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Membrane Potassium Channels and Human Bladder Tumor Cells: II. Growth Properties

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Abstract. These experiments were done to determine the effect of glibenclamide and diazoxide on the growth of human bladder carcinoma (HTB-9) cells in vitro. Cell growth was assayed by cell counts, protein accumulation, and ^{3}H-thymidine uptake. Glibenclamide added at 75 and 150 μM for 48 hr reduced cell proliferation. Dose-inhibition curves showed that glibenclamide added for 48 hr reduced cell growth at concentrations as low as 1 μM (IC_{50} = 73 μM) when growth was assayed in the absence of added serum. This μM-effect on cell growth was in agreement with the dose range in which glibenclamide decreased open probability of membrane K_{ATP} channels. Addition of glibenclamide for 48 hr also altered the distribution of cells within stages of the cell cycle as determined by flow cytometry using 10^{-5} M bromodeoxyuridine. Glibenclamide (100 μM) increased the percentage of cells in G_0/G_1 from 33.6% (vehicle control) to 38.3% ($P < 0.05$), and it reduced the percentage of cells in S phase from 38.3% to 30.6%. On the other hand, diazoxide, which opens membrane K_{ATP} channels in HTB-9 cells, stimulated growth measured by protein accumulation, but it did not increase the cell number. We conclude that the sulfonylurea receptor and the corresponding membrane K_{ATP} channel are involved in mechanisms controlling HTB-9 cell growth. However, K_{ATP} is not rate-limiting among the signaling mechanisms or molecular switches that regulate the cell cycle.

Key words: K^+ channels — Bladder carcinoma — Cell cycle — Glibenclamide — Charybdotoxin — Iberiotoxin — ^{3}H-Thymidine

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Introduction

Membrane K^+ channels play a significant role in controlling cell proliferation (Dubois & Rouzaire-Dubois, 1993; Wonderlin & Strobl, 1996). Nevertheless, efforts to delineate a specific mechanism for K^+ channels in this regard have been inconclusive; presumably this results from differences among the various cell types studied. For example, voltage-gated K^+ channels contribute to the control of T-lymphocyte proliferation by their role in the elaboration and secretion of interleukin-2 (IL-2), which acts as an autocrine activator (Chandy et al., 1984; Freedman, Price & Deutsch, 1992). Ca^{2+}-activated K^+ channels are involved in growth regulatory mechanisms of B-lymphocytes (Partiseti et al., 1992), and Ca^{2+}-activated K^+ channels are upregulated in *ras* transformed fibroblasts (Huang & Rane, 1993, 1994). On the other hand, all voltage-activated and Ca^{2+}-activated K^+ channels have been excluded from growth regulatory mechanisms in human melanoma and breast tumor cells (Woodfork et al., 1995; Lepple-Wienhues et al., 1996). Instead, growth of these cells depends on the function of an inwardly rectifying K^+ channel (Lepple-Wienhues et al., 1996) and a ATP-sensitive K^+ channel (Woodfork et al., 1995), respectively.

Human bladder tumor cells (HTB-9) have two predominant membrane K^+ channels: a Ca^{2+}- and voltage-dependent K^+ channel (K_{Ca}) and a ATP-sensitive K^+ channel (K_{ATP}) (Monen, Schmidt & Wondergem, 1998). The functional significance of these K^+ channels in HTB-9 cells is unknown. However, these cells grow rapidly in vitro as monolayers, and they secrete various cytokines and growth factors (Otsuka et al., 1991; Kaashoek et al., 1991). Thus, we performed the present experiments with the aim of determining whether these membrane K^+ channels affect the growth rate of HTB-9 cells. We show here that inhibition of K_{ATP} markedly

slows the rate of cell growth prior to the G_1/S phase transition of the cell cycle, and that inhibition of K_{Ca} accelerates cell growth at low cell density. Moreover, activation of K_{ATP} stimulated accumulation of cell protein, but it had no effect on cell number. Preliminary reports of these findings have appeared elsewhere (Cregan et al., 1997).

Materials and Methods

Cell Culture

Human urinary bladder carcinoma cells of the HTB-9 cell line (Mochizuki et al., 1987) were obtained from the ATCC (American Type Culture Collection, Rockville, MD) and were cultured in RPMI-1640 medium as described elsewhere (Monen et al., 1998). All organic chemicals were purchased from Sigma Chemical unless stated otherwise.

