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ATP-sensitive K^+ Channel Opener Acts as a Potent Cl^- Channel Inhibitor in Vascular Smooth Muscle Cells

K.O. Holevinsky,[2] Z. Fan,[1] M. Frame,[3] J.C. Makielski,[1] V. Groppi,[3] D.J. Nelson[1,2]

[1]Department of Medicine, The University of Chicago, Chicago, Illinois 60637
[2]Department of Neurology, University of Chicago, Hospital Box 2030, 5841 S. Maryland Avenue, Chicago, Illinois 60637
[3]Department of Cell Biology, Upjohn Laboratories, Kalamazoo, Michigan 49001

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Abstract. We describe the activation of a K^+ current and inhibition of a Cl^- current by a cyanoguanidine activator of ATP-sensitive K^+ channels (K_{ATP}) in the smooth muscle cell line A10. The efficacy of U83757, an analogue of pinacidil, as an activator of K_{ATP} was confirmed in single channel experiments on isolated ventricular myocytes. The effects of U83757 were examined in the clonal smooth muscle cell line A10 using voltage-sensitive dyes and digital fluorescent imaging techniques. Exposure of A10 cells to U83757 (10 nM to 1 μM) produced a rapid membrane hyperpolarization as monitored by the membrane potential-sensitive dye bis-oxonol ([diBAC$_4$(3)], 5 μM). The U83757-induced hyperpolarization was antagonized by glyburide and tetrapropylammonium (TPrA) but not by tetraethylammonium (TEA) or charybdotoxin (ChTX). The molecular basis of the observed hyperpolarization was studied in whole-cell, voltage-clamp experiments. Exposure of voltage-clamped cells to U83757 (300 nM to 300 μM) produced a hyperpolarizing shift in the zero current potential; however, the hyperpolarizing shift in reversal potential was associated with either an increase or decrease in membrane conductance. In solutions where $E_K = -82$ mV and $E_{Cl} = 0$ mV, the reversal potential of the U83757-sensitive current was approximately −70 mV in those experiments where an increase in membrane conductance was observed. In experiments in which a decrease in conductance was observed, the reversal potential of the U83757-sensitive current was approximately 0 mV, suggesting that U83757 might be acting as a Cl^- channel blocker as well as a K^+ channel opener. In experiments in which Cl^- current activation was specifically brought about by cellular swelling and performed in solutions where Cl^- was the major permeant ion, U83757 (300 nM to 300 μM) produced a dose-dependent current inhibition. Taken together these results (i) demonstrate the presence of a K^+-selective current which is sensitive to K_{ATP} channel openers in A10 cells and (ii) indicate that the hyperpolarizing effects of K^+ channel openers in vascular smooth muscle may be due to both the inhibition of Cl^- currents as well as the activation of a K^+-selective current.

Key words: Smooth muscle — Glyburide — Pinacidil — Chloride channels — Membrane potential — Vasodilation

Introduction

Potassium channels that are inhibited by intracellular ATP (K_{ATP}) were first characterized in vertebrate cardiac muscle (Noma, 1983) and have since been described in skeletal muscle (Spruce, Standen, & Stanfield, 1985; Quasthoff et al., 1990), and in smooth muscle (Standen et al., 1989). The vasorelaxation in vascular smooth muscle produced by compounds which act as K_{ATP} channel agonists in cardiac and β-cells has been attributed to specific effects on K_{ATP} channels in smooth muscle (Quast & Cook, 1989; Standen et al., 1989). We have used a potent cyanoguanidine K_{ATP} channel opener, U83757, which activates K_{ATP} in cardiac ventricular myocytes to determine whether K_{ATP} is expressed in cells from the smooth muscle cell line A10. The clonal line A10 was derived from the thoracic aorta of the

Correspondence to: D.J. Nelson

rat and possesses many of the properties characteristic of native smooth muscle cells (Kimes & Brandt, 1976).

Whole-cell, voltage-clamp studies were carried out to determine the ionic selectivity of the currents underlying a cellular hyperpolarization induced by U83757. Our results indicate that antihypertensive agents which cause membrane hyperpolarization and subsequent relaxation of vascular smooth muscle cells may produce their effects through the simultaneous inhibition of a Cl^- conductance as well as the activation of a K^+-selective conductance.

Materials and Methods

Cell Preparation

Cardiac myocytes were enzymatically dissociated from the heart of adult rabbits using a modification of the method of Poole et al. (1989). Hearts were retrogradely perfused via the aorta at 37°C with a buffer gassed with 100% O_2 containing (in mM): 130 NaCl, 5 HEPES, 10 glucose, 20 taurine, 10 creatine, 5.4 KCl, 3.5 $MgCl_2$, and 0.4 NaH_2PO_4; pH 7.25. The heart was first perfused for 4 min with buffer plus 0.75 mM $CaCl_2$, and then for 4 min with buffer plus 0.1 mM EGTA. Finally, the heart was perfused for 10 min with buffer plus 0.08 mM $CaCl_2$ plus 1 mg/ml Type II collagenase (Worthington Biochemical, Freehold, NJ) plus 0.1 mg/ml Type XIV protease (Sigma Chemical, St. Louis, MO). The heart was removed from the perfusion apparatus and coarsely chopped. Selected pieces were shaken at 37°C for four 5-min periods in flasks containing the collagenase solution with 1 mg/ml bovine serum albumin added. After each 5-min incubation, tissue from one flask was filtered through a nylon gauze (pore size, 200 μm). The filtered material was centrifuged at low speed, and the harvested cells were transferred to a solution containing 150 mM K^+ glutamate and 10 mM HEPES (pH 7.2) and stored at room temperature or refrigerated. Cells were used within 24 hr of isolation. Small aliquots of cells were added to a 200 μl glass-bottom chamber mounted on the stage of an inverted microscope. Only cells with crisp edges and rod-shaped morphology were selected for study.

Cell Culture

The A10 cell line was obtained from American Type Culture Collection (ATCC CRL 1476) and studied between passages 15 and 30. Cells were plated at a density of 2.5×10^4 cells/ml on uncoated 35-mm tissue culture dishes and maintained in Dulbecco's modified Eagle's medium with high glucose (4,500 mg/l) supplemented with 10% fetal bovine serum, penicillin (100 U/ml and streptomycin (100 μg/ml). The medium was replaced on cells to be used for the electrophysiological experiments 4 hr prior to recording.

