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Inhibition of angiogenesis by blockers of volume-regulated anion channels

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Abstract

Osmotic cell swelling activates an outwardly rectifying Cl^- current in endothelial cells that is mediated by volume-regulated anion channels (VRACs). In the past, we have shown that serum-induced proliferation of endothelial cells is arrested in the presence of compounds that potently block the endothelial VRACs. Here we report on the effects of four chemically distinct VRAC blockers [5-nitro-2-(3-phenylpropylamino)benzoic acid] (NPPB), mibefradil, tamoxifen, and clomiphene—on several models of experimental angiogenesis. Mibefradil (20 μM), NPPB (100 μM), tamoxifen (20 μM), and clomiphene (20 μM) inhibited tube formation by rat microvascular endothelial cells plated on matrigel by 42.9 ± 8.8%, 25.3 ± 10.4%, 32.2 ± 4.5%, and 20 ± 5.8%, respectively ($p < 0.05$). Additionally, NPPB (50–100 μM) and mibefradil (10–30 μM) significantly inhibited bFGF (10 ng/ml) + TNFα (2.5 ng/ml)-stimulated microvessel formation by human microvascular endothelial cells plated on fibrin by 30–70%. Furthermore, NPPB, mibefradil, and clomiphene concentration dependently inhibited spontaneous microvessel formation in the rat aorta-ring assay and vessel development in the chick chorioallantoic membrane assay. These results suggest that VRAC blockers are potent inhibitors of angiogenesis and thus might serve as therapeutic tools in tumor growth and other angiogenesis-dependent diseases.

Keywords: Volume-regulated anion channels; Angiogenesis; Endothelial cells; Tamoxifen; Clomiphene; Mibefradil; NPPB

1. Introduction

Angiogenesis, the formation of new blood vessels, plays an important role in a variety of physiological processes such as wound healing, corpus luteum formation, endometrium formation, and embryonic development (Folkman and Klagsbrun, 1987). Additionally, it constitutes an integral part of many pathological processes including diabetic retinopathy, arthritis, inflammation, and the growth of several types of solid tumors (Folkman, 1995; Folkman and Klagsbrun, 1987). The angiogenic cascade consists of several sequential and highly coordinated steps, including basement membrane proteolysis, migration of individual endothelial cells, and endothelial cell proliferation (D'Amore and Thompson, 1987).

Inhibition of angiogenesis has emerged as a promising strategy for the treatment of pathological conditions that critically depend on new vessel formation (Folkman, 1995). Several endogenous and exogenous molecules with antiangiogenic properties have been identified, and some of them are currently in phase II–III clinical trials (Augustin, 1998). Recently, Gagliardi and Collins (1993) reported that the estrogen antagonists tamoxifen and clomiphene inhibit angiogenesis in the chick chorioallantoic membrane (CAM) assay. The same investigators later showed that antiestrogens inhibit endothelial cell proliferation stimulated by bFGF and VEGF

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A preliminary account of these results was presented in the NATO ASI "Vascular endothelium: mechanisms of cell signaling" Crete, Greece, June 20–29, 1998.

This paper is dedicated to the memory of Dr. Eva Pipili-Synetos (1951–1999).

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(Gagliardi et al., 1996). Tamoxifen was also shown to inhibit tumor-induced angiogenesis in nude mice in vivo (Furman Haran et al., 1994; Lindner and Borden, 1997). Interestingly, in all these cases, the antiangiogenic action of the antiestrogens was independent of their antagonistic action on the estrogen receptors. Overall, the mechanism of the antiestrogen-induced angiogenesis inhibition remains unknown.

Volume-regulated, outwardly rectifying Cl^- channels (VRACs) are present in most mammalian cells and participate in the regulation of the membrane potential, the transport of osmolytes, and cell-volume regulation [for a review, see Nilius et al. (1997a)]. In endothelial cells, we have identified and extensively characterized an outwardly rectifying Cl^- current that is activated by osmotic cell swelling (Nilius et al., 1994a, 1994b, 1997a; Szücs et al., 1996). The molecular identity of the endothelial VRAC has not yet been identified, but the evidence so far indicates that it differs from the known volume-regulated anion channels present in other cell types (Nilius et al., 1997a).

We have identified several structurally unrelated compounds that potently block the endothelial VRAC, including the arylaminobenzoate NPPB [5-nitro-2-(3-phenylpropylamino)benzoic acid], the phenol derivatives mibefradil, gossypol, and quinine, and the antiestrogens tamoxifen and clomiphene (Nilius et al., 1997a, 1997b). In addition, we have shown that serum-induced proliferation in calf pulmonary artery endothelial cells is arrested in the presence of VRAC blockers (Nilius et al., 1997b; Voets et al., 1995). On the basis of this finding and the previously reported action of antiestrogens on angiogenesis, we recently put forward the hypothesis that VRACs may play a role in angiogenesis (Nilius et al., 1997a). To test this hypothesis, we examined the effect of the VRAC blockers mibefradil, NPPB, tamoxifen, and clomiphene on new microvessel formation in four distinct models of angiogenesis. We report here that VRAC blockers inhibit new vessel formation in all four models. These results suggest that VRACs may be implicated in angiogenesis and that blockers of VRACs may be useful in the therapy of angiogenesis-dependent tumor growth and other angiogenesis-dependent diseases.

