J. Membrane Biol. 165, 133–143 (1998)

The Journal of
Membrane
Biology

The Action of Blocking Agents Applied to the Inner Face of Ca^{2+}-activated K^+ Channels from Human Erythrocytes

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Received; 18 February 1998/Received: 5 June 1998

Abstract. The actions of clotrimazole and cetiedil, two drugs known to inhibit the Gardos channel, have been studied on single intermediate conductance calcium-activated potassium (IK_{Ca}) channels in inside out patches from human red blood cells, and compared with those of TEA and Ba^{2+} applied to the cytoplasmic face of the membrane. TEA produced a fast block which was observed as a reduction in the amplitude of the single channel current. This effect was weakly voltage dependent with the fraction of the membrane potential sensed by TEA at its binding site (δ) of 0.18 and a K_d at 0 mV of 20.5 mM. Ba^{2+} was a very potent blocker of the channel, breaking the single channel activity up into bursts, interspersed with silent periods lasting several seconds. The effect of Ba^{2+} was very voltage sensitive, $\delta = 0.44$, and a K_d at 0 mV of 0.15 μM. Clotrimazole applied to the inner face of the membrane at a concentration $\leqslant 1$ μM produced a slow block resulting in bursts of channel activity separated by quiescent periods lasting many seconds. The effect of clotrimazole was mimicked by a quaternary derivative UCL 1559, in keeping with an action at the cytoplasmic face of the channel. A high concentration of cetiedil (100 μM) produced only a weak block of the channel. The kinetics of this action were very slow, with burst and inter-burst intervals lasting several minutes. While inhibition of the Gardos channel by cetiedil is unlikely to involve an intracellular site of action, if clotrimazole is able to penetrate the membrane, part of its effect may result from binding to an intracellular site on the channel.

Key words: Calcium activated — Potassium channel — Erythrocyte — Clotrimazole — Cetiedil

Introduction

Ca^{2+}-activated K^+ channels are found in many cell types and have been divided into three main groups (small, intermediate and large conductance) on the basis of their single channel conductance and sensitivity to blocking agents (for review *see* Haylett & Jenkinson, 1990). Recent advances in molecular biology have lead to the cloning of the small conductance (SK_{Ca}) channel (Kohler et al., 1996) and two subunits of the large conductance (BK_{Ca}) channel (Adelman et al., 1992; Knaus et al., 1995). Although there is considerable similarity in the presumed tertiary structure between the BK_{Ca}, SK_{Ca} and the K_v family of voltage-activated K^+ channels, there is only limited sequence homology, and there are a number of pharmacological agents which can distinguish between them. Very recently, an intermediate conductance Ca^{2+}-activated K^+ channel, which exhibits some of the pharmacological characteristics of the channel found in human erythrocytes has been cloned from a human pancreatic cDNA library (Ishii et al., 1997). This channel is predicted to have the same, 6 trans-membrane domain, topology as the other Ca^{2+}-activated K^+ channels.

In erythrocytes, the elevation of intracellular Ca^{2+} leads to a loss of K^+ through Ca^{2+}-activated K^+ channels. This is accompanied by the efflux of Cl^- and osmotically obliged water, leading to a reduction in cell volume (Gardos, 1958; Sarkadi et al., 1985). This mechanism of cellular dehydration may be an important factor in the initiation of sickling crises in patients suffering from sickle cell disease (*see* Joiner, 1993). Drugs which can selectively block these channels may therefore be useful in the

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management of this disease. Two compounds that have recently aroused interest, and have shown some benefit in clinical trials, are the smooth muscle relaxant cetiedil, and the antifungal agent clotrimazole (Benjamin et al., 1986; Brugnara et al., 1996).

A better understanding of the mechanisms by which these compounds block Ca^{2+}-activated K^+ efflux from red blood cells may help in the development of more potent and more selective agents. Both cetiedil and clotrimazole are lipophilic molecules and may readily penetrate cell membranes. A recent study which tested a series of cetiedil analogues as blockers of the IK_{Ca} channel found a strong correlation between potency and lipophilicity (Benton et al., 1994; Benton, 1995). This has raised the possibility that these compounds may penetrate the membrane and act from within it, or at the cytoplasmic face of the channel. To date, pharmacological studies on the IK_{Ca} channel have been carried out mainly using measurement of net K^+ efflux (Benton et al., 1995; Benton, 1995) or ^{86}Rb uptake (Ellory et al., 1992; Brugnara et al., 1993). However there have also been some reports of the fundamental properties of this channel as determined by single channel recording from isolated patches of human erythrocytes (Grygorczyk & Schwartz, 1983, 1985; Christopherson, 1991; Leinders et al., 1992*a*,*b*). Very recently the effect of clotrimazole on Ca^{2+}-activated K^+ channels in outside-out patches from mouse erythroleukemia cells has been described (Rittenhouse et al., 1997*b*).

In this study, the action of cetiedil and clotrimazole at the cytoplasmic side of the Ca^{2+}-activated K^+ channels from human erythrocytes has been investigated directly, using single channel recording, and compared with the actions of two well characterized K^+ channel blockers, TEA and Ba^{2+}.

Materials and Methods

Preparation of Cells

A sample of blood (1–5 μl) was obtained from healthy male volunteers by fingertip puncture and diluted in 1 ml of extracellular solution (*see below*) at 37°C. A small volume (50–100 μl) of this suspension was then plated out in a 35 mm plastic culture dish (Falcon) containing 2 ml of extracellular solution at 37°C. The dish was then left to cool to room temperature (19–22°C). This procedure produced turgid erythrocytes that adhered to the culture dish and were suitable for patch clamping.

Electrophysiology

The culture dish was placed on the stage of an inverted microscope (Diaphot, Nikon) and cells visualized under phase contrast at 600× magnification. The culture dish was perfused with Ca^{2+}-free intracellular solution at a rate of 0.5 ml min^{-1} while solution was applied to isolated patches using a microperfusion device (*see* Dunn et al., 1996). Recordings were carried out at room temperature, using the inside out configuration of the patch-clamp technique (Hamill et al., 1981). Patch electrodes were fabricated from thin wall borosilicate glass capillaries (Clark GC 150TF), Sylgard coated and fire polished. When filled with extracellular solution they had a resistance of 10–15 MΩ. After formation of a gigaseal, the cell was detached by gently touching the bottom of the dish, to yield an inside-out patch. Single K_{Ca} channel currents were recorded using a List EPC 7 amplifier. The measured junction potential between the pipette and bath solution was 2 mV (pipette positive), and has not been corrected for. Data were recorded on a chart recorder (Gould TA240) and also stored on digital audio tape using a DTR-1204 (Biologic, Claix, France) recorder for subsequent off line computer analysis.

