Chloride Intracellular Channel Protein-4 Functions in Angiogenesis by Supporting Acidification of Vacuoles Along the Intracellular Tubulogenic Pathway

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Endothelial cells form capillary tubes through the process of intracellular tubulogenesis. Chloride intracellular channel (CLIC) family proteins have been previously implicated in intracellular tubulogenesis, but their specific role has not been defined. In this study, we show that disruption of the Clic4 gene in mice results in defective angiogenesis in vivo as reflected in a Matrigel plug angiogenesis assay. An angiogenesis defect is also apparent in the retina, both in the decreased spontaneous development of retinal vasculature of unstressed mice and in the dramatically decreased angiogenic response of retinal vessels to an oxygen toxicity challenge. We found that endothelial cells derived from Clic4−/− mice demonstrated impaired tubulogenesis in three-dimensional fibrin gels compared with cells derived from wild-type mice. Furthermore, we found that tubulogenesis of wild-type cells in culture was inhibited by both an inhibitor of CLICs and an inhibitor of the vacuolar proton ATPase. Finally, we showed that vacuoles along the endothelial tubulogenesis pathway are acidic in wild-type cells, and that vacuolar acidification is impaired in Clic4−/− cells while lysosomal acidification is intact. We conclude that CLIC4 plays a critical role in angiogenesis by supporting acidification of vacuoles along the cell-hollowing tubulogenic pathway. (Am J Pathol 2009, 174:1084–1096; DOI: 10.2353/ajpath.2009.080625)

The ability of endothelial cells to organize into multicellular capillary tubes is essential to normal development and is important in disease states such as cancer and diabetes mellitus. Endothelial cells can form capillary tubes through the process of cell-hollowing tubulogenesis that involves the coordinated formation and fusion of specialized intracellular vacuoles.1,2 During cell-hollowing tubulogenesis, cells form a large intracellular lumen through the fusion of multiple intracellular vesicles.1 This lumen then fuses with those of neighboring cells and what was the lumen of the intracellular compartment becomes the extracellular lumen of the multicellular tube. Thus tubulogenesis of capillaries requires organized traffic of intracellular membranes. In addition to numerous other proteins,3 ion transporters have been implicated in intracellular membrane traffic. In particular, acidification by the electrogenic vacuolar proton ATPase (vH-ATPase) coupled with a short-circuiting chloride conductance is important in trafficking of several intracellular compartments.4

CLICs are a family of proteins that have been implicated as intracellular chloride channels, but whose physiological roles remain uncertain.5 A CLIC family member has been shown to be essential for the cell-hollowing tubulogenesis of the excretory cell of Caenorhabditis elegans6 in which it is necessary for intracellular vesicles to fuse into an intact intracellular tube although its specific role in this process is unknown. Furthermore, CLIC4 (one of six mammalian CLICs) has been implicated in endothelial cell tubulogenesis in a proteomics study.7

To explore the physiological roles of CLIC4 more directly, we generated mice in which the Clic4 gene has been disrupted. In this study, we report that Clic4−/− mice demonstrate decreased angiogenesis activity in
two independent in vivo assays. Endothelial cells derived from these mice show decreased ability to proceed through the cell-hollowing tubulogenesis pathway, and cell-hollowing tubulogenesis of wild-type endothelial cells is inhibited both by IAA-94, a CLIC inhibitor, and by bafilomycin A1, an inhibitor of the vacuolar proton ATPase. We directly measured the pH of intracellular vacuoles along the cell-hollowing tubulogenesis pathway, showing that they acidify and that acidification is impaired in Clic4\(^{-/-}\) cells. We conclude CLIC4 plays a critical role in angiogenesis by supporting acidification of vacuoles along the cell-hollowing tubulogenic pathway, perhaps by providing a short-circuiting ion conductance to permit transport by the electronegic vacuolar proton pump. These data firmly identify CLIC4 as a key player in angiogenesis, identify vacuolar acidification as a necessary process in cell-hollowing tubulogenesis, and establish a novel specific physiological function for a member of a protein family whose cellular roles have been uncertain.

