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Properties of the ATP-sensitive K^+ Current Activated by Levcromakalim in Isolated Pulmonary Arterial Myocytes

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Abstract. Tension and patch clamp recording techniques were used to investigate the relaxation of rabbit pulmonary artery and the properties of the K^+ current activated by levcromakalim in isolated myocytes. Under whole-cell voltage clamp, holding at −60 mV in symmetrical 139 mM K^+, levcromakalim (10 μM) induced a noisy inward current of -116 ± 19 pA ($n = 13$) which developed over 1 to 2 min. This current could be blocked by either glibenclamide (10 μM) or phencyclidine (5–50 μM) and was unaffected when extracellular Ca^{2+} was removed. Both these drugs inhibited the levcromakalim-induced relaxation of muscle strips precontracted with 20 mM $[K^+]_o$. Application of voltage ramps in symmetrical 139 mM K^+ confirmed that the levcromakalim-induced current was carried by K^+ ions and was weakly voltage dependent over the potential range from −100 to +40 mV.

The unitary current amplitude and density of the channels underlying the levcromakalim-activated whole-cell K^+ current was estimated from the noise in the current record. We estimate that levcromakalim caused activation of around 300 channels per cell, with a single channel current of 1.1 pA, corresponding to a slope conductance of about 19 pS. Furthermore, cells dialyzed with an ATP-free pipette solution developed a large noisy inward current at −60 mV, which could subsequently be blocked by flash photolysis of caged ATP. Analysis of the noise associated with this current indicated that the single channel amplitude underlying the ATP-blocked current was 1.4 pA, a value similar to that estimated for the levcromakalim-induced current. We conclude that the conductance of this ATP-sensitive channel is likely to be small under physiological conditions and that it is present at low density.

Key words: Potassium channels — Whole-cell recording — BRL 38227 — Pulmonary artery — K_{ATP} channels — Noise analysis

Introduction

Cromakalim and its more active enantiomer, levcromakalim (BRL 38227) hyperpolarize and relax a wide variety of mammalian smooth muscles by a mechanism that appears to rely heavily on an increase in K^+ conductance (Cook & Quast, 1990; Standen et al., 1989; Longman & Hamilton, 1992). These and other chemically diverse vasodilator compounds, commonly referred to as K^+ channel openers (KCOs), are known to activate K^+ channels in a number of excitable tissues including cardiac muscle, skeletal muscle and pancreatic β cells (*for review, see* Ashcroft & Ashcroft, 1990; Longman & Hamilton, 1992). Their effects appear to be primarily on a type of K^+ channel inhibited by intracellular ATP and blocked by the sulfonylurea, glibenclamide. These and other observations formed the basis of the hypothesis that ATP-sensitive K^+ (K_{ATP}) channels are responsible for the hyperpolarization and vasodilation produced by KCOs in vascular smooth muscle (Quast & Cook, 1989; Standen et al., 1989).

A number of electrophysiological studies on isolated smooth cells have described the effects of KCOs both on single K^+ channels and whole-cell K^+ currents. Single channel experiments have indicated that KCOs can activate several K^+ channels. These include K_{ATP} channels with a large conductance (~130–140 pS at 0 mV in a 60 mM $[K^+]_o$/120 mM $[K^+]_i$ gradient;

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Standen et al., 1989; Kovacs & Nelson, 1991) or small conductance (10–30 pS, close to 0 mV in a quasi-physiological K^+ gradient; Kajioka, Kitamura & Kuryiama, 1991; Kajioka, Oike & Kitamura, 1990; Miyoshi et al., 1992; Beech et al., 1993) as well as large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}; Gelband, Lodge & van Breemen, 1989; Klöckner, Trieschmann & Isenberg, 1989). Furthermore, K_{ATP} channels in rat portal vein (Kajioka et al., 1990) or pig coronary artery (Miyoshi et al., 1992) were reported to be Ca^{2+} sensitive, while in rabbit portal vein, the channel was reported to be Ca^{2+} insensitive but required GDP for the action of pinacidil (Kajioka et al., 1991). However, there seems to be broad agreement from whole-cell, voltage-clamp data that cromakalim (or levcromakalim) activates a glibenclamide-sensitive, time-independent background K^+ current that is relatively insensitive to tetraethylammonium ions (TEA^+; Beech & Bolton, 1989; Clapp & Gurney, 1992, 1993; Noack et al., 1992*a;* Russell, Smirnov & Aaronson, 1992; Silberberg & van Breemen, 1992).

The apparent diversity of the K^+ channels targeted by KCOs in different vascular smooth muscle preparations may reflect either a real heterogeneity between K^+ channels targeted by these agents in different vascular tissues or that KCOs can activate more than one K^+ channel in the same preparation. In pulmonary arterial myocytes it has recently been shown that levcromakalim activates an ATP-sensitive, background K^+ current which contributes to the resting potential of pulmonary arterial cells and shares a similar pharmacology to muscle relaxation induced by levcromakalim in the whole tissue (Clapp, Davey & Gurney, 1993; Clapp & Gurney, 1993). The present paper further characterizes the mechanism by which levcromakalim relaxes rabbit pulmonary artery muscle. In particular, by analysis of the whole-cell current noise elicited by levcromakalim and blocked by inhibitors of the levcromakalim-induced relaxation, we have estimated the unitary current amplitude underlying the response. We suggest that the conductance of this channel is likely to be small under physiological conditions and that channels are present at quite low density. Preliminary reports of some of these data have been presented (Clapp & Gurney, 1993; Langton et al., 1993*a,b*).