Data Analysis

Data were subsequently played back, filtered at 1.0 KHz (-3dB, 8 pole Bessel filter) and digitally sampled at 4 KHz with a Digidata 1200 data acquisition system and pClamp 6.0 software (Axon Instruments, Foster City, CA). The same software was used for analysis of the digitized data. For illustrations of long duration, data were filtered and digitized at much lower frequencies (0.2 and 0.4 KHz respectively). For slow blocking effects, blocked and unblocked durations were measured directly from the chart record. Concentration response data were fitted with the Hill equation using commercially available least squares minimization software (Origin v 4, Microcal Software). Values are expressed as mean ± SEM and *n* indicates the number of patches. For burst analysis of blocking events the inter-burst intervals were determined in two ways. For the very slow blocking by clotrimazole, any closed time longer than the longest closed interval in control conditions (usually ≈ 1 sec) was accepted as an inter-burst interval. For the action of Ba^{2+}, the optimal inter-burst interval was determined from a graph of closings per burst as a function of minimum closed interval delimiting a burst. As this minimum interval increases, the number of closings increases then reaches a plateau at the optimum inter-burst interval (*see* Sigurdson et al., 1987).

Solutions and Chemicals

The normal extracellular solution had the following composition (mM): NaCl 145, KCl 5.0, HEPES 10, $CaCl_2$ 1.8, $MgCl_2$ 0.8. The pH was adjusted to 7.4 by addition of NaOH. Unless stated otherwise, recording electrodes were filled with a similar solution, but containing 0.1 mM KCl and 0 mM $CaCl_2$. The 'intracellular' solution bathing the cytoplasmic side of the patch contained (mM): KCl 120, K_3 citrate 10, HEPES 10, adjusted to pH 7.2 with KOH. The amount of $CaCl_2$ added to give the required free Ca^{2+} concentration was determined from the stability constants given in Sillen and Martell (1964 for H^+ K_1 = 5.84, K_2 = 4.36, K_3 = 2.88; for Ca^{2+} K_1 = 3.4) by an iterative computer program (REACT, written by G.L. Smith, Dept. of Chemistry, University College London). Citrate was used as the Ca^{2+} buffer because the range of free [Ca^{2+}] required (1–100 μM) is outside the effective buffering ranges of EGTA and BAPTA. Because the IK_{Ca} channel blocking action of cetiedil is sensitive to the extracellular [K^+], and K^+ concentration of 0.1 mM in the pipette solution was used to maintain consistency with previous studies of the effects of blocking agents on the $P_{K(Ca)}$ of erythrocytes (Benton et al., 1994, Benton, 1995) and also has the advantage of increasing the amplitude of the single channel currents at the routine holding potential of 0 mV.

Cetiedil was a gift from Innothera (Arcueil, France). UCL 1559 (I-[o-chloro-α,α-diphenylbenzyl] 3-methylimidazolinium iodide) was synthesized by Dr. S. Athmani, Department of Chemistry, University

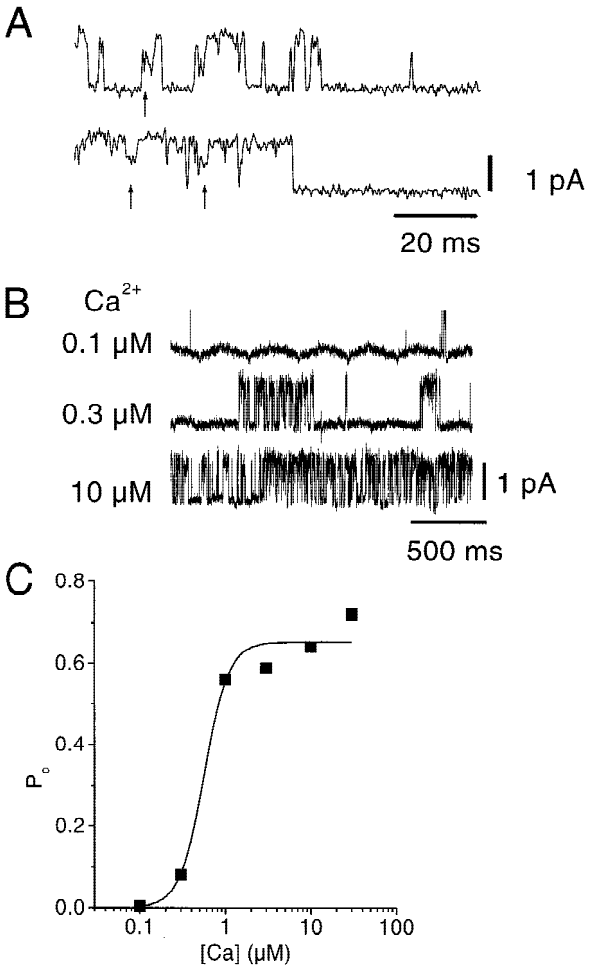

Fig. 1. Properties of IK_{Ca} channels from human red blood cells recorded from inside out patches (*A*) Channel activity recorded at 0 mV in the presence of 3 μM Ca^{2+} on a fast time base to show the flickery nature of the channel openings (upwards) and an apparent subconductance level (arrows). (*B*) Recording from a different patch at 0 mV, showing the effect of changing the Ca^{2+} concentration bathing the cytoplasmic face of the membrane (*C*) The relationship between P_o and Ca^{2+} concentration for the patch shown in *B*. Fitting the Hill equation to these data yielded an EC_{50} of 0.6 μM and a Hill coefficient of 3.