Materials and Methods

Animal studies were performed in accordance with the regulations of the institutional animal care and use committees. Matrigel plug experiments, preparation of retinal flat mounts, induction of retinal angiogenesis after hyperoxygen exposure, isolation of heart endothelial cells, and vacuolization assay in fibrin gel were performed using published methods with slight modifications as described below.

Generation of New Antisera

The peptide CMALSMPNLGLKEED, which represents the N-terminal sequence of mouse CLIC4 with an N-terminal cysteine added, was covalently linked to keyhole limpet hemocyanin and the conjugate used to immunize a rabbit. The peptide CMALSMPLNGLKEED, which represents the N-terminal sequence of mouse CLIC4 with an N-terminal cysteine added, was covalently linked to keyhole limpet hemocyanin and the conjugate used to immunize a rabbit. The resulting affinity-purified antibody is named AP255. Western blot of glutathione S-transferase fusion proteins with CLIC1, CLIC4, and CLIC5 were performed as previously described.

Generation of Clic4\(^{-/-}\) Mice

Nucleotide coordinates are numbered from NCBI mouse genome sequences NT_039267. Fragments of CLIC4 gene were amplified from mouse genomic DNA using polymerase chain reaction (PCR). An upstream fragment (position 31,018 to 33,277) from intron 1 and downstream fragment (33,924 to 37,925) from intron 2 were inserted into the KpnI-XbaI and XhoI-NotI sites of pNT-Cass-loxA (gift of Drs. Shunji Tomatsu and William Sly, St. Louis University, St. Louis, MO). The targeting vector was linearized at the NotI site and introduced into embryonic stem cells (line W9.5 derived from 129/Sv mice; Gene Targeting Services, Yale University, New Haven, CT) by electroporation using standard methods (Siteman Cancer Center Murine Embryonic Stem Cell Core, St. Louis, MO). Cells were selected for growth in G418 (400 \(\mu\)g/ml) and ganciclovir (2 \(\mu\)mol/L). Individual colonies were selected after 1 week, DNA isolated, and screened for homologous recombination by PCR. Positive colonies were confirmed bySouthern blotting of NheI-digested genomic DNA. The Southern blot probe consisted of fragments 30,021 to 30,840 from the CLIC4 gene. Blastocyst injection and generation of chimeric offspring were performed by the Transgenic Core Facility at Washington University, St. Louis, MO. Animals were genotyped by PCR amplification of tail DNA. Primers for genotyping the CLIC4 lineage were 5'-TGAC-CACGGCAACTCCTAGAAGGACCGG-3' and 5'-AGGAC TCGGGGTGACACTGTAATCGAC-3'.
paraformaldehyde in saline for 2 hours. Retinal whole mounts were incubated in ice-cold 70% methanol for 10 minutes, rinsed with phosphate-buffered saline (PBS), incubated in PBS with 1% Triton X-100 for 30 minutes, rinsed with PBS, incubated in Alexa Fluor 565-ILB4 at 10 μg/ml in PBS with 1 mmol/L CaCl₂ (PBS + Ca) overnight at 4°C, rinsed with PBS + Ca, incubated in PBS + Ca with 1% Triton X-100 for 20 minutes, and washed three times for 5 minutes in PBS + Ca. Images were collected on an Olympus (Center Valley, PA) FV500 laser-scanning confocal microscope using a ×4 objective.

For immunostaining of flat mounts, unfixed eyes were dissected, flat mounts prepared and fixed on the slide by immersion in 100% methanol at −20°C for 5 minutes. Blocking and incubations were as described 18 using AP255 or rat anti-CD31 (Pharmingen, La Jolla, CA) as primary antibodies and 1:200 dilutions of Alexa Fluor 488 anti-rat (Invitrogen) and Cy3 anti-rabbit (Jackson Immunoresearch, West Grove, PA) as secondary antibodies. Images were collected on a Zeiss (Thornwood, NY) laser-scanning confocal microscope under ×40 objective using sequential mode to eliminate potential bleed through.

