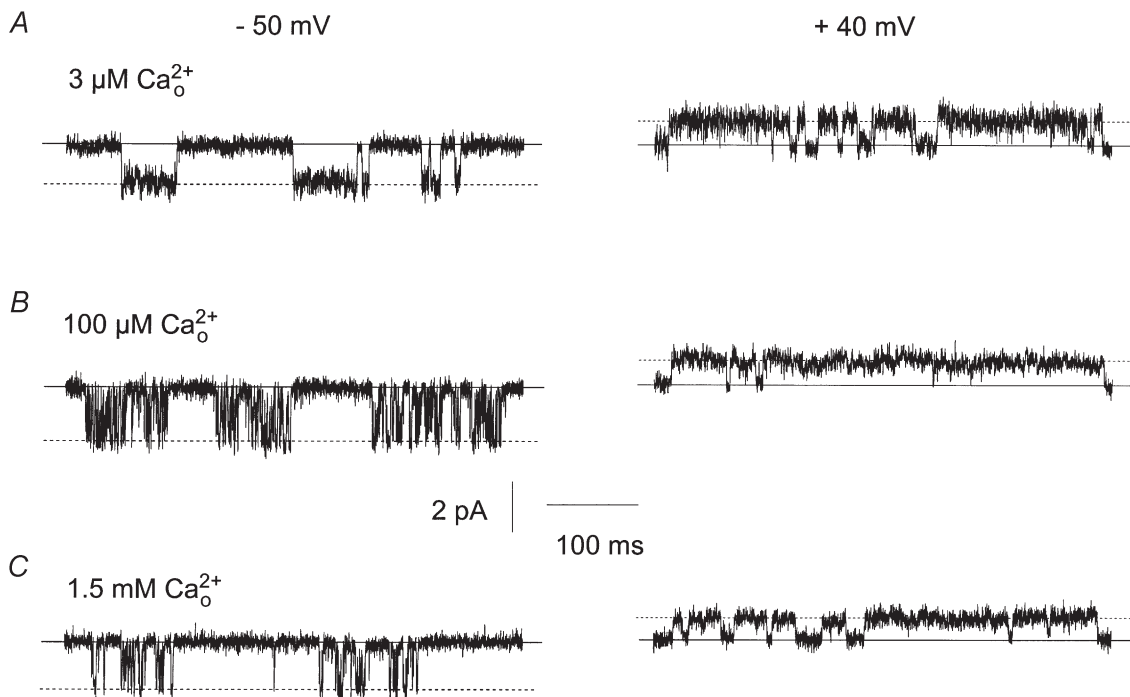


Figure 5. Effect of membrane potential on single channel currents in different Ca_o^{2+} concentrations

A, B and C, effects of different Ca_o^{2+} concentrations on single channel currents recorded from three different patches at -50 mV and $+40$ mV. Note that single channel currents behave similarly at $+40$ mV in 3 μM (A), 100 μM (B) and 1.5 mM (C) Ca_o^{2+} .

Table 3. Comparison of the properties of the noradrenaline-evoked I_{cat} obtained from spectral density functions and single spontaneous cation channel currents in the presence of various external divalent cations concentrations