Measurement of Cell Number

HTB-9 cells were plated in 60 mm tissue culture dishes at 1×10^5 cells per dish in 5 ml medium. Cells were counted daily on a hemocytometer by trypan blue exclusion to determine cell growth. Cells grew uninterrupted until the beginning of log phase growth (day 3), at which time the medium was changed to one containing glibenclamide at concentrations indicated in the results. Glibenclamide was dissolved (10 mM) in dimethyl sulfoxide (DMSO) for a stock solution, which was diluted by culture medium to obtain desired final concentrations. DMSO was added in an equivalent amount in all control measurements to exclude effects of the solvent.

Protein Determinations and ^{3}H-Thymidine Uptake Measurements

HTB-9 cells were plated in 96-well tissue culture plates at 1×10^4 cells per well. These grew for two days, at which time the medium was aspirated and replaced with either control (DMSO) medium or medium plus either glibenclamide or diazoxide. The cells grew for an additional 48 hr, at which time the medium was aspirated and each well was rinsed with 0.2 ml of ice cold phosphate-buffered saline (PBS; pH = 7.41). PBS was replaced with 0.2 ml of 0.25 N NaOH. The plates were shaken slowly for 4 hr to solubilize cellular protein, and the amount of protein in each well was determined by the BCA protein assay (Pierce, Rockford, IL) (Smith et al., 1985).

HTB-9 cells were plated in a 96-well tissue culture plate at 2×10^3 cells/well and grew for two days. Cells were washed three times with sterile PBS to remove serum and endogenous growth factors. Peptide K^+ channel blockers, charybdotoxin (ChTx; Accurate Chem., Westbury, NY) and iberiotoxin (IbTx; LC Laboratories, Woburn, MA) were added to separate wells on the third day in culture. After 48-hr exposure to the inhibitors, these cells plus controls (serum-free) were incubated with ^{3}H-thymidine (0.5 μCi/well; spec. act. 20 Ci/mmol) for 3-hr pulse labeling. The cells were harvested using a multichannel cell harvester (Cambridge Technology, Watertown, MA) and placed onto glass fiber filters. Radioactive decay was counted by liquid scintillation spectrometry.

Cell Cycle Analysis

Vehicle control and glibenclamide-treated (100 μM) cells were grown in 60-mm tissue culture dishes. After 48-hr incubation, bromodeoxyuridine (BrdU; 10^{-5} M) was added for 1 hr. Cells were then harvested by trypsinization and fixed with 70% (v/v) ice cold ethanol for 15 m. Cells were pelleted by centrifugation and resuspended by vortex in 2N HCl (final) and incubated for 30 min at 21°C. The cells were washed with PBS and centrifuged three times, after which they were labeled with FITC-conjugated anti-BrdU for 30 m in the dark at 21°C. The cells were washed by centrifuging through an underlayer of fetal bovine serum, and 10^6 cells were resuspended in 2 ml of 10 μg/ml propidium iodide. These were filtered through nylon mesh and analyzed by a FACS as described previously (Stout & Suttles, 1992). Bivariate distributions of DNA/BrdU from 10^4 cells were analyzed for allocation into G_1, S, and G_2/M phases of the cell cycle according to procedures of Dolbeare et al. (1983) and Stout and Suttles (1988).

Statistical Analysis

Cell growth rates in control, glibenclamide-treated or diazoxide-treated cells were compared by plotting $\log_{10}$ cell number *vs.* days in culture (days 3–5). Slopes of linear functions fitted to these plots were determined by regression analysis utilizing the method of least squares. Growth rates of cells in experimental treatment groups differed from those of the control if the numerical values of the experimental slopes were outside the 95% confidence interval of the control slope. Differences among means were determined by either the paired *t*-test where applicable, or by 95% confidence interval in cases of unpaired comparisons. Dose effects also were determined either by linear regression analysis utilizing the method of least squares, or by fitting the data to a sigmoidal function by means of an iterative, nonlinear curve fit (SlideWrite; Advanced Graphics Software), which employed a Levenberg-Marquardt algorithm.