2. Materials and methods

2.1. Materials

Tamoxifen, clomiphene citrate, bovine fibrinogen, cortisone acetate, and collagenase (type IA) were purchased from Sigma (Bornem, Belgium). Crude endothelial cell growth factor (ECGF) was either supplied by Sigma or made from bovine brains. NPPB was obtained from RBI (Natick, MA). Mibefradil (Ro 40-5967) was kindly provided by Dr. J.-P. Clozel (Hoffmann-La Roche, Basel, Switzerland). Stock solutions of bFGF, TNFα, and mibefradil were prepared in H_2O, and those of tamoxifen, clomiphene, and NPPB were prepared in DMSO. bFGF was obtained from Pepro Tech, Inc. (Rocky Hill, NJ), thrombin from Leo Pharmaceutical Products (Weesp, The Netherlands), and human fibrinogen from Chromogenix AB (Molndal, Sweden). Factor XIII was kindly provided by Drs. H. Boeder and P. Kappas (Centeon Pharma, Marburg, Germany). Human recombinant TNFα was a gift from Dr. J. Tavernier (Biogent, Gent, Belgium) and contained 2.45×10^7 U/mg of protein and 40 ng lipopolysaccharide per microgram of protein. Matrigel was from Becton Dickinson (Aalst, Belgium). Agarose was obtained from Pharmacia Biotech (Uppsala, Sweden). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), HEPES, trypsin-EDTA, penicillin, streptomycin, and L-glutamine were supplied by Life Technologies (GIBCO BRL, Merelbeke, Belgium), and M199 was from BioWhittaker (Servier, Belgium).

2.2. Culture of endothelial cells

Microvascular endothelial cells from the rat adrenal medulla (RAMECs) were isolated, grown, and characterized as previously described (Manolopoulos et al., 1997a, 1997b). The cells were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin), at 37°C, 10% CO_2 in air, and used at passages 17–19. Human foreskin microvascular endothelial cells (HMVECs) were isolated and grown as previously described (Koolwijk et al., 1996). The cells were cultured in gelatin-coated dishes in M199 supplemented with 20 mM HEPES (pH 7.3), 10% human serum, 10% newborn calf serum (NBCS), 150 μg/ml ECGF, 5 IU/ml heparin, 2 mM L-glutamine, and antibiotics, at 37°C, 5% CO_2 in air, and used at passage 10 or 11. Both cell types were passaged by brief exposure to 0.5 g/l trypsin-EDTA in a Ca^{2+}/Mg^{2+}-free solution.

2.3. Matrigel in vitro tube-formation assay

The matrigel assay was performed according to a published protocol (Kubota et al., 1988). Matrigel, a tumor extract containing basement-membrane components at 15.8 mg/ml, was applied to 1-cm^2 wells (120 μl/well) and allowed to solidify at 37°C for 1 h. Subsequently, 50,000 RAMECs were seeded in each well and incubated with complete DMEM containing the drugs under study at 37°C for 8 h. The incubation period chosen (8 h) was found in preliminary studies to be the minimal necessary for optimal tube formation under our experimental conditions (data not shown). The total length of the tubular structures formed in each well was measured in six microscopic fields (at 2.5× magnification) covering the entire well surface by using a microscope equipped with a video camera connected to a computer with OPTIMAS image analysis software (Tokyo, Japan).

2.4. Fibrin gel assay

The fibrin gel assay was performed as recently described (Koolwijk et al., 1996). Briefly, human fibrin matrices were prepared by the addition of 0.1 U/ml thrombin to a mixture of 2.5 U factor XIII, 2 mg human fibrinogen, 2 mg Na citrate, 0.8 mg NaCl, and 3 μg plasminogen per milliliter of M199 medium without indicator. A total of 300 ml of this mixture was added to 1-cm^2 wells. After clotting at room temperature, fibrin matrices were soaked with 0.5 ml M199 supplemented with 10% human serum, 10% NBCS, and antibiotics. Endothelial cells were seeded at high density to obtain confluent monolayers and were cultured in M199 medium without indicator supplemented with 20 mM HEPES (pH 7.3), 10% human serum, 10% NBCS, 2 mM L-glutamine, antibiotics, 10 ng/ml bFGF, and 2.5 ng/ml TNFα. Incubations were allowed to proceed for 10 days, with fresh medium and test compounds being added every 2 to 3 days. The tubular structures formed by endothelial cells in the three-dimensional fibrin matrix were observed by phase-contrast microscopy, and their total length in each well was measured in six microscopic fields covering the entire well surface by using an Olympus microscope equipped with a monochrome CCD camera (MX5) connected to a computer with OPTIMAS image analysis software.

2.5. Rat aorta-ring assay

The rat aorta-ring assay of angiogenesis originally developed by Nicosia and Ottinetti (1990) was performed as recently described (Liekens et al., 1997). Briefly, a sterile 1.5% solution of agarose was poured into culture dishes and allowed to gel. Agarose rings were obtained by punching two concentric circles, with diameters of 10 and 17 mm, respectively, in the agarose gel. The rings were transferred to six-well plates, three rings in each well. Thoracic aortas were obtained from adult male Wistar rats, cleaned from fat and connective tissue, and sectioned in 0.5-mm rings. Each aortic ring was positioned at the center of an agarose well, the bottom of which had already been coated with 150 μl of clotting fibrinogen, and then the agarose well was completely filled with clotting fibrinogen. The fibrinogen solution used was obtained by dissolving partly purified bovine fibrinogen in serum-free medium to obtain a concentration of 3 mg/ml. The fibrin gel formed within 30 s at room temperature. After fibrin gelation, each well was filled with M199 medium supplemented with 20% FCS, 10 mM HEPES, 1 mM glutamine, and antibiotics, and the test compounds were added. Cultures were examined daily and scored under an inverted microscope. Formation of more than 200 microvessels is common, owing to the three-dimensional complexity of the microvascular network, and the margin of error for the observer who is counting the microvessels is high; therefore, the formed microvessels were scored on a scale from 0 (no vessels) to 10 (maximum vessel number) by two independent observers.

