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Endothelial Cells Contain a Glycine-Gated Chloride Channel

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Abstract: *Glycine inhibited growth of B16 melanoma tumors in vivo most likely because of the inhibition of angiogenesis. Here, the hypothesis that the anticancer effect of glycine in vivo is due to expression of a glycine-gated Cl^- channel in endothelial cells was tested. First, the effects of glycine on vascular endothelial growth factor-induced increases in intracellular Ca^{2+} concentration in a bovine endothelial (CPA) cell line were studied. Vascular endothelial growth factor (1 ng/ml) increased intracellular Ca^{2+} concentration, with peak values reaching 141 ± 11 nM. Glycine blunted this increase dose dependently. Furthermore, the inhibitory effects of glycine were prevented by 1 μM strychnine, a glycine receptor antagonist, or when cells were incubated in Cl^--free buffer. Moreover, glycine increased influx of ^{36}Cl into CPA cells ~10-fold; this reaction was also strychnine sensitive. Furthermore, mRNA similar to the β-subunit of the glycine-gated Cl^- channel from spinal cord was identified in endothelial cells by reverse transcription-polymerase chain reaction. In addition, Western analysis using antibody for the glycine receptor demonstrated expression of the β-subunit of the glycine receptor. Importantly, glycine diminished serum-stimulated proliferation and migration of endothelial cells. Collectively, these data indicate that the inhibitory effect of glycine on growth and migration of endothelial cells is due to activation of a glycine-gated Cl^- channel. This hyperpolarizes the cell membrane and blocks influx of Ca^{2+}, thereby minimizing growth factor-mediated signaling.*

Introduction

Angiogenesis plays a major role in the progressive growth of solid tumors, which are dependent on the supply of oxygen and nutrients from new blood vessels (1). Indeed, inhibition of angiogenesis is an important target for tumor treatment (2). Angiogenesis is a multistage process that involves release of angiogenic factors from several cell types, including tumor cells, proliferation and migration of endothelial cells, and synthesis of the vascular basement membrane (3). The vascular endothelial growth factor (VEGF) family of cytokines plays a central role in these processes (4). VEGF is secreted by various tumor cells, is expressed by several normal cells during development, and has a highly specific mitogenic effect on endothelial cells (5,6). Activation of the VEGF receptor has been shown to result in tyrosine phosphorylation, activation of phospholipase C-γ, and increases in inositol 1,4,5-trisphosphate and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Moreover, VEGF-induced cell proliferation is dependent on $[Ca^{2+}]_i$ (7,8).

Glycine, a simple nonessential amino acid, is an inhibitory neurotransmitter in the central nervous system that acts on a glycine-gated Cl^- channel (9). Recently, a similar Cl^- channel was discovered in Kupffer cells (10,11), alveolar macrophages (12), and neutrophils (13). In vitro, addition of glycine inhibits endotoxin-induced increases in $[Ca^{2+}]_i$ and release of tumor necrosis factor-α by Kupffer cells (10,12). The mechanism of this effect involves hyperpolarization of the cell membrane by increased Cl^- influx. This makes voltage-dependent Ca^{2+} channels more difficult to open and prevents increases in $[Ca^{2+}]_i$ theoretically from a variety of agents (10).

Dietary glycine blunted the growth of B16 melanoma tumors in mice (14) and the development of liver tumors caused by the nongenotoxic carcinogen and peroxisome proliferator WY-14643 in rats (15). It was hypothesized that the effect of glycine in these in vivo models was due to inhibition of proliferation of endothelium, thus preventing neovascularization and growth of tumors. Indeed, proliferation of endothelial cells in vitro has been shown to be inhibited by glycine (14). Therefore, the purpose of this study was to determine whether endothelial cells contain a glycine-gated Cl^- channel.

Materials and Methods

Cell Culture

VEGF was obtained from Chemicon (Temecula, CA) and strychnine from Sigma Chemical (St. Louis, MO). Other chemicals were of analytic grade and were obtained from standard commercial sources. Bovine endothelial (CPA) cells were obtained from the University of North Carolina Tissue