Materials and Methods

Preparation

Male New Zealand rabbits (2–2.5 kg) were killed by i.v. injection of sodium pentobarbitone (60 mg/kg; May & Baker, Dagenham, Essex) and exsanguinated. The main pulmonary artery was removed and placed in physiological salt solution (PSS) with the following composition (in mM): 112 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 15 $NaHCO_3$, 0.5 KH_2PO_4, 0.5 NaH_2PO_4, 10 glucose, 5 *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES), 0.03 phenol red (pH 7.4 with 95% O_2/5% CO_2). The connective tissue was then carefully removed and the cleaned vessel opened along its longitudinal axis. Strips of muscle (between 1 and 1.5 mm wide and 10 mm long) were cut transversely and used either for tension or isolated cell experiments.

Tension Experiments

Muscle strips were mounted in a small chamber (~0.3 ml), with one end fixed and the other end attached to an isometric transducer (Harvard Apparatus) connected to a chart recorder (Labdata Instrument Services). No attempt was made to retain or remove the endothelium. A basal tension of 1 g was applied to the strip, which was continuously perfused at 1.2 ml/min with PSS at 36°C. A 45 min equilibration period was allowed before the addition of any drugs. Muscle strips were precontracted by raising the external K^+ concentration to 20 mM by equimolar replacement of NaCl for KCl in the PSS. Phentolamine (1 μM) was included to block the effects of noradrenaline released from surviving nerve endings. The effects of levcromakalim were measured as the percent inhibition of the total contraction existing before its application.

Whole-Cell Recording Methods

Vascular myocytes were isolated as previously described (Clapp & Gurney, 1991). Briefly, strips of muscle were stored in the refrigerator overnight in a low Ca^{2+} (160 μM) medium containing around 0.27 mg ml^{-1} papain (type IV, Sigma) and 0.02% bovine serum albumin (fraction V, fatty acid free, Sigma). Single cells were isolated the next day by warming up the tissue in the enzyme solution to 36°C for 10 min in the presence of 0.2 mM dithiothreitol (Sigma). Afterwards, the tissue was removed and put in 5 ml of the low Ca^{2+} medium. Cells, released from the tissue by trituration, were stored at 4°C and used within 12 hr.

Cells were allowed to settle on the bottom of a small chamber before being perfused at a rate of 0.5 ml/min with either standard PSS or one containing (mM): 139 KCl, 1 $MgCl_2$, 5 glucose, 10 HEPES (pH 7.4 with NaOH). In some experiments, the high K^+ solution also contained 1.8 mM Ca, with no added glucose. Recording pipettes, unless otherwise stated contained (mM): 130 KCl, 1 EGTA, 1 $MgCl_2$, 0.5 guanosine triphosphate-Na_3 (GTP, Sigma), 1 adenosine triphosphate-Na_2 (ATP, Sigma), 20 HEPES (pH 7.2 with KOH).Under these conditions, the free intracellular Ca^{2+} concentration is estimated to be <15 nM (Fabiato, 1991). A similar solution was used for flash photolysis experiments, except that ATP was omitted from the pipette and it also contained 20 mM 4,5-dimethoxy-1(2-nitrophenyl) ethyl ATP (caged ATP; Molecular Probes). Light flashes, delivered from a Xenon flash lamp were directed through the microscope objective and focused onto the tip of the recording pipette. Using HPLC methods described in Clapp and Gurney (1992), the amount of ATP liberated from caged ATP by a single flash was estimated to be at least 0.5 mM.

Standard patch clamp techniques were used to record whole-cell membrane currents under voltage clamp using either an Axopatch-1A or 200 amplifier (Axon Instruments, Foster City, CA), or a List-electronic EPC-7 amplifier. Patch pipettes, pulled from 1.5 mm OD borosilicate capillaries (Clark Electromedical, Pangbourne, UK) using a Narashige puller, were fire-polished and coated with beeswax. These electrodes had resistances of 4–8 MΩ when filled with electrolyte. The junction potential between the electrode and the bath solution was subtracted using the DC offset on the amplifier. Series resistance was calculated from the decay time constant of capacity

transients, recorded in response to 10 mV hyperpolarizing steps. All experiments were performed at room temperature (21–25°C).

Data Collection and Noise Analysis

For on-line data collection, currents were filtered at 2 kHz, digitized at 1–10 kHz with a Labmaster TM-40 or TL-125 interface (Scientific Solutions), and stored on a PC-clone computer for analysis using pCLAMP software (version 5.5, Axon Instruments, Foster City, CA). For off-line analysis, data were stored on either FM tape (Racal 4500) or on video tape using a pulse code modulator (Sony). For display purposes, current traces were played back onto a Gould chart recorder (filtered at 100 Hz).