Divalent cation concentration	Single channel data							Spectral density analysis of whole-cell current		
	γ (pS)	P_o	O_{τ_1} (ms)	O_{τ_2} (ms)	B_{τ_1} (ms)	B_{τ_2} (ms)	Frequency (bursts s^{-1})	γ (pS)	τ_1^\dagger (ms)	τ_2^\dagger (ms)
0 Ca_o^{2+}	$12 \pm 2^{**}$	0.02 ± 0.01	$0.7 \pm 0.1^*$	$3.9 \pm 0.4^*$	3.9 ± 0.7	$5.8 \pm 1^*$	12 ± 3	11 ± 2	1.4 ± 0.1	11 ± 1
3 μM Ca_o^{2+}	22 ± 2	$0.11 \pm 0.03^{**}$	1.3 ± 0.3	$17 \pm 2.3^{**}$	2.8 ± 0.4	$43 \pm 4^{**}$	$17 \pm 4^*$	22 ± 2	1.2 ± 0.2	44 ± 3
100 μM Ca_o^{2+}	26 ± 2	$0.15 \pm 0.03^{**}$	1.1 ± 0.2	6.2 ± 1.1	2.9 ± 1.4	$42 \pm 9^*$	$45 \pm 8^{**}$	24 ± 2	1.6 ± 0.4	45 ± 4
1.5 mM Ca_o^{2+}	23 ± 1	0.05 ± 0.02	1.2 ± 0.3	7.4 ± 0.2	3.1 ± 0.3	16 ± 1	14 ± 1	19 ± 2	1.6 ± 0.3	15 ± 1
1.5 mM Sr_o^{2+}	25 ± 2	$0.14 \pm 0.04^*$	1.1 ± 0.2	6.0 ± 0.7	3.6 ± 0.4	$30 \pm 5^*$	19 ± 3	22 ± 1	1.5 ± 0.1	24 ± 2
1.5 mM Ba_o^{2+}	22 ± 1	0.06 ± 0.02	1.0 ± 0.1	5.3 ± 0.8	3.7 ± 0.4	13 ± 2	19 ± 4	20 ± 1	1.8 ± 0.3	12 ± 1

\dagger These values were obtained from $\tau = \frac{1}{2}\pi f_c$, where f_c is the corner frequency (Helliwell & Large, 1998; Albert *et al.* 2001). All mean values are calculated from at least five patches. * $P < 0.05$, ** $P < 0.01$ compared to values in 1.5 mM Ca_o^{2+} .

the I - V relationship of the single channel currents shown in Fig. 7A. The relationship had a unitary conductance of 20 pS and V_r of +11 mV. Figure 7C and D shows the open time and burst duration distributions of the single

channel currents shown in Fig. 7A. Both distributions could be described by the sum of two exponentials with time constants of 1.4 ms (O_{τ_1}) and 7 ms (O_{τ_2}) for open times and 2.6 ms (B_{τ_1}) and 19 ms (B_{τ_2}) for burst durations

