British Journal of Pharmacology (1997) 122, 1119–1126

Binding and effects of K_{ATP} channel openers in the vascular smooth muscle cell line, A10

[1]Ulrich Russ, Friedrich Metzger, Elisabeth Kickenweiz, Annette Hambrock, *Peter Krippeit-Drews & Ulrich Quast

Departments of Pharmacology and *Physiology, Medical Faculty, University of Tübingen, D-72074 Tübingen, Germany

1 The ATP-sensitive K^+ channel (K_{ATP} channel) in A10 cells, a cell line derived from rat thoracic aorta, was characterized by binding studies with the tritiated K_{ATP} channel opener, [^{3}H]-P1075, and by electrophysiological techniques.

2 Saturation binding experiments gave a K_D value of 9.2 ± 5.2 nM and a binding capacity (B_{Max}) of 140 ± 40 fmol mg^{-1} protein for [^{3}H]-P1075 binding to A10 cells; from the B_{Max} value a density of binding sites of 5–10 per μm^2 plasmalemma was estimated.

3 K_{ATP} channel modulators such as the openers P1075, pinacidil, levcromakalim and minoxidil sulphate and the blocker glibenclamide inhibited [^{3}H]-P1075 binding. The extent of inhibition at saturation depended on the compound, levcromakalim inhibiting specific [^{3}H]-P1075 binding by 85%, minoxidil sulphate and glibenclamide by 70%. The inhibition constants were similar to those determined in strips of rat aorta.

4 Resting membrane potential, recorded with microelectrodes, was -51 ± 1 mV. P1075 and levcromakalim produced a concentration-dependent hyperpolarization by up to -25 mV with EC_{50} values of 170 ± 40 nM and 870 ± 190 nM, respectively. The hyperpolarization induced by levcromakalim (3 μM) was completely reversed by glibenclamide with an IC_{50} value of 86 ± 17 nM.

5 Voltage clamp experiments were performed in the whole cell configuration under a physiological K^+ gradient. Levcromakalim (10 μM) induced a current which reversed around -80 mV; the current-voltage relationship showed considerable outward rectification. Glibenclamide (3 μM) abolished the effect of levcromakalim.

6 Analysis of the noise of the levcromakalim (10 μM)-induced current at -40 and -20 mV yielded estimates of the channel density, the single channel conductance and the probability of the channel to be open of 0.14 μm^{-2}, 8.8 pS and 0.39, respectively.

7 The experiments showed that A10 cells are endowed with functional K_{ATP} channels which resemble those in vascular tissue; hence, these cells provide an easily accessible source of channels for biochemical and pharmacological studies. The density of binding sites for [^{3}H]-P1075 was estimated to be one order of magnitude higher than the density of functional K_{ATP} channels; assuming a plasmalemmal localization of the binding sites this suggests a large receptor reserve for the openers in A10 cells.

Keywords: K_{ATP} channels; A10 cells; potassium channel openers; P1075; levcromakalim; glibenclamide; [^{3}H]-P1075 binding; smooth muscle

Introduction

K_{ATP} channels, a group of K^+ channels inhibited by intracellular adenosine 5′-triphosphate (ATP) and opened by nucleoside diphosphates like adenosine 5′-diphosphate (ADP) couple the membrane potential to the metabolic state of the cell (reviews: Ashcroft & Ashcroft, 1990; Edwards & Weston, 1993; Quast, 1996a). In many tissues, these channels are opened by K_{ATP} channel openers such as levcromakalim, pinacidil, P1075 and minoxidil sulphate, and blocked by sulphonylureas like glibenclamide. Functionally, the openers (with the exception of diazoxide) show the greatest potency in vascular smooth muscle where they produce hyperpolarization and relaxation of the tissue (Edwards & Weston, 1993; Quast, 1996b).

Coexpression of tissue specific sulphonylurea binding proteins (sulphonylurea receptors, SURs) with certain weakly inwardly rectifying K^+ channels (K_{ir}) produces channels with the characteristics of the native K_{ATP} channel in the respective tissue (Inagaki *et al*., 1995; 1996; Sakura *et al*., 1995; Isomoto *et al*., 1996). Matching the sequences of different SURs, with the pharmacological properties of the respective K_{ATP} channels, Isomoto *et al*. (1996) have proposed that the openers may bind to different sites at the SUR protein. However, this hypothesis has not yet been tested experimentally. Binding studies with K_{ATP} channel modulators in vascular smooth muscle have been performed in rings of rat aorta with the tritiated pinacidil analogue, [^{3}H]-P1075 (Bray & Quast, 1992; Quast *et al*., 1993) or [^{3}H]-glibenclamide (Löffler & Quast, 1997). These studies suggest that structurally different K_{ATP} channel openers bind to the same target, but possibly to different sites at this target, to elicit their effects (Bray & Quast, 1992; Quast *et al*., 1993). The sulphonylurea, glibenclamide, binds to a site different from and negatively allosterically coupled to the [^{3}H]-P1075 binding site (Bray & Quast, 1992). Further progress has been precluded by the lack of opener binding after homogenization of the tissue (Quast *et al*., 1993) and the difficulty in homogenizing aortic tissue may be one factor contributing to this failure. For easy homogenization and reduction of experimental scatter it would be preferable to perform binding studies with K_{ATP} channel modulators in a homogeneous population of isolated cells.

Recently, evidence has been presented that the A10 cell, a cell line from embryonic rat thoracic aorta (Kimes & Brandt, 1976), is endowed with K_{ATP} channels (Holevinski *et al*., 1994). Optical measurements indicated that P1075 hyperpolarizes these cells in a glibenclamide-sensitive manner and in voltage-clamp experiments it was observed that P1075 elicits a current which reverses at -67 mV. In the same cells, P1075

[1] Author for correspondence.

was shown also to block a swelling-activated Cl^- conductance. This block also hyperpolarizes the cell membrane. However, this effect is not blocked by glibenclamide (Holevinski *et al*., 1994).

It was the aim of this study to investigate the properties of the K_{ATP} channel in A10 cells and to see whether it had retained the characteristics of a vascular K_{ATP} channel. To this end, binding studies with [^{3}H]-P1075 as the radiolabel, membrane potential measurements and voltage-clamp experiments were performed. The results show that the channel in A10 cells resembles the one described in rat aorta and suggest that the density of binding sites for the openers in the cell line is at least one order of magnitude higher than the density of functional K_{ATP} channels. Some of the results have been presented in abstract form to the German Pharmacological Society (Kickenweiz *et al*., 1996; Russ *et al*., 1997).

Methods

Cell culture

A10 cells were obtained at passage 16 from American Type Culture Collection (ATCC No. CRL 1476; lot No. 5590, Rockville, MD, U.S.A.). They were cultured in 10 cm plastic culture dishes (Primaria, Falcon, Heidelberg, Germany) at 37°C in a humidified atmosphere with 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% foetal calf serum and penicillin (100 u ml^{-1})/streptomycin (100 μg ml^{-1}). Cells were subcultured every 2 to 4 days at a split ratio of 1:3 to 1:5 by use of trypsin/EDTA (0.15%/0.06%) and used 3–5 days later when they had reached a confluency of 80–100%; maximum passage number was 60. The cell number in each culture dish was determined by counting the cells in representative areas of defined size. For electrophysiological measurements cells were seeded in small Primaria culture dishes (diam. 3.5 cm), sometimes supplied with a glass cover slip.