Estimates for i, the single channel amplitude, and N, the number of channels, were made from measurements of mean current, $\bar{I}$, and the current variance, σ_I^2. The variance associated with the open/closed transitions of a homogeneous population of N single channels is given by (e.g., Sigworth, 1980):

$$\sigma_I^2 = Ni^2p - Ni^2p^2 \quad (1)$$

where p is the open probability. Since $\bar{I}$ is given by Nip the above expression for current variance can be simplified to:

$$\sigma_I^2 = i\bar{I} - \frac{\bar{I}^2}{N} \quad (2)$$

Analysis of current noise was done either with a PDP 11/73 computer, using a program (Noise) provided by Prof. D. Colquhoun to calculate variance directly, or with a PC-clone computer, using pCLAMP to form histograms of current amplitude. In the first case, currents were replayed, low-pass filtered (8-pole Butterworth, −3 dB at 512 Hz), digitized at 1,024 Hz with a CED 502 A-D converter and stored on computer. Occasionally, the current signal was also high-pass filtered at 5 Hz to remove any DC changes in the current record. Records were 200–400 sec in duration and contained either increases in current in the presence of levcromakalim or decreases in current following application of glibenclamide or phencyclidine. These records were divided into subsets 2 to 4 sec in duration. Values for mean current and the variance about the mean were determined for between 10 and 20 selected subsets. Using pCLAMP, currents were filtered (8-pole Bessel, −3 dB at 2 kHz), digitized at 5–10 kHz (Labmaster TM-40 or TL-125), and stored in files containing 2–3 sec of record. Mean current and current variance were obtained for each file by fitting Gaussian functions to all-points amplitude histograms to give values for $\bar{I}$ and SD (σ_I^2 calculated as SD^2). With either method, estimates of i and N were obtained by fitting values of $\bar{I}$ and σ_I^2 to Eq. (2) using a least-squares routine within the KaleidaGraph graphics package (Synergy Software, Reading, PA) or the Sigmaplot graphics software (version 5.1, Jandel Scientific, Corte Madera, CA).

Drugs

Glibenclamide and phencyclidine (PCP) were purchased from Sigma Chemical and phentolamine mesylate from Ciba. Levcromakalim (BRL 38227, the active enantiomer of cromakalim) was a gift from SmithKline & Beecham, Harlow, Essex and charybdotoxin was a gift from ICI Pharmaceuticals. Levcromakalim and glibenclamide were normally prepared as 10 mM stocks in a 50:50 mixture of dimethylsulfoxide (DMSO; Sigma) and polyethylene glycol (MW 200) and diluted in bath solution on the day of the experiment. For some experiments, the levcromakalim stock solution was simply made up in DMSO. The final concentration of the vehicle (up to 0.3%) had no effect on tension. For single cell experiments, drugs were applied either by pressure ejection from a micropipette or from a flow pipe system that allowed the cell to be superfused (Langton, 1993). In the former, the micropipette was placed >100 μm from cells, where control application of DMSO (0.05–0.2%) or bath solution had no significant effect on membrane potential or current. With this method, drug concentrations refer to those in the pipette, although some dilution is likely to have occurred.

Results in the text are expressed as mean ± SEM (no. of observations). Statistical analysis was performed using Student's unpaired or paired t-test.

Results

Effects of Phencyclidine on Levcromakalim-induced Relaxations

Several studies in systemic blood vessels have used the effects of K^+ channel blockers on KCO-induced vasorelaxation or hyperpolarization to characterize the K^+ channel underlying these effects. In rabbit pulmonary artery, Clapp et al. (1993) have shown that relaxation to levcromakalim is blocked by glibenclamide and tetrapentylammonium ions, but not by low concentrations of TEA^+ ions. The K^+ current activated by cromakalim in rabbit portal vein (Beech & Bolton, 1989) or the relaxation induced by levcromakalim in guinea-pig coronary artery (Eckman, Frankovich & Keef, 1992) are also blocked by PCP. In the present study, we have investigated the effect of PCP on both relaxations and membrane currents induced by levcromakalim in rabbit pulmonary artery.

The effect of 10 μM PCP on relaxations evoked by levcromakalim (0.3–30 μM) in strips of rabbit pulmonary artery is shown in Fig. 1. Levcromakalim (1 and 10 μm) significantly relaxed muscle strips precontracted with 20 mM K^+ in the absence (Fig. 1*A*) but not in the presence (Fig. 1*B*) of 10 μM PCP. In a series of experiments, PCP was found to block the effect of levcromakalim at all doses studied, reducing the relaxation induced by 10 μM levcromakalim from 42 ± 2% (n = 10) to 14 ± 2% (n = 10; $P < 0.01$) (Fig. 1*C*). Thus, PCP is an effective inhibitor of the relaxing effects of levcromakalim in the pulmonary artery.

Whole-Cell Recording of Steady-State Current

It has recently been shown in pulmonary arterial myocytes that levcromakalim activates a glibenclamide-sensitive, time-independent background K^+ current (Clapp et al., 1993). In this paper, we have investigated the current induced by levcromakalim in more detail, using cells bathed in symmetrical K^+ (139 mM) solutions to increase inward current, and clamped to −60 mV to minimize activation of other voltage-dependent K^+ channels. With 1.8 mM Ca in the bathing solution,