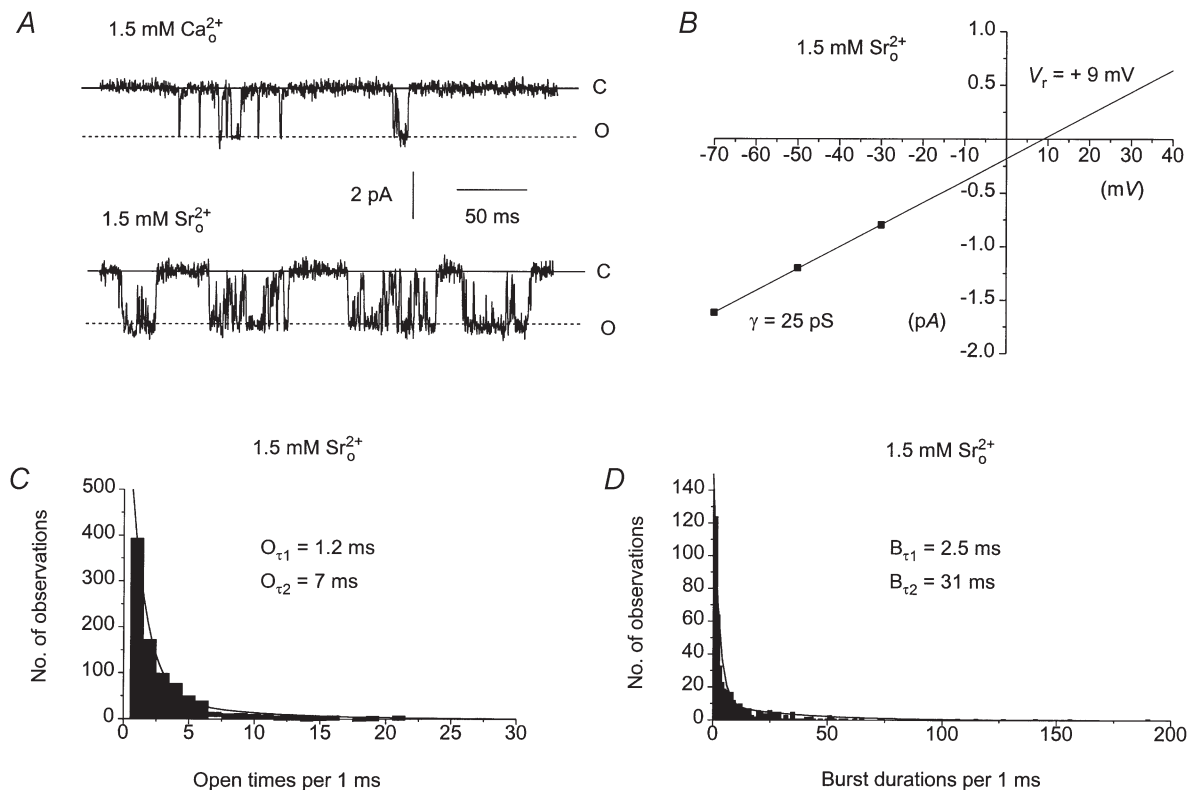


Figure 6. The effect of replacing of 1.5 mM Ca_o^{2+} by 1.5 mM Sr_o^{2+} on the properties of single cation channel currents

A, effects of replacing 1.5 mM Ca_o^{2+} by 1.5 mM Sr_o^{2+} on single channel currents recorded from the same patch at -50 mV. B, I - V relationship of the single channel currents shown in A in 1.5 mM Sr_o^{2+} had an unitary conductance of 25 pS and an extrapolated V_r of +9 mV. C, the open time distribution of the single channel currents in Sr_o^{2+} shown in A could be described by two exponentials with time constants of 1.2 ms (O_{τ_1}) and 7 ms (O_{τ_2}). D, the burst duration distribution of the single channel currents in Sr_o^{2+} shown in A could be described by two exponentials with time constants of 2.5 ms (B_{τ_1}) and 31 ms (B_{τ_2}).

respectively; i.e. the open time and burst duration time constants are similar to the values in 1.5 mM Ca_o^{2+} . The mean data are shown in Table 3.

DISCUSSION

Previously we have shown that external divalent cations have a profound effect on the amplitude of the noradrenaline-evoked whole-cell current (I_{cat}) in rabbit portal vein smooth muscle cells (Helliwell & Large, 1996, 1998; Aromolaran & Large, 1999). The present work was undertaken to elucidate the basis of these actions at the single channel current level. In the present study we have shown that external divalent cations have a marked effect on the amplitude and kinetic behaviour of non-selective cation channel currents recorded with isolated patches in the outside-out configuration. In external divalent cation-free conditions the channel currents have a number of distinguishing properties with two conductance levels, at least two open states and a bursting behaviour. Increasing the concentration of external divalent cations alters these properties to produce facilitatory and inhibitory effects on the single channel currents. Here we will discuss the effect

of external divalent cations on the properties of single channel currents and how this relates to both the amplitude of I_{cat} and the properties of the unitary conductance estimated by noise analysis of the whole-cell current.