For immunostaining of retinal cross sections, freshly dissected whole eyes were embedded in Tissue Tek and quick-frozen. Frozen sections were prepared and fixed on the slide with methanol at −20°C for 5 minutes. Slides were blocked by incubation in Superblock (Pierce Biochemicals) for 1 hour. All subsequent incubations including primary and secondary antibody incubations (diluted as in previous paragraph) and washes were done in TNT (200 mmol/L NaCl, 50 mmol/L Tris, pH 7.5, 0.1% Tween 20).

**Endothelial Cell Culture**

Endothelial cells were prepared from mouse heart as reported. 15 Growth medium consisted of Dulbecco’s modified Eagle’s medium (high glucose, with glutamine and pyruvate) with 20% heat inactivated fetal calf serum, 100 μg/ml heparin, 100 μg/ml endothelial cell growth supplement (Biomedical Technologies, Stoughton MA), 1× nonessential amino acids, 25 mmol/L HEPES, pH 7.4, 15 μg/ml ascorbic acid, 5 μmol/L β-mecaptoethanol, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Tubulation medium consisted of Dulbecco’s modified Eagle’s medium (high glucose, with glutamine and pyruvate), 20% heat inactivated fetal calf serum, 50 μmol/L ascorbic acid, 40 ng/ml vascular endothelial growth factor, 40 ng/ml basic fibroblast growth factor (both from R&D Systems, Minneapolis, MN), and 100 μmol/L phorbol 12-myristate 13-acetate. IAA-94 and Bafilomycin A1 (both from Biomol, Plymouth Meeting, PA) were dissolved in dimethyl sulfoxide at 200 mmol/L and 100 μmol/L, respectively, and diluted 1000-fold into medium.

Quantitation of vacuolization was performed with cells grown in 50 μl fibrin gels in 96-well plates. Formalin-fixed, toluidine blue-stained cultures were examined. All cells in all focal planes of randomly selected microscope fields were scored for vacuolization. To be counted as vacuolated, at least one-third of the area of the cell had to be occupied by well-defined vacuolar space. To be counted as multivacuolated, the cell had to contain at least three well-defined vacuoles that together occupied at least one-third of the area of the cell. At least 400 cells were scored at each data point. The experiment was performed three times with independent cell preparations and the results averaged. P values were determined using analysis of variance. 20

**Immunostaining Cells in Fibrin Gels**

Five μl drops of cell/fibrin suspension were allowed to solidify in eight-well Lab-Tek chambered coverslips (Nunc, Rochester, NY) and incubated in tubulation medium. At 18 hours, the fibrin gel was fixed in PLP (75 mmol/L sodium phosphate, pH 7.4, 75 mmol/L lysine, 10 mmol/L sodium periodate, 2% paraformaldehyde) for 1 hour, permeabлизed in Superblock (Pierce Biochemicals) with 0.05% saponin for overnight, incubated with AP255 diluted 1:100 in TNT for 6 hours, washed overnight with four changes of TNT, incubated in 1:200 dilution of Cy3 goat anti-rabbit secondary antibody in TNT for 6 hours, and then washed overnight with four changes of TNT.