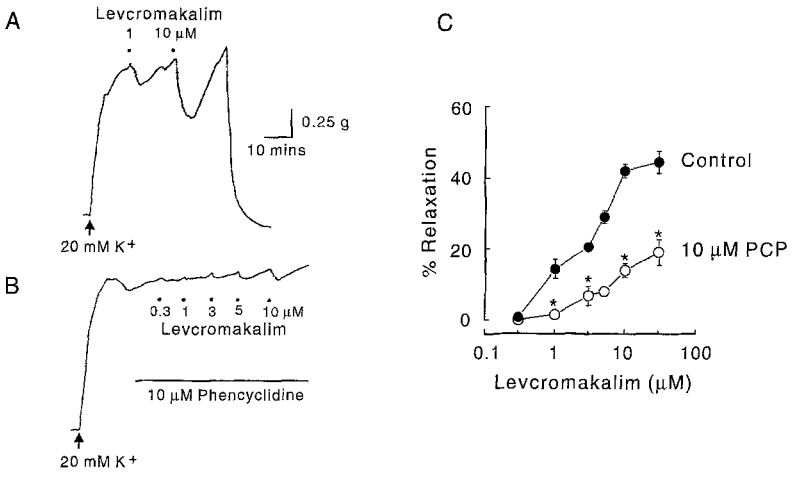

Fig. 1. Phencyclidine blocks relaxations evoked by levcromakalim in rabbit pulmonary artery. Strips were precontracted by elevating extracellular K^+ to 20 mM and levcromakalim was applied for 30 sec before (*A*) and in the presence of 10 μM PCP (*B*). Records in *A* and *B* are from the same strip of muscle. (*C*) Concentration-response curves for levcromakalim in the absence (Control; ○) and in the presence of 10 μM phencyclidine (○). Results are expressed as the percent relaxation of the total contraction existing before the application of levcromakalim. Each point represents the mean ± SEM, n = 4–10, with * = $P < 0.01$.

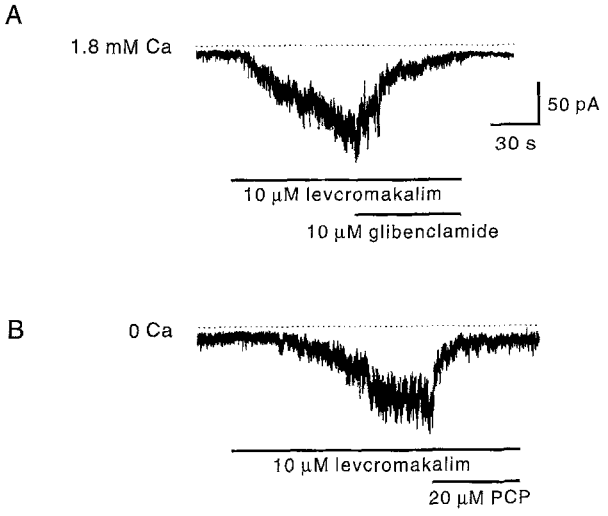

Fig. 2. The effect of levcromakalim on whole-cell current recorded with 1 mM ATP in the pipette, in the presence (*A*) and absence (*B*) of 1.8 mM extracellular Ca. The levcromakalim-induced current was subsequently blocked by the addition of glibenclamide (*A*) or PCP (*B*). In each case, the internal and external solutions contained 139 mM K^+, and the cell was voltage-clamped at −60 mV. The dashed line represents the zero current level. For display purposes, current recordings were played back onto a Gould chart recorder and filtered at 100 Hz.

and 1 mM ATP in the pipette solution, the holding current at −60 mV was found to be on average −39 ± 6 pA (n = 12). This steady-state holding current presumably represents leakage current and basal activity of K_{ATP} channels (*see later*). Upon application of levcromakalim, the current required to clamp the cells at −60 mV became increasingly negative and noisy with respect to control (Fig. 2*A*), taking over 2 min to reach a maximum. The levcromakalim-induced current reversed only slowly on washout (*not shown*), but could be rapidly blocked by glibenclamide (10 μM; Fig. 2*A*) and PCP (20 μM; Fig. 2*B*) but not by charybdotoxin (100 nM, *data not shown*). In a series of experiments, the mean current induced after superfusion with 10 μM levcromakalim was −116 ± 19 pA (n = 13). However, the magnitude of the current blocked by glibenclamide was slightly larger than this value (−127 ± 21 pA; n = 13), indicating some basal K_{ATP} channel activity, even in the presence of 1 mM $[ATP]_i$ (*see* Clapp & Gurney, 1992). The magnitude of the levcromakalim-induced current was essentially unaltered in the absence of extracellular Ca^{2+} (Fig. 2*B*). Under these conditions, the peak response to levcromakalim (10–20 μM) was −98 ± 22 pA (n = 11), which was not significantly different from the value obtained in 1.8 Ca. Levcromakalim was only used at 20 μM in experiments where it was applied by pressure ejection, where dilution of the drug almost certainly occurred. This is unlikely to have affected the present results, since both these concentrations produce near maximal effects on tension (Fig. 1*C;* and Clapp et al., 1993). Since the Ca^{2+} in the pipette solution is estimated to be <15 nM (*see* Fabiato, 1991), our results strongly suggest that the levcromakalim-activated current that we observe does not flow through Ca^{2+}-activated K^+ channels.

Voltage Dependence

To assess the voltage dependence of the current activated by levcromakalim, cells were held at −60 mV and ramped from −100 mV to +30 or +40 mV over 1.5 sec (Fig. 3). In the absence of any drug, little inward current was detected negative to 0 mV (Fig. 3A and *D*). Positive to this potential, the current-voltage (*I-V*) relation rectified in the outward direction. In some cells an additional inward current was observed, activating between −50 and −40 mV (*see* Fig. 3*D*), whose mag-