Binding experiments

For saturation experiments, 0.8 to 1.2 million cells were incubated in their culture dishes (55 cm^2 in surface) with 2 ml DMEM containing [^{3}H]-P1075 (0.5–20 nM) for 60 min at 37°C; determination of nonspecific binding was done in the presence of 10 μM unlabelled P1075. Aliquots of 50 μl were removed for the determination of total radioactivity. Incubation was stopped by removal of the incubation medium and exposing the cells to 10 ml of an ice-cold quench solution (50 mM Tris, 154 mM NaCl, pH 7.4) for 90 s. After a further short wash cells were broken by addition of 1.5 ml of 10 mM ice-cold HEPES (pH 7.4) and scraped off. Three samples of 0.45 ml were transferred from each culture dish into scintillation vials and supplemented with 3 ml of scintillant (Ultima gold, Packard, Groningen, NL) and counted for ^{3}H with an efficiency of 0.5. From each dish, 50 μl were taken to determine the cell protein content according to the method of Lowry (Lowry *et al*., 1951) with bovine serum albumin as standard. In the concentration range from 0.5 to 5 μM, [^{3}H]-P1075 was used in undiluted form, for higher concentrations it was diluted 1:10 with unlabelled P1075.

Nonspecific binding (B_{NS}) was proportional to the free label concentration, L, and was fitted to the equation, $B_{NS} = a * L$, where a is a proportional constant. Total binding (B_{Tot}) was then analysed as the superposition of specific + nonspecific binding and was fitted to the equation

$$(1) \qquad B_{Tot} = B_{Max} \times L \times (L + K_D)^{-1} + a \times L$$

to estimate the values of the equilibrium dissociation constant (K_D) and the maximum concentration of binding sites (B_{Max}, fmol mg^{-1} protein) by the method of least squares.

Inhibition of [^{3}H]-P1075 binding was studied in the presence of [^{3}H]-P1075 (0.55 nM) and the inhibitor (I) of interest as described above. B_s in the presence of inhibitor I was normalized to % of B_s in the absence of I and fitted to the Law of Mass Action,

$$(2) \qquad B_S = 100 - A \times I \times (I + IC_{50})^{-1},$$

in order to obtain estimates for the midpoint (concentration of 50% inhibition, IC_{50}) and the degree of inhibition at saturation (A, in % B_s) of the inhibition curve. Since the label concentration, L = 0.55 nM, was much smaller than the IC_{50} value for P1075 (= 14 nM), the IC_{50} values for the inhibitors can be equated with their inhibition constant K_i (Cheng-Prusoff equation; Tallarida, 1995).

Membrane potential measurements

Membrane potential was measured with microelectrodes. A recording chamber (diam. 1.3 cm) was inserted into the culture dish and continuously perfused with a buffer containing (in mM): NaCl 115, KCl 5, $MgCl_2$ 1, NaH_2PO_4 2, $NaHCO_3$ 24, $CaCl_2$ 1.3, glucose 5 and gassed with 95% O_2/5% CO_2 in order to maintain a pH of 7.4 at 37°C. The bath perfusion system allowed for a solution change within less than 1 min. Microelectrodes were drawn from filament borosilicate glass capillaries (GC 150F-15, Clark Electromedical Instruments, Pangbourne, U.K.) on a horizontal microelectrode puller (Zeitz, Augsburg, Germany) and filled with 1 M KCl solution; electrode resistance was 90–130 MΩ. Cells at the edge of an agglomeration were impaled from the side with a piezomanipulator (PM20N, Frankenberger, Gilching, Germany). The microelectrode was connected to an amplifier (Duo 773, World Precision Instruments, Hamden, CT, U.S.A.) and the time course of the voltage was continuously recorded on a chart recorder. Concentration-response curves were established either cumulatively or by a protocol in which each drug application was followed by a wash period which allowed membrane potential to return to the control value.

Patch clamp experiments

The patch-clamp technique was used in the whole-cell configuration as described by Hamill *et al*. (1981). The bath solution was (in mM): NaCl 139, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 1.2, HEPES 5, glucose 11, gassed with 95% O_2/5% CO_2 titrated to pH 7.4 with NaOH at 37°C. Patch-pipettes were pulled from filament borosilicate glass capillaries as described above and heat polished. After filling with (in mM) K-glutamate 100, KCl 35, NaCl 10, $MgCl_2$ 1.5, HEPES 10, EGTA 10, Na_2ATP 0.5, titrated to pH 7.2 with KOH, they had a resistance of 3–5 MΩ. Series resistance (5 to 12 MΩ) and cell capacitance (60–180 pF) were compensated (series resistance by 70%) with the compensation circuit of the EPC9 patch-clamp amplifier (HEKA, Lambrecht, Germany). Signals were filtered at 5 kHz by use of the 4-pole Bessel filter of the EPC9 amplifier; data recording and analysis were performed with the 'E9 Screen' and 'M2 Lab Analysis' software (HEKA, Lambrecht, Germany).

Since cells with contact to neighbouring cells could not be voltage-clamped, isolated cells were chosen for measurement. Cells tended to depolarize during experiments (40 min); only experiments were accepted where the resting membrane potential at the end of the experiment was negative to −30 mV. Capacitance measurements were performed before the start of each pulse train with the EPC9 amplifier-system ('Time Domain' technique, Gilles, 1995, Sigworth *et al*., 1995) and gave constant values during the experiment. Current-voltage (*I*/V) relationships were measured by applying voltage steps of 200 ms duration from a holding potential of −50 mV to a series of test potentials from −100 mV to 20 mV in 20 mV increments; alternatively, voltage ramps from −100 to +50 mV were applied with a slope of 1.5 V s^{-1}. Membrane conductance was calculated from the current difference at −60 and −40 mV recorded in the last 50 ms of a 200 ms pulse. Cell size was calculated assuming a specific membrane capacitance of 1 μF cm^{-2} (Hille, 1992).

For noise analysis, cells were clamped at −40 mV or −20 mV and the LCRK-induced current increase was recorded continuously, filtered at 2 kHz with the 4-pole Butterworth filter of the EPC9 amplifier and sampled with 5 kHz. Segments of 250 ms were used for calculation of variance (σ^2) and mean current (I); during this time interval, the maximum changes in I were smaller than $0.1 \times \sigma$. For estimation of the single channel current (i) and the number of channels (N) the equation.

(3) $$\sigma^2 = i \times I - I^2 \times N^{-1}$$ (Heinemann, 1995)

was fitted to the data. The channel open probability (p_o) is given by

(4) $$p_o = I \times (i \times N)^{-1}$$

Chemicals

DMEM was purchased from Serva (Heidelberg, Germany), foetal calf serum from Gibco (Eggenstein, Germany), penicillin and streptomycin from Boehringer (Mannheim, Germany). [^{3}H]-P1075 (specific activity 107 or 121 Ci mmol^{-1}) was from Amersham Buchler (Braunschweig, Germany) and glibenclamide from Sigma (Deisenhofen, Germany). The following drugs were kind gifts of the pharmaceutical companies given in parentheses: P1075 ((*N*-cyano-*N*′-(1,1-dimethylpropyl)-*N*″-3-pyridyl-guanidine) Leo Pharmaceutical, Ballerup, Denmark), levcromakalim (SmithKline-Beecham, Harlow, U.K.). Minoxidil sulphate and pinacidil were synthesized by Dr W.P. Manley (Sandoz, Basel, Switzerland). The other reagents and chemicals were from Merck (Darmstadt, Germany).