2.6. Chorioallantoic Membrane assay

The chorioallantoic membrane vessel development assay was performed as recently described (Liekens et al., 1997). Briefly, fresh fertilized eggs were incubated at 37°C (humidity 55–60%) for 4 days before a window was opened on the eggshell, exposing the CAM. The window was covered with cellophane tape, and the eggs were returned to the incubator. On day 9, plastic discs (10-mm diameter), on which the test compounds had been allowed to dry under sterile conditions, were applied to selected areas of the CAM, one disc in each CAM. In addition, a control disc (containing PBS or DMSO) was placed on each CAM, 1 cm away from the disc containing the test compounds. A sterile solution of cortisone acetate (100 μg/disc) was incorporated in all discs to prevent an inflammatory response. Thereafter, the windows were covered, and the eggs were incubated at 37°C for 48 h. Incubation was terminated by flooding of the eggs with 10% buffered formalin, and the plastic discs were removed. Usually, no microvessel branches were broken in the removal of the discs. In rare cases, when big branches at the edge of the CAM were broken, resulting in extensive bleeding of the CAM, this CAM was not included in the results.

The eggs were kept at room temperature for at least 4 h, and then a large area around the discs was cut off and placed on a glass slide. The vascular density index under the discs (expressed as number of blood vessels) was measured by the method of Harris-Hooker et al. (1983). Briefly, membranes were fixed in 10% buffered formalin, excised, and laid flat on a glass slide. The vessel density was determined by covering with a grid the spot where the disc had been. The grid contained three concentric circles (1 mm apart) that covered the area of interest. The vessels intersecting the circles were counted. This method allows for an objective evaluation of microvessel formation, taking into account the small, recently formed microvessels. Overall chick embryo survival until disc implantation was 92%. Control discs received the same volume of DMSO as the discs containing the compounds. Application of as much as 150 μg/disc of tamoxifen, mibefradil, or clomiphene or 1 mg of NPPB to the CAM did not cause any apparent toxicity.

2.7. Statistics

The statistical significance was determined by using simple Student's t-test in the matrigel and the fibrin gel assays and by using Student's t-test with paired samples in the CAM assay.

3. Results

3.1. Effect of anion channel blockers on tube formation in the matrigel assay

Matrigel, a matrix derived from the basement membrane of the murine Engelbrecht-Holm-Swarm tumor, is a complex mixture of basement membrane proteins including laminin, collagen type IV, entactin/nidogen, and proteoheparan sulfate, as well as several growth factors including EGF, TGFβ, PDGF, IFG-1, and nerve growth factor (Kubota et al., 1988; Baatout, 1997). Endothelial cells plated on matrigel undergo within hours differentiation toward an angiogenic phenotype, as evidenced by morphologic changes, a reduction in proliferation, and the formation of tubular networks. This in vitro assay is a simple and fast model for the study of biochemical and molecular events associated with some particular aspects of the angiogenic cascade.

RAMECs plated on matrigel attached rapidly and started immediately to migrate and orient toward one another. Within 2 h, elongation of the cells could be observed, and, at 8 h after plating, abundant networks of branching and anastomosing cords of cells had been formed on the surface of the gel (Fig. 1a, panel A). VRAC inhibitors significantly attenuated this process: in the presence of 20 μM mibefradil (Fig. 1a, panel B), 20 μM tamoxifen (Fig. 1a, panel C), 20 μM clomiphene or 100 μM NPPB (Fig. 1a, panel D), tube formation was reduced by 42.9 ± 8.8%, 32.2 ± 4.5%, 20 ± 5.8%, or 25.3 ± 10.4%, respectively (Fig. 1b).

3.2. Effect of anion channel blockers on tube formation in the fibrin gel assay

The fibrin gel assay is based on the fact that human foreskin microvascular endothelial cells seeded on top of a three-dimensional fibrin matrix can be induced to invade the matrix and to form capillary-like tubular structures. Both TNFα and an angiogenic growth factor such as bFGF and VEGF are required for the formation of tubular structures (Koolwijk et al., 1996). The requirement of both fibrin and inflammatory mediators makes this assay a suitable human in vitro model for pathological angiogenesis that takes place at sites of chronic inflammation and tumor stroma (Dvorak et al., 1992).

In unstimulated cultures, confluent monolayers of HMVECs were quickly formed and maintained on top of the three-dimensional fibrin matrix, and no invading cells and tubular structures penetrating the matrix could be observed (Fig. 2a, panel A). None of the VRAC blockers used had any effect on the HMVEC monolayers under unstimulated conditions (data not shown). However, in the continuous presence of 10 ng/ml bFGF and 2.5 ng/ml TNFα, formation of tubular structures by cells invading the matrix was detectable within 3 days, and this process was complete by day 10 from initiation of the culture (Fig. 2a, panel B). In bFGF+TNFα-stimulated cultures, mibefradil, 10 or 30μM (Fig. 2a, panel C; Fig. 2b), or NPPB, 50 or 100 μM (Fig. 2a, panel D; Fig. 2b), significantly reduced the total length of the tubular structures formed as measured at day 10. At higher concentrations, the two compounds were toxic for the cells. Of the two antiestrogens used, clomiphene (20 μM) exhibited no significant inhibitory effect (Fig. 2b), whereas tamoxifen (also 20 μM) not only did not inhibit bFGF+TNFα-stimulated tube formation, but even increased it by 30.6 μ 11.4%, $n = 12$, $p < 0.05$ (Fig. 2b). The stimulatory effect of tamoxifen and the lack of effect of clomiphene most likely result from VRAC-independent actions of these antiestrogens (Section 4). However, the results obtained with mibefradil and NPPB support the idea that VRAC channels have a role in bFGF+TNFα-induced capillary tube formation in a human in vitro model of pathological angiogenesis.