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Culture Facility (original source American Type Culture Collection, CCL 207). Cells were seeded onto 25-mm glass coverslips in 60-mm plastic dishes at a density of 20,000 cells/ml in minimum essential medium (MEM; GIBCO BRL) supplemented with 10% fetal bovine serum, antibiotics, and nonessential amino acids (100 μM alanine, asparagine, aspartic acid, glutamic acid, proline, and serine) in the absence or presence of 0.1–10 mM glycine. Cells were maintained at 37°C in a humidified 95% air-5% CO_2 atmosphere. To test whether glycine changes morphology of CPA cells, they were cultured in MEM as described above without or with 3 mM glycine for up to 7 days, and light microscopy was performed daily. No changes in morphology were observed (data not shown). For measurements of $[Ca^{2+}]_i$, cells were cultured for 24 h after they were plated. For proliferation assays, cells were trypsinized, seeded onto 60-mm tissue culture dishes (Corning) at a density of 5×10^4 cells/dish, and cultured in MEM as described above. After 24 h, medium was changed and cells were incubated in the absence or presence of 1, 3, 5, and 10 mM glycine without or with VEGF (1 ng/ml). On Days 2, 4, 6, and 8, cells were trypsinized and counted. All experiments were performed in triplicate.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured fluorometrically using the fluorescent Ca^{2+} indicator dye fura 2 and a microspectrofluorometer (InCyt Im2, TM Imaging, Cincinnati, OH) interfaced with an inverted microscope (TMS-F, Nikon, Japan). Endothelial cells were incubated in modified Hanks' buffer (115 mM NaCl, 5 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5.6 mM glucose, 0.8 mM $MgSO_4$, 1.26 mM $CaCl_2$, 15 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4) containing 5 μM fura 2-acetoxymethyl ester (Molecular Probes, Eugene, OR) and 0.03% Pluonic F127 (BASF, Wyandotte, MI) at room temperature for 60 min. Coverslips plated with endothelial cells were rinsed and placed in chambers with buffer at room temperature. Changes in fluorescence intensity of fura 2 at excitation wavelengths of 340 and 380 nm and emission at 510 nm were monitored in individual cells. Each value was corrected by subtracting the system dark noise. $[Ca^{2+}]_i$ was determined from the following equation: $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)]/(F_o/F_s)$, where F_o/F_s is the ratio of fluorescent intensities evoked by 380-nm light from fura 2 pentapotassium salt loaded in a Ca^{2+} buffer (C-3008, Molecular Probes), R is the ratio of fluorescent intensities at excitation wavelengths of 340 and 380 nm, and R_{max} and R_{min} are calculated from a standard curve. The values of these constants were determined at the end of each experiment, and a dissociation constant (K_d) of 135 nM was used (16).

Measurement of ^{36}Cl Uptake by Endothelial Cells

Assays for uptake of ^{36}Cl employed an adaptation of a method for neurons described by Schwartz et al. (17) modified by Morrow and Paul (18). Briefly, endothelial cells (2×10^6 cells/ml) were plated on glass coverslips and incubated in glycine-free MEM (see above) for 24 h. Medium was replaced with buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 118 mM NaCl, 4.7 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 10 mM glucose) and allowed to equilibrate for 10 min at room temperature. Coverslips were gently blotted dry and incubated in a petri dish with 2 ml of buffer containing 2 μCi/ml ^{36}Cl in the presence of 1 mM glycine and/or 1 μM strychnine for 5 s, a time point where influx of Cl^- was linear. Strychnine was dissolved in dimethyl sulfoxide (final concentration 0.002%), which had no effect on ^{36}Cl movement in these studies. Cl^- flux was terminated by washing the coverslip with ice-cold buffer for 3 s and again for 7 s (18). Coverslips were placed in scintillation vials, biodegradable scintillation solution (10 ml) was added, and radioactivity was determined by scintillation spectroscopy. Counts were normalized to the amount of protein determined by the Lowry assay (19).

Reverse Transcription-Polymerase Chain Reaction and Western Blotting

Total RNA from cultured endothelial cells and rat spinal cord was isolated by phenol-chloroform extraction with 1 mM guanidinum isothiocyanate as described previously (20). cDNA was synthesized using avian myeloblastosis virus reverse transcriptase following the manufacturer's recommendations. cDNA was added to a standard polymerase chain reaction (PCR) mixture containing 1 mM dNTPs, 0.4 μM primers, and 1.25 U of DNA polymerase, and amplification was initiated for 4 min at 94°C, followed by 40 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The amplified products were analyzed by 1.4% agarose gel electrophoresis.

Crude membranes from endothelial cells and rat spinal cord were obtained by differential centrifugation after lysis in Triton X-100 buffer as described previously (21). Briefly, CPA cells were washed in cold phosphate-buffered saline and harvested by scraping in phosphate-buffered saline after culture. Rat spinal cord and CPA cells were pelleted by centrifugation at 500 *g* and resuspended in lysis buffer (150 mM NaCl, 25 mM Tris, and 1.0% Triton X-100 with protease inhibitor cocktail). The samples were agitated for 10 min at 4°C for cell lysis, then the lysate was centrifuged at 12,000 *g* to pellet the nuclei and cellular debris. The supernatant was collected and was the source of membrane protein for Western blotting. The protein was analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose (Hybond ECL, Amersham) using a semidry transfer in 20% methanol. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TTBS) for 1 h and blotted for 6–8 h with a 1:100 dilution of mouse anti-rat glycine receptor subunit 4α monoclonal antibody (Cederlane, PQ, Canada) in 1% nonfat dry milk-TTBS. The membrane was then blotted for 2 h with a 1:5,000 dilution of goat anti-mouse IgG-horseradish peroxidase-conjugated antibody (Boehringer, Mannheim, Germany) in 1% nonfat dry milk-

TTBS. The membrane was washed three times for 10 min between blotting steps with TTBS. After it was incubated in secondary antibody and washed, the protein was detected by enhanced chemiluminesence (Amersham) and exposure to X-ray film (Kodak, Rochester, NY).