Statistics

Results are expressed as mean ± s.e.mean. Individual binding experiments were fitted to the Hill equation and to the Law of Mass Action according to the method of least squares by use of the programme Figure P (Biosoft, Cambridge, U.K.). Statistical comparison of the fits according to the 'extra sum of squares principle' and to the 'minimum Akaike information criterion' as described in Quast & Mählmann (1982) indicated that the fit to Law of Mass Action (eqn. 1) was superior. Errors in the fitting parameters were estimated assuming that the amplitudes follow a normal and IC_{50} values of log-normal distribution. Data from membrane potential measurements were pooled and concentration-response curves were heuristically fitted to a sigmoidal function as given by the Law of Mass Action; errors in the parameters were estimated by use of the univariate approximation (Draper & Smith, 1981). In calculations involving two mean values with standard errors, propagation of errors was taken into account according to Bevington (1969). Statistical significance of differences was assessed by ANOVA followed by an appropriate post hoc test (t test, Dunnett's or Newman-Keuls test) by use of the Instat programme (Graphpad Software, San Diego, U.S.A.). Patch-clamp data were not normally distributed; data are presented as median with the 95% confidence interval in parentheses. ANOVA was performed by the Friedman Nonparametric Repeated Measures test followed by the Dunn's Multiple Comparisons test by use of the Instat programme.

Results

Binding experiments

For these experiments (which were performed in culture dishes), cell at 80–100% confluency were used in order to minimize nonspecific binding. Figure 1 illustrates total and nonspecific binding of [^{3}H]-P1075 to A10 cells as a function of the free label concentration. Specific binding (S_B), calculated as the difference between total and nonspecific binding, is shown in the inset. The fit of the data to the Law of Mass Action gave an equilibrium dissociation constant (K_D) of 9.2 ± 5.2 nM and a binding capacity (B_{Max}) of 140 ± 40 fmol mg^{-1} protein for specific [^{3}H]-P1075 binding to the cells. The protein content of an A10 cell was determined to 630 ± 40 pg; from this the number of binding sites per cell was estimated to be 49000 ± 14000.

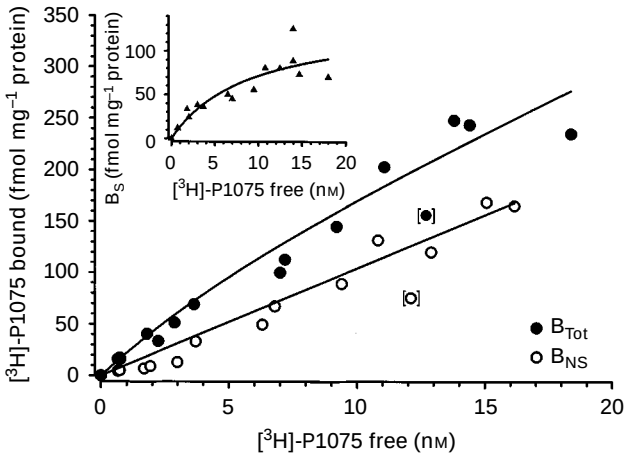

Figure 1 Total (B_{Tot}) and nonspecific (B_{NS}) binding of [^{3}H]-P1075 to A10 cells as a function of the free label concentration. B_{NS} increased linearly with the free label concentration with a slope of 10.4 ± 0.3 fmol mg^{-1} nM^{-1}. B_{Tot} as the superposition of specific binding (B_s) + B_{NS} was fitted to equation (1) and gave for B_s a K_D value of 9.2 ± 5.2 nM and a binding capacity (B_{max}) of 140 ± 40 fmol mg^{-1} protein. Inset: specific [^{3}H]-P1075 binding with the fit to the Law of Mass action giving the parameters listed above. Data points in parentheses were not used for evaluation.

Figure 2a illustrates the inhibition of specific [^{3}H]-P1075 binding by several K_{ATP} channel modulators. P1075 inhibited [^{3}H]-P1075 binding with an IC_{50} value of 14 ± 1 nM in reasonable agreement with the K_D value obtained in the saturation experiments. Pinacidil, another cyanoguanidine, displaced specific [^{3}H]-P1075 binding completely whereas the chemically unrelated openers, levcromakalim and minoxidil sulphate, and the K_{ATP} channel blocker, glibenclamide, inhibited specific [^{3}H]-P1075 binding by 85 and 70%, respectively (Figure 2a and Table 1). Inhibition curves were in agreement with the Law of Mass Action (Figure 2a); the IC_{50} values derived from these experiments are listed at pIC_{50} ($= -\log IC_{50}$) in Table 1. Comparison of these pIC_{50} values with those determined previously in [^{3}H]-P1075 binding assays in rat aortic strips (Bray & Quast, 1992; Quast *et al*., 1993) gave a correlation coefficient of 0.93 (Figure 2b). The correlation line with a slope not different from unity was shifted rightwards by about 0.5, indicating that, on average, the compounds were about 3 times weaker in A10 than in rat aortic strips.

Membrane potential measurements

Two to five minutes after impalement of the cells with microelectrodes, the recording stabilized for a period of at least 120 min. Resting membrane potential was −51 ± 1 mV ($n = 29$). Spontaneous action potentials as described by Kimes & Brandt (1976) and Korbmacher *et al*. (1989) were observed in only one experiment out of 29. Figure 3 presents an original trace illustrating the effect of P1075 on membrane potential. Cumulative application of the opener induced a concentration-dependent hyperpolarization of the cell. Glibenclamide (1 μM), applied in the presence of P1075 (1 μM), reversed the hyperpolarizing effect of P1075 completely; after washout of glibenclamide by P1075 (1 μM) alone, membrane potential returned to the value reached before the application of glibenclamide (Figure 3). Glibenclamide alone did not affect