3.3. Effect of anion channel blockers on microvessel formation in the rat aorta-ring assay

Similarly to the fibrin gel assay described in Section 3.2, the rat aorta-ring assay is based on the formation of capillary structures by endothelial cells originating from the inner surface of rings of rat aorta placed on a three-dimensional gel of fibrin. A major advantage of this assay is that the aortic cells have not been modified by repeated passages in culture and generate vascular outgrowths that strongly resemble those formed during in vivo angiogenesis. Such "physiologic" features of rat aortic angiogenesis include the formation of microvessels composed of both endothelial cells and pericytes, the self-limiting nature of the angiogenic response, and a dynamic regression and remodeling of the neovasculature. Also, the addition of external growth factors is not required to achieve vessel growth. Finally, the aorta ring assay provides a model for all the steps of angiogenesis, including endothelial cell invasion, migration, proliferation, differentiation, and new vessel formation (Nicosia and Ottinetti, 1990; Nicosia and Villaschi, 1999).

The first microvascular sprouts from the aortic rings placed on fibrin appeared after 3 days, followed by a growth phase and the obtainment of a plateau usually at day 9. A dose-dependent inhibition of sprouting was observed for NPPB, mibefradil, and clomiphene (Fig. 3). The concentrations required to inhibit microvessel formation by 50% (calculated on day 10, when control cultures had reached a maximum of vessel density) was 41.0 μM, 16.3 μM, and 4.2 μM, respectively, for NPPB, mibefradil, and clomiphene, similar to the concentrations of these drugs required to inhibit VRACs (Nilius et al., 1997a, 1997b; and Maertens, Manolopoulos, and Nilius, unpublished observations). Surprisingly, tamoxifen had no effect on microvascular sprout formation at concentrations as high as 10 μM and had only a modest effect at 30 μM (data not shown). The lack of inhibitory