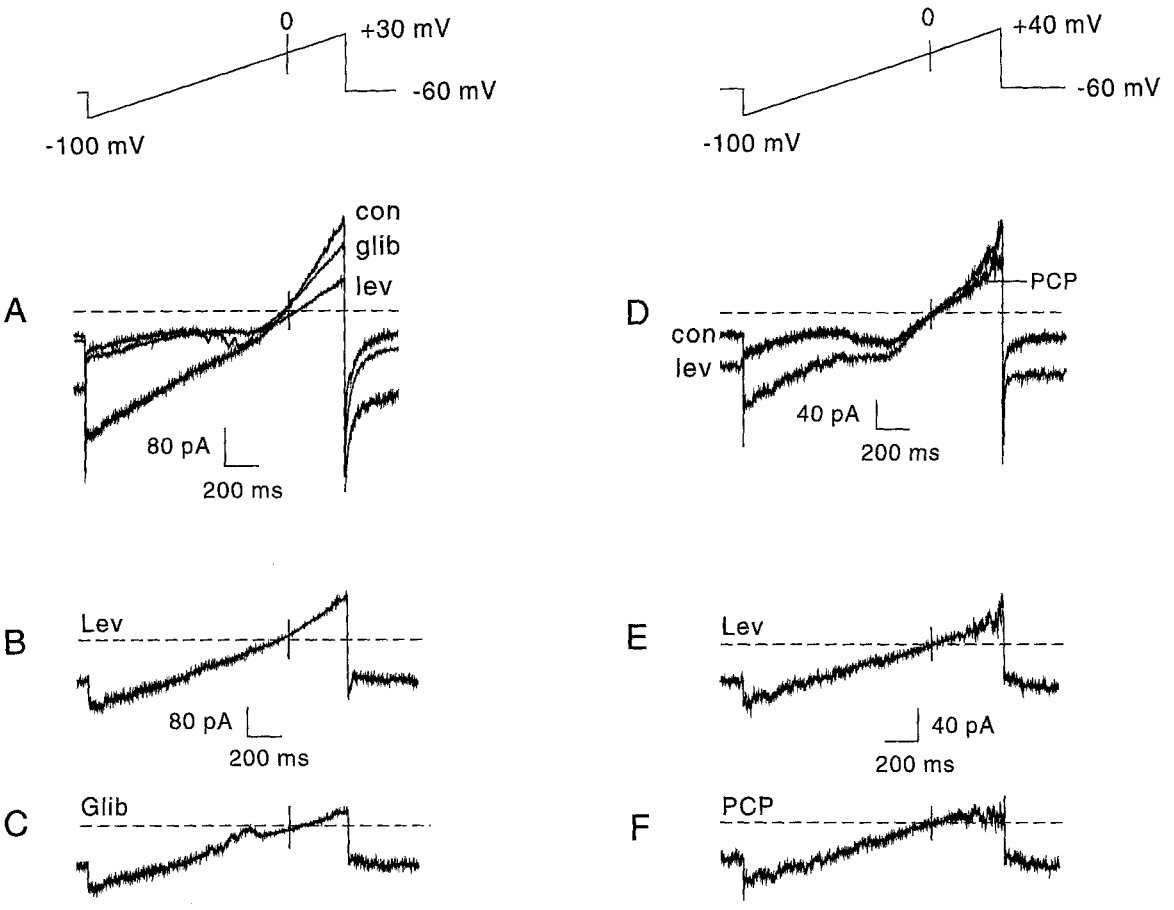

Fig. 3. Levcromakalim activates an essentially voltage-insensitive K^+ current that is blocked by PCP and glibenclamide. (*A*) Whole-cell current traces recorded in response to 1.5 sec voltage ramps from −100 to +30 mV under control conditions (*con*), in the presence of 10 μM levcromakalim (*lev*), and after the application of 10 μM glibenclamide (*glib*). The cell, which was bathed in symmetrical 140 K^+ with extracellular Ca^{2+} present, was held at −60 mV, and the ramps repeated at 4 sec intervals. Each current record represents an average of four consecutive traces. Difference currents for the levcromakalim-induced (*B*) and the glibenclamide-blocked (*C*) current were obtained by digitally subtracting the appropriate records. (*D*) current records generated in the absence of extracellular Ca^{2+} using the same protocol as described in *A* obtained under control conditions, in the presence of 20 μM levcromakalim and in the presence of levcromakalim and 50 μM PCP. (*E* and *F*) Difference currents for the levcromakalim-induced current and the PCP-blocked current, respectively.

nitude was dependent on extracellular K^+ but not extracellular Ca^{2+}. This current has not been characterized in the present study, but its properties resembled that of a delayed rectifier current recently described in this preparation (Evans, Clapp & Gurney, 1993). Following the application of 10–20 μM levcromakalim, the holding current became more negative, and significant inward current was now activated at negative voltages. The current activated by levcromakalim was obtained by subtracting the current seen in response to a voltage ramp in the absence of levcromakalim from that in its presence. The *I-V* relation of the levcromakalim-activated current showed little voltage sensitivity, being essentially linear over the voltage range from −100 to 0 mV either in the presence (Fig. 3*B*) or absence (Fig. 3*E*) of extracellular Ca^{2+}. In some (e.g., Fig. 3*B*) but not all cells, the current showed some outward rectification positive to 0 mV. It is possible that this reflects small time-dependent changes in other currents that occur during whole-cell recording rather than rectification of the levcromakalim-induced current. The reversal potential for the levcromakalim-induced current was 0.6 ± 1.1 mV (n = 11), under conditions where the equilibrium potential for K^+ (E_K) was 0 mV, consistent with the current being carried by K^+ ions.

The current activated by levcromakalim was almost completely abolished by the addition of either 10 μM glibenclamide (Fig. 3*A*) or 50 μM PCP (Fig. 3*D*). With PCP, blocking effects were observed with concentrations as low as 5 μM. The *I-V* relations for the

glibenclamide- or PCP-sensitive components of current (Figs. 3*C* and *F*) were essentially the same as those for the levcromakalim-activated current itself, suggesting that these agents all act on the same K^+ current, which shows little voltage or Ca^{2+} dependence.