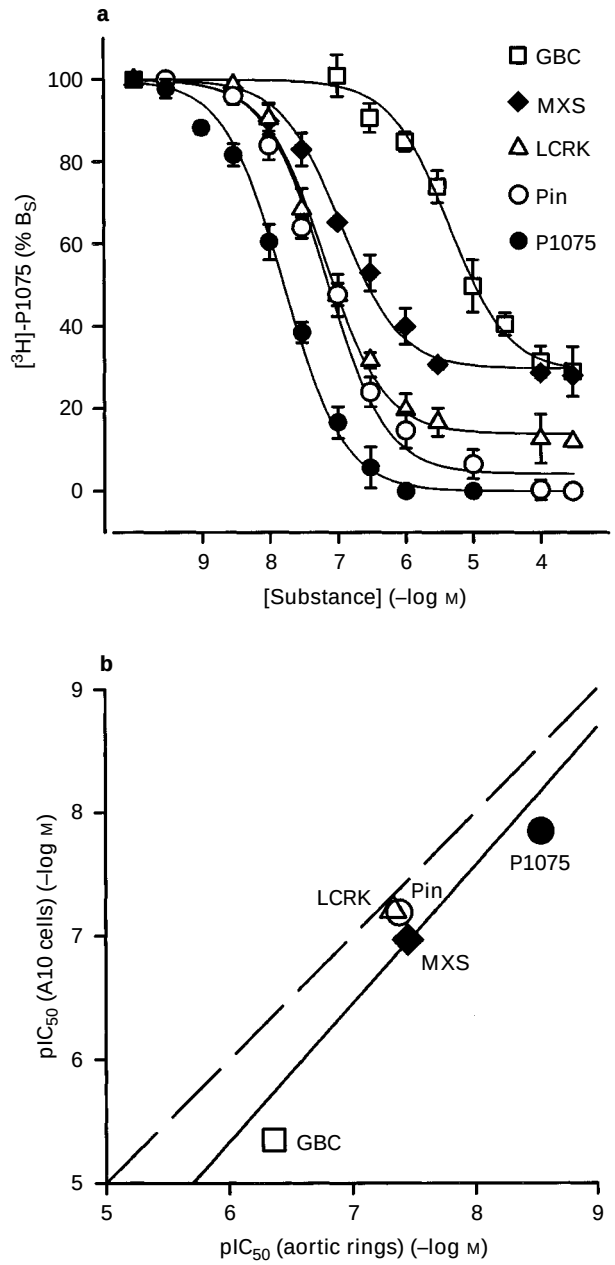

Figure 2 Inhibition of specific [^{3}H]-P1075 binding to A10 cells by K_{ATP} channel modulators. (a) Concentration-inhibition curves for the openers P1075, pinacidil (Pin), levcromakalim (LCRK) and minoxidil sulphate (MXS) and the blocker glibenclamide (GBC). Specific binding (B_s) at [^{3}H]-P1075 = 0.55 nM was 2.9 ± 0.1 fmol mg^{-1} protein, corresponding to 48 ± 1% of total binding. Data (as % B_s) were fitted to the Law of Mass Action; the fitting parameters are listed in Table 1. (b) Comparison of the pIC_{50} (= −log IC_{50}) values from [^{3}H]-P1075 binding assays in A10 cells with those determined previously in rat aortic rings (Bray & Quast, 1992). Correlation analysis gave a slope of 1.1 ± 0.3 and an ordinate intercept of −1.4 ± 2.0 with a correlation coefficient of 0.93 (solid line); the broken line is the line of identity.

membrane potential (not shown). The concentration-dependent hyperpolarizing effect produced by the two K_{ATP} channel openers, P1075 and levcromakalim, is shown in Figure 4. Both compounds induced the same maximum hyperpolarization (−24 ± 1 mV) with EC_{50} values of 170 ± 40 nM and 870 ± 190 nM for P1075 and levcromakalim, respectively. The hyperpolarization induced by levcromakalim (3 μM) was concentration-dependently reversed by glibenclamide with an IC_{50} value of 87 ± 17 nM (Figure 4).

Table 1 Inhibition of specific [^{3}H]-P1075 binding to A10 cells by K_{ATP} channel modulators[1]

Substance	pIC_{50}[2]	A[3]
P1075	7.85 ± 0.03	100
Levcromakalim	7.21 ± 0.04	85 ± 1*
Pinacidil	7.19 ± 0.06	94 ± 2
Minoxidil sulphate	6.97 ± 0.06	70 ± 2***
Glibenclamide	5.35 ± 0.05	71 ± 2***

[1]The parameters describing the inhibition curves were obtained from the fit of the data in Figure 2a to equation (1). [2]pIC_{50} = −log IC_{50} (M), where IC_{50} is the midpoint of the inhibition curve. [3]A is the maximum extent of inhibition as % of specific binding. *P < 0.05; ***P < 0.001 compared to control (ANOVA followed by Newman-Keuls test).

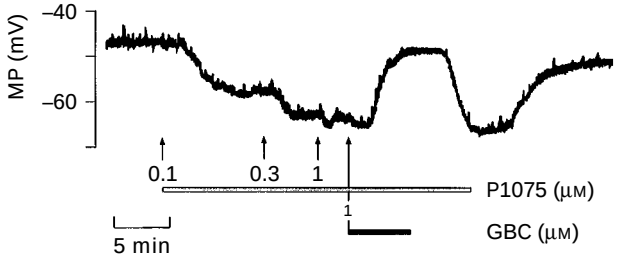

Figure 3 Original trace illustrating the effects of P1075 and glibenclamide on the membrane potential of an A10 cell. Resting membrane potential was −48 mV; drugs were applied as indicated by the bar. Superfusion of glibenclamide (GBC, 1 μM) in the presence of P1075 (1 μM) abolished the hyperpolarizing effect of P1075 alone; the inhibition by glibenclamide was reversed by wash out with P1075. The relatively small maximum hyperpolarizing effect of P1075 (−15 mV at 1 μM) was due to the cumulative application of the opener.

Patch clamp experiments

The currents underlying the hyperpolarizing effects of the openers were investigated in voltage clamp experiments in the whole cell configuration. Isolated cells were chosen for clamping; these cells were flat and had a large surface (8761; 7830, 11095 μm^2, median with 95% confidence limits, n = 18) as calculated from cell capacitance. Surprisingly we did not observe any effect of levcromakalim at room temperature (1 μM, n = 4; 10 μM, n = 4); hence, further experiments were conducted at 37°C. Figure 5a shows the response of a cell to voltage steps from −100 to 20 mV; the data from 18 experiments are summarized in Figure 6. In the region from −100 to −20 mV the current-voltage relationship was approximately linear with a slope (which represents membrane conductance) of 0.1 pS μm^{-2} and the current reversed at potentials around −50 mV. In 80% of the cells, application of levcromakalim (10 μM) increased membrane conductance and shifted the zero current potential to values negative to −60 mV (Figures 5b and 6); these effects were abolished by addition of glibenclamide (3 μM) in the continued presence of levcromakalim (Figure 5c and Figure 6). The current elicited by levcromakalim and blocked by glibenclamide, I_{KCO}, is shown in Figure 6. The I_{KCO}-voltage relationship was well fit by the Goldman-Hodgkin-Katz equation. This fit gave a zero current potential of I_{KCO} of −79 mV in agreement with a K^+-selective conductance and a value of 2.4 10^{-7} cm s^{-1} for the levcromakalim-induced increase in membrane K^+ permeability; in addition, I_{KCO} showed the outward rectification expected for a K^+ conductance without intrinsic rectification under the physiological K^+ gradient. Similar current-voltage relationships were obtained in response to voltage ramps (not illustrated).

From the voltage steps to −60 mV and −40 mV, membrane conductance under control conditions was calculated to be 0.12 (0.10, 0.17; $n=18$) pS μm^{-2} (median with 95% confidence limits in parentheses). Addition of the opener increased this value three fold (0.38; 0.33, 0.62); from the original currents one then calculates the opener-induced conductance to 0.23 (0.12, 0.43) pS μm^{-2}. The effects of both levcromakalim and glibenclamide were completely reversible upon washout of the drugs (not shown).