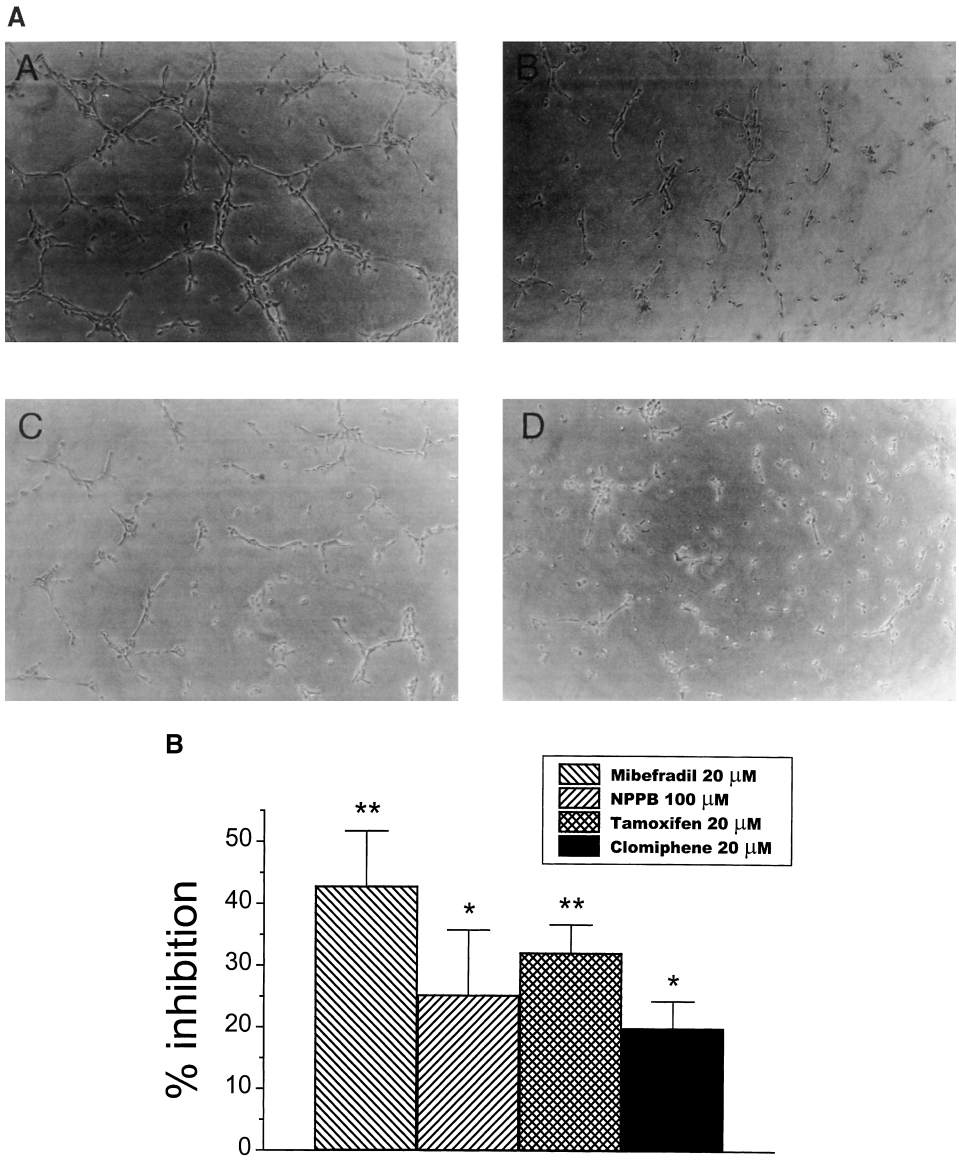

Fig. 1. Inhibitory effect of VRAC blockers on tube formation in the matrigel assay. (a) RAMECs were seeded on matrigel and cultured at 37°C for 8 h in the presence of: (A) vehicle (DMSO); (B) mibefradil, 20 μM; (C) tamoxifen, 20 μM; and (D) NPPB, 100 μM. Photographs were taken in a phase-contrast microscope at 4× magnification. (b) RAMECs were seeded on matrigel and cultured at 37°C for 8 h in the presence of the indicated VRAC blockers. The length of tubes present in each well was measured by using OPTIMAS image analysis software. Results are expressed as percent inhibition in the length of tubes formed in experimental wells compared with tubes in the control wells. Each bar represents the mean value ± SE from 6–9 wells, from three different experiments. One asterisk and two asterisks denote statistically significant inhibition, $p < 0.05$ and $p < 0.01$, respectively.

effect of tamoxifen at the expected concentration range probably results from VRAC-independent actions of this compound (see Section 4). However, the results obtained with mibefradil, NPPB, and clomiphene support the idea that VRAC channels have a role in the spontaneous formation of capillary structures by rat aortic rings placed on a three-dimensional fibrin matrix.

3.4. Effect of anion channel blockers on vessel development in the CAM assay

The CAM assay in the chick embryo is perhaps the most widely used vessel development assay in vivo. Although it was originally developed as a qualitative assay, ways were later reported for reliably quantifying the effects of activators and inhibitors of angiogenesis on vessel development in this assay (Harris-Hooker et al., 1983; Nguyen et al., 1994). It is particularly suitable for the initial screening of potential inhibitors of angiogenesis in a living organism because it is simple and quick and vessels grow spontaneously, with no need for the addition of external growth factors.

All the compounds tested caused the inhibition of vessel development in the CAM. Figure 4a depicts the maximum inhibitory effects of mibefradil, NPPB, and