Digital Fluorescent Imaging

A10 cells were subcultured in 2 × 2 cm coverglass chambers and grown to confluence. Immediately prior to analysis, cells were washed two times with Earle's balanced salt solution that was buffered to pH 7.4 with 20 mM HEPES and then placed in the same buffer containing 5 μM $DiBAC_4$ (3). Fluorescent imaging of membrane potential was carried out using a modification of the procedure described by Bräuner and colleagues (1984). Briefly, cells were equilibrated with 5 μM $DiBAC_4$ (3) for 15 min at 37°C and then placed in a 35°C temperature-regulated mini-incubator that was mounted on the stage of a laser-based imaging cytometer (ACAS 570, Meridian Instruments). The ACAS 570 was configured to excite the cells at 488 nm using an argon laser. The fluorescent emission was collected at 525 nm using a 495 nm LP dichroic mirror and a 10 nm band pass filter centered at 525 nm. In all cases, data were collected every 60 sec for at least 25 min using the Kinetic program within the ACAS software. Changes in fluorescence were computed using a logistical model developed at Upjohn Laboratories. Previous studies established a linear relation between changes in fluorescence and changes in membrane potential indicating that the optical imaging protocol described is quantitative (D. Epps, M.L. Wolfe, and V. Groppi; *unpublished observations*). Control experiments established that the addition of solvents such as dimethylsulfoxide and ethanol had no effect at concentrations up to 0.8% (v/v).

Electrophysiology

Single channel and whole-cell recordings from both cardiac myocytes and individual A10 cells were obtained using the techniques of Hamill et al. (1981). A dish containing cultured cells was placed in a chamber on the movable stage of an inverted microscope equipped with phase-contrast optics. Recording pipettes were formed from soda lime glass (Blue-Dot Hematocrit Glass; Fisher Scientific, Pittsburgh, PA or Drummond Scientific, Broomall, PA) on a horizontal microelectrode puller in a three-stage process (Model P-87, Sutter Instrument, Novato, CA). Pipettes were fire-polished to a final tip diameter of approximately 0.5 μm just before use. Filled pipettes had resistances of 5–10 mΩ. Experiments were performed at room temperature (21–23°C).

Data Acquisition and Analysis

Whole-Cell Currents

Whole-cell currents on the A10 cells were obtained using a List EPC-7 (List Electronic, Darmstadt, Germany) voltage clamp. The voltage commands were provided via the output of a Metrabyte D/A converter; currents were sampled with a Data Translation DT2818 A/D converter and analyzed using a PC-386. Current records were sampled and filtered at 1 kHz, unless stated otherwise. Currents were not leak or capacity corrected.

Single Channel Recordings

Single channel currents were recorded in either the cell-attached or inside-out patch configuration (Hamill et al., 1981) using an Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, CA). The current signals were analog-filtered at a −3 dB cutoff frequency of 1 kHz through a built-in 4-pole Bessel filter and digitized onto the Winchester disk of a microcomputer (Masscomp 5450) through a 16-bit analog-to-digital converter at a sampling frequency of 5 kHz. Single channel records were analyzed by pCLAMP 5.5 software on an IBM 386SX-compatible microcomputer.

Solutions

In the single channel recording experiments on the isolated ventricular cells, the bath solution had the following composition (mM):

140 KCl, 5.5 glucose, 2 ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA-KOH), and 5 *N*-2-hydroxy-ethyl-piperazine-*N′*-2-ethanesulfonic acid (HEPES); pH 7.3. The composition of the pipette solution was (mM): 140 KCl, 1.8 $CaCl_2$, 0.53 $MgCl_2$, 5 HEPES, pH 7.3. In the whole-cell recordings made on A10 cells, the standard internal (pipette) solution with 38 nM free Ca^{2+} contained (in mM): 140 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 11 EGTA-KOH, 0.1 to 2 ATP and 10 HEPES buffered to pH 7.2. The bath solution contained (in mM): 140 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1 g/liter glucose, and 10 HEPES buffered to pH 7.4. Pipette and bath solutions were isosmotic (280 ± 10 mOs/kgH_2O). Solution osmolarities were monitored using a vapor pressure osmometer (Model 5500, Wescor, Logan, UT), ATP was added to the pipette solution and the pH readjusted immediately prior to use.

In those experiments investigating drug effects on volume-regulated Cl^- current, the bath solution contained (in mM): 140 N-methyl-D-glucamine (NMDG)-Cl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and 1 gm/liter glucose; buffered to pH 7.4. The pipette solution contained 40 NMDG-Cl, 100 NMDG-glutamate, 1 EGTA, 0.2 $CaCl_2$ (20 nM calculated free Ca), 2 $MgCl_2$ and 10 HEPES; pH = 7.2. ATP (0.1 mM) was added to the pipette solution and the pH readjusted immediately prior to use. DIDS (4′,4′-diisothiocyanatostibene-2′,2′-disulfonic acid) was purchased from Sigma.

Volume-regulated Cl^- current was activated in some experiments by exposure of the cells to a bathing solution in which the osmolarity had been reduced from 280 to 190 mOs by a decrease in the NMDG-Cl concentration. In other experiments cellular swelling and, therefore, Cl^- current activation, was alternatively brought about by increasing the concentration of NMDG-OH in the pipette solution by 70 mM bringing the pipette solution osmolarity to 350 mOs. There was no significant difference in the magnitude of the current activated by either experimental manipulation.

A bath perfusion system was used which allowed for sequential solution changes. Bath solution changes were made at the rate of 2 cc/min. In those experiments in which the bath solution was changed, the bath ground electrode was connected to the bath solution via an agar/saline bridge to prevent electrode offset potentials.

Where a multiple number of experiments were performed for a given experimental condition, data are expressed as means ± SEM with the number of experiments in parentheses.

Results

U83757 Opens Single K_{ATP} Channels in Cardiac Muscle

The efficacy of the compound U83757 as a K_{ATP} opener was established in cell-attached and excised patches from isolated rabbit ventricular myocytes. Figure 1*A* illustrates single channel currents obtained from a cell-attached patch on a cardiac ventricular myocyte in the presence of high K^+ in the bath and in the pipette solution. Under control conditions, single channels with a conductance of approximately 35 pS could be recorded at negative potentials with no activity observable at positive potentials. These baseline currents were identified as inwardly rectifying K^+ currents due to their conductance and strong inward rectification. Following the addition of U83757 (5 μM) to the bathing solution, a second population of channels with unitary currents more than twice that of the inwardly rectifying K^+ channel was observed. The second population of channels also showed prominent inward rectification with a single channel conductance of approximately 75 pS suggesting the channels might be K_{ATP}. The activation of the larger amplitude channel was not influenced by changes in membrane potential, also like K_{ATP}.

To confirm that the U83757-activated currents were K_{ATP}, inside-out patches were used to control the ATP concentration at the inner surface of the membrane patch (Fig. 1*B*). Following patch excision, single channel currents were recorded in the absence of ATP in the bath solution with a conductance similar to the U83757-activated current observed in cell-attached mode. Currents disappeared upon exposure of the cytoplasmic surface of the patch to 0.5 mM ATP. U83757 (5 μM) reversibly activated the current in the presence of ATP. Similar results were obtained in two other experiments.