Noise Analysis of the Levcromakalim-activated Current

To obtain information about the single channel currents that underlie the whole-cell current activated by levcromakalim, we have analyzed the increase in current noise that occurs at −60 mV following application of levcromakalim. Levcromakalim-induced changes in variance and mean current were obtained by subtracting values of these two parameters made in the absence of levcromakalim from those made in its presence. Data from such experiments were then fit with the hyperbolic function of Eq. (2) to obtain estimates for the single channel current *i* and the number of channels, *N*. We have also analyzed the reduction in current noise observed following block of the levcromakalim-induced current with either glibenclamide or PCP. Measurements were obtained in a similar way except that baseline values of variance and mean current were taken during maximum block of the holding current at −60 mV. Figure 4 shows that both the increase in current, in response to levcromakalim, and the decrease in current during block by PCP (or glibenclamide, *not shown*), gave similar values for *i* and *N* (*see* figure legend). Subsequent estimates were therefore made by combining data obtained during the levcromakalim-induced and PCP- or glibenclamide-blocked current responses. We also found that calculating variance, either directly using the Noise program or by fitting Gaussian curves to amplitude histograms using the pCLAMP software, gave similar results (Fig. 4*B*), so that estimates returned by the two methods have been combined.

Our estimates of variance will be affected both by the length of the current segments sampled, and by low-pass filtering due to the combination of the pipette series resistance and the cell capacity. Assuming that excess current variance in the presence of levcromakalim reflects the transitions of many channels between the open and closed states, measured variance will be reduced if the current segment sampled is not several times longer than the mean open channel lifetime (*see* Silberberg & Magleby, 1993). Under conditions where mean current was relatively stable (Fig. 5*A*, inset), we found that measured variance increased markedly as sample duration was increased from 300 msec to 2 sec but remained relatively constant with sample durations longer than this (Fig. 5*A*), consistent with channels having relatively long openings. Such loss of measured variance through inadequate sample length has the

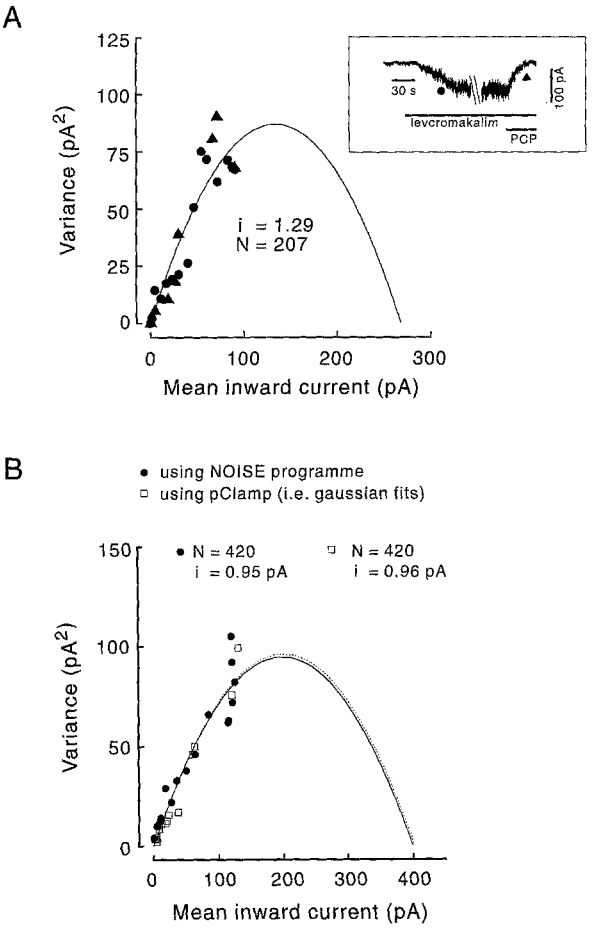

Fig. 4. (*A*) Plot of the levcromakalim-induced (filled circles) and phencyclidine-blocked current (filled triangles) and the associated changes in variance. The current record shown in the inset was split into 3 sec blocks, sampled at 5 kHz and digitally filtered at 2 kHz. The relation between variance and mean current was obtained by fitting a Gaussian curve to the amplitude histogram measured from each 3 sec block. The fitted curve drawn in the plot shows the best fit of Eq. (2) to all the data with $i = 1.29$ pA and $N = 207$ channels. Fitting the data for either the levcromakalim-induced or PCP-blocked current separately, gave values for *i* of 1.49 or 1.2 pA with *N* equal to 260 or 276, respectively. (*B*) Comparison from a different cell of the estimates of *i* returned by direct calculation of mean and variance (filled circles) and by fitting Gaussian functions to the data (open squares). In fitting Eq. (2) to the data obtained from Gaussians, the value of *N* was fixed at 420, the value returned from the free fit to the directly calculated data, because the fitting routine occasionally failed to detect a limit for *N*.

effect of underestimating *i* and overestimating *N*. On the other hand, filtering did not greatly affect measured variance. The cutoff frequency (f_c) of the whole-cell recording is given by the expression $f_c = 1/2\pi\tau$, where τ is the product of cell capacitance and series resistance. In our experiments, we calculate this filtering to be 484 ± 59 Hz ($n = 13$). Data from a representative cell, with an intrinsic cutoff frequency of 338 Hz, were analyzed after low-pass filtering (−3 dB) at 500 or 63