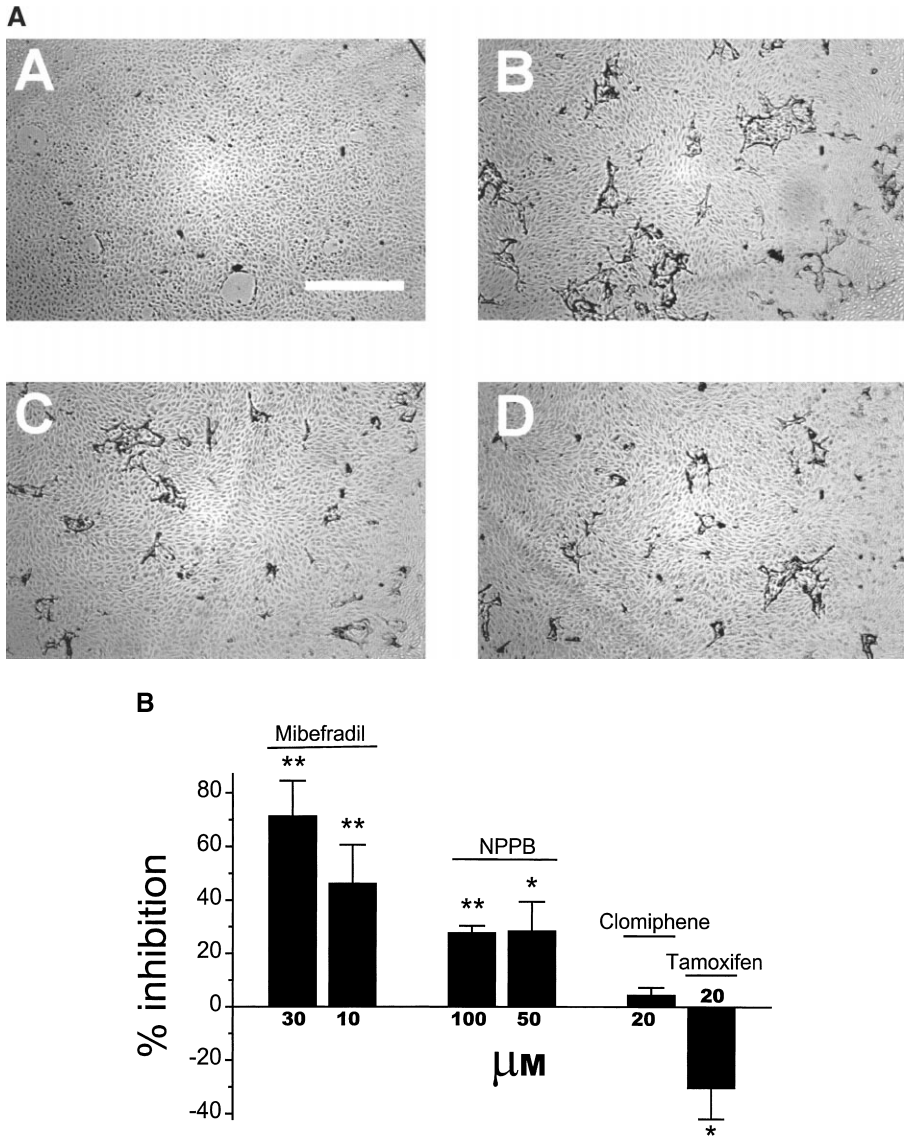

Fig. 2. Inhibitory effect of VRAC blockers on tube formation in the fibrin gel assay. (a) HMVECs were cultured on a fibrin gel in the presence of: (A) vehicle (DMSO); (B) bFGF, 10 ng/ml, and TNFα, 2.5 ng/ml; (C) bFGF, TNFα, and mibefradil, 30 μM; and (D) bFGF, TNFα, and NPPB, 50 μM; at 37°C for 10 days. Photographs were taken in a phase-contrast microscope at 2.5× magnification. (b) HMVECs were cultured on a fibrin gel in the presence of 10 ng/ml bFGF + 2.5 ng/ml TNFα and the indicated VRAC blockers at 37°C for 10 days. The length of tubular structures present in each well was measured by using OPTIMAS image analysis software. Results are expressed as percent inhibition in the length of tubes formed in experimental wells compared with tubes in the control wells. Each bar represents the mean value ± SE from 9–12 wells, from three or four different experiments. One asterisk and two asterisks denote statistically significant inhibition, $p < 0.05$ and $p < 0.01$ respectively.

clomiphene. No thrombi and no changes in the growth patterns of the microvessels were observed with any of the compounds. Mibefradil inhibited vessel growth by 31.3 ± 7.9%, 60 ± 4.6%, and 69.4 ± 2.7% at the concentrations of 20, 50, and 150 μg/disc, respectively (Fig. 4b). NPPB had a similar dose-dependent inhibitory effect, ranging from 25.6 ± 6.2% inhibition at 100 μg/disc to 63.3 ± 4.9% at 1 mg/disc (Fig. 4b). Dose-dependent inhibition was also caused by clomiphene, with the highest amount used (150 μg/disc) causing 52.4 ± 7% inhibition (Fig. 4b). Tamoxifen was less potent than clomiphene: only at 150 μg/disc did it cause significant inhibition (24.6 ± 8.8%, $n = 7$, $p < 0.05$); whereas, at 20 or 50 μg/disc, it had no inhibitory effect on vessel growth in the CAM (Fig. 4b).

It should be noted that the evaluation method used does not distinguish between newly formed microvessels (after application of the drug-containing discs on the CAM) and those already present at day 9. Therefore, 100% reduction of vascular density is not attainable by this method and the 69% and 63% reductions seen with mibefradil and NPPB can be considered the maximum achievable inhibition of new vessel growth. In fact, no microvessels could be detected on the CAM

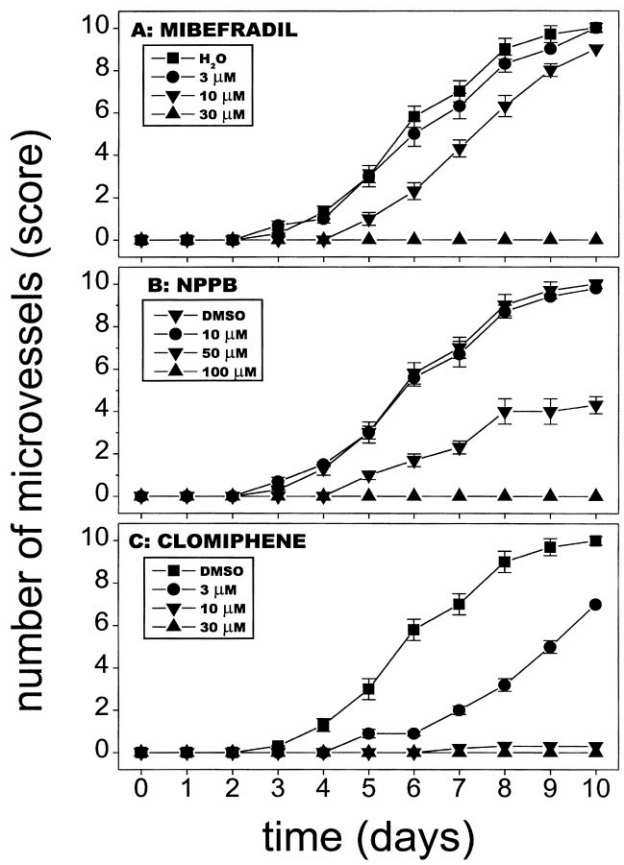

Fig. 3. Inhibitory effect of VRAC blockers on microvessel growth in the rat aorta-ring assay. Aortic rings were placed inside a fibrin gel, and the growth of microvessels in the presence and absence of a range of concentrations of mibefradil, NPPB, or clomiphene was monitored daily for 10 days and scored as described in Section 2. Each point represents the mean value ± SE from three different experiments, each performed in triplicate wells.

under the disc containing 150 μg of mibefradil or 1 mg of NPPB (Fig. 4a, panels B and C), suggesting that these compounds not only inhibit formation of new microvessels, but also cause collapse or regression of existing capillaries or both.

4. Discussion

Volume-regulated anion channels are present in most mammalian cell types including endothelial cells, but little is known about their physiological functions in each cell type (Nilius et al., 1997a). We report here that blockers of the channels belonging to three different chemical classes inhibit the formation of microvessels and tubular structures in four experimental models of angiogenesis. These results suggest that VRACs may play a role in the angiogenic cascade.