Results

HTB-9 cells grew to confluence within 5–6 days and then continued growing in multilayers. Log phase growth began after three days in culture. The effect of glibenclamide (75 and 150 μM), added at the beginning of log phase growth, is shown in Fig. 1. Increasing concentrations of glibenclamide markedly reduced the rate HTB-9 cell growth. In contrast, the ratio of trypan blue-stained cells to total cells, corresponding to all data points shown in Fig. 1 showed no functional relation with time, nor were there differences in this ratio between control and treatment groups (*not shown*). Thus, we excluded cell toxicity in accounting for the reduced growth rates with added glibenclamide. A similar decrement in HTB-9 cell growth rate occurred (*results not shown*) with increasing concentrations of either quinine (1, 10, and 100 μM) or barium (100 μM, 1 and 2 mM). Examination by light microscopy showed that the morphology of all cells exposed to a K^+ channel blocker was unremarkable compared with that of control cells, except for the Ba^{2+} treatment group, in which there occasionally appeared round, large (30–100 μm diameter) highly refractile structures with no apparent nuclear membranes

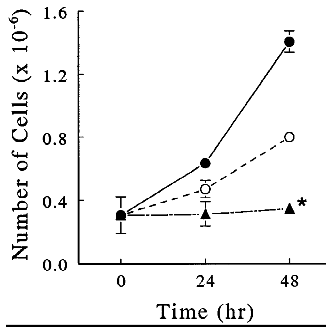

Fig. 1. Effects of glibenclamide on proliferation of cultured human bladder tumor (HTB-9) cells. ●, Control; ○, glibenclamide (75 μM); ▲, glibenclamide (150 μM). Cells were seeded at 1×10^5 cells per dish. Glibenclamide was added at the beginning of log phase growth (day 3 in culture). Each point shows the mean ± SE ($n = 3$). *Growth rate differs from control, $P < 0.05$.

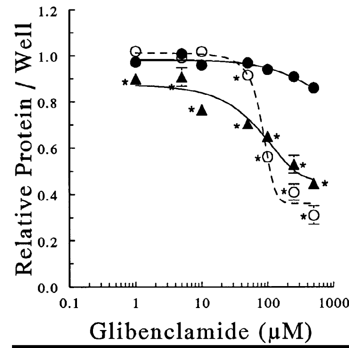

Fig. 2. Dose effects of glibenclamide on HTB-9 cell growth over 48 hr measured by total protein. ●, Control (DMSO vehicle); ○, Glibenclamide added to cells cultured with 5% fetal clone serum; ▲, Glibenclamide added to cells cultured without added serum. Glibenclamide was added at the beginning of log phase growth (day 3 in culture). All values are relative to 48-hr protein accumulation (day 5 in culture) without added inhibitor or DMSO vehicle. Relative protein/well of total cells at time zero (day 3 in culture) ≈0.3. Data were fitted to a sigmoidal plot using a Levenberg-Marquardt algorithm. Each point is the mean ± SE (●, ○ $n = 8$; ▲ $n = 4$). *Differs from control, $P < 0.05$.

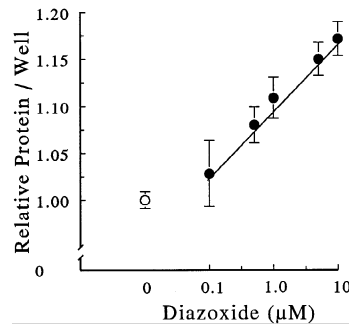

Fig. 3. Dose effect of diazoxide on HTB-9 cell growth measured by total protein. ○, Glibenclamide (10 μM); ●, Diazoxide plus glibenclamide (10 μM). Drugs were added at the beginning of log phase growth (day 3 in culture). Cells grew without added serum. Each point is the mean ± SE ($n = 5$). Slope of the linear regression differed from zero; $P > 0.05$ for H_o: slope = 0.

nor Feulgen-positive staining (B. Stiefel and R. Wondergem, *unpublished observations*).