Facilitatory effect of Ca_o^{2+} on single non-selective cation channel currents

In all Ca_o^{2+} concentrations we observed two conductance states, one of about 12 pS and another of approximately 23 pS. In 0 Ca_o^{2+} the majority of the cation channels opened to a conductance state of about 12 pS whereas in higher Ca_o^{2+} concentrations most of the channels opened to the higher conductance state. Therefore there seems to be an equilibrium between the two conductance levels which is shifted in favour of the higher conductance state by increasing Ca_o^{2+} . Table 3 shows that increasing Ca_o^{2+} from 0 to 3 μM almost doubled the unitary conductance to 22 pS. Fluctuation analysis of the noradrenaline-evoked whole-cell current with $\text{Ca}_o^{2+} \geq 3 \mu\text{M}$ yields an underlying unitary conductance value of 19–24 pS (Table 3) which suggests that the higher conductance state is predominant in these conditions. Moreover, increasing Ca_o^{2+} from 0 to

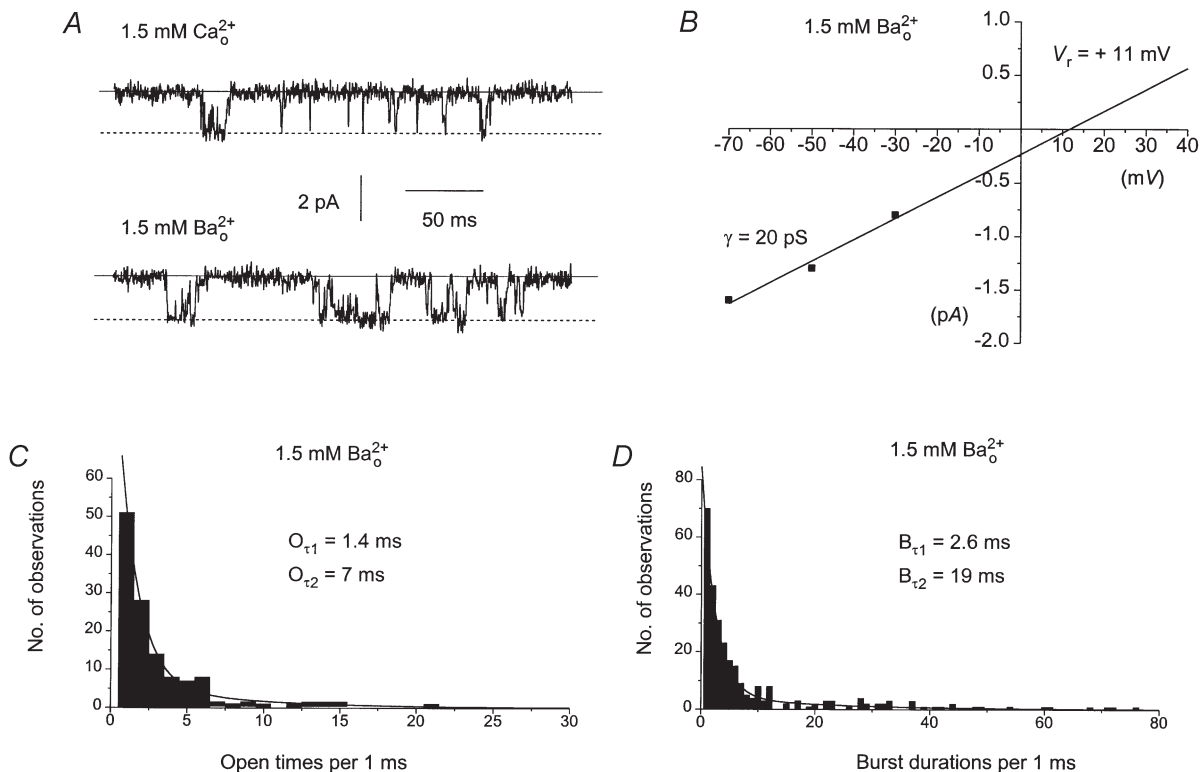


Figure 7. The effect of replacing 1.5 mM Ca_o^{2+} by 1.5 mM Ba_o^{2+} on the properties of single cation channel currents