The angiogenic cascade consists of several sequential and highly coordinated steps, including basement membrane proteolysis, migration of individual endothelial cells, and endothelial cell proliferation (D'Amore and Thompson, 1987). VRAC channels may have a role in endothelial-specific functions that are relevant to distinct steps in the angiogenic cascade (e.g., expression of cell-adhesion molecules, metalloproteinases, or metalloproteinase inhibitors, or all three), or in some general cellular functions that may also be important in angiogenesis. We have already shown that VRAC blockers inhibit endothelial cell proliferation at similar concentrations as those reported here to be effective in the angiogenesis models (Nilius et al., 1997b; Voets et al., 1995), and this may be one of the mechanisms responsible for their inhibitory effect on angiogenesis.

In the fibrin gel assay, inhibition of endothelial cell proliferation by the addition of the tyrosine kinase inhibitor tyrphostin A47 had only a moderate inhibitory effect (Koolwijk et al., 1996), suggesting that no significant proliferation takes place in this system. Additionally, in the matrigel assay, tube formation was completed within 6–8 h, before any proliferation could occur. However, in both these assays, VRAC blockers significantly attenuated tube formation. These results suggest that, in addition to inhibiting proliferation, VRAC blockers may also interfere with other steps of the angiogenic cascade. Likely candidates are the migratory, adhesive, and fibrinolytic–matrix-degrading abilities of the angiogenic endothelial cell, and research is warranted in all these directions.

Puzzling results were obtained in the fibrin gel assay with the two antiestrogens: clomiphene showed no effect, whereas tamoxifen not only did not inhibit, but even significantly potentiated tube formation induced by bFGF+TNFα. Recently, we showed that the bFGF+TNFα-induced tube formation in this assay is inhibited by estrogens (Lansink et al., 1998). It is possible that tube formation may not be maximal under these conditions, inasmuch as estrogens contained in the human serum routinely used in this assay may suppress it. The addition of tamoxifen may eliminate the inhibitory effect of the naturally occurring estrogens, resulting in an enhancement of tube formation. This is the most likely explanation for the potentiating effect of tamoxifen. Clomiphene, on the other hand, is a weaker estrogen antagonist, and its antiestrogenic activity may have been buffered by its VRAC-blocking activity, producing a neutral net result on tube formation.

In addition to blockade of VRAC that may result in inhibition of angiogenesis, antiestrogens have other cellular actions, some of which could lead to a stimulation of angiogenesis. For example tamoxifen stimulates the expression of the potent angiogenic factor VEGF in the rat uterus (Hyder et al., 1997). Moreover, the same compound down-regulated CD36 expression in normal and neoplastic human breast tissues (Silva et al., 1997). This glycoprotein acts as a receptor for thrombospondin-1, a potent endogenous antiangiogenic molecule. Thus, inhibition of CD36 could result in a proangio-