We found that measuring total cellular protein provided greater precision in assessing cell growth. Hence, inhibitory dose effects of glibenclamide (48 hr) on cells grown in the presence serum were evident at 50 μM with an $IC_{50} = 85$ μM, Fig. 2. However, the initial inhibitory dose fell to 1 μM with an $IC_{50} = 73$ μM for cells grown without serum, Fig. 2. The apparent slope of the dose response determined in the presence of serum was considerably greater than that determined in the absence of serum.

We have shown (Monen, Schmidt & Wondergem, 1998) that glibenclamide blocks K_{ATP} channels in HTB-9 cells. We surmised that, if the inhibitory effect of glibenclamide on cell growth resulted from block of membrane K_{ATP}, activation of K_{ATP} might promote growth of HTB-9 cells. Thus, we determined the effect of diazoxide, an agent shown to open K_{ATP} channels in HTB-9 cells (Monen et al., 1998), on growth of HTB-9 cells. Diazoxide (1–10 μM) added for 48 hr increased total cellular protein of HTB-9 cells (grown without serum) over and above that of cells grown with added glibenclamide (10 μM), Fig. 3. In contrast, 10-μM diazoxide, added for 48 hr either to cells (grown without serum) alone or to cells whose growth was inhibited by 10-μM glibenclamide, had no effect on cell numbers compared with the corresponding paired controls, Fig. 4.

To exclude general toxicity of glibenclamide on HTB-9 cells, we determined whether the inhibitory effect of glibenclamide on cell growth affected HTB-9 cells in a specific phase of the cell cycle. We assumed that glibenclamide toxicity would affect cells in all phases of the cell cycle. Flow cytometry, Fig. 5, showed that a 48-hr treatment of HTB-9 cells with glibenclamide (100 μM) increased the percentage of cells in G_1/G_0 from 33.6 ± 3.1% (vehicle control) to 38.3 ± 2.2% ($P < 0.05$; $n = 4$), and it reduced the percentage of cells in S phase from 38.3 ± 0.0% (vehicle control) to 30.6 ± 2.9% ($P = 0.05$; $n = 4$). The treatment did not alter the number of cells in G_2.

Previous studies (Huang & Rane, 1993, 1994) show Ca^{2+}-activated K^+ channels play a role in regulating the growth of various cells. Moreover, we have demonstrated that K_{Ca} channels are prevalent in HTB-9 cells (Monen et al., 1998). Thus, we measured the effects of both ChTx and IbTx on HTB-9 cell proliferation with the aim of determining whether the growth inhibiting effects of broad-acting K^+ channel blockers may be attributable to their block of the K_{Ca} channels. Neither peptidyl inhibitor added for 48 hr to cells at moderate densities, which comprised protein content of >22 μg/well of a

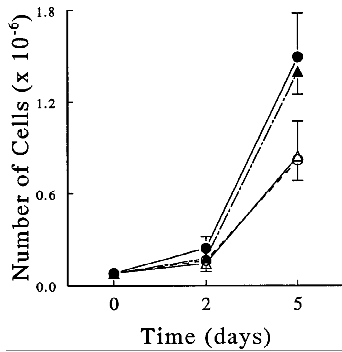

Fig. 4. Effect of either glibenclamide, diazoxide, or the combination on the proliferation of HTB-9 cells. ●, Control; ○, Glibenclamide (10 μM); ▲, diazoxide (10 μM); △, Glibenclamide (10 μM) plus diazoxide (10 μM). Cells were seeded at 1×10^5 cells per dish. Drugs were added at the beginning of log phase growth (day 3 in culture). Each point shows the mean ± SE ($n = 3$).

96-well culture plate, affected the growth of HTB-9 cells compared to vehicle-treated controls (*not shown*). Cell confluence corresponded to ~50 μg of cellular protein/well. However, at low cell density (<22 μg protein/well) both ChTx and IbTx with increasing doses stimulated incorporation of ^{3}H-thymidine into HTB-9 cells, Fig. 6.