Orientating experiments in the cell attached configuration suggested that the K_{ATP} channel density in A10 cells was low. Therefore noise analysis was performed to estimate the single channel current (i) and the number of channels (N) in the plasmamembrane underlying I_{KCO} at −20 and −40 mV. The data from a cell clamped at −20 mV and superfused with 10 μM levcromakalim are illustrated in Figure 7; in this experiment (cell capacitance: 45 pS corresponding 4500 μm^2 cell surface), noise analysis gave $i=0.20\pm0.01$ pA and $N=2365\pm522$. The slight decrease of the current in the continued presence of 10 μM LCRK, as visible in Figure 7a, was seen frequently. The median values (confidence intervals and number of experiments, n, in parentheses) in this series of experiments were $i=0.32$ (0.21, 0.40; $n=6$) pA at −20 mV and $i=0.15$ (0.03, 0.29; $n=4$) pA at −40 mV; N was 1164 (203, 3665; $n=10$). From these values it was calculated that the channel has a conductance of 8.8 pS in this voltage range at

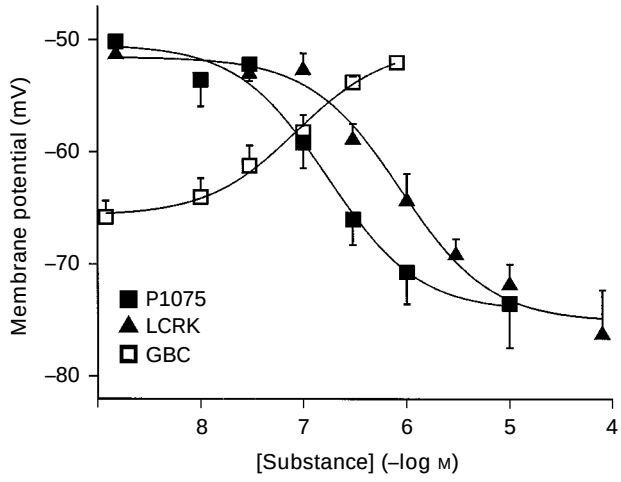

Figure 4 Concentration-dependent hyperpolarizing effects of P1075 and levcromakalim (LCRK) and reversal by glibenclamide (GBC). Superfusion of P1075 ($n=4-9$ per concentration) and levcromakalim ($n=5-7$) produced a concentration-dependent hyperpolarization with a maximum effect of -24 ± 1 mV and EC_{50} values of 170 ± 40 nM and 870 ± 190 nM for P1075 and levcromakalim, respectively. For the glibenclamide experiments, the cells were hyperpolarized from -51 ± 1 mV to -66 ± 2 mV by superfusion of levcromakalim (3 μM) ($n=5$). Glibenclamide reversed the hyperpolarizing effect of levcromakalim completely with an IC_{50} value of 86 ± 17 nM. The curves represent heuristic fits of the data to the Law of Mass Action from which the parameters were derived.

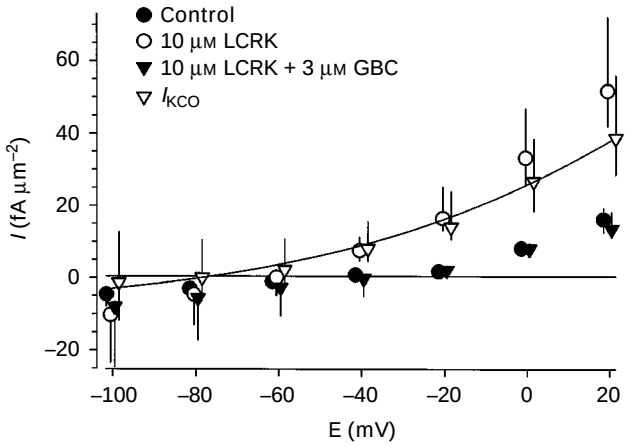

Figure 6 Current-voltage relationships for the median whole cell currants from 18 A10 cells under control conditions, after application of levcromakalim (LCRK, 19 μM) and in the presence of glibenclamide (GBC, 3 μM) plus LCRK (10 μM). The median current induced by levcromakalim, I_{KCO}, was calculated by subtracting the original currents in the presence of LCRK + GBC from the currents in the presence of LCRK alone. The solid curve represents a fit of the Goldman-Hodgkin-Katz current equation for a K^+ conductance under the physiological K^+ gradient to I_{KCO}, giving a current reverse at −79 mV and a value of 2.4 10^{-7} cm s^{-1} for the levcromakalim-induced increase in membrane K^+ permeability. Data points are slightly shifted around the abscissae values for reasons of clarity; the vertical lines show the 95% confidence limits. The voltage protocol was as in Figure 5 and the last 50 ms of the pulses were used for calculation of the data.

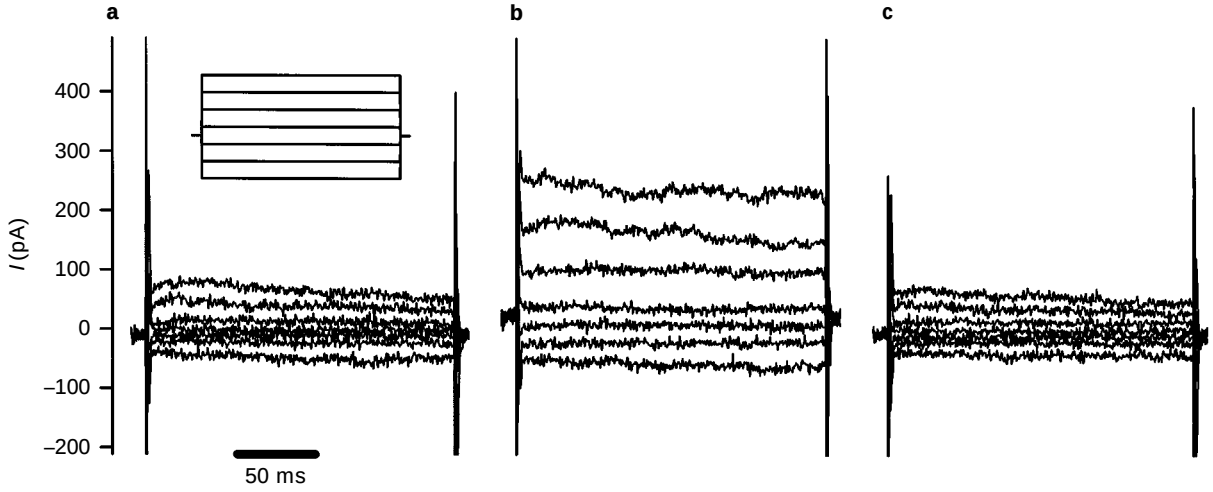

Figure 5 Whole cell currents of an A10 cell. The cell was held at −50 mV and stepped for 200 ms to a series of test potentials from −100 mV to 20 mV in 20 mV increments (inset in a). (a) Current under control conditions, (b) in the presence of levcromakalim (10 μM) and (c) in the presence of levcromakalim (10 μM) and glibenclamide (3 μM). Note also the shift in the holding current at −50 mV in (b) indicating a hyperpolarizing current induced by levcromakalim.