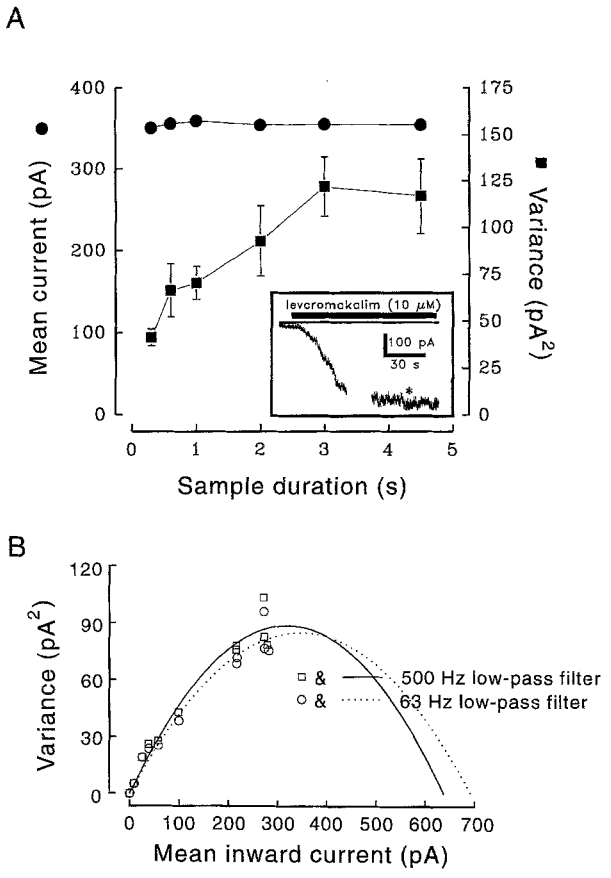

Fig. 5. The effect of sample duration (*A*) and applied filtering (*B*) on measured variance. (*A*) Individual determinations of mean current (filled circles) and current variance (filled squares) measured using the pCLAMP method. Data files 4.5 sec in duration, filtered at 2 KHz and digitized at 10 KHz, were recorded during a sustained levcromakalim-activated current in which the mean current was relatively constant (*A*, inset, *). From four such files, all-points histograms were constructed from either 0.3 sec of the record or longer samples, up to 4.5 sec. Values for mean current and variance were obtained from the Gaussian fits to these histograms and the mean ± sem for these files plotted as a function of the sample duration. (*B*) Effect that low-pass filtering the data has on the measured current variance. Data were analyzed using Bessel filter cutoffs of 500 (open square) or 63 (open circle) Hz. The calculated cutoff frequency of the whole-cell recording was 338 Hz.

Hz. If the current fluctuations underlying the variance were of relatively high frequency, we would expect the variance to be substantially reduced by filtering at 63 Hz, but to be much less affected by 500 Hz filtering. In fact Fig. 5*B* shows that variance measured after filtering at 63 Hz was not greatly different from the variance measured after filtering at 500 Hz. This might suggest that the mean open time of the channels underlying the levcromakalim response is relatively long, and that intrinsic filtering by the whole-cell recording configuration does not much affect the variance measured in our experiments. However, we cannot exclude the possibility that the mean open time of the channel is short, but that openings occur in long bursts.

From a series of experiments, the unitary current amplitude and channel number were estimated to be -1.14 ± 0.07 pA ($n = 16$) and 330 ± 44 ($n = 16$), respectively. Assuming a linear unitary current voltage relation reversing around 0 mV, our estimate of unitary current amplitude in symmetrical 139 mM K^+ at -60 mV corresponds to a slope conductance of 19 pS.

The Current Inhibited by the Photorelease of ATP

Previous reports have shown that the zero current potential of pulmonary arterial smooth muscle cells is sensitive to pipette ATP and glibenclamide (Clapp & Gurney, 1992). Taken together with other evidence, the interpretation was that the levcromakalim-induced and glibenclamide-blocked background K^+ current was ATP sensitive. In this study, we have recorded the current required to voltage-clamp isolated cells to -60 mV with symmetrical 143 mM K^+, when the pipette contained 20 mM caged ATP, but no free ATP. After whole-cell access, the holding current grew more negative and became noisy, averaging -136 ± 17 pA ($n = 5$) after 3–5 min of whole-cell recording. This current was quickly and substantially blocked after flash photolysis of caged ATP (Fig. 6*A*), which we estimate liberated at least 0.5 mM free ATP. With time, this inward current developed again, presumably as the released ATP diffused away or was metabolized, since a further light flash similarly blocked the inward current. Analysis of the current noise during recovery from the flash-induced block, using Gaussian fits to amplitude histograms, gave estimates for i and N which were very similar to those estimates from the levcromakalim-induced current (Fig. 6*B*). For a series of three cells, the mean values for i and N were -1.38 ± 0.01 pA and 198 ± 32, respectively.

Discussion

The results of the present study lend further support to the suggestion that levcromakalim activates ATP-sensitive K^+ channels in pulmonary arterial smooth muscle, as it does in smooth muscle from the systemic circulation (Clapp & Gurney, 1993). Whole-cell currents induced by levcromakalim were previously shown to be inhibited by glibenclamide, but to be relatively insensitive to TEA^+ (Clapp et al., 1993). Here, we show that the current and relaxation can also be blocked by PCP, and that the underlying channels show little or no voltage dependence. Further, the channels activated by levcromakalim do not appear to be Ca^{2+} sensitive, since