Discussion

These results show that glibenclamide inhibits the growth in vitro of urinary bladder carcinoma cells. The inhibition increased with the drug dose, and the range of inhibitory concentrations compared well with the range of concentrations required to reduce the K^+ channel open time in HTB-9 cells (Monen et al., 1998). Taken together, these dose responses are consistent with a receptor-mediated phenomenon. An isoform of the sulfonylurea receptor (SUR) has been found in urinary bladder (Isomoto et al., 1996). Thus, we conclude that prolonged inhibition by glibenclamide of the SUR in bladder tumor cells results in reduced cell proliferation and growth. This effect of glibenclamide on cell proliferation is consistent with previous investigations, which have shown that either glibenclamide or broad acting K^+ channel blockers inhibit proliferation of breast tumor cells (Woodfork et al., 1996), melanoma cells (Nilius & Wohlrab, 1992), neuroblastoma cells (Rouzaire-Dubois & Dubois, 1990, 1991) and other tumor cells (Nilius & Droogmans, 1994).

The specific effect of glibenclamide in a growth bioassay cannot be assumed. For example, sulfonylureas also inhibit CFTR Cl^- currents (Sheppard & Welsh, 1992; Venglarik et al., 1996) with K_is in the range of the IC_{50} reported here. Moreover, sulfonylureas also affect intracellular enzymes, such as protein kinase A (Okuno et al., 1988). Nonetheless, the dose range in which glibenclamide significantly inhibited HTB-9 cell growth agreed well with the dose range that decreased open probability of membrane K_{ATP} channels (Monen et al., 1998), when both measurements were accomplished without added serum.

We attribute the apparent steepness of the inhibitory dose-response curve in the presence of serum to the high-affinity binding of glibenclamide by serum protein. It is well documented that serum proteins, particularly albumin, bind sulfonylureas (Crooks & Brown, 1974; Brown & Crooks, 1974), and the percentage of free drug not bound by albumin increases markedly in the range of 50–100 μM (Panten et al., 1989). Hence, the steep slope of the dose-response curve with an IC_{50} of 85 μM for glibenclamide inhibition of cells grown with added serum most likely reflects the complex binding of glibenclamide to serum protein and the SUR. In contrast, the significant inhibition by glibenclamide at lower doses with an IC_{50} of 73 μM for HTB-9 cells grown in the absence of serum most likely results from glibenclamide binding only to the SUR.

We exclude general toxicity of glibenclamide in accounting for its inhibition of cell proliferation, because glibenclamide-treated cells were blocked prior to the G_1/S phase transition of the cell cycle. From this we conclude that block of membrane K_{ATP} channels affects regulatory mechanisms that control early progression through the cell cycle. We contend that toxic effects of glibenclamide would have affected all cells, regardless of their phase in the cell cycle. Hence, there would have been no difference in the distribution into various stages of the cell cycle of control cells compared with glibenclamide-treated cells. Woodfork et al. (1995) used similar reasoning to distinguish specific from nonspecific effects of various K^+ channel blockers, including glibenclamide, in inhibiting growth of breast carcinoma cells.

The diminution of cell proliferation by glibenclamide was evident over days; whereas, the time course for its inhibition of membrane K^+ channels and currents was immediate. Thus, we infer a complex link between functional membrane K_{ATP} channels and anabolic metabolism related to cell growth. Moreover, the differential effects of diazoxide on cell proliferation and protein synthesis indicate that although opening of K_{ATP} may stimulate the growth of protein accumulation, the function of these channels are not rate-limiting for the mechanisms controlling cell proliferation.

The functional role of K_{ATP} in cellular mechanisms that affect cell growth and proliferation remains unclear. We cannot exclude the possibility that K_{ATP} channels are involved in cytokine secretion and, thereby, affect HTB-9 cell growth; however, we have not examined this. Nevertheless, an emerging literature suggests a significant involvement of inwardly rectifying K^+ channels in growth and differentiation of various type of cells. Ce-