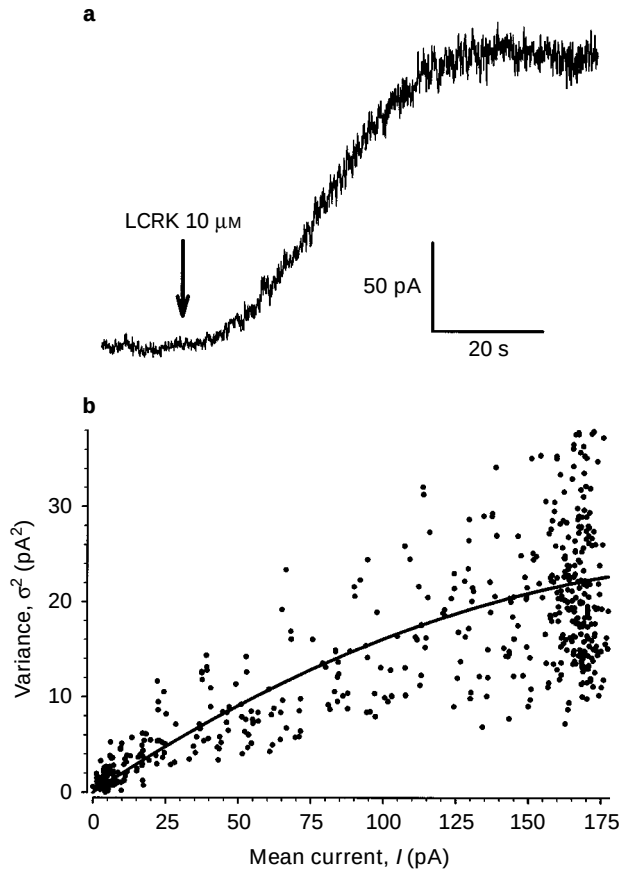

Figure 7 Noise analysis of the levcromakalim (LCRK)-induced current. (a) Whole cell current of an A10 cell. The cell was clamped at −20 mV; after application of LCRK (10 μM) the current increased by ≈167 pA. (b) Relationship between mean current I and variance σ^2 calculated from 250 μs segments of the trace in (a). The least squares fit of equation 3 to the data gave a single channel current (i) of 0.20±0.01 pA and number of channels (N) of 2365±522. Cell capacitance was 45 pS corresponding 4500 μm^2 cell surface. The slight decrease of the current in the continued presence of 10 μM LCRK was seen frequently.

physiological K^+ concentrations and, from equation 4, the open probability of the channel at 10 μM levcromakalim was estimated to be 0.39 (0.26, 0.53; $n=10$) at the maximum of the effect (1 min after the application of the drug). Channel density was calculated from N and the cell surface (see above) to 0.14 (0.07, 0.37; $n=10$) μm^{-2}.

Discussion

[^{3}H]-P1075 binding experiments

Evidence for K_{ATP} channels in A10 cells was found in binding studies and electrophysiological experiments. Saturation experiments showed that [^{3}H]-P1075 bound to A10 cells with a K_D value of 9.2±5.2 nM and a binding capacity (B_{Max}) of 140±40 fmol mg^{-1} protein. The large errors in these values were due to the scatter of the original data (Figure 1) which reflect the difficulties in these experiments performed in culture dishes at cell numbers varying from 0.8 to 1.2 million per dish. The K_D value determined here agrees well with that obtained earlier in rings of rat aorta (6 nM); the B_{Max} value is about two times higher ($B_{Max}=77$ fmol mg^{-1} protein, Bray & Quast, 1992; Quast *et al*., 1993), reflecting the fact that aorta is rich in protein not stemming from smooth muscle cells. These experiments also allow an estimation of the density of P1075 sites on the A10 cells. The number of binding sites present per cell was estimated to be 49,000 and, the cells being flat cylinders, their surface at confluency was estimated to be 10,000 μm^{-2}, taking both the upper and the lower surface into account. Hence the density of binding sites was estimated to be 5 or 10 μm^{-2}, depending on whether the binding sites are located on both or only on the upper surface of the cell.

The openers P1075, pinacidil, levcromakalim and minoxidil sulphate as well as the blocker glibenclamide inhibited [^{3}H]-P1075 binding according to the Law of Mass Action. The potency of the compounds is, on average, 3 times lower in A10 cells than in rat aorta (Bray & Quast, 1992). In contrast to the earlier results in aortic strips, some of the K_{ATP} channel modulators did not displace specific [^{3}H]-P1075 binding completely; for minoxidil sulphate and glibenclamide maximum inhibition was 70%, for levcromakalim 85%. Similarly, partial inhibition of specific [^{3}H]-P1075 binding has been observed recently with glibenclamide in rat glomeruli (Metzger & Quast, 1996), and with the openers minoxidil sulphate and aprikalim in primary cultures of rat aortic myocytes (Lemoine *et al*., 1996). The most plausible explanation for these results is that these K_{ATP} channel modulators bind to different binding sites which are coupled to a varying degree by negative allosteric interactions. Direct evidence for a negative allosteric coupling between the sites for P1075 and glibenclamide had been obtained before in rat aorta (Bray & Quast, 1992). The recent cloning results suggest that the glibenclamide binding site is located in the N-terminal portion of the SUR protein (Aguilar-Bryan *et al*., 1995); the site for diazoxide is hypothesized to be located in the C-terminal part and the pinacidil site elsewhere on SUR (Isomoto *et al*., 1996). With the availability of different SUR clones these questions can be tackled now by use of mutational analysis.

Collectively, the binding data show that the [^{3}H]-P1075 binding sites in A10 cells are qualitatively similar to those described in vascular tissues; this suggests that the drug receptor in this cell line has essentially retained the pharmacological characteristics of the vascular K_{ATP} channel. A10 cells therefore represent an easily accessible source of the vascular type drug receptor for the openers and should be useful in biochemical characterization.

Electrophysiological experiments

P1075 and levcromakalim produced concentration-dependent hyperpolarizations of A10 cells from −50 mV up to −75 mV; clearly, the maximum hyperpolarization was about 15 mV positive to the K^+ equilibrium potential. The current measurements have shown that levcromakalim at 10 μM, a maximally effective concentration (Noack *et al*., 1992; Ruβ *et al*., unpublished data), increased membrane conductance about three fold by inducing a glibenclamide-sensitive K^+ conductance (see below). Apparently, this increase was not sufficient to shift the membrane potential completely to the K^+ equilibrium potential; indeed, the density of open K_{ATP} channels was estimated to be very low under these conditions (0.056 μm^{-2}, $p_o \times N \times \mu m^{-2}$). The midpoints of the concentration-hyperpolarization curves were approximately 0.2 and 1 μM for P1075 and levcromakalim, respectively. Due to the nonlinear relationship between membrane potential and membrane K^+ permeability these EC_{50} values are difficult to relate to the K_i values determined in the binding assay. However, the value for P1075 is more than 10 times higher than that determined by Holevinski *et al*. (1994) with a fluorescent dye for measurement of membrane potential, perhaps reflecting nonlinearities of the optical signal.