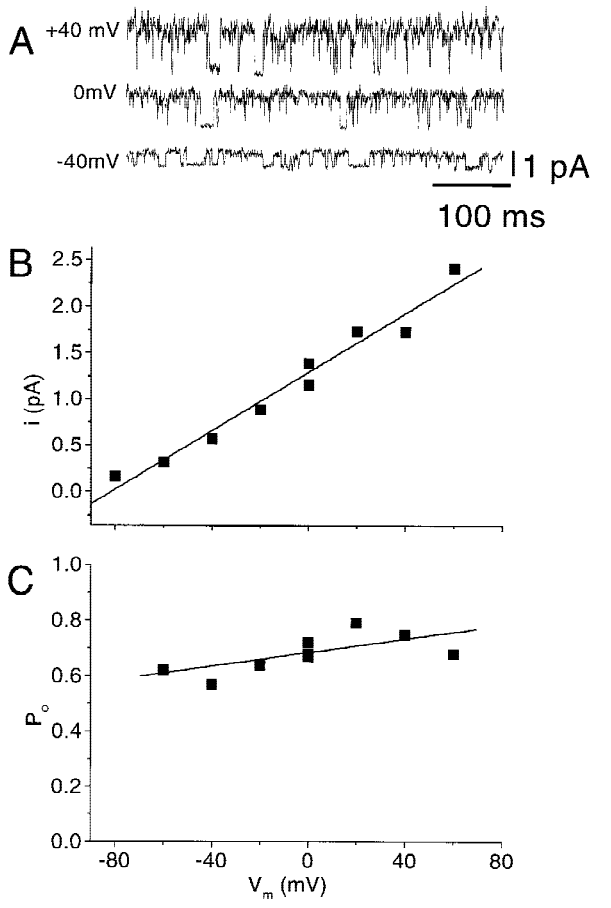

Fig. 2. Effect of membrane potential on IK_{Ca} channels (*A*) Single channel records obtained in 3 μM Ca^{2+} at three different membrane potentials. The current voltage relationship in *B* is linear for membrane potentials between −80 and 80 mV, giving a slope conductance for this channel of 15.9 pS. (*C*) A graph of open probability as a function of membrane potential for the same patch, showing only very weak dependence of P_o on V_m (correlation coefficient = 0.67).

College London. Clotrimazole and all other reagents were purchased from Sigma.

Results

General Properties of the Channels

In approximately 10% of patches, application of Ca^{2+} (0.1 to 30 μM) to the cytoplasmic face activated K^+ channels (Fig. 1*A* and *B*). Usually only a single channel was evident, although occasionally up to 3 channels were observed in a patch. Channel openings were generally very noisy, presumably reflecting rapid, unresolved transitions between the open and closed states. Some channels also appeared to show brief transitions to a subconductance level at approximately 50% of the fully open channel (Fig. 1*A*). However, because of the flickery nature of the channel openings, this was difficult to analyze further.

Open channel probability increased with increasing Ca^{2+} concentration (Fig. 1*B* and *C*). There was considerable variation in the sensitivity to Ca^{2+} between patches, with EC_{50} values ranging from 0.6 to 6.5 μM, and the maximum P_o varied between 0.4 and 0.8. Similar variation in EC_{50} and maximum P_o has previously been described for the BK_{Ca} channel (McManus & Magleby, 1991). Under the ionic conditions used in these experiments, the channels had a linear *I-V* relationship between −80 and +80 mV and a conductance of 13.5 ± 0.5 pS (n = 12). This is close to the values of 15 to 20 pS measured in asymmetrical K^+ conditions by Grygor-

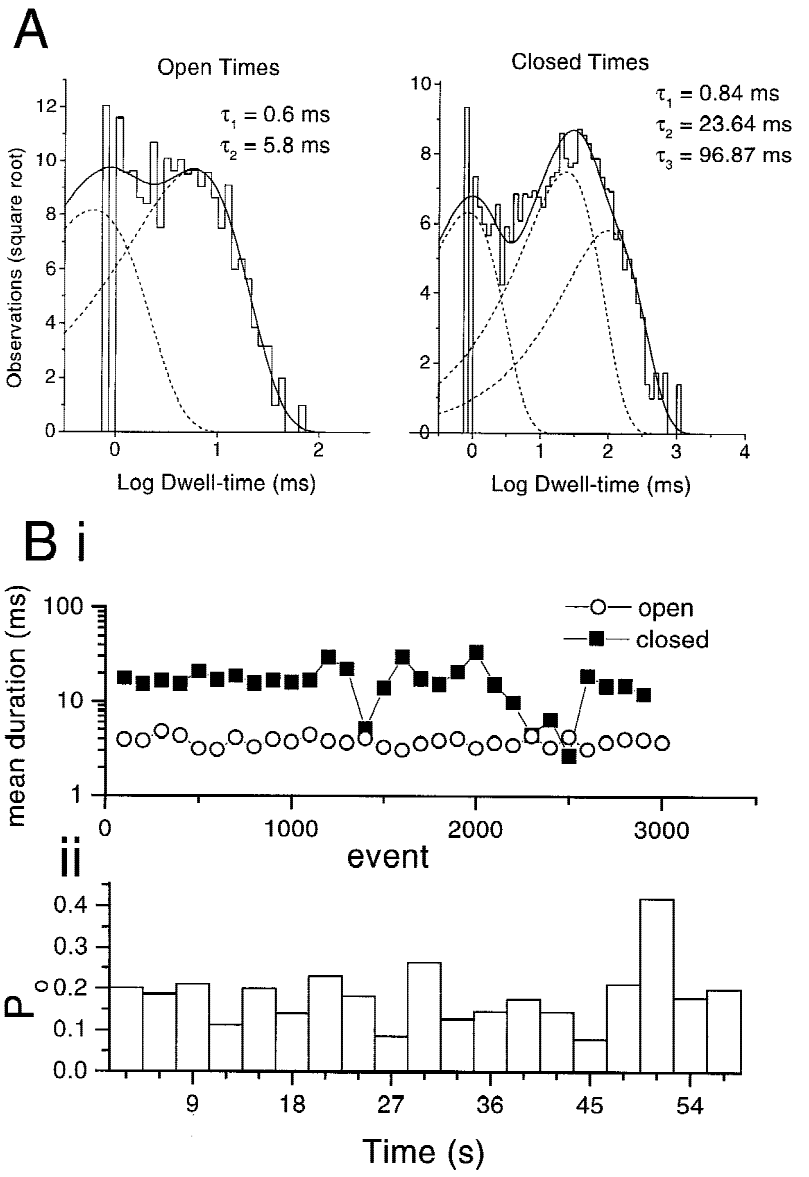

Fig. 3. Kinetic properties of IK_{Ca} channels from human erhtyrocytes. (*A*) Log-dwell time distributions of the open and closed times for a single channel recorded in the presence of 3 μM Ca^{2+} at 0 mV. Dwell times were determined by a 50% threshold crossing method. The histograms have been fitted with multiple exponentials using the method of maximum likelihood. Comparison between models was made using the log likelihood ratio, with values greater than 2 taken to indicate a significantly improved fit. The open time distribution was best fitted with two components having time constants of 0.6 and 5.8 msec, while 3 components (0.84, 23.6 and 96 msec were required to adequately fit the closed time distribution. (*B*) Stability plots for channel activity recorded in a different patch. *i,* mean open and closed times calculated for 100 successive events plotted against event number. While the mean open time remained quite constant for many seconds, there were two periods when there was a dramatic reduction in the mean closed time. *ii,* shows the change in open probability with time for the same patch. Columns represent open probability determined over successive 3-sec periods.