U83757 Acts to Hyperpolarize Membrane Potential in Cultured Cells from the Vascular Smooth Muscle Cell Line A10

The anti-hypertensive action of compounds such as cromakalim (BRL 34915), pinacidil, minoxidil sulfate, nicroandil, and diazoxide on arterial smooth muscle is thought to be mediated by the opening of a diversity of K^+ channels giving rise to membrane hyperpolarization (for review, *see* Nelson et al., 1990). To determine whether U83757 acts as a K^+ channel opener in vascular smooth muscle cells, we performed digital fluorescent imaging experiments on A10 cells, a vascular smooth muscle cell line, in the presence of the potential sensitive dye $diBAC_4$ (3). Fluorescence data obtained in A10 cells in the presence of U83757 are presented in Fig. 2*A*. U83757 caused a pronounced loss of $diBAC_4$ (3) fluorescence, indicating that A10 cells undergo membrane hyperpolarization in the presence of the drug. The data in Fig. 2*F* demonstrate that U83757 (10 nM–1 μM) induced membrane hyperpolarization in a concentration-dependent manner with maximal effects observed at 300 nM. To determine if the hyperpolarization was due to a drug-induced increase in the open-state probability of K_{ATP} channels, we attempted to reverse the effect of U83757 with a variety of K^+ channel blockers known to inhibit K_{ATP} or calcium-activated K^+ channels (Fig. 2*B–E*). Data obtained in these experiments demonstrated that glyburide (10 μM) and tetrapropylammonium (TPrA, 1 mM) reversed the U83757-induced hyperpolarization, whereas the calcium-activated K^+ channel inhibitors tetraethylammonium (TEA, 10 mM) and charybdotoxin (ChTX, 100 nM) were without effect. Pretreatment of cells with 100 nM glyburide completely inhibited the U83757-induced membrane hyperpolarization (*data not shown*). Much

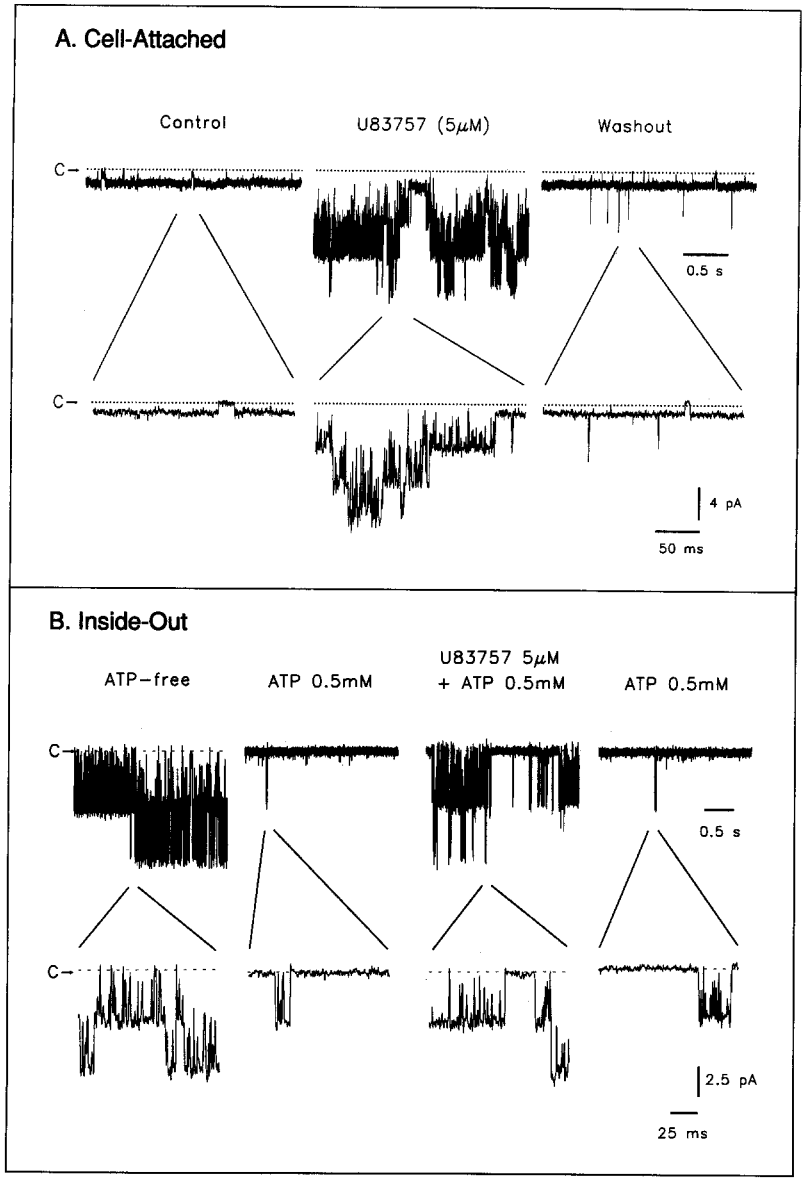

Fig. 1. Activation of K_{ATP} channel activity by U83757 in isolated rabbit cardiac ventricular myocytes. (*A*) Recording of single channel currents from a cell-attached membrane patch under control conditions, in the presence of U83757 (5 μM), and following removal of the drug. The drug was applied to the bath (extracellular) solution. Low amplitude single channel events with prolonged open times are presumed to be inwardly rectifying K^+ channels whose activation was unaffected by the drug. (*B*) Recording of single channel currents from an excised, inside-out membrane patch. Changes in the frequency of channel opening were observed through two single K_{ATP} channels recorded in the absence of ATP at the internal surface of the membrane, in the presence of 0.5 mM internal ATP, and in the presence of ATP plus 5 μM U83757, as well as upon removal of the drug in the presence of 0.5 mM ATP. A significant increase in the probability of channel opening was observed in the absence of ATP and in the presence of the drug in ATP-free solutions. Both ATP and the drug were applied to the bath (cytoplasmic) solution. The membrane potential was held at −60 mV in both *A* and *B*. The letter *C* to the left of each trace indicates the closed state of the channels.

higher concentrations of glyburide (10 μM), however, were required to reverse the U83757-induced membrane hyperpolarization. These observations are consistent with those of Bray and Quast (1992) demonstrating negative allosteric coupling between P1075 (U83757) and glyburide in binding assays to rat aorta.