The hyperpolarizing effect of both openers was entirely abolished by glibenclamide (1 μM). This rules out the possibility that a block of swelling-activated Cl^- channels by P1075 contributed significantly to the hyperpolarizing effect of this drug under our conditions since the Cl^- channel block by P1075 was not affected by glibenclamide (Holevinski *et al*., 1994). The IC_{50} value of 86 nM for glibenclamide acting against levcromakalim (3 μM) is the potency typical for this

sulphonylurea in the presence of a rather high concentration of an opener (Quast, 1996b). Collectively, the membrane potential experiments suggest that the hyperpolarization produced by P1075 and levcromakalim in A10 cells was due to the opening of K_{ATP} channels.

The whole cell current measurements showed that levcromakalim induced a current, I_{KCO}, which was well fit by the Goldman-Hodgkin-Katz (GHK) current equation for a K^+-selective conductance under the physiological K^+ gradient. The current reversed at -79 mV and was abolished by 3 μM glibenclamide These properties suggest that levcromakalim opens K_{ATP} channels in A10 cells. From noise analysis, the single channel conductance was estimated to be 8.8 pS in the range from -40 to -20 mV and under a physiological K^+ gradient; this value is similar to that obtained recently by others under such conditions (Bonev & Nelson, 1993; Bychkov *et al*., 1997). Using the Goldman-Hodgkin-Katz current equation and extrapolating to positive voltages, where conductance should approach the values observable in symmetrical high K^+ concentrations, we estimated a single channel conductance of 23 pS. This value is somewhat lower than those determined by single channel experiments in vascular smooth muscle for the channel sensitive to the K_{ATP} channel openers (Wakatsuki *et al*., 1992; Beech *et al*., 1993; Zhang & Bolton, 1996; for review see Quayle & Standen, 1994; Nelson & Quayle, 1995). However, the fact that noise analysis is known to underestimate the unitary current by about 20%, due to filtering of the recorded current by segment length, cell capacitance and series resistance, should be considered (Dart & Standen, 1993; Clapp *et al*., 1994; Quayle & Standen, 1994). The values for channel density (0.14 μm^{-2}) and for p_o(0.39) determined here are also in good agreement with those given in the literature (density: Bonev & Nelson, 1993; Clapp *et al*., 1994; Xu & Lee, 1994; p_o: Wakatsuki *et al*., 1992; Bonev & Nelson, 1993; Bychkov *et al*., 1997; for review see Nelson & Quayle, 1995). Finally, the density of K_{ATP} channels opened by levcromakalim (10 μM) estimated by noise analysis ($p_o \times N \times \mu m^{-2} = 0.056\ \mu m^{-2}$) is consistent with the result obtained from the opener-induced increase in specific conductance (0.23 pS μm^{-2}) and the single channel conductance (6.7 pS between -60 and -40 mV calculated by extension of the GHK current equation); from these values the density of open channels was estimated to be 0.034 μm^{-2}.

P1075 binding sites vs functional channels

The binding experiments have demonstrated that A10 cells are endowed with binding sites for the K_{ATP} channel openers and glibenclamide; the electrophysiological measurements have shown that these cells possess functional K_{ATP} channels. The density of binding sites for P1075 was estimated to be of the order of 5–10 μm^{-2}, whereas the density of K_{ATP} channels was around 0.14 μm^{-2}, i.e. there are about 30 to 60 times more binding sites than channels. This number must be considered with caution since the binding experiments were performed with cells at high confluency whereas the voltage-clamp experiments were performed with isolated cells; another reason is, that a functional K_{ATP} channel contains four SUR subunits and may therefore bind up to four molecules of the opener (Clement IV *et al*., 1997). Nevertheless, even in the case, the experiments do suggest that density of binding sites exceeds that of plasmalemmal K^+ channels by at least one order of magnitude. This finding may be interpreted in two ways depending on the localization of the binding sites. Assuming that they are located on the plasmalemmal SUR (Isomoto *et al*., 1996), one would have to conclude that only a small fraction of the SUR is coupled to K_{ir} channels to form the K_{ATP} channel, i.e. that there is a considerable number of 'spare receptors'. An alternative explanation is that the major part of the binding sites is located intracellularly, e.g. on the mitochondria, where they could participate in K_{ATP} channel formation (Paucek *et al*., 1992; Garlid *et al*., 1996). Experiments to define of the localization of the binding sites are underway in this laboratory.

The study was supported by Deutsche Forschungsgemeinschaft, grant Qu 100/2-1.

References

AGUILAR-BRYAN, L., NICHOLS, C.G., WECHSLER, S.W. CLEMENT, J.P., BOYD, A.E., GONZÁLES, G., HERRERA-SOSA, H., NGUY, K., BRYAN, J. & NELSON, D.A. (1995). Cloning of the β cell high-affinity sulfonylurea receptor: A regulator of insulin secretion. *Science*, **268,** 423–426.

ASHCROFT, S.J.H. & ASHCROFT, F.M. (1990). Properties and functions of ATP-sensitive K-channels *Cell. Signal*., **2,** 197–214.

BEECH, D.J., ZHANG, H., NAKAO, K. & BOLTON, T.B. (1993). K channel activation by nucleoside diphosphates and its inhibition by glibenclamide in vascular smooth muscle cells. *Br. J. Pharmacol*., **110,** 573–582.

BEVINGTON, P.R. (1969). *Data Reduction and Error Analysis for the Physical Sciences*. pp. 55–65 and 92–118. New York: McGraw-Hill.

BONEV, A.D. & NELSON, M.T. (1993). ATP-sensitive potassium channels in smooth muscle cells from guinea pig urinary bladder. *Am. J. Physiol*., **264,** C1190–C1200.

BRAY, K.M. & QUAST, U. (1992). A specific binding site for K^+ channel openers in rat aorta. *J. Biol. Chem*., **267,** 11689–11692.

BYCHKOV, R., GOLLASCH, M., RIED, C., LUFT, F.C. & HALLER, H. (1997). Effects of pinacidil on K^+ channels in humans coronary artery vascular smooth muscle cells. *Am. J. Physiol*., **273,** in press.

CLAPP, L.H., GURNEY, A.M., STANDEN, N.B. & LANGTON, P.D. (1994). Properties of the ATP-sensitive K^+ current activated by levcromakalim in isolated pulmonary arterial myocytes. *J. Membrane Biol*., **140,** 205–213.

CLEMENT IV, J.P., KUNJILWAR, K., GONZALEZ, G., SCHWANSTECHER, M., PANTEN, U., AGUILAR-BRYAN, L. & BRYAN, J. (1997). Association and stoichiometry of K_{ATP} channel subunits. *Neuron*, **18,** 827–838.

DART, C. & STANDEN, N.B. (1993). Adenosine-activated potassium current in smooth muscle cells isolated from the pig coronary artery. *J. Physiol*., **471,** 767–786.

DRAPER, N.B. & SMITH. H. (1981). *Applied Regression Analysis*. pp. 85–96, 458–517. New York: Wiley.

EDWARDS, G. & WESTON, A.H. (1993). The pharmacology of ATP-sensitive potassium channels. *Annu. Rev. Pharmacol. Toxicol*., **33,** 597–637.