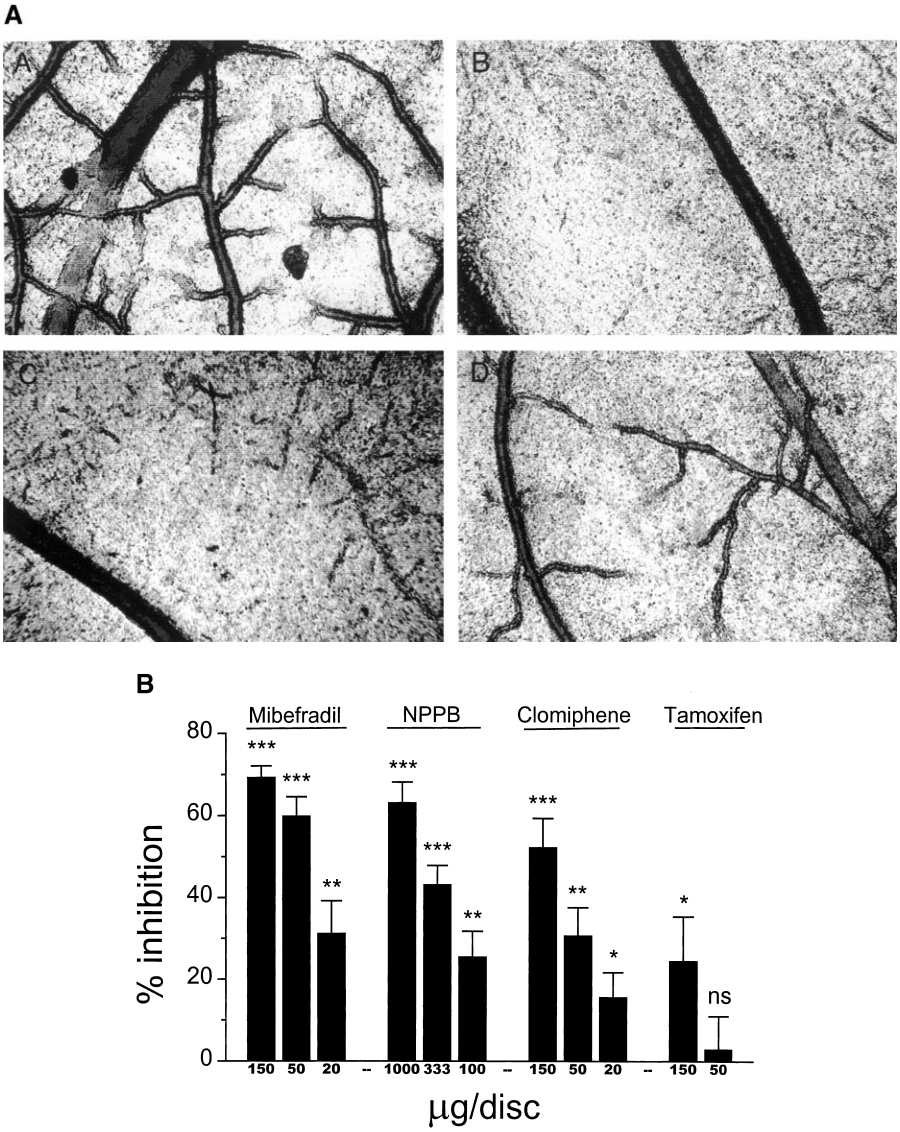

Fig. 4. Inhibitory effect of VRAC blockers on neovascularization in the CAM assay. Discs containing either vehicle or the indicated VRAC blockers were placed on selected areas of the CAM on day 9, and the eggs were further incubated at 37°C for 48 h. One control and one drug-containing disc were placed on every CAM. (a) Discs contained (A) vehicle (DMSO or PBS); (B) mibefradil, 150 μg/disc; (C) NPPB, 1000 μg/disc; and (D) clomiphene, 150 μg/disc. Photographs were taken 48 h later at 40× magnification. (b) Dose-dependent inhibitory effect of VRAC blockers. Results are expressed as percent inhibition in the number of microvessels under the drug-containing disc compared with the control disc in the same CAM. Each bar represents the mean value ± SE from 7–10 eggs. One, two, or three asterisks denote statistically significant inhibition, $p < 0.05$, $p < 0.01$, or $p < 0.001$, respectively.

genic response to tamoxifen in tissues where CD36 participates in the angiogenic cascade. Furthermore, tamoxifen was shown to stimulate TGF-β production in rat vascular smooth muscle cells (Grainger et al., 1993). TGF-β often has a stimulatory effect on angiogenesis. Overall, it appears that tamoxifen, and presumably other antiestrogens as well, may have concurrent pro- and antiangiogenic actions in each tissue. The net final effect may be the sum of these actions, and it may vary among tissues, depending on which action is predominant in each case. Such a dual pro–antiangiogenic action has recently been found also in the case of NO (Pipili-Synetos et al., 1994; Ziche et al., 1994). This diversity in the effects of the antiestrogens on angiogenesis exemplifies the necessity and merit of using more than one angiogenesis model for screening compounds with suspected antiangiogenic activity.

In light of the preceding findings, the observed differences in the effects of tamoxifen and clomiphene most likely result from the differential potency of each of them to activate specific pro- and antiangiogenic mechanisms. In support of this explanation, much higher doses of clomiphene as compared with tamoxifene were required to achieve similar levels of VEGF production in the rat uterus (Hyder et al., 1997).

The compounds used in the present study are not

specific for VRACs, and they may inhibit other types of anion channels as well, such as the Ca^{2+}-activated, the cAMP-dependent, and the voltage-dependent CIC-type Cl^- channels. However, to date, no evidence has been found for the presence of cAMP-dependent and voltage-dependent Cl^- channels in endothelial cells. In contrast, we have clearly demonstrated that no such channels are present in bovine pulmonary artery endothelial cells (Nilius et al., 1997c). In addition, we have shown that Ca^{2+}-activated Cl^- channels are expressed in macrovascular but not in microvascular endothelial cells (Nilius et al., 1997c). On the other hand, VRACs are present in virtually every endothelial cell type that has been investigated, including microvascular (Manolopoulos et al., 1997b; Nilius et al., 1994b, 1997c). The fact that three of the four assays that we used involve microvascular endothelial cells strengthens our notion that the inhibitory action of the anion channel blockers on angiogenesis is mediated by specific inhibition of the endothelial VRACs.

Each of the compounds used in this study reportedly affects other targets in the cell in addition to their common action on anion channels: NPPB may also inhibit certain anion cotransporters (Cabantchik and Greger, 1992), mibefradil is a T-type selective Ca^{2+} channel blocker (Mishra and Hermsmeyer, 1994), and tamoxifen and clomiphene are well-known estrogen receptor antagonists and have a variety of other cellular actions (as discussed already). However, to the best of our knowledge, the inhibition of VRACs is the only common property of these chemically unrelated compounds. Thus, it is unlikely that their effect on angiogenesis results from an action different from the inhibition of VRAC.

Blockers of other types of ion channels also have been shown to inhibit angiogenesis: the Na^+-channel blocker amiloride inhibited capillary morphogenesis in a model for angiogenesis employing vascularized tissue explants (Alliegro et al., 1993). The Ca^{2+}-channel blocker nicardipine inhibited tube formation of bovine endothelial cells (Kaneko et al., 1992), and the inhibitor of Ca^{2+} influx, carboxyamidotriazole (CAI), was shown to inhibit tube formation on matrigel by human umbilical vein endothelial cells, as well as angiogenesis in the CAM assay (Kohn et al., 1995). As a result of VRAC activation, the driving force for Ca^{2+} entry into the cell increases, and that may affect the intracellular Ca^{2+} concentration. Such a mechanism could also explain the involvement of VRACs in angiogenesis.

In conclusion, we have shown that VRAC blockers are potent inhibitors of angiogenesis. These results unravel a possible physiological role for VRAC in angiogenesis and add a new dimension to antiangiogenesis research by suggesting that cell membrane anion channel blockers may be active players in the angiogenic process. Some of the known compounds with VRAC-blocking properties (such as clomiphene and tamoxifen) are already in use in the clinic for angiogenesis-unrelated indications. Additionally, intensive research by several groups to identify molecules that target VRACs with very high affinity and specificity is in progress. Our results suggest that such VRAC-blocking compounds may serve as therapeutic tools in angiogenesis-dependent tumor growth or other angiogenesis-dependent diseases or both.

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