Endothelial Cell Migration Assay

CPA cells were preincubated in modified MEM as described above in the absence or presence of 3 mM glycine for 48 h. The migration of endothelial cells was quantitated using a Transwell culture chamber (Costar, Cambridge, MA), where the upper and lower culture chambers are separated by a polycarbonate filter with 8-μm pores. Each membrane was precoated with rat tail collagen (30 μg/ml; Collaborative Biochemical Research) in 0.2 M acetic acid for 24 h before each chemotactic assay. Cultured CPA cells without or with 3 mM glycine were trypsinized and suspended at 1×10^5 cells/ml. A volume of 50 μl of the CPA cell suspension was seeded in the upper chamber. The culture medium without or with 3 mM glycine containing 10% serum was placed in the bottom chamber. After 5 h of incubation at 37°C, the filters were removed and cells on the upper side of the filter were removed by scraping. Cells that migrated to the bottom of the filter were fixed in methanol and stained with Diff-Quick staining solution (Dade International, Miami, FL). The number of migrating cells was determined microscopically by counting an average of 10 high-power (×200) fields of constant area per well.

Statistics

Values are means ± SEM; $n = 4$–5 in each group. Treatment groups were compared using analysis of variance on ranks with Dunn's post hoc test or analysis of variance with Bonferroni's post hoc test where appropriate. $P < 0.05$ was selected before the study to define statistical differences between groups.

Results

Addition of VEGF to cultured endothelial cells causes a rapid increase in $[Ca^{2+}]_i$ (22). Indeed, after the addition of VEGF (1 ng/ml) to endothelial cells in vitro, $[Ca^{2+}]_i$ levels increased quickly to 141 ± 11 nM from a basal level of 10 ± 1 nM and then rapidly returned to basal levels within 3 min, confirming work by Brock et al. (7) (Fig. 1). The dose of VEGF (1 ng/ml) was determined to be maximal in pilot studies (data not shown). Glycine (1 mM), when added before VEGF to CPA cells cultured in a glycine-free medium, abolished the increase in $[Ca^{2+}]_i$ due to VEGF. The effect of glycine on VEGF-stimulated increases in $[Ca^{2+}]_i$ was dose dependent, with a 50% inhibitory concentration of ~0.3 mM (Fig. 2). Similar data were obtained using fibroblast growth

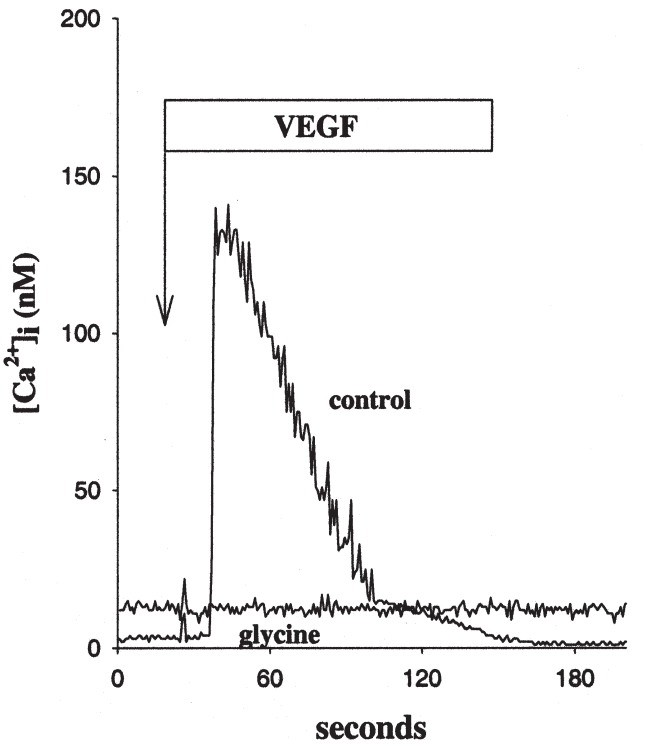

Figure 1. Effect of glycine on vascular endothelial growth factor (VEGF)-induced increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured endothelial cells. $[Ca^{2+}]_i$ was measured in cultured endothelial cells fluorometrically using fluorescent Ca^{2+} indicator fura 2. VEGF (1 ng/ml) was added in Hanks' balanced salt solution; 1 mM glycine was added in modified Hanks' balanced salt solution 3 min before VEGF. Typical traces.