czyk & Schwarz (1983) and Leinders et al., (1992*a*). Also in agreement with the findings of Leinders et al., (1992*a*) open channel probability showed little if any change with membrane potential (Fig. 2*C*). As with the BK_{Ca} channels, the gating of these channels appeared to be complex and sometimes changed dramatically during the course of a recording. In agreement with previous observations, open time distributions usually revealed 2 components, while 2 or 3 components were required to adequately fit the closed time distributions (Leinders et al., 1992*a;* Somasundaram et al., 1997; *see* Fig. 3). The mean time constants obtained from 5 patches are given in the Table. There was considerable variation in the stability of channel activity from patch to patch. Some channels became quiescent within a few minutes, while others remained consistently active for tens of minutes (*see* e.g., Fig. 9). In a proportion of patches, channel activity fluctuated with large changes in open probability associated with changes in the gating behavior of the channel. Figure 3 shows stability plots for one channel which showed such changes in P_o attributable to transient reductions in the mean closed time. Thus the IK_{Ca} channel appears to exhibit modal behavior similar to that seen with the BK_{Ca} channel (McManus & Magleby, 1988). Of the 1 in 10 patches possessing Ca^{2+}-activated channels, in less than 10% was the channel activity stable enough and sufficiently long lasting for pharmacological studies. This severely limited the amount of data that could be obtained.

TEA

In agreement with the observations of Grygorczyk & Schwarz (1985), the application of millimolar concentra-

Table. Time constants for the open and closed time distributions for IK_{Ca} channels recorded from inside-out patches of human erythrocytes, in the presence of 3 μM Ca^{2+}

	Mean (Range) (msec) ($n = 5$)
Open times τ_1	1.27 (0.6 − 3.7)
τ_2	18.96 (5.8 − 32.6)
Closed times τ_1	1.23 (0.1 − 4.1)
τ_2	23.12 (4.7 − 23.6)
τ_3	155.5 (42.8 − 347)

tions of TEA to the cytoplasmic face of the patch produced similar effects to those seen in BK_{Ca} channels (Yellen, 1984; Benham et al., 1985; Smart, 1987), namely a concentration-dependent reduction in the amplitude of the single channel currents (Fig. 4). The data were well fitted by the Hill equation giving a K_d of 22.6 ± 0.2 mM and a Hill coefficient of 1.0 ± 0.1 (fitted value ± SEM. $n = 8$; Fig. 4*B*). This effect of TEA was weakly voltage dependent. It is generally accepted that the current amplitude observed corresponds to the time averaged value of the current through the channel (Woodhull, 1973; Yellen, 1984) which is given by:

$$i(0)(V)\left[1+\frac{B}{K_0\, exp\left(\frac{F\delta\cdot V}{RT}\right)}\right]^{-1}$$

where $i_o(V)$ is the current through the unblocked channel at membrane potential (V), K_o is the equilibrium constant at 0 mV, B is the blocker concentration, δ is the fraction of the membrane voltage sensed at the binding site (or the effective valence for a monovalent blocker). From the graph of $\ln(I_o/I - 1)$ *vs.* membrane potential (Fig. 4*C*) values for δ of 0.18 and K_d at 0 mV of 20.5 mM were obtained. In addition, TEA produced a small reduction in P_o. However, this amounted to only a 30% decrease at 60 mM, a concentration which greatly attenuated the amplitude of the currents, so this action of TEA was not investigated further.

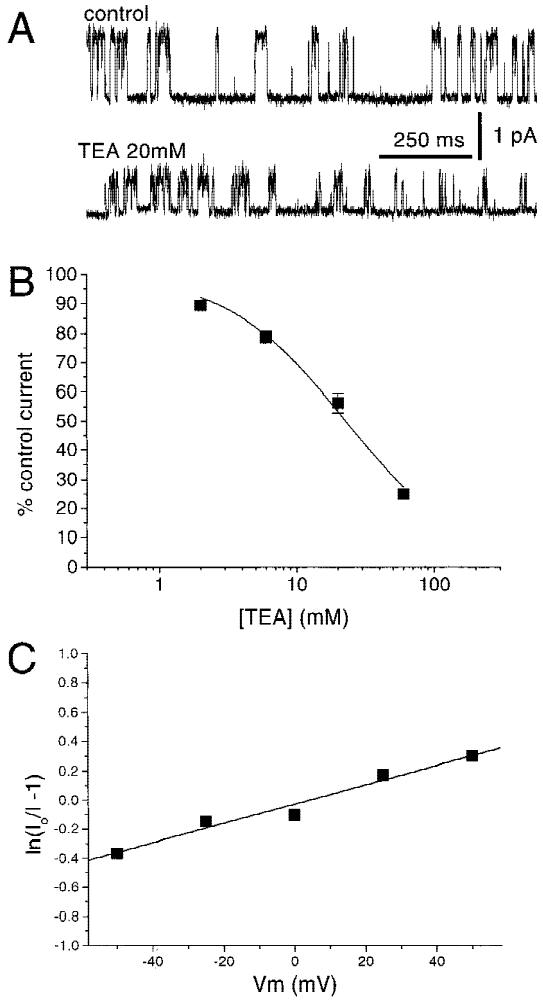

Fig. 4. Effect of internal TEA on IK_{Ca} channels. (*A*) Single channel activity in the presence of 3 μM Ca^{2+} in the absence and presence of 20 mM. TEA, which produced a rapid and reversible reduction in the amplitude of the single channel currents. (*B*) Concentration response curve for the reduction in current amplitude by TEA at 0 mV. Points represent the mean and SEM from 5 patches. The line has been drawn according to the Hill equation with a value for K_d of 22.6 mM and a Hill coefficient of 1. (*C*) Voltage dependence of the action of TEA. This was examined by plotting ln $(I_o/I - 1)$ *vs.* membrane potential, where I_o and I are the apparent single channel current amplitudes in the absence and presence of TEA respectively. From this the K_d at 0 mV was found to be 20.5 mM, and δ, the fraction of the membrane potential sensed by the drug at its binding site within the channel, was 0.18.