**Vacular pH Measurement**

Cells were plated in 12-μl fibrin gels in glass-bottomed dishes with fluorescein/tetramethylrhodamine dextran (Invitrogen) at 2 μg/ml in the gel mixture and the tubulation medium. After 8 to 12 hours, plates were mounted on the heated stage of an Olympus FV500 inverted laser-scanning microscope under 5% CO₂ atmosphere. Vacuolated cells were identified under differential interference contrast (DIC) optics. Paired 16-bit images were obtained using a 560- to 600-nm band pass emission filter and with excitation at either 488 or 543 nm. Areas of interest were defined over fluorescently-labeled intracellular compartments and ratio of average fluorescence intensity determined. Average ratios from samples obtained at pH 5.5 and 7.0 in highly buffered, high potassium/nigericin solutions were used to obtain a two point standardization curve for each fibrin gel, which was then used to calculate a pH value for each compartment. Statistical significance between average pH values of each group was determined using Student’s unpaired t-test. 20

**Results**

A new antiserum was raised against a CLIC4 N-terminal peptide. Characterization of the affinity-purified antibody, named AP255, is shown in Figure 1. Western blot of crude bacterial lysates of *Escherichia coli* expressing glutathione S-transferase fusion proteins of CLIC1, CLIC4, and CLIC5 probed with AP255 is shown in Figure 1A. The antibody only recognizes the CLIC4 fusion protein (and associated degradation products running as smaller bands). Whole mouse kidney homogenate (which contains CLIC1, CLIC4, and CLIC5) was separated by SDS-PAGE and probed with antibody. A single band with the mobility of CLIC4 was detected and this band disap-
peared with preabsorption of the diluted antibody with antigenic peptide for 1 hour at 20 μg/ml (Figure 1B). Thus, this antibody is specific for CLIC4.

Disruption of Clic4 Gene

A map of the Clic4 gene, targeting vector, and structure of the recombinant chromosomal DNA are shown in Figure 2A. This construct eliminates exon 2 in the recombinant product. Splicing from exon 1 to exon 3 would put the 31-kDa molecular weight standard is indicated by the arrow.

Characterization of CLIC4−/− Mice

Total protein and RNA was prepared from wild-type and CLIC4−/− kidney and liver. Quantitative RT-PCR using a TaqMan 7700 QPCR assay and GAPDH message as the internal standard detected no intact CLIC4 mRNA from the tissues of the CLIC4−/− mice whereas CLIC4 transcript in the wild-type mice was readily detected. On Western blots of total protein, CLIC4 was undetectable in tissues from the CLIC4−/− mice (Figure 2D).

CLIC4−/− mice were mated and genotypes of 198 consecutive offspring determined, yielding 60 wild type, 110 heterozygotes, and 28 CLIC4−/−. The knockout mice are significantly underrepresented (28 observed, 49.5 predicted; P < 0.02). Because the observed number of heterozygotes (n = 110) is almost double the observed number of wild-types (n = 60) the most direct explanation for the discrepancy is that the CLIC4−/− embryos have a tendency to prenatal mortality.

Cohorts of littermates from heterozygote crosses were observed for abnormalities in growth and development. CLIC4−/− mice tend to weigh less than wild-type or heterozygous littermates, but among this cohort, the difference only reached statistical significance among males.
(at 12 weeks: +/+ males, 39.4 ± 0.9 g, n = 8; +/- males, 39.4 ± 1.1, n = 12; -/- males, 34.9 ± 1.6 g, n = 6; P < 0.05 between +/- and either +/− or +/+).

Standard blood and urine chemistries were performed on cohorts of young adults 6 to 12 weeks of age. No systematic differences in serum electrolytes, acid-base status, hemoglobin, urine chemistries, urine volume, or total urine protein were found among the groups of mice. Standard screening histology of a wide range of organs did not identify any structural abnormality. No differences in behavior, spontaneous activity, or gait were noted. The mice appear to have a normal lifespan and do not appear to accumulate any unusual tumors or growths.

Both male and female homozygous knockouts are fertile. However, Clic4−/− females have an increased rate of stillbirth among their offspring, independent of the genotype of the father or the offspring. In 15 consecutive litters from 9 different Clic4−/− females yielding 111 pups, there were 25 (22.5%) nonviable pups. In contrast, in 12 consecutive litters from wild-type or heterozygous females yielding 146 pups, there was 1 still birth (0.68%). In summary, Clic4−/− mice have a modest overt phenotype: they are viable but small, are under-represented among offspring, and females show a high stillbirth rate among their pups.

Matrigel Angiogenesis Assay

We investigated whether Clic4−/− mice have a defect in angiogenesis using the Matrigel plug assay.10,11,22 Four hundred μl of Matrigel supplemented with 200 ng/ml basic fibroblast growth factor were injected subcutaneously. Sections of plugs recovered after 2 weeks from wild-type mice (Figure 3, A–C) show typical features of induced angiogenesis reported by others:12–14 invading cells form linear structures, some with well-formed lumens that occasionally are filled with red blood cells, demonstrating
that these are vascular structures in continuity with the host’s own blood stream. Matrigel plugs from Clic4^{-/-} mice show a dramatically different pattern (Figure 3, D–F) with extensive areas filled with large cystic structures that appear to be hugely dilated cells. There are also abundant large multivesicular cells that are very rarely seen in wild type. Almost the entire cytoplasm of these cells appears to be replaced with large dilated vesicles. In addition, some structures similar to those seen in wild-type mice were also found in the plugs from Clic4^{-/-} mice. Parallel experiments using vascular endothelial growth factor instead of basic fibroblast growth factor as the inducing agent gave essentially identical results.

To characterize the cells invading the Matrigel further, sections were stained with fluorescently-labeled endothelial-specific marker, isolectin B4 from G. simplicifolia (ILB4) and counterstained with the nuclear marker Sytox Green. As expected in plugs from wild-type mice, most of the cells are in linear or tubular structures that stain with the lectin and thus represent developing endothelial cells along the pathway of angiogenesis (Figure 3, G and H). In plugs from Clic4^{-/-} mice, some structures similar to those in the wild-type plugs are seen (Figure 3I). In addition, the numerous multivacuolated structures also stain with the lectin, indicating these also represent endothelial structures (Figure 3, J and K).

Hemoglobin content of the Matrigel plugs was used as a measure of angiogenesis. As determined by densitometry of Western blots, plugs from wild-type mice contained 431 ± 61 (mean ± SE) arbitrary units of hemoglobin/mg protein (n = 6) whereas plugs from Clic4^{-/-} animals contained 172 ± 56 (n = 7). Overall development of blood-filled vessels was decreased by 60% (P < 0.01).

**Retinal Vasculature in Clic4^{-/-} Mice**

To determine whether the absence of CLIC4 affected a naturally occurring vascular bed, we examined the eye.23 We first determined whether CLIC4 is expressed in the retinal vasculature. Whole mounts of wild-type adult mouse retina were stained with antibodies to both CLIC4 and the endothelial marker, CD31 (Figure 4A), revealing CLIC4 is present in retinal blood vessels. In addition, frozen sections of adult eyes from wild-type and Clic4^{-/-} mice were stained for CLIC4 and CD31. The vascular pattern in these cross sections is similar between wild-type and Clic4^{-/-} mice with both superficial and deeper capillaries present in both retinas (Figure 4, B and C). CLIC4 clearly co-localizes with CD31 in vessels in the wild-type eye and is absent from the Clic4^{-/-} eye.

The superficial vasculature of the mouse retina develops postnatally, expanding from the central retinal vessels to cover the entire retina by approximately postnatal day 7. This primary plexus undergoes further maturation to yield a fully mature retinal vasculature by approximately day 21. Retinal whole mounts were prepared from mice at age 4, 7, and 21 days. The retinal vascular tree was visualized by staining with the endothelial-specific marker, ILB4. Fluorescence images were collected and composites covering the entire retina were generated.

Images from day 4 retinas are shown in Figure 5. The amount of retina covered by the advancing vascular tree is approximately comparable between wild-type and Clic4^{-/-} mice (Figure 5, A and B). Higher-power images from the advancing edge of the vascular plexus are shown in