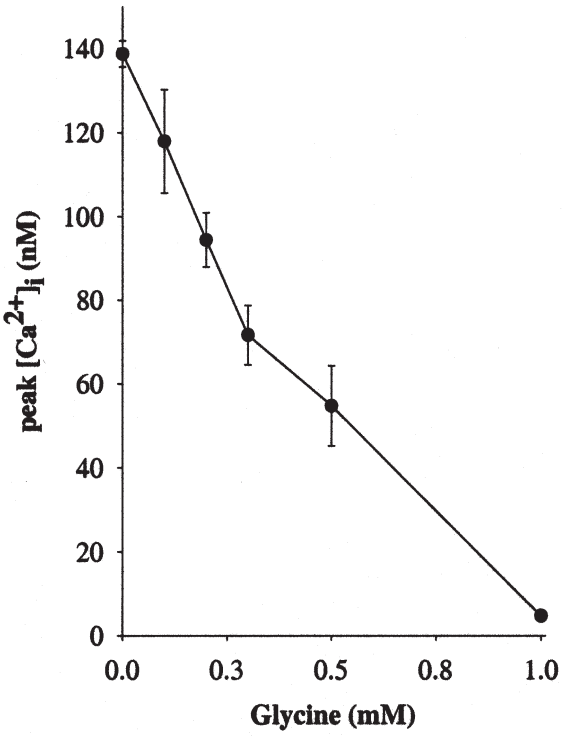

Figure 2. Glycine inhibits VEGF-induced increases in $[Ca^{2+}]_i$ in a dose-dependent manner. Experimental conditions are described in Fig. 1 legend. Endothelial cells were incubated with glycine for 3 min before addition of VEGF (1 ng/ml), and peak values of $[Ca^{2+}]_i$ were plotted. Glycine concentration was varied as indicated. Values are means ± SE of 31 individual measurements. *, $P < 0.05$ [analysis of variance (ANOVA) on ranks with Dunn's post hoc test].

factor (FGF). Addition of FGF (1 ng/ml) to cultured endothelial cells caused a rapid increase in $[Ca^{2+}]_i$, which was abolished by 1 mM glycine (data not shown).

Glycine acts as an inhibitory neurotransmitter in the central nervous system via activation of a glycine-gated Cl^- channel, which increases intracellular Cl^- and hyperpolarizes the plasma membrane (23). The hypothesis that the inhibitory effect of glycine on VEGF-stimulated increases in $[Ca^{2+}]_i$ in endothelial cells occurs via mechanisms dependent on a glycine-gated Cl^- channel was tested. First, because it is known that low doses of strychnine antagonize the glycine receptor in the central nervous system and hepatic macrophages (10,24), low concentrations of strychnine (1 μM) were added before glycine. Indeed, the inhibitory effect of glycine on the elevation of $[Ca^{2+}]_i$ by VEGF was blocked by 1 μM strychnine (Fig. 3A). Second, cultured endothelial cells were incubated in Cl^--free buffer, where equimolar amounts of gluconate were substituted for Cl^- for 2 min before the addition of 1 mM glycine. Under these conditions, the VEGF-induced increase of $[Ca^{2+}]_i$ was not inhibited by glycine, with peak values reaching 106 ± 9 nM (Fig. 3B). Thus the inhibitory effect of glycine was dependent on extracellular Cl^-. To further test the idea that glycine causes movement of Cl^- from the extracellular to the intracellular space, ^{36}Cl was used. Accordingly, endothelial cells were incubated with ^{36}Cl in glycine-free medium, and 1 mM glycine was added. Indeed, glycine increased ^{36}Cl influx into endothelial cells ~10-fold (Fig. 4), an effect that was nearly completely blocked by strychnine.