U83757 Modulation of Whole-Cell Currents in Cultured A10 Cells

To determine the molecular basis for the membrane hyperpolarization seen in the fluorescence studies, we carried out whole-cell voltage-clamp experiments on A10 cells using standard pipette and bath solutions where $E_K = -82$ mV and $E_{Cl} = 0$. Figure 3 demonstrates whole-cell currents prior to and following sequential exposure of a voltage-clamped cell to 0.3 and 300 μM U83757. A concentration-dependent increase in outward current with a concomitant hyperpolarizing shift in the zero current potential in the presence of the drug was consistent with the activation of a K^+ conductance. The reversal potential of the U83757-sensitive current in Fig. 3 was −67 mV indicating that the current was primarily K^+ selective. In all cells examined, exposure to U83757 was associated with a shift in the zero current potential in the hyperpolarizing direction. The mean shift in zero current potential in seven cells was −36.5 ± 10.1 ($n = 4$) mV and −25.0 ± 9.2 mV ($n = 5$) in the presence of 300 nM and 300 μM U83757, respectively. However, of the seven cells studied in K^+-containing solutions, only three responded with an increase in outward current when exposed to the drug. In

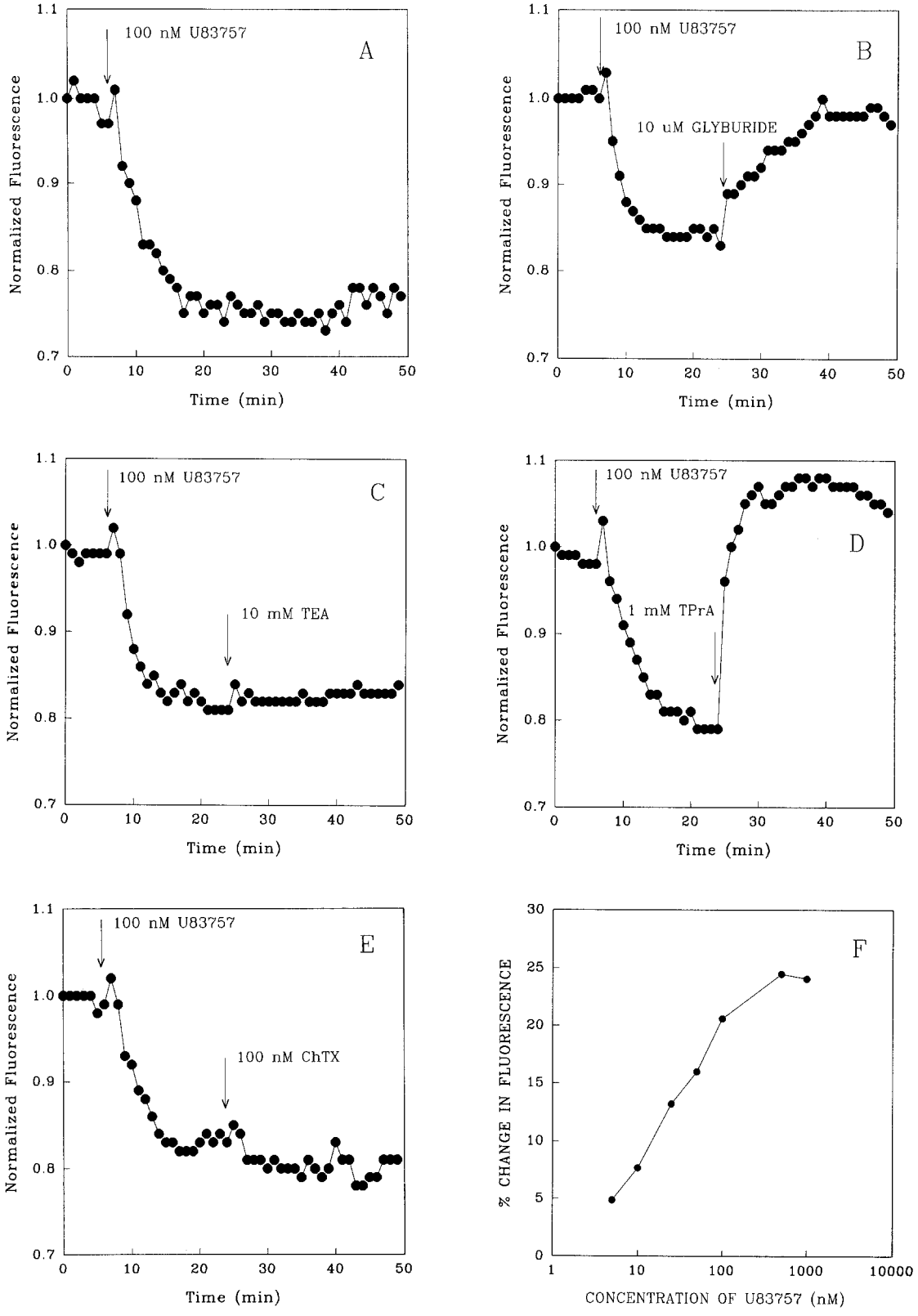

Fig. 2. U83757-induced membrane hyperpolarization in cultured A10 cells is inhibited by glyburide and TPrA but not by TEA and charybdotoxin (ChTX). (*A*) The effect of 100 nM U83757 on the membrane potential of A10 cells. In panels *B–E*, A10 cells were first treated with 100 nM U83757 and then challenged with 10 μM glyburide (*B*), 10 mM TEA (*C*), 1 mM TPrA (*D*) or 100 nM ChTX (*E*). (*F*) The response of A10 cells to 1 nM–1 μM U83757. In all cases, changes in membrane potential were measured using the voltage sensitive dye, $diBAC_4$ (3).