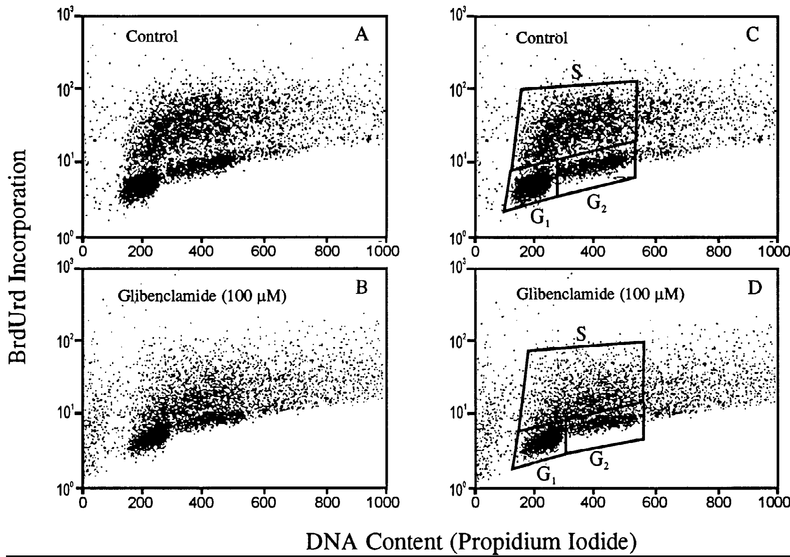

Fig. 5. Bivariate distribution of DNA content (propidium iodide) and BrdUrd incorporation. (*A*) Vehicle-treated control HTB-9 cells. (*B*) Cells treated for 48 hr with glibenclamide (100 μM). (*C*) Brackets according to the phases of the cell cycle of the distribution of cells shown in *A*. (*D*) Brackets according to the phases of the cell cycle of the distribution of cells shown in *B*.

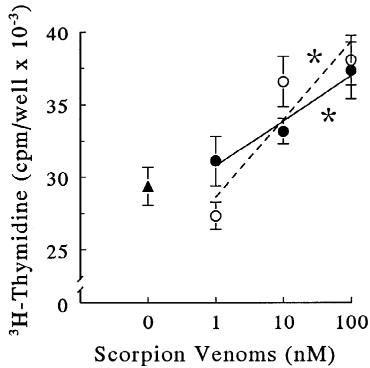

Fig. 6. ^{3}H-thymidine uptake into HTB-9 cells vs external concentration of added scorpion venom peptides, charybdotoxin (●), iberiotoxin (○) and vehicle-treated control (▲). Peptides were present for 48 hr prior to 3-hr pulse with ^{3}H-thymidine. *Regression coefficient differs from zero; $P > 0.05$ for H_o: slope = 0.

sium blockade of inwardly rectifying currents in neuroblastoma cells inhibits their integrin-mediated neurite outgrowth (Arcangeli et al., 1993), as well as the phosphorylation of $pp125^{FAK}$ and associated proteins (Bianchi et al., 1995). In contrast, activation of glibenclamide-sensitive K^+ channels in follicular cells promotes *Xenopus* oocyte maturation (Wilbrand, Honore & Lazdunski, 1992). Moreover, minoxidil, in the absence of streptomycin, potentiates the mitogenic effects of fetal calf serum in vitro on NIH 3T3 fibroblasts by opening K^+ channels, and it also is able to potentiate the mitogenic effects of platelet derived growth factor and insulinlike growth factor (Sanders et al., 1996).

We conclude by exclusion that growth inhibition of HTB-9 cells by either glibenclamide, Ba^{2+} or quinine does not result from their potential inhibitory effects on K_{Ca} channels. These channels are abundant in HTB-9 cells (Monen et al., 1998). Nonetheless, the petidyl scorpion venoms ChTx and IbTx, which markedly inhibit K_{Ca} channels in HTB-9 cells (Monen et al., 1998), did not inhibit cell growth. In contrast, they stimulated growth of HTB-9 cells at low density. Whether this growth enhancement is attributable to block of K_{Ca} channels by these agents or to some heretofore unknown peptide effect cannot be determined from these results.

In summary, the present findings show that membrane K_{ATP} channels are linked to mechanisms that control cell proliferation of human bladder carcinoma cells. HTB-9 cells proliferate rapidly, and glibenclamide, in a range of concentrations that blocks K_{ATP} channels in these cells, inhibits their progression through the G_1 phase of the cell cycle. Activation of K_{ATP} by diazoxide increased protein synthesis by HTB-9 cells; however, diazoxide did not stimulate cell proliferation per se. Thus, we conclude that glibenclamide's block of K_{ATP} in HTB-9 cells is not toxic but prevents the normal progression through the cell cycle. However, open K_{ATP} channels are not rate limiting in their role in controlling HTB-9 cell proliferation.

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