The glycine receptor is known to be a pentameric assembly of three α-subunits and two β-subunits. For the α-subunit, four isoforms exist, whereas only one isoform exists for the β-subunit (25). Thus it is reasonable to determine whether the β-subunit is expressed in CPA cells. Total RNA was isolated from cultured endothelial cells and rat spinal cord as a positive control. RT-PCR amplification of endothelial cell mRNA revealed a 124-bp fragment (Fig. 5A, Lane 4), as predicted from the known sequence of the spinal cord glycine receptor β-subunit (Lane 2). Furthermore, the PCR-amplified fragment was purified, sequenced, and found to be similar to the β-subunit of the spinal cord glycine receptor (data not shown). Additionally, Western blot for spinal cord and CPA cell membrane extracts was performed using antiglycine receptor subunit 4α monoclonal antibody, which detects the glycine receptor in the brain (26). In purified spinal cord membranes, the ~58-kDa β-subunit of the glycine receptor was detected (Fig. 5B, Lane 1). Moreover, in purified CPA cell membranes, a similar ~58-

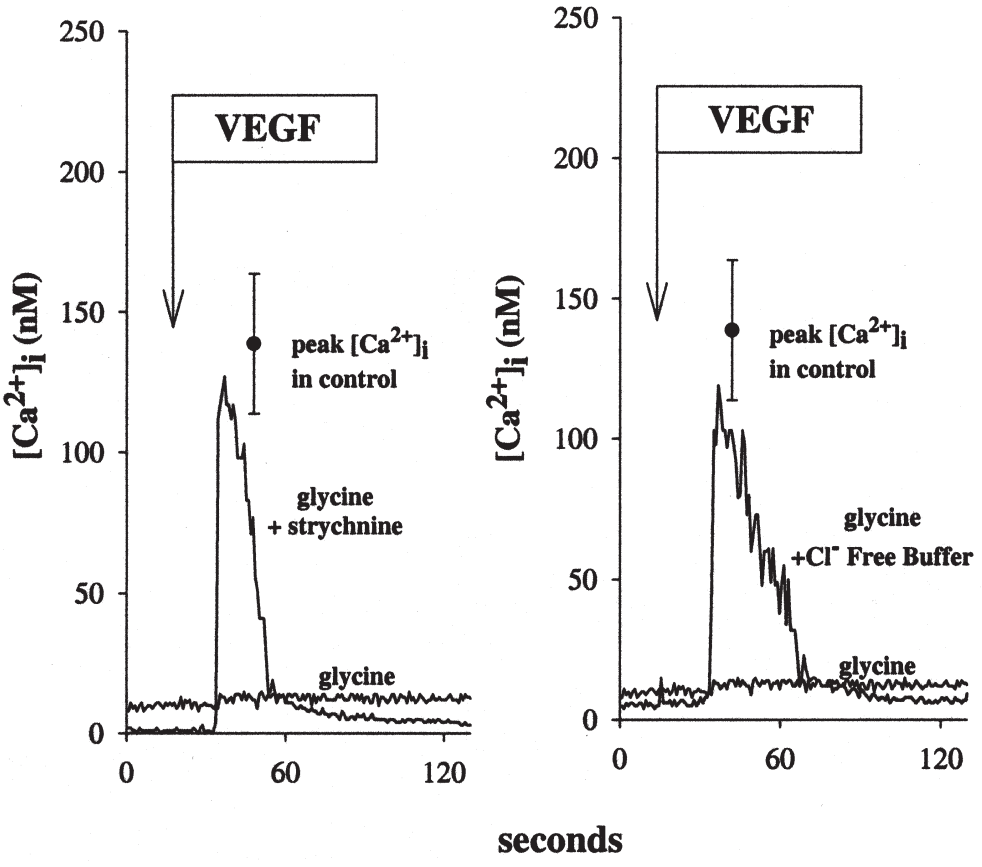

Figure 3. Effect of Cl^--free buffer and low-dose strychnine on effects of glycine on $[Ca^{2+}]_i$. Experimental conditions are described in Fig. 1 legend. A: 1 μM strychnine was added to buffer 3 min before 1 mM glycine. After incubation with strychnine and glycine for 3 min, VEGF (1 ng/ml) was added. B: endothelial cells were incubated in Cl^--free buffer by substitution with gluconate for 2 min before addition of 1 mM glycine. After 3 min, VEGF (1 ng/ml) was added. Typical traces.

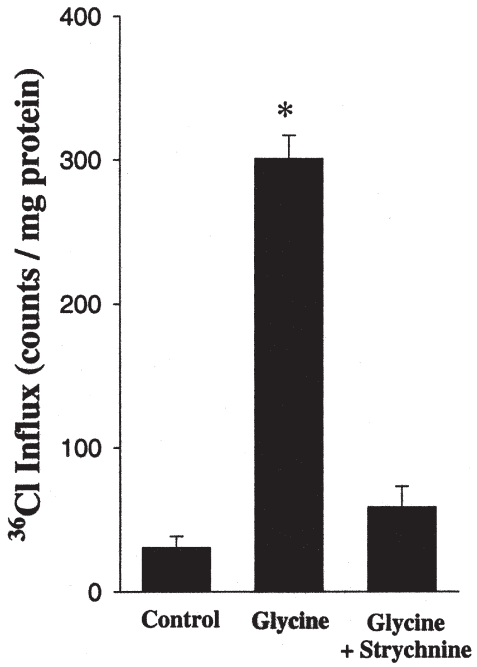

Figure 4. Effect of glycine and low-dose strychnine on ^{36}Cl influx in endothelial cells. Cultured endothelial cells were incubated with ^{36}Cl (2 μCi/ml) in presence or absence of 1 mM glycine or 1 μM strychnine. Amount of intracellular ^{36}Cl was measured. Values are means ± SEM; $n = 8$ in each group. *, $P < 0.05$ (ANOVA on ranks and Dunn's post hoc test).

kDa band was detected (Fig. 5B, Lane 2). Therefore, it is concluded that endothelial cells express the β-subunit of the glycine receptor.