Barium

Barium has been shown to block the Ca^{2+}-activated K^+ channel in erythrocytes (Grygorczyk & Schwarz, 1985). These authors applied a single high concentration of Ba^{2+} simultaneously to both sides of the membrane. In the present study, addition of barium at a total concentration of 20 μM to the solution bathing the cytoplasmic side of the patch produced a dramatic reduction in P_o (Fig. 5*A*). In the presence of 10 mM citrate, the concentration of free Ba^{2+} in the solution was calculated to be only 2.4 μM. The effect of Ba^{2+} was similar to the ''slow block'' described for the BK_{Ca} channel (Vergara & Latorre, 1983), with bursts of channel activity separated by longer closures. The effect of Ba^{2+} was greatly reduced at negative membrane potentials (Fig. 5*B*), with longer bursts of channel activity, although there was little change in the duration of the long closures. Because only a limited range of Ba^{2+} concentrations was examined, it was not possible to estimate K_d values at different membrane potentials from concentration-response curves. Instead, the mean burst and inter-burst durations were determined as described by Sigurdson et al., (1987)

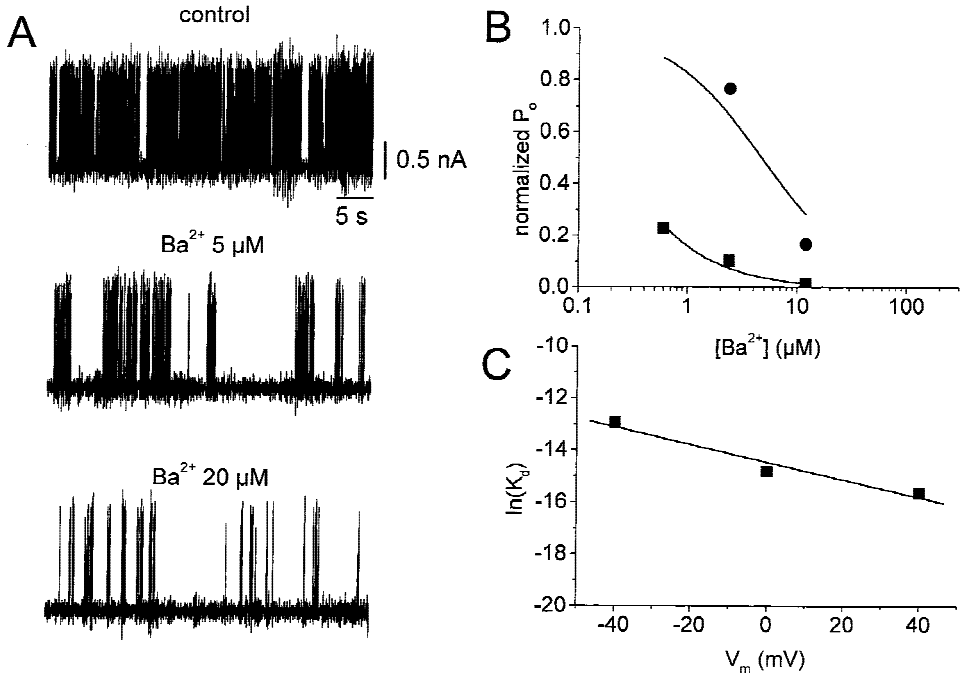

Fig. 5. Blockade of the IK_{Ca} channel by Ba^{2+}. (*A*) Effect of Ba^{2+} on single-channel activity evoked by 3 μM Ca^{2+}. Addition of 5 μM Ba^{2+} (free concentration 0.6 μM) to the solution bathing the cytoplasmic face of the membrane caused a reduction in open probability, with the channel activity being broken up into bursts. Increasing the Ba^{2+} concentration to 20 μM (2.4 μM free) reduced the duration of the bursts (*B*) Concentration dependence of the reduction of P_o by Ba^{2+} at two membrane potentials (0 mV ■; −40 mV, ●), for the patch shown in *A*. The data were fitted simultaneously with the Hill equation (solid lines). P_o was normalized with respect to the open probability in the absence of Ba^{2+}. Hyperpolarization from 0 to −40 mV dramatically reduced the blocking action of Ba^{2+}. (*C*) Quantification of the voltage sensitivity of the block by internal Ba^{2+} for the same patch. Values for the K_d for Ba^{2+} at 3 different membrane potentials were determined from analysis of the burst and interburst durations. The plot of $\ln(K_d)$ *vs.* membrane potential yielded a value for δ, the fraction of the membrane potential sensed by Ba^{2+} at its binding site in the channel, of 0.44 and a K_d at 0 mV of 0.15 μM. Similar voltage and concentration dependence were observed in 3 other patches.

and used along with P_o, in the absence of Ba^{2+}, to determine k_{+1} and k_{-1} for the blocking reaction, and thus K_d values.

$$K_d = \frac{P_o K_{-1}}{K_{+1}} \text{ where } K_{-1} = \frac{1}{\tau_{interburst}} \text{ and } K_{+1} = \frac{1}{[Ba^{2+}]\tau_{burst}}$$

The model of Woodhull, (1973) was again used to analyze the voltage sensitivity of the blocking action of Ba^{2+}. From a graph of $\ln(K_d)$ against membrane potential a K_d at 0 mV of 0.15 μM and a value for δ of 0.44 were obtained (Fig. 5*C*).

Clotrimazole

Application of 1 μM clotrimazole to the cytoplasmic face of the patch in the presence of 3 μM Ca^{2+} produced a dramatic change in channel activity, with the appearance of long closed periods interspersed with intervals of normal channel activity (Fig. 6). This effect reversed rapidly on removing clotrimazole. The action of clotrimazole is unlikely to result from competition for the Ca^{2+} binding site since it is very different from the effect of reducing the Ca^{2+} concentration (*see* Fig. 1*B*), when openings occur with a relatively uniform, albeit lower, frequency. Dwell time distributions (for example *see* Fig. 6.*B* and *C*) for the bursts of ''normal'' channel activity and the long closed (inter-burst) intervals in the presence of clotrimazole could be fitted with single exponentials with time constants of 2.8 ± 1.3 and 9.3 ± 3.9 s (n = 5), respectively.