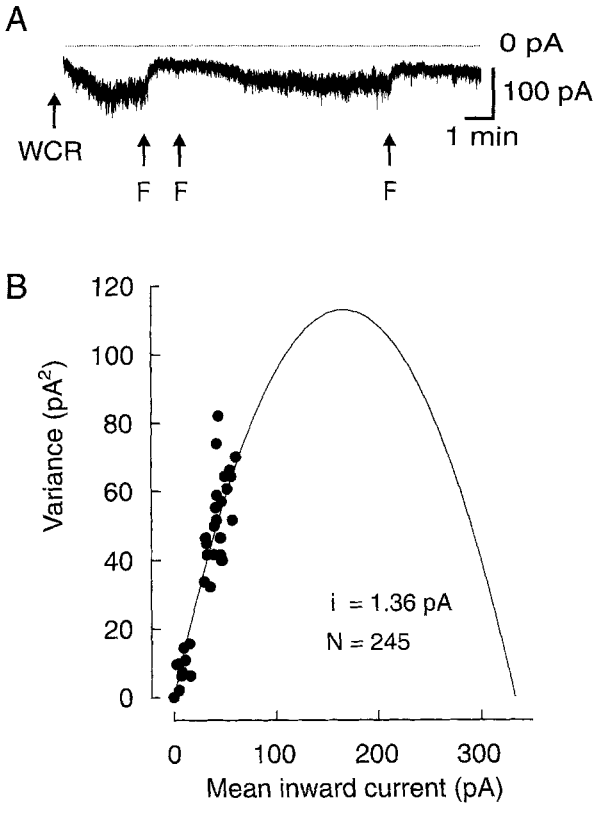

Fig. 6. Noise analysis of current blocked by photolytically released ATP. (*A*) Record of whole-cell current from a cell dialyzed with 20 mM caged ATP but no free ATP and held at −60 mV in symmetrical 143 mM K^+. After access to the cell was gained (at WCR), a noisy inward current developed which was blocked by flash-released ATP (at the arrow labeled *F*). The bath solution contained in mM: 143 KCl, 10 NaCl, 15 $NaHCO_3$, 1 NaH_2PO_4, 5 HEPES, 0.05 phenol red, bubbled with 95% O_2/5% CO_2, pH 7.4. (*B*) Analysis of the current noise seen in *A* during the redevelopment of inward current following the flash-induced blocked. The pCLAMP method was applied to 3 sec current segments filtered at 2 KHz and sampled at 5 KHz, giving estimates for *i* of 1.36 pA and a value for *N* of 245. The baseline variance and mean current were measured from the fully blocked current, when a second flash had no further effect.

the current is activated when the calculated $[Ca^{2+}]_i$ should be <15 nM, and is unaltered by the presence or absence of external Ca^{2+}. These findings are consistent with activation of K_{ATP} channels. Additional support comes from the observation that estimates of the single channel current and channel number, obtained from noise analysis of the levcromakalim-induced current, are in good agreement with those measured from the current activated by ATP depletion that could be inhibited by photorelease of intracellular ATP.

Our estimates of single channel current from the current fluctuations induced by levcromakalim, suggest that the channels underlying the current are of quite small conductance, around 19 pS in symmetrical high K^+. Our estimate from the current inhibited by photoreleased ATP after intracellular ATP depletion gives a similar estimate of 23 pS. These are probably lower limits since both sample length and intrinsic filtering in the whole-cell recording configuration can reduce the measured variance, as we have discussed in the text. Our results suggest that sample length effects may be important, while filtering is probably less so. Single K_{ATP} channels of small or intermediate conductance have been reported in rabbit portal vein cells by Kajioka et al. (1991, 50 pS in symmetrical high K^+), while Beech et al. (1993) have described a nucleotide diphosphate-dependent, ATP-sensitive K^+ channel with a conductance of 24 pS in 60 mM $[K^+]_o$ in the same preparation. Noise analysis of currents induced by levcromakalim or ATP depletion in rat portal vein suggests unitary conductances in the range 10–20 pS in physiological $[K^+]_o$ (Noack et al., 1992*a,b*). In pig coronary arterial myocytes, single K_{ATP} channels with conductances of 30 pS in symmetrical high (150 mM) $[K^+]$ have been reported (Miyoshi et al., 1992). The conductance we estimate in pulmonary arterial cells is rather lower than those described above, taking into account the ionic conditions. This could reflect a somewhat lower unitary conductance in the pulmonary artery, but may also reflect that noise analysis gives a lower limit for unitary conductance. In pig coronary myocytes, noise analysis of the adenosine-activated current gave an estimate for the underlying single channel current about 20% lower than the amplitude measured directly (Dart & Standen, 1993). Estimates of channel number from fits to the variance mean relation give quite a wide variation, but our results suggest a channel number of a few hundred per cell.

In summary, we conclude from our experiments that the density and single channel conductance underlying the ATP- and levcromakalim-sensitive current are low in pulmonary artery. Given that the average membrane capacitance of single pulmonary artery cells is 28 pF (Clapp & Gurney, 1991), this corresponds to a channel density of <1 per μm^2, assuming a specific capacitance of 1.0 μFcm^{-2} for cell membranes. Taking this into account, together with the prediction that the conductance of this channel is likely to be even smaller under physiological conditions, this might explain why K_{ATP} channels have been hard to study in smooth muscle. However, it is clear from recent studies in isolated pulmonary artery cells that ATP-sensitive K^+ channels do contribute to the resting conductance (Clapp & Gurney, 1992), thus allowing membrane potential and hence vascular tone, to be modulated by local changes in metabolism. While in coronary arteries these channels appear to be responsible for hypoxia-induced vasodilation (Daut et al., 1990), it is not yet established whether they play any role in the constriction response to hypoxia in pulmonary circulation. The different mechanical response to hypoxia between the pulmonary

and systemic arteries may reflect an intrinsic difference in the regulation of ATP-sensitive channels in the two tissues.

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