When 10 mM glycine was added to cultured endothelial cells, cell growth was inhibited in a time-dependent manner (Fig. 6A). Furthermore, when VEGF (1 ng/ml) was added, glycine produced an antiproliferative effect (Fig. 6B). Significant differences between groups in the presence and absence of VEGF were observed as early as Day 4 of culture. Glycine blunted cell proliferation in a dose-dependent manner with or without VEGF (1 ng/ml; Fig. 6, C and D). FGF (1 ng/ml) also stimulated cell proliferation, an effect inhibited by glycine (data not shown).

As reported previously, cultured endothelial cells migrate because of the chemotactic action of growth factors present in serum (27). Glycine (3 mM) added to the culture medium inhibited serum-induced migration of endothelial cells by ~50% (Fig. 7), suggesting that glycine suppresses neovascularization of tumors, at least partially, by inhibiting endothelial cell migration.

Discussion

It is generally recognized that >2-mm-diameter tumors require de novo development of vasculature to provide oxygen and nutrients to maintain further growth (28). Tumor angiogenesis is evident during dysplasia or in situ carcinoma, the early stages of tumor development, and is initiated by positive mediators produced directly by malignant cells or by host cells recruited to the peritumoral space (29,30). The effect of angiogenic factors on endothelial cell behavior has been extensively studied using in vivo cultured endothelial cells of different origin (31). For instance, it was shown recently that spontaneous growth of endothelial cells was inhibited dramatically by glycine (14). Furthermore, dietary supplementation with glycine was shown 1) to slow growth of B16 melanoma cells transplanted to the mouse (14) and 2) to prevent development of liver tumors caused by the peroxisome proliferator WY-14643, a rodent nongenotoxic carcinogen (15).

The question then arises, how can glycine have such beneficial effects? Recent work from this group suggests that it may have a universal inhibitory effect via activation of a glycine-gated Cl^- channel, which disrupts Ca^{2+}-dependent cell-signaling mechanisms (32). Indeed, VEGF-induced proliferation and migration of endothelial cells, a central process in tumor angiogenesis, is dependent on $[Ca^{2+}]_i$ (8). Therefore, it was hypothesized that endothelial cells express a glycine-gated Cl^- channel similar to that described in neurons and several other cell types (9,10). Indeed, pharmacological and molecular data in support of this hypothesis were obtained. First, glycine blocked VEGF-stimulated increases in $[Ca^{2+}]_i$ in endothelial cells (Figs. 1 and 2). Furthermore, the inhibitory effect of glycine was reversed by low concentrations of strychnine, an antagonist of the receptor (Fig. 3A). Second, no effect of glycine was observed when cells were incubated in a Cl^--free buffer (Fig. 3B), and addition of glycine caused transmembrane movement of ^{36}Cl (Fig. 4). For direct molecular evidence, a 124-bp fragment nearly identical to the β-subunit of the spinal cord glycine receptor was identified in endothelial cells by RT-PCR (Fig. 5A). Furthermore, expression of the glycine receptor was also demonstrated by Western blot analysis (Fig. 5B). Collectively, pharmacological and molecular data provide strong evidence for the presence of a glycine-gated Cl^- channel in endothelial cells.

Proliferation of endothelial cells is a key step in the process by which new blood vessels grow from established ones (33), and it was suggested that glycine may inhibit angiogenesis by preventing endothelial cell proliferation. To test the hypothesis that antiproliferative effects of the nutrient glycine observed in a B16 melanoma tumor model (14) are due to inhibition of growth factor-dependent proliferation and migration of endothelial cells, thus slowing neovascularization of the tumor, a series of experiments were performed. Indeed, glycine inhibited endothelial cell proliferation in a dose-dependent manner in these studies (Fig. 6). Furthermore, growth factor-induced migration was also blunted significantly by glycine (Fig. 7). Glycine inhibits agonist-induced increases in $[Ca^{2+}]_i$ in endothelial cells by activating a Cl^- channel (see above), and since regulation of the cell cycle is Ca^{2+} dependent (34), glycine may inhibit endothelial cell proliferation via inhibition of Ca^{2+} signaling.