Though these results clearly show that clotrimazole applied to the inner face of the membrane causes ''slow'' channel block (*see* e.g., Hille, 1992), the magnitude of the time constants made further analysis difficult. Many minutes of recording were needed because of the long dwell times in the burst and inter-burst states and very few patches possessed single channel activity of the required stability. Five such patches provided the following information. i. As shown in Fig. 7*A* and *B*, raising the clotrimazole concentration from 0.1 to 1 μM reduced the burst duration without changing the inter-burst intervals. ii. Although clotrimazole is a very weak base ap-

proximately 25% will be protonated at neutral pH. However there was no consistent change in P_o on stepping the membrane potential from −40 to 40 mV in the presence of clotrimazole (1 μM) in the two patches tested. Unfortunately, both patches contained two channels, so that burst and inter-burst dwell times could not be analyzed. iii. In a single patch, reducing P_o by lowering $[Ca^{2+}]_i$ from 1 to 0.2 μM in the presence of clotrimazole (1 μM) increased the duration of the inter-burst intervals, while having little effect on the duration of the bursts (Fig. 7*C*). However much more data will be required to determine the mechanism of the channel block by clotrimazole.

Blocking Action of a Quaternary Analogue of Clotrimazole

To determine whether the action of clotrimazole was due to an action at the cytoplasmic face of the channel, or resulted from the penetration of the drug into or through the membrane, a quaternary derivative of clotrimazole (UCL 1559) was also tested. This compound again produced a 'slow block' similar to that observed in the presence of clotrimazole (Fig. 8). The burst and inter-burst intervals in the presence of 1 μM UCL 1559 appeared to be exponentially distributed with time constants of 47 ± 22 sec and 35 ± 14 sec ($n = 5$), respectively.

Cetiedil

In preliminary experiments the application of cetiedil (100 μM) to the cytoplasmic face of the patch in the presence of 3 μM Ca^{2+} for periods of 1 to 3 min gave inconsistent results. In some instances there was little or no change in channel activity, while other patches became quiescent, and remained so for several minutes. Subsequent experiments in which cetiedil was applied for longer periods resolved the discrepancy. Figure 9 shows the effect of 100 μM cetiedil on P_o measured over 30-sec intervals during the course of a 20 min recording. Like clotrimazole, cetiedil also appears to produce a 'slow block,' but with exceptionally slow kinetics, so that there are very long silent periods lasting several minutes separated by bursts of apparently normal channel activity of similar duration. However, even during these bursts, P_o was somewhat lower than the control value. Examination of the open and closed time distributions (*not shown*) revealed that this resulted from a decrease in the mean open time. Because this effect was relatively small (<20%), and only apparent at such a high concentration of cetiedil it was not investigated further. Analysis of the burst and inter-burst distributions was made difficult by their long durations, and consequently the need for very long recordings, from patches with very consistent channel activity. For those patches in which long recordings were obtained, the burst and inter-burst intervals appeared to be exponentially distributed with time constants of 64 ± 18 and 93 ± 36 sec ($n = 6$), respectively.

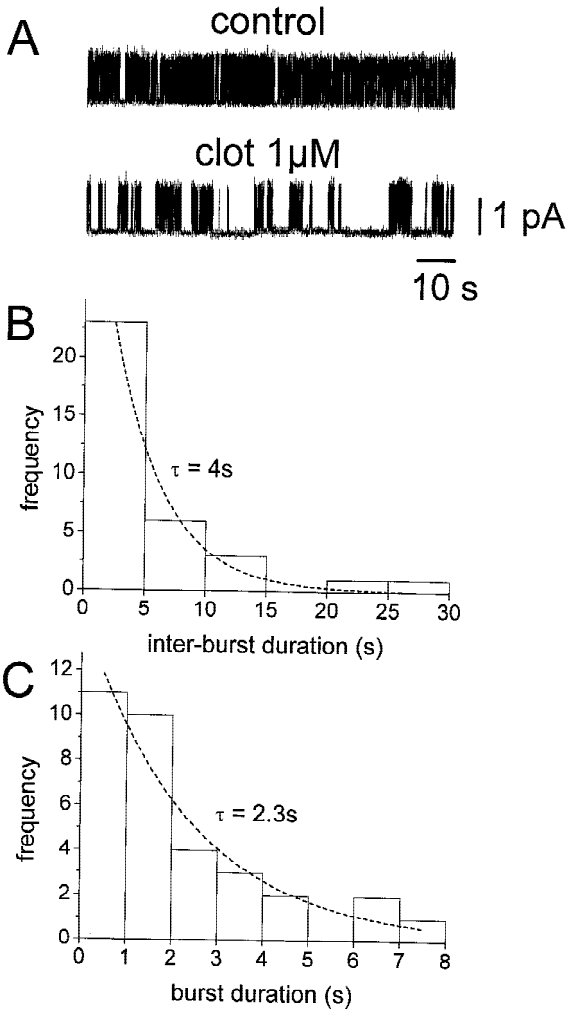

Fig. 6. Blockade of IK_{Ca} channels by clotrimazole applied to the inner face of the membrane (*A*) Single channel activity recorded in the presence of 3 μM Ca^{2+} in the absence and presence of 1 μM clotrimazole. In the presence of clotrimazole, the channel activity was broken up into bursts. Dwell time histograms for the inter-burst (*B*), burst durations (*C*) for this patch in the presence of clotrimazole were well fitted by single exponentials with time constants of 4 and 2.3 sec, respectively.