A, effects of replacing 1.5 mM Ca_o^{2+} by 1.5 mM Ba_o^{2+} on single cation currents recorded from the same patch at -50 mV. *B*, I - V relationship of the single cation currents shown in *A* in 1.5 mM Ba_o^{2+} had a unitary conductance of 20 pS and an extrapolated V_r of +11 mV. *C*, the open time distribution of the single channel currents shown in *A* could be described by two exponentials with time constants of 1.4 ms (O_{r1}) and 7 ms (O_{r2}). *D*, the burst duration distribution of the single channel currents shown in *A* could be described by two exponentials with time constants of 2.6 ms (B_{r1}) and 19 ms (B_{r2})

3 μM increased P_o , mainly due to marked increases in the longer open time (O_{r2}), from 4 ms to 17 ms, and the longer burst duration (B_{r2}), from 6 ms to 43 ms (Table 3). The increases in single channel conductance and P_o on increasing Ca_o^{2+} from 0 to 3 μM accounts for the 4- to 5-fold increase in the amplitude of the noradrenaline-evoked whole-cell current shown previously (Helliwell & Large, 1998).

Interestingly, in 0 Ca_o^{2+} the smaller conductance channel current had a V_r of about +4 mV whereas the V_r of the higher conductance state was about +10 mV. Thus the larger conductance state in 0 Ca_o^{2+} had similar characteristics to channels recorded in $\text{Ca}_o^{2+} \geq 3 \mu\text{M}$. It is possible that with 1 mM BAPTA in the bathing solution there may still be pockets of Ca^{2+} ions in microdomains surrounding the external mouth of some of the channels. In such cases the Ca_o^{2+} causes the channel to open to the full conductance state and also Ca^{2+} permeates the channel with the result that V_r is shifted to more positive values. However, in the majority of cases there are no Ca^{2+} ions near the channel mouth and therefore the channel only opens to the lower conductance level and no Ca^{2+} ions permeate the channel with the consequence that the V_r is relatively negative. According to the Boltzmann principle, it would not be expected that the Ca_o^{2+} concentration at the channel mouth would be similar in bathing solutions containing 0 Ca_o^{2+} and 1.5 mM Ca_o^{2+} . However, there might be structural characteristics of the external surface of the channel causing regional differences in Ca_o^{2+} concentration at the channel mouth compared to the bulk solution, i.e. a form of compartmentalisation.

With whole-cell recording, the amplitude of noradrenaline-evoked I_{cat} in 100 μM Ca_o^{2+} is about double the value in 3 μM Ca_o^{2+} (Helliwell & Large, 1998). With single channel recording there are marked changes in channel activity and compared to 3 μM Ca_o^{2+} there was an increase in burst frequency, from 17 bursts s^{-1} to 45 bursts s^{-1} , and a reduction in the longer open time (O_{r2} , Table 3) from 17 ms to 6 ms in 100 μM Ca_o^{2+} . Presumably, therefore, the doubling of the amplitude of I_{cat} on changing from 3 μM to 100 μM is accounted for by the increase in burst frequency which outweighs the reduction in O_{r2} .

Inhibitory effect of Ca_o^{2+} on single cation channel currents

With whole-cell recording, increasing Ca_o^{2+} above 100 μM reduces the amplitude of I_{cat} and the apparent equilibrium constant for the inhibitory effect of Ca_o^{2+} is about 400 μM (Helliwell & Large, 1996). In the present study with single channel recording, the main effect of increasing Ca_o^{2+} from 100 μM to 1.5 mM Ca_o^{2+} is to reduce the long burst duration (B_{r2}) from 42 ms to 16 ms and the burst frequency from 45 bursts s^{-1} to 14 bursts s^{-1} (Table 3). This produces a marked reduction of P_o (Table 3) which is responsible for the smaller amplitude of I_{cat} in 1.5 mM compared to 100 μM Ca_o^{2+} .