After the initial step of vessel wall formation via proliferation of endothelial cells, vascular smooth muscle cells are recruited to the endothelium to form a multilayered vessel

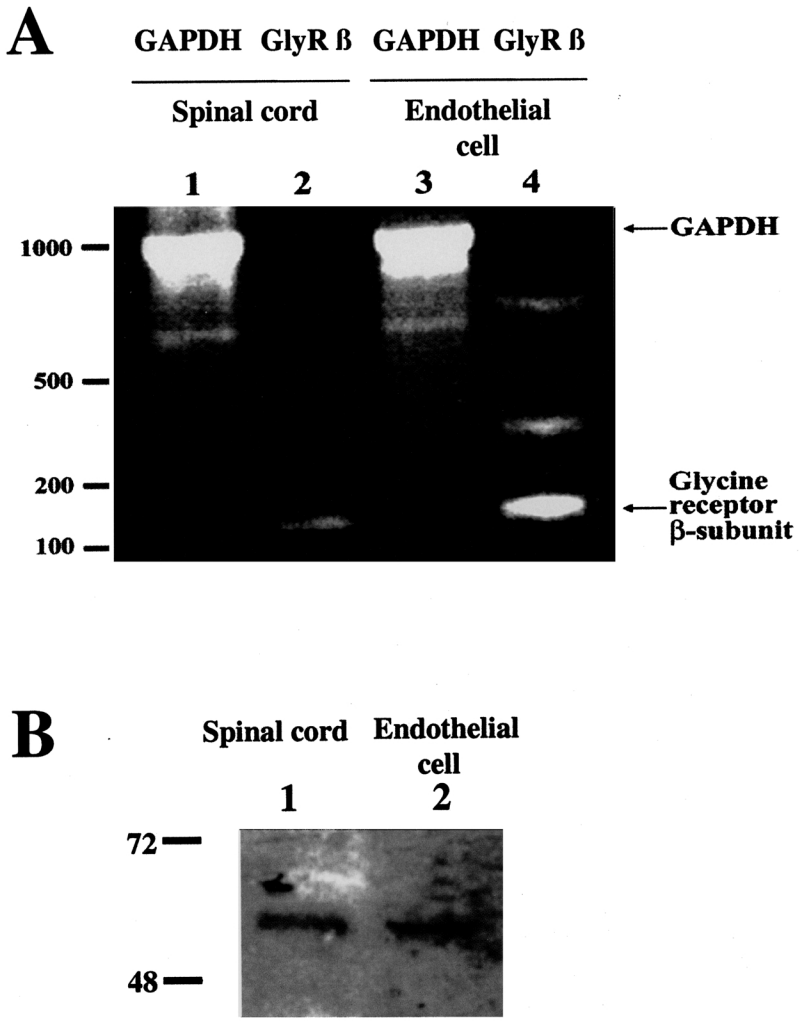

Figure 5. Reverse transcription (RT)-polymerase chain reaction (PCR) and Western blotting of glycine receptor in spinal cord and endothelial cells. A: RNA isolated from cultured endothelial cells was subjected to RT-PCR using PCR primers specific for glycine receptor β-subunit (GlyRβ) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Spinal cord RNA was used as a positive control. Lane 1, GAPDH of spinal cord; Lane 2, GlyRβ of spinal cord; Lane 3, GAPDH of endothelial cells; Lane 4, GlyRβ of endothelial cells. Position of molecular weight markers is shown. B: Western blot of spinal cord membrane extract (Lane 1) and CPA cell membrane extract (Lane 2) using antiglycine receptor subunit 4α monoclonal antibody.

wall (35). Importantly, it was reported recently that glycine also inhibited proliferation and migration of smooth muscle cells (36). Because interactions among endothelial cells, smooth muscle cells, and fibroblasts play a critical role in the regulation of angiogenesis and signaling processes in these cells require Ca^{2+}, glycine, which is a simple nontoxic nutrient that affects Ca^{2+} signaling in endothelial and smooth muscle cells, may be an intriguing antiangiogenic agent.

Acknowledgments and Notes

This work was supported, in part, by grants from the National Institute of Alcohol Abuse and Alcoholism. Address correspondence to R. G. Thurman, Laboratory of Hepatobiology and Toxicology, Dept. of Pharmacology, CB 7365, Mary Ellen Jones Bldg., The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7365. FAX: (919) 966-1893. E-mail: Thurman@med.unc.edu.