Discussion

Action of TEA and Ba^{2+}

In this study, the actions of two widely studied K^+ channel blockers were examined at the internal face of the Ca^{2+}-activated K^+ channel from human erythrocytes. TEA produced very fast block, unresolved by the recording technique and giving rise to an apparent reduction in

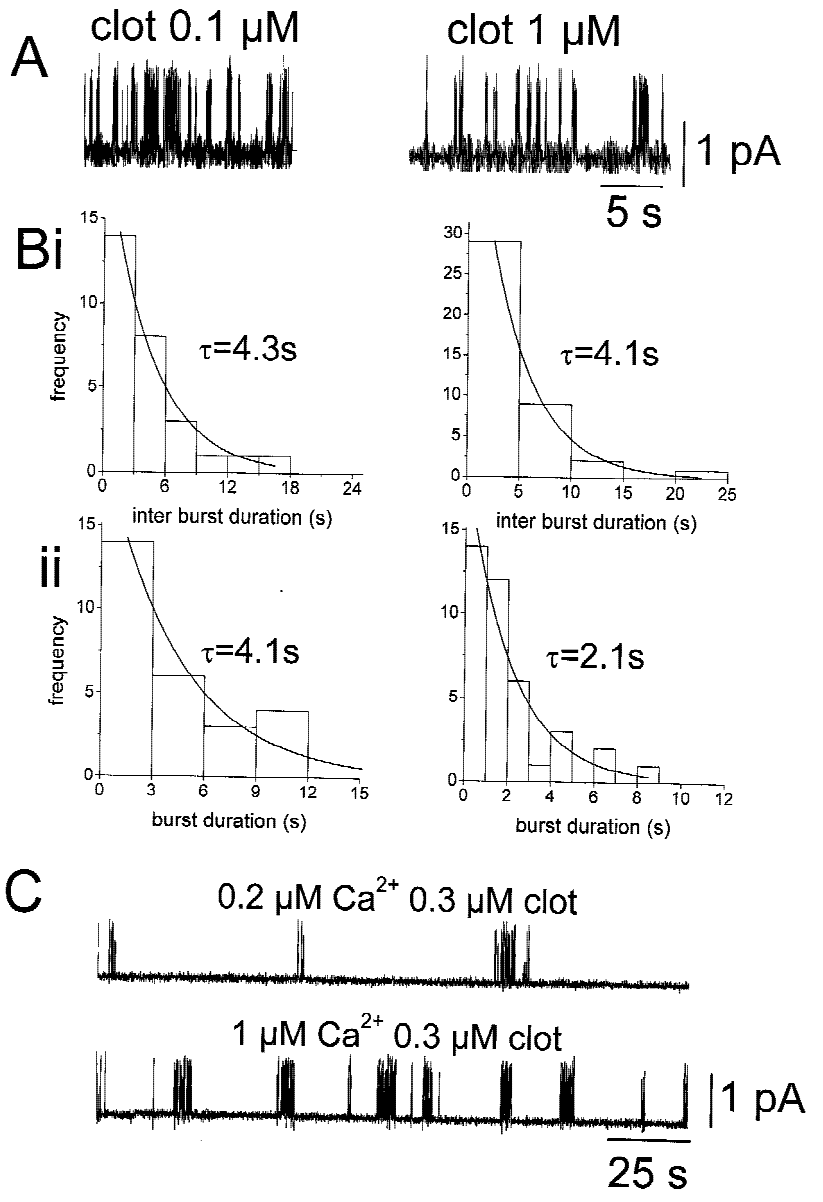

Fig. 7. Dependence of the blocking action on the clotrimazole and Ca^{2+} concentration (*A*) Records from a single channel activated by 3 μM Ca^{2+} from one patch in the presence of 0.1 and then 1 μM clotrimazole. Dwell time distributions for the burst and inter-burst intervals are shown in *Bi* and *ii*. While the time constant for the inter-burst intervals remained constant at approximately 4 sec, the mean burst duration decreased from 4.6 to 2.1 sec on increasing the clotrimazole concentration. (*C*) Single-channel activity recorded from another patch in the presence of 0.2 μM Ca^{2+} and 0.3 μM clotrimazole and then in the presence of 0.3 μM clotrimazole but on raising the Ca^{2+} concentration to 1 μM. Note the dramatic reduction in the length of the interburst intervals.

the unitary current amplitude. This is similar to its action at BK_{Ca} channels (Yellen, 1984; Benham et al., 1985; Smart, 1987). The weak voltage dependence for this action suggests that TEA is binding at a site close to the inner mouth of the channel. The existence of a second slower blocking action is reminiscent of the action of TEA on K_{ATP} (Davies et al., 1989). In contrast, Ba^{2+} produced a slow block having much greater voltage sensitivity, with a δ value of 0.44, indicating a binding site approximately half way through the membrane. Although some competition of Ba^{2+} for the Ca^{2+} binding site cannot be excluded, the voltage sensitivity would suggest that the predominant effect is the result of channel block. The affinity of the IK_{Ca} channel for Ba^{2+} is considerably higher than that reported for the BK_{Ca} channel (K_d = 36 μM; Vergara & Latorre, 1983), but is similar to the value of 0.1 μM reported for the dealyed rectifier of the squid giant axon (Eaton & Brodwick, 1980), and makes this K^+ channel one of the most sensitive to internally applied Ba^{2+}.

Clotrimazole and Cetiedil

Previous work on analogues of cetiedil as blockers of the IK_{Ca} channel in intact erythrocytes found a strong correlation between potency and lipophilicity (Benton et al., 1994; Benton, 1995). One interpretation of this finding is that these compounds penetrate the membrane and act at the cytoplasmic face of the channel. This hypothesis

has now been directly tested. Cetiedil at 100 μM produced relatively little block of the channel when applied to inside-out patches, yet this concentration would abolish Ca^{2+}-activated K^+ efflux from intact cells when applied externally (Benton et al., 1994; Benton, 1995). This suggests that inhibition of the Gardos channel by cetiedil is likely to be due to an action either at an extracellular site on the IK_{Ca} channel, or within the channel though at a site not accessible from its inner face.

In contrast, clotrimazole was able to block the channel at the cytoplasmic face at concentrations comparable to those required to inhibit K^+ efflux in intact cells (Alvarez, Garcia-Sancho & Herreros, 1992; Brugnara et al., 1993). Furthermore, the IC_{50} for the quaternary compound UCL 1559 when applied externally to intact cells is 16 μM (Benton, 1995), making it at least 10 times less potent than when applied to the inner surface of the membrane. These results suggest that clotrimazole analogues may be able to act at both an internal and an external site on the channel. Whether enough clotrimazole enters the cell when applied externally to contribute to the overall block remains to be determined.