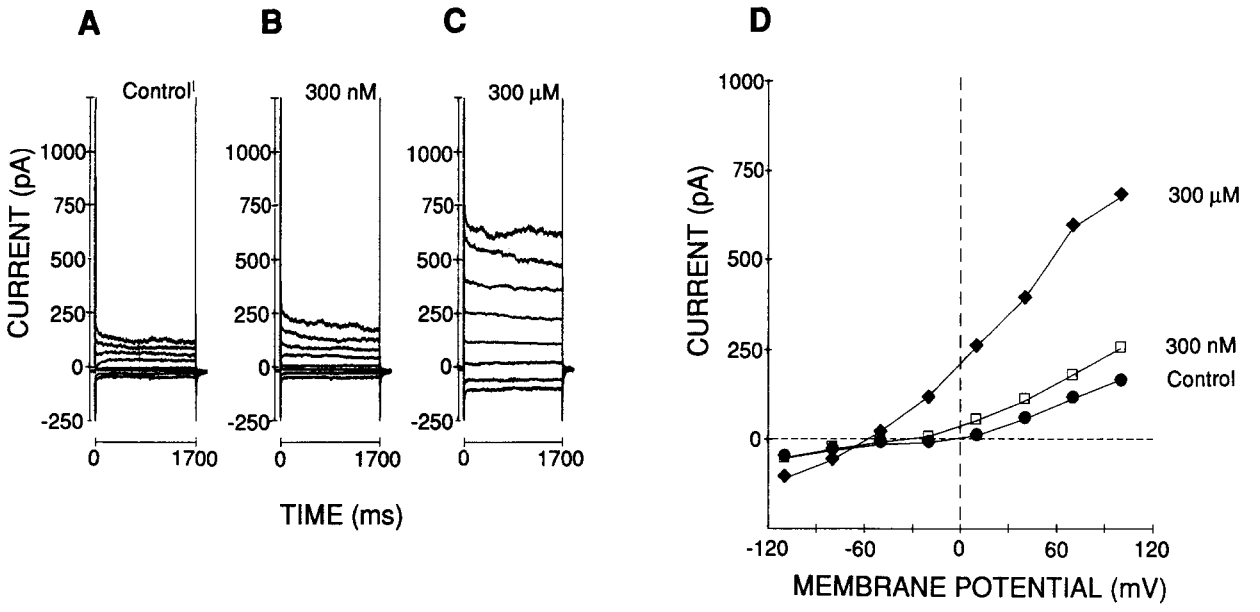

Fig. 3. U83757-induced whole-cell current activation in a cultured A10 cell. (*A*) Control currents in standard pipette and bath solutions. Currents were elicited from a holding potential of −60 mV to potentials between −110 and 100 mV. The pipette solution contained, in addition, 0.1 mM ATP. (*B*) Current recording following the addition of 300 nM U83757 to the bathing solution. (*C*) Current recording following exposure of the voltage-clamped cell to 300 μM U83757. (*D*) Current-voltage relationship for the currents depicted in *A* through *C*. The zero current potential was −7 mV for the control, −34 mV in the presence of 300 nM U83757, and −57 mV following treatment of the cell with 300 μM U83757.

those cells which responded with an increase in outward current, the mean increase in current amplitude at the maximal depolarization of 100 mV was 405 ± 50 pA which was associated with a hyperpolarizing shift in the zero current potential of −51.8 ± 4.3 mV.

In four cells, a hyperpolarizing shift in reversal potential was associated with a decrease in the magnitude of both outward and inward current as seen in Fig. 4 and similar to that observed following exposure of the voltage-clamped cell to the Cl^- channel blocker DIDS (100 μM, *data not shown*). The zero current potential was consistently near zero under control conditions in the cells which responded to U83757 with a decrease in outward current. This was consistent with the expected zero current potential in symmetrical Cl^- solutions if a Cl^- conductance predominated in the resting state. The reversal potential of the U83757 sensitive-current in those cells exhibiting a conductance decrease in response to the drug was also approximately 0 mV (mean value for two cells was 5 mV) suggesting that U83757 was inhibiting a Cl^- conductance (gCl) prominent in some cells in the resting state. Based on data from cells in which we observed a decrease in current (n = 4), we postulated that U83757 was acting simultaneously as a Cl^- channel blocker and a K^+ channel opener. We, therefore, sought to determine (i) whether we could consistently activate a Cl^- conductance in A10 cells and (ii) whether we could inhibit this conductance with the K_{ATP} opener U83757.

U83757 Inhibits a Volume-Regulated Anion Conductance in A10 Cells

A volume-regulated gCl has been reported in human colonic T84 cells (Worrell et al., 1989; Chan et al., 1992), cultured human airway epithelial cells (McCann, Li & Welsh, 1989), cell lines derived from normal and CF-airway epithelia (Wagner et al., 1991) and both T and B lymphocytes (Lewis, Ross & Cahalan, 1993). Current recordings were made prior to and following the sequential exposure of a cell to hypotonic bathing solutions (*see* Materials and Methods) in which the major permeant ion was Cl^- (Fig. 5). An outwardly rectifying current was activated following osmotic challenge (Fig. 5*B*). Current inactivation was occasionally observed during depolarizing voltage pulses to potentials greater than +50 mV (Fig. 5*B* and *C*). Voltage-dependent inactivation of volume-activated Cl^- channels has been observed in a number of investigations (Worrell et al., 1989; Solc & Wine, 1991; Wagner et al., 1991; Chan, Goldstein & Nelson, 1992; Diaz et al., 1993). The volume-regulated current was inhibited in a concentration-dependent manner by the Cl^- channel blocker DIDS (100 μM), as summarized in the Table. Upon replacement of the bath Cl^- with aspartate, the reversal potential of the whole-cell current shifted in the depolarizing direction indicating that the current was primarily Cl^- selective. The shift in reversal potential was also associated with a decrease in the conductance