The effects of Sr_o^{2+} and Ba_o^{2+} on single cation channel currents

Table 3 shows that compared to 0 Ca_o^{2+} the single cation channel currents in 1.5 mM Sr_o^{2+} or 1.5 mM Ba_o^{2+} had a 2-fold increase in unitary conductance, indicating that Sr_o^{2+} and Ba_o^{2+} can substitute for Ca_o^{2+} in potentiating single channel conductance. Compared to 0 Ca_o^{2+} in 1.5 mM Sr_o^{2+} there is a marked increase in P_o associated with an increase in the longer burst duration (B_{r2}) from 6 ms to 30 ms (Table 3). With whole-cell recording the amplitude of I_{cat} in 1.5 mM Sr_o^{2+} is about 7 times larger than in 0 Ca_o^{2+} (Aromolaran & Large, 1999) which is accounted for by the increase in single channel conductance and B_{r2} . In contrast, I_{cat} is only 2- to 3-fold larger in 1.5 mM Ba_o^{2+} compared to 0 Ca_o^{2+} (Aromolaran & Large, 1999) which is mainly due to the increase in single channel conductance and a doubling of the longer burst duration (B_{r2}).

Mechanisms of action of the facilitatory and inhibitory effects of external divalent cations

The present study shows unequivocally that the facilitatory effect of Ca_o^{2+} , Sr_o^{2+} and Ba_o^{2+} is associated with two distinct effects on single cation channel currents, namely increasing single channel conductance and altering kinetic behaviour. As the experiments were carried out using isolated outside-out patches these effects are probably due to Ca_o^{2+} , Sr_o^{2+} and Ba_o^{2+} binding to sites on the external surface of the membrane. Changing from external divalent-free conditions to 1.5 mM Ba_o^{2+} increased single channel conductance but had little effect on the kinetic properties of the cation channels which was also apparent with noise analysis of whole-cell currents (Aromolaran & Large, 1999). It is therefore tempting to speculate that the facilitatory effects of external divalent cations on single channel conductance and kinetic behaviour are mediated by two distinct binding sites. Spectral density analysis of the noradrenaline-evoked I_{cat} also suggested that the facilitatory effect of Ca_o^{2+} may be due to Ca^{2+} binding to two sites since in 1 μM Ca_o^{2+} single channel conductance was the same as in 0 Ca_o^{2+} but there were marked differences in kinetics (Helliwell & Large, 1998).

A notable feature was that at positive membrane potentials the single cation currents had similar amplitudes and kinetic properties in 3 μM , 100 μM and 1.5 mM Ca_o^{2+} . In our experiments the internal Ca^{2+} concentration was buffered to about 14 nM with 10 mM BAPTA. Consequently the inhibitory effects of Ca_o^{2+} on channel activity at negative potentials is due to external Ca^{2+} ions affecting inward movement of cations through the channel. At -50 mV increasing Ca_o^{2+} from 3 μM to 100 μM induced a reduction in mean open time and increased flickerings between the open and closed states during bursts of channel activity. Increasing Ca_o^{2+} further from 100 μM to 1.5 mM reduced P_o . These are characteristics of a molecule causing channel block (see Hille, 1992). Since we have shown that divalent

cations can permeate this conductance (Byrne & Large, 1988; Wang & Large, 1991) it is possible that Ca^{2+} ions produce an apparent block as they permeate the channel.

Comparison of the single channel data with spectral density analysis data

Table 3 compares the single channel data from the present study with the characteristics of the unitary conductance estimated by spectral density analysis of the whole-cell current evoked by noradrenaline. The values of the single channel conductance predicted by noise analysis of whole-cell current were extremely accurate in all the divalent cation concentrations used. The 'noise' spectrum also was described by two Lorentzian components predicting two open states. Single channel analysis confirms this prediction. The fast open time was the same in the two studies but the corner frequency of the second Lorentzian reflects the burst duration and not the longer of the mean open times in all divalent solutions (Table 3).

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