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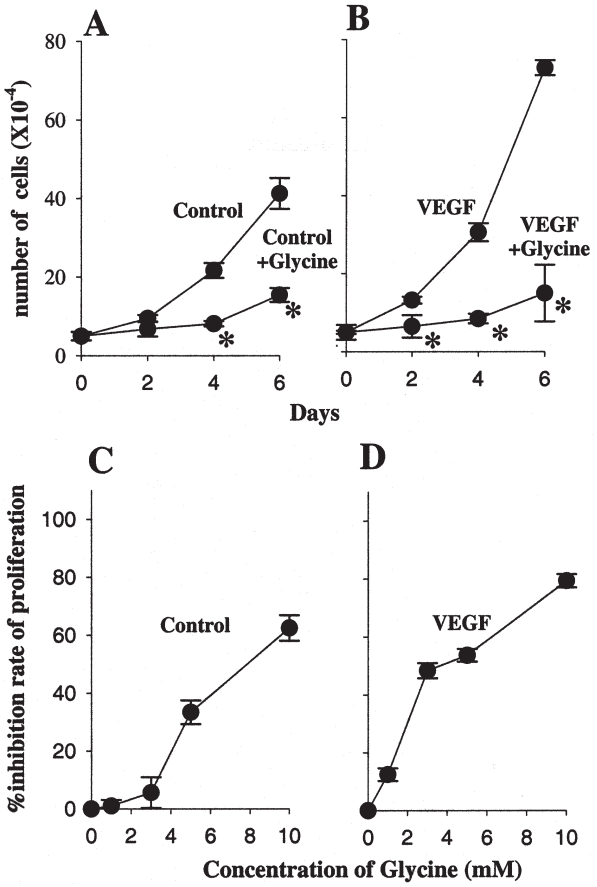

Figure 6. Effect of glycine on endothelial cell proliferation. Endothelial cells were cultured without (A and C) or with (B and D) VEGF (1 ng/ml). Glycine (0, 1, 3, 5, and 10 mM) was added, and cell growth was assessed by counting number of cells per plate with a hemocytometer. Percent inhibition of cell proliferation caused by glycine was determined for each concentration of glycine (0, 1, 3, 5, and 10 mM) without (C) or with (D) VEGF (1 ng/ml). Values are means ± SEM for 4 different cell passages in each group. *, Significant difference from control (ANOVA with Bonferroni's post hoc test).

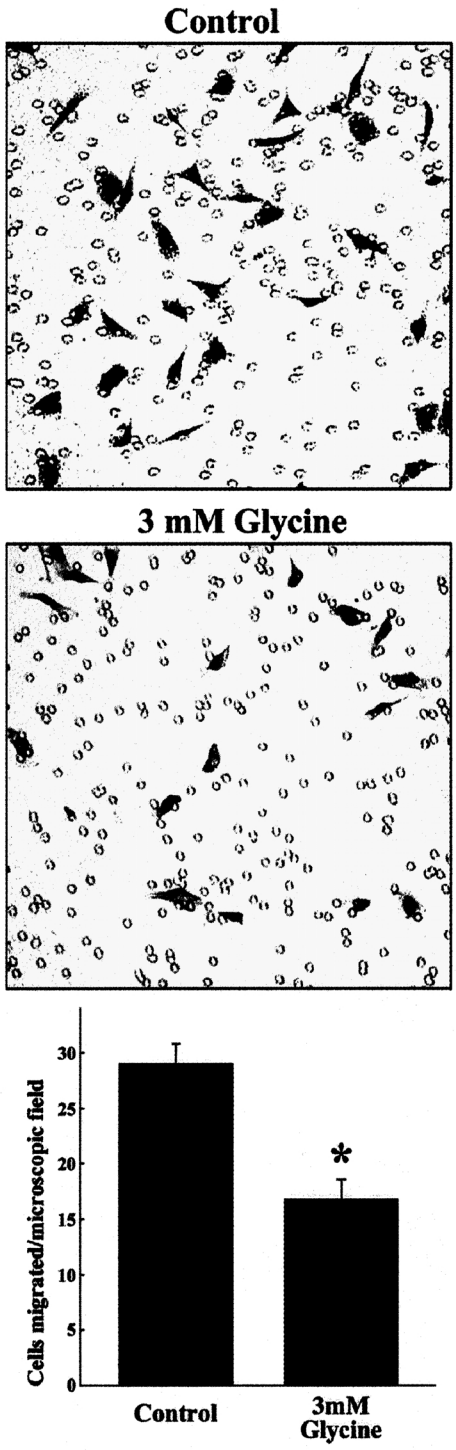

Figure 7. Inhibitory effect of glycine on migration of endothelial cells. Endothelial cell migration was determined as described in **Materials and Methods**. Cells were cultured in presence or absence of 3 mM glycine for 5 h and allowed to migrate in serum-containing medium. Representative photomicrographs (original magnification ×200). *, $P < 0.05$ compared with control (ANOVA with Bonferroni's post hoc test).

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