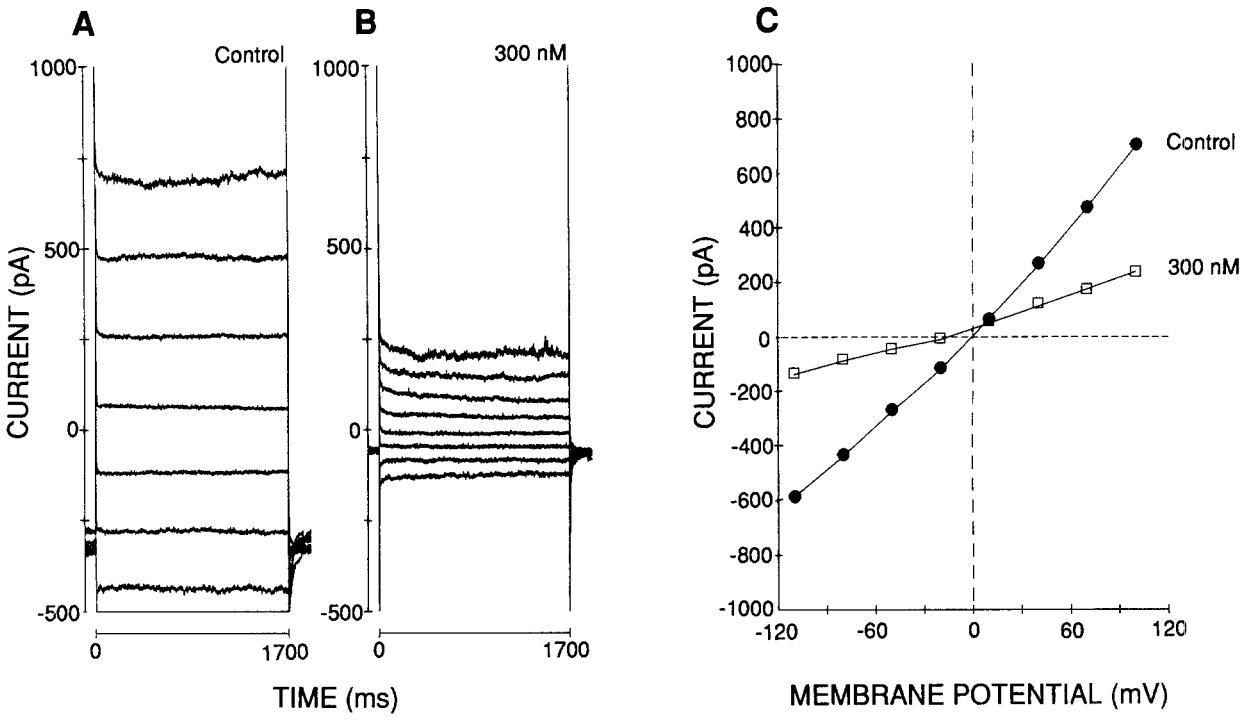

Fig. 4. Heterogeneity in A10 whole-cell currents and the response to U83757. (*A*) Whole-cell currents in standard pipette and bath solutions. The voltage protocol was the same as that described in Fig. 2. (*B*) Current recording in the presence of 300 nM U83757. (*C*) Current-voltage relationship for the currents in *A* and *B*. Note the decrease in outward and inward current in the presence of U83757. The zero current potential was −1 mV under control conditions and −15 mV in the presence of 300 nM U83757.

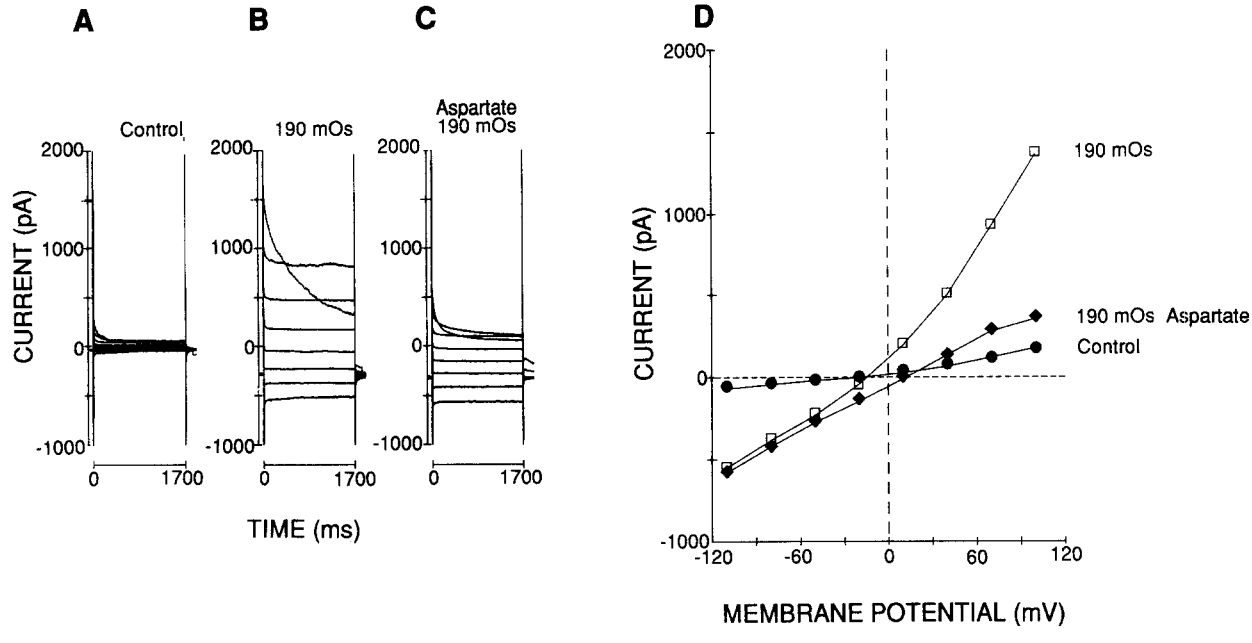

Fig. 5. Selectivity of swelling-induced Cl^- current. (*A*) Current recordings in isosmotic NMDG pipette and bath solutions. (*B*) Current recording following the exposure of the same cell to a 90 mM NMDG-Cl (190 mOs) bathing solution. (*C*) Current recording after substitution of the bathing solution Cl^- with aspartate. (*D*) Corresponding current-voltage relationship measured 20 msec following the onset of the voltage pulse. The predicted Cl^- equilibrium potential was −31 mV in these experiments. The zero current potential in isosmolar bath and pipette solutions was −26 mV. The zero current potential following current activation resultant to cellular swelling was 14 mV. Replacement of most of the Cl^- with aspartate shifted the reversal potential by 27 mV indicating that the volume-regulated conductance is primarily Cl^- selective.

Table. Comparative effect of U83757, glyburide, and DIDS on volume-regulated Cl^- current activation in cultured A10 cells

Compound	Concentration (μM)	Change in current at +100 mV (pA) ($\Delta I_{blocker}$)	$\Delta I_{blocker}/\Delta I_{control}$ (in percent)
U83757	0.3	−1,263 ± 351 (n = 7)	54.7
U83757	1.0	−1,287 ± 353 (n = 13)	53.9
U83757	300	−2,105 ± 403 (n = 5)	24.6
Glyburide	0.5	−17 ± 384 (n = 3)	99.4
U83757 + Glyburide	0.3 + 0.3	−1,275 ± 335 (n = 3)	54.3
DIDS	1	272 ± 75 (n = 8)	109.7
DIDS	100	−2,269 ± 417 (n = 3)	18.7

The mean peak current activated at +100 mV under control conditions was 4,098 ± 555 pA (n = 36). The mean volume-induced increase in current determined in control experiments ($\Delta I_{control}$) was 2,790 ± 363 pA (n = 16) where the pipette solution was 70 mOs hyperosmolar as compared to the bathing solution. This average increase was used to calculate the fraction volume-induced current remaining after U83757-induced inhibition ($\Delta I_{blocker}/\Delta I_{control}$). In experiments using standard pipette solutions and hypotonic bathing solutions (190 mOs), the increase in volume-activated current (2,946 ± 484 pA (n = 31)) was not significantly different from that seen with hyperosmolar pipette solutions.