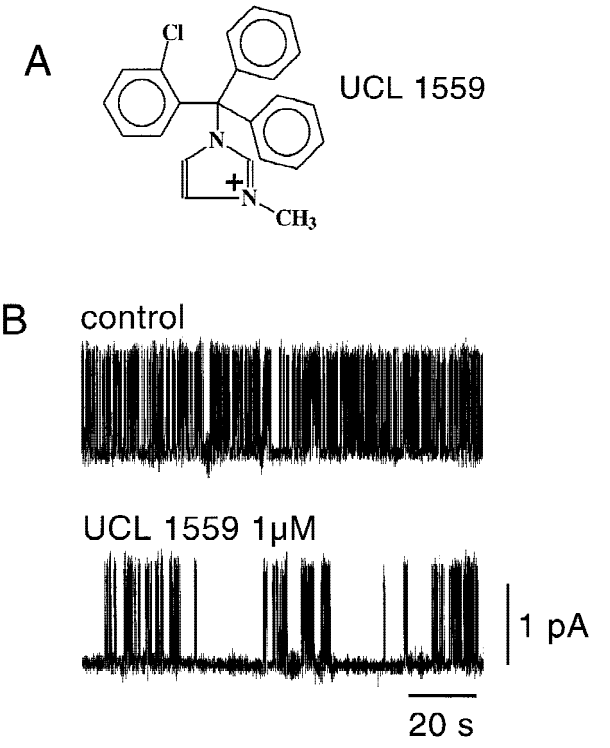

Fig. 8. Effect of internal application of a quaternary analogue of clotrimazole (UCL 1559). (*A*) Chemical structure of UCL 1559 (*B*) Single channel activity recorded in the same patch in response to 3 μM Ca^{2+} in the absence and presence of 1 μM UCL 1559. In the presence of this compound, channel activity was broken up into bursts, as seen with clotrimazole itself, while the amplitude of the single channel current remained unchanged.

Mechanism of Clotrimazole Action

It has been suggested that the activity of the IK_{Ca} channel requires the presence of some cytoplasmic factor (Moore et al., 1991). However, the ability to record single channel activity in excised membrane patches for many minutes would suggest that this requirement is not absolute. The activity of this channel may also be dependent upon its redox state (Garcia-Sancho, Sanchez & Herreros 1979; Alvarez et al., 1984), though treatment of inside out patches with the oxidising agent 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) or the electron donor system ascorbate + phenazine methosulphate was not found to modify channel activity (P.M. Dunn, *unpublished observations*).

Nevertheless, the processes regulating this channel in the intact cell may be complex, and the behavior of a channel in an excised patch may be dependent on the state of the channel immediately prior to patch isolation. Clotrimazole is known to inhibit cytochrome P450, and it has been suggested that its inhibition of K^+ efflux from erythrocytes is secondary to this action (Alvarez et al., 1992). However, the results obtained in isolated membrane patches indicate another mode of action, as do studies with clotrimazole analogues that do not inhibit cytochrome P450 but are still effective blockers of IK_{Ca} (Brugnara et al., 1993).

Many drugs prevent the flux of ions through channels by binding to a site within the pore and physically occluding it. In such cases, the block is usually voltage dependent, as has been observed in this study for the action of Ba^{2+} and to a lesser extent TEA. In contrast, larger blocking molecules such as charybdotoxin bind at a superficial site in the channel mouth and show no voltage sensitivity (MacKinnon & Miller, 1988). The observed lack of voltage dependence in the action of internally applied clotrimazole thus suggests either that it does not bind within the pore of the channel, or that it only does so at a site very close to the inner surface.

Attempts at a detailed kinetic analysis of the action of clotrimazole were thwarted by the slow kinetics of action and the rarity of channels with a stable level of activity. While the data obtained may indicate that clotrimazole does not distinguish between open and closed states of the channel, confirmation of this remains for further study.

Recently, the action of clotrimazole on the IK_{Ca} channels in outside out patches from mouse erythroleukemia cells has been described (Rittenhouse et al., 1997*b*). It was found that nanomolar concentrations of clotrimazole applied to the external surface of the patch reduced P_o, though a kinetic analysis has yet to be attempted. Externally applied clotrimazole has also been shown to block BK_{Ca} channels from portal vein smooth

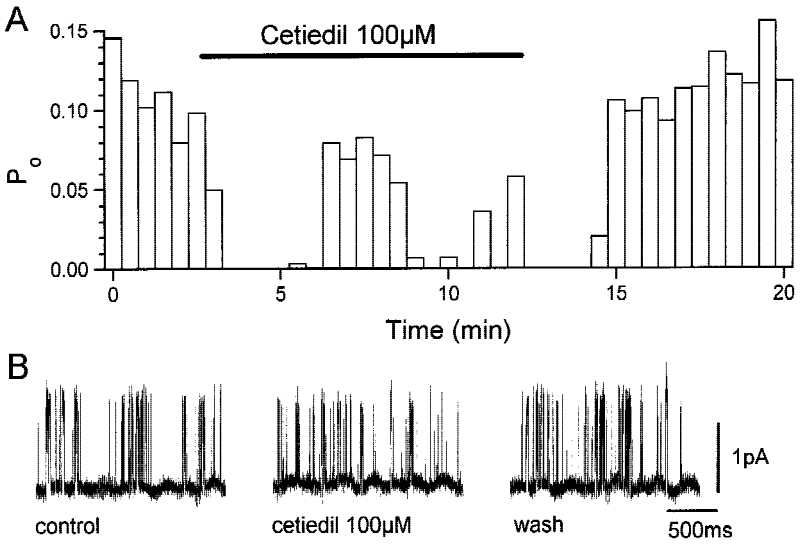

Fig. 9. Action of internal cetiedil on IK_{Ca} channels. (*A*) Record of channel open probability measured over 30 sec intervals for a 20-min recording, during which 100 μM cetiedil was applied for 10 min. (*B*) Sections of channel activity recorded before, during and after washout of cetiedil. In the presence of cetiedil, there were prolonged silent periods interspersed with long spells of apparently normal channel activity.

muscle (Rittenhouse et al., 1997*a*) and carotid body cells (Hatton & Peers, 1996) where a fast open channel blocking mechanism appears to be implicated.

In conclusion, several well established inhibitors of the Gardos channel in red cells have been examined for their action when applied to the inner surface of the membrane. The findings with cetiedil suggest that its effect on the intact cell is either at, or requires access from, the extracellular surface. In contrast, internally applied clotrimazole is a potent blocker, though whether this action contributes the inhibitory action of externally applied clotrimazole remains to be determined.

I am grateful to the Wellcome Trust for financial support, to Dr. D.H. Jenkinson for encouragement and advice throughout this work, and to Dr. A.J. Gibb and Dr. D.G. Haylett for many helpful discussions.

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