GARLID, K.D., PAUCEK, P., YAROV-YAROVOY, V., SUN, X. & SCHINDLER, P.A. (1996). The mitochondrial K_{ATP} channel as a receptor for potassium channel openers. *J. Biol. Chem*., **271,** 8796–8799.

GILLES, K.D. (1995). Techniques for membrane capacitance measurements. In *Single-Channel Recording*. Second edition, ed. Sakmann, B. & Neher, E. pp. 155–198. New York: Plenum Publishing Corporation.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch*., **391,** 85–100.

HEINEMANN, S.H. (1995). Guide to data acquisition and analysis. In *Single-Channel Recording*. Second edition, ed. Sakmann, B. & Neher, E. pp. 53–91. New York: Plenum Publishing Corporation.

HILLE, B. (1992). *Ionic Channels of Excitable Membranes*. Second edition, Sunderland, Massachusetts: Sinauer Associates Inc.

HOLEVINSKY, K.O., FAN, Z., FRAME, M., MAKIELSKI, J.C., GROPPI, V. & NELSON, D.J. (1994). ATP-sensitive K^+ channel opener acts as a potent Cl-channel inhibitor in vascular smooth muscle cells. *J. Membrane Biol*., **137,** 59–70.

INAGAKI, N., GONOI, T., CLEMENT IV, J.P., NAMBA, N., INAZAWA, J., GONZALES, G. AGUILAR-BRYAN, L., SEINO, S. & BRYAN, J. (1995). Reconstitution of I_{KATP}: An inward rectifier subunit plus the sulfonylurea receptor. *Science*, **270,** 1166–1170.

INAGAKI, N., GONOI, T., CLEMENT IV, J.P., WANG, C.-Z., AGUILAR-BRYAN, L., BRYAN, J. & SEINO, S. (1996). A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K^+ channels. *Neuron*, **16,** 1011–1017.

ISOMOTO, S., KONDO, C., YAMADA, M., MATSUMOTO, S. HIGASHIGUCHI, O., HORIO, Y., MATSUZAWA, Y. & KURACHI, Y. (1996). A novel sulfonylurea receptor forms with BIR (Kir6.2) a smooth muscle type ATP-sensitive K^+ channel. *J. Biol. Chem.*, **217,** 24321–24324.

KICKENWEIZ, E., METZGER, F., KRIPPEIT-DREWS, P. & QUAST, U. (1996). Evidence for the existence of ATP-sensitive K^+ channels in a vascular smooth muscle cell line. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **353** (suppl.), R55.

KIMES, B.W. & BRANDT, B.L. (1976). Characterization of two putative smooth muscle cell lines from rat thoracic aorta. *Exp. Cell Res.*, **98,** 349–366.

KORBMACHER, C., HELBIG, H., STAHL, F., CORONEO, M., HALLER, H., LINDSCHAU, C., QUASS, P. & WIEDERHOLT, M. (1989). Continuous membrane voltage recordings in A10 vascular smooth muscle cells: effect of AVP. *Am. J. Physiol.*, **257,** C323–C332.

LEMOINE, H., MANNHOLD, R. & GRITTNER, D. (1996). Binding studies with potassium channel openers (KCO) in smooth muscle cells of calf coronary artery as compared to rat cardiomyocytes. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **353** (suppl.), R54.

LÖFFLER, C. & QUAST, U. (1997). Pharmacological characterization of the sulphonylurea receptor in rat isolated aorta. *Br. J. Pharmacol.*, **120,** 476–480.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193,** 265–275.

METZGER, F. & QUAST, U. (1996). Binding of [^{3}H]-P1075, an opener of ATP-sensitive K^+ channels, to rat glomerular preparations. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **354,** 452–459.

NELSON, M.T. & QUAYLE, J.M. (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *Am. J. Physiol.*, **268,** C799–C822.

NOACK, T., DEITMER, P., EDWARDS, G. & WESTON, A.H. (1992). Characterization of potassium currents modulates by BRL 38227 in rat portal vein. *Br. J. Pharmacol.*, **106,** 717–726.

PAUCEK, P., MIRONOVA, G., MAHDI, F., BEAVIS, A.D., WOLDEGIORGIS, G. & GARLID, K.D. (1992). Reconstitution and partial purification of the glibenclamide-sensitive, ATP-dependent K^+ channel from rat liver and beef heart mitochondria. *J. Biol. Chem.*, **267,** 26062–26069.

QUAST, U. (1996a). ATP-sensitive K^+ channels in the kidney. *Naunyn Schmiedeberg's Arch. Pharmacol.*, **354,** 213–225.

QUAST, U. (1996b). Effects of potassium channel activators in isolated blood vessels. In *Potassium Channels and their Modulators: From Synthesis to Clinical Experience*. ed. Evans, J.M., Hamilton, T.C., Longman, S.D. & Stemp, G. pp. 173–195. London: Taylor & Francis.

QUAST, U., BRAY, K.M., ANDRES, H., MANLEY, P.W., BAUMLIN, Y. & DOSOGNE, J. (1993). Binding of the K^+ channel opener [^{3}H]P1075 in rat isolated aorta: relationship to functional effects of openers and blockers. *Mol. Pharmacol.*, **43,** 474–481.

QUAST, U. & MÄHLMANN, H. (1982). Interaction of [^{3}H]flunitrazepam with the benzodiazepine receptor: evidence for a ligand-induced conformation change. *Biochem. Pharmacol.*, **31,** 2761–2768.

QUAYLE, J.M. & STANDEN, N.B. (1994). K_{ATP} channels in vascular smooth muscle. *Cardiovasc. Res.*, **28,** 797–804.

RUSS, U., METZGER, F. & QUAST, U. (1997). Effect of K_{ATP}-channel modulators on whole cell ion current in a smooth muscle cell line (A10) compared with binding studies. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **355** (suppl.), R74.

SAKURA, H., ÄMMÄLÄ, C., SMITH, P.A., GRIBBLE, F.M. & ASHCROFT, F.M. (1995). Cloning and functional expression of the cDNA encoding a novel ATP-sensitive potassium channel subunit expressed in pancreatic β-cells, brain, heart and skeletal muscle. *FEBS Lett.*, **377,** 338–344.

SIGWORTH, F.J., AFFOLTER, H. & NEHER, E. (1995). Design of the EPC9, a computer controlled patch-clamp amplifier. 2. Software. *J. Neurosci. Meth.*, **56,** 203–215.

TALLARIDA, R.J. (1995). Receptor discrimination and control of agonist-antagonist binding. *Am. J. Physiol.*, **269,** E379–E391.

WAKATSUKI, T., NAKAYA, Y. & INOUE, I. (1992). Vasopressin modulates K^+-channel activities of cultured smooth muscle cells from porcine coronary artery. *Am. J. Physiol.*, **263,** H491–H496.

XU, X. & LEE, K.S. (1994). Characterization of the ATP-inhibited K^+ current in canine coronary smooth muscle cells. *Pflügers Arch.*, **427,** 110–120.

ZHANG, H.-L. & BOLTON, T.B. (1996). Two types of ATP-sensitive potassium channels in rat portal vein smooth muscle cells. *Br. J. Pharmacol.*, **118,** 105–114.

(Received July 7, 1997
Revised August 21, 1997
Accepted August 29, 1997)