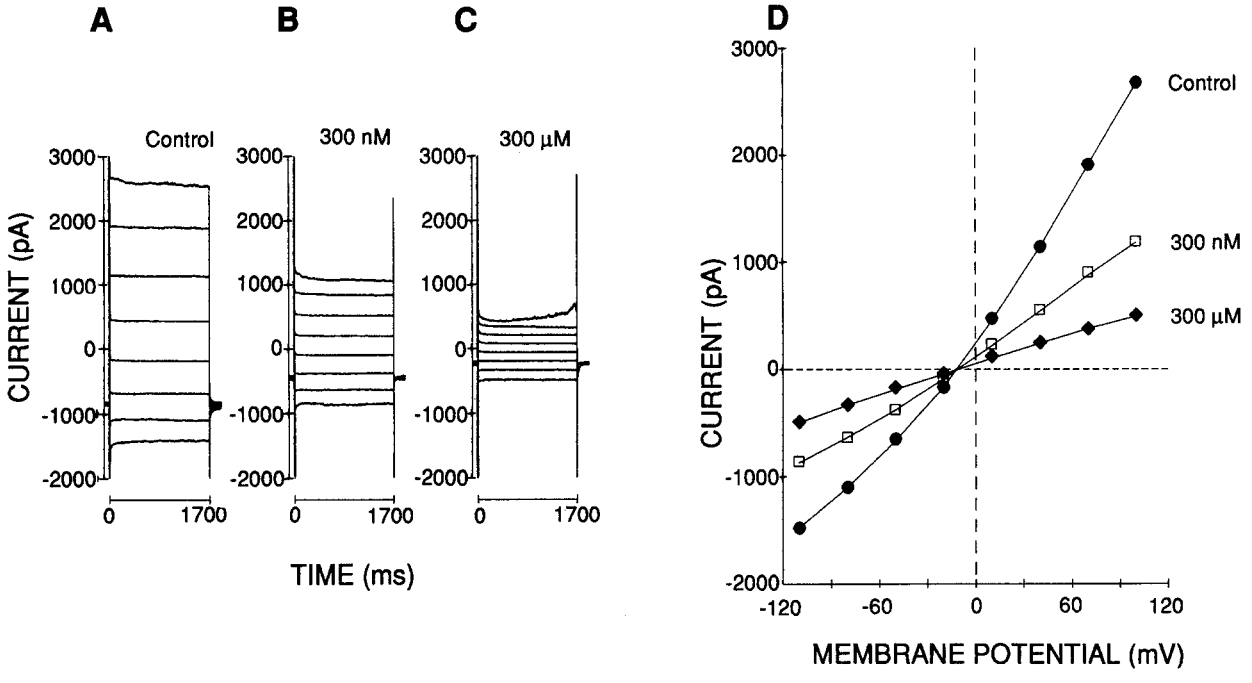

Fig. 6. U83757 inhibits volume-regulated Cl^- current. (*A*) Whole-cell current recordings in the presence of pipette and bathing solutions in which the Cl^- was the major permeant ion. Cellular swelling was induced with a pipette solution which contained a 70 mM excess of NMDG-OH. (*B*) Current inhibition in the presence of 300 nM U83757. (*C*) Further inhibition of both inward and outward current in the presence of 300 μM U83757. (*D*) Current-voltage relationship for the currents in *A* through *C*. U83757 inhibited current activation in a concentration-dependent manner throughout the voltage range without a significant change in current reversal potential. Zero current potential was −12 mV under control conditions and −10 mV following maximal current inhibition.

ward current range (Fig. 5*C*) consistent with an anion-selective current.

Once a stable level of current activation had been achieved in experiments in which Cl^- current activation was brought about by an increase in the osmolarity of the pipette solution, the bathing solution was changed to one which contained increasing concentrations of U83757. The results of a representative experiment can be seen in Fig. 6. Following volume-regulated anion current activation, U83757 reduced both outward and inward current in a concentration-dependent manner without accompanying changes in current reversal potential (Fig. 6*B, C,* and *D*). The average reversal potential of the U83757-sensitive current in asymmetrical Cl^- solutions was -14 ± 1.1 ($n = 7$). The experimentally observed potential was in agreement with the predicted value of -6 mV for a perfectly Cl^--selective current. A summary of the comparative inhibition of volume-regulated Cl^- current in A10 cells can be seen in the Table.

Discussion

Our results demonstrate that U83757, a potent analogue of pinacidil, activates K_{ATP} channels in cardiac cells and inhibits Cl^- channels in cells from the smooth muscle cell line, A10. U83757 (also termed P1075) is one of the most potent derivatives in the cyanoguanidine class of K^+ channel openers (Smallwood & Steinberg, 1988). Single channel studies on isolated cardiac myocytes established the efficacy of U83757 as an activator of K_{ATP}.

U83757, a K_{ATP} Channel Opener in Cardiac Cells, Induces Membrane Hyperpolarization in Smooth Muscle Cells

U83757-induced membrane hyperpolarization in smooth muscle cells was reversibly inhibited in the presence of compounds known to inhibit K_{ATP}. Analysis of a broad concentration range of U83757 indicated that A10 cells responded with membrane hyperpolarization at concentrations as low as 10 nM with a maximal effective drug concentration of 300 nM. The increase in membrane hyperpolarization as a function of increasing U83757 concentration (10 mM to 1 μM) was well described by a single site binding curve with a K_D of 14.3 nM. Bray and Quast (1992) developed a binding assay for U83757 in vascular smooth muscle and found that it binds to endothelium-denuded rings of rat aorta with a K_D of 6 nM similar to the K_D of 14.3 which we observed in the fluorescence studies.

Membrane hyperpolarization in the A10 cells was likely due to the activation of a K_{ATP}-like conductance. The drug response was reversed in the presence of the K_{ATP} inhibitors glyburide and TPrA. Blockers of calcium-activated K^+ channels, TEA and ChTX, were ineffective in reversing the U83757-induced hyperpolarization.

A10 Cell Line as a Model for Native Vascular Smooth Muscle Cells

Early electrophysiological studies characterizing A10 cells in culture revealed that at confluency the cells are electrically coupled and capable of generative spontaneous action potentials (Kimes & Brandt, 1976). We have demonstrated in this study that A10 cells have electrophysiological properties similar to those reported for native and cultured smooth muscle cells. A10 cells respond to K_{ATP} agonists with membrane hyperpolarization and increase in a K^+-selective current as do native vascular smooth muscle cells (Noack et al., 1992; Ibbotson et al., 1993). Volume-regulated Cl^- currents have not previously been described in native vascular smooth muscle cells. This study is the first description of volume-regulated gCl in a smooth muscle-derived cell. The large basal Cl^- currents observed in a subpopulation of the cells may represent partial activation of volume-regulated anion conductance. Alternatively, the large basal conductance may represent a Ca^{2+}-activated Cl^- current similar to that described in cultured smooth muscle cells of rat portal vein (Pacaud et al., 1989, 1992) which has been proposed to modulate action potential duration. The threshold for activation of the Ca^{2+}-activated Cl^- current in rat portal vein cells maintained in short-term tissue culture is reported to be around 180 nM (Pacaud et al., 1992). Heterogeneity in basal Cl^- conductance in A10 cells may therefore be due to differences in Ca_i.

Cl^- Channels Involved in Volume Regulation

Our study of the volume-regulated current was prompted by the observation that P-glycoprotein, a membrane-associated active transport protein that utilizes ATP hydrolysis to pump cytotoxic drugs out of cells, has been shown to be associated with the expression of a volume-regulated Cl^- channel (Gill et al., 1992; Valverde et al., 1992; Diaz et al., 1993). The simplest interpretation of this linkage is that P-glycoprotein is itself a Cl^- channel or closely associated regulatory component. In that intracellular ATP regulates both K_{ATP} channels and P-glycoprotein functioning as a volume-activated Cl^- channel, we examined the modulatory effect of U83757 on swelling-activated Cl^- current.

K^+ Channel Openers Act as Inhibitors of Cl^- Current

Recent experiments using cells expressing both human wild-type and mutant forms of the cystic fibrosis trans-

membrane conductance regulator (CFTR), a Cl^- channel that is regulated by cAMP, have shown both tolbutamide and glyburide to be effective inhibitors of whole-cell, cAMP-activated CFTR Cl^- currents (Sheppard & Welsh, 1992). A number of sulfonylureas and K^+ channel openers were investigated in the studies of Sheppard and Welsh (1992); the rank order of potency for inhibition of CFTR Cl^- currents was glyburide > BRL 38227 ≈ minoxidil sulfate > tolbutamide > diazoxide. CFTR Cl^- current inhibition was > 90% complete at glyburide concentrations of 100 μM with a half-maximal concentration of 20 μM. In the experiments described in this study, the cyanoguanidine K^+ channel opener U83757 induced half-maximal volume-regulated *g*Cl inhibition at concentrations which were approximately two orders of magnitude lower than glyburide concentrations necessary to induce half-maximal inhibition of CFTR Cl^- current activation. Glyburide (300–500 nM) did not produce inhibition of the volume-regulated *g*Cl in the smooth muscle cells nor did it prevent current inhibition induced by U83757.

Comparison of the U83757-mediated Change in Membrane Hyperpolarization and Whole-Cell Current

The volume-regulated *g*Cl in A10 cells was inhibited by U83757 over the same concentration range observed to produce membrane hyperpolarization in the fluorescent studies. The U83757-induced inhibition of the volume-regulated *g*Cl was half maximal at 1 μM, in a concentration range where the Cl^- channel blocker DIDS was ineffective in inducing current inhibition. The inhibitory effect of U83757 on volume-regulated *g*Cl was relatively flat from 0.3 to 1 μM with a significant increase in current inhibition observed at 300 μM (*see* the Table). This relationship is not well described by a single-site binding curve, but does suggest the possibility that channels or associated regulatory molecules with different affinities for U83757 may be involved in the volume-regulated *g*Cl. A two site curve with K_D's of 1 nM and 11 μM fit the inhibitory data in our studies; however, more data would be required to support this suggestion.

Our inability to observe consistent activation of a K^+ conductance in the voltage-clamped cells which would correlate well with the glyburide-sensitive U83757-induced membrane hyperpolarization observed in the fluorescent experiments might be the result of dilution of cellular cytoplasmic constituents brought about by internal dialysis with the pipette solution in the electrophysiological experiments. The activation of K_{ATP} channels by K_{ATP} agonists is dependent upon the presence of cytosolic factors (Kozlowski, Hales & Ashford, 1989; Dunne, Aspinall & Petersen, 1990) which may or may not be disrupted by internal dialysis with the pipette solution. The activation of K_{ATP} channels in pancreatic β-cells by sulfonylureas is sensitive to the cytosolic concentration of ADP, while other nucleotides are without effect (Zünkler et al., 1988). K_{ATP} channel openers are either ineffective or inhibit channel opening in the absence of Mg-ATP (Kozlowski, et al., 1989; Dunne et al., 1990). Although cytosolic Mg-ATP concentrations in our whole-cell experiments should have mimicked those found in the intact cell, it is possible that the U83757-activated *g*K in smooth muscle cells is associated with a closely associated regulatory protein which is dialyzable and disrupted in the voltage-clamp experiments.

Data obtained in the cardiac cells suggest that U83757 permeates the plasma membrane and may exert its effect on K_{ATP} channels at a cytoplasmic site. Sulfonylureas are equally effective in inhibiting K_{ATP} channels when applied to either the intracellular or extracellular membrane surface (Belles, Hescheler & Trube, 1987; Gillis et al., 1989).

It should be noted that the U83757-sensitive *g*K which we infrequently observed in the smooth muscle cells may not be due to the activation of the identical channel subtype as characterized in the cardiac cells. We were unable to observe single channel events in cell-attached or excised patches from the smooth muscle cells under the identical experimental conditions used in the studies on the isolated ventricular cells. This may well reflect a significant difference in channel density between the cell types but may also indicate that the U83757-sensitive K^+ channel in the smooth muscle cell is not identical to the K_{ATP} found in cardiac cells. The pharmacology of the reversal of the U83757-induced membrane hyperpolarization observed in the fluorescent experiments is consistent with the known drug sensitivities of K_{ATP} channels in a variety of tissues including the vasculature (Ashcroft & Ashcroft, 1990). K_{ATP} channel agonists appear to increase the open probability of a diversity of K^+ channels in vascular smooth muscle (Beech & Bolton, 1989; Gelband, Lodge & van Breemen, 1989; Standen et al., 1989; Kajioka, Kitamura & Kuriyama, 1991).

In summary, data obtained in this study demonstrate that the cyanoguanidine, U83757, activates K_{ATP} channels in cardiac preparation and a K_{ATP}-like conductance in a smooth muscle cell line. In addition, we have shown that U83757 inhibits a swelling-activated *g*Cl in smooth muscle cells. Therefore, U83757-induced membrane hyperpolarization in smooth muscle cells may well be due to the simultaneous activation of *g*K and inhibition of *g*Cl. There are at least two different models which could explain these data. In the first model, we make the assumption that the volume-regulated Cl^- and K_{ATP} channels have no regulatory proteins or structural domains in common. This assumption then demands that U83757 must bind to two distinct sites. One

site regulates the open-state probability of K_{ATP} and the other site inhibits activation of the volume-regulated Cl^- channels. In the second model, we make the assumption that the volume-regulated Cl^- channel and the K_{ATP} channel share a common regulatory protein or subunit. This model requires a single U83757 binding protein but demands that it interacts with at least two different ion channels. In support of the second model, it is important to note that Quast and colleagues (1993) have developed a binding assay for P1075 (U83757) which is functional in intact strips of rat aorta but is ineffective in isolated membranes implying perhaps a soluble U83757 binding protein. Using a similar binding assay on A10 cells, it may be possible to determine whether a similar binding protein is present in the smooth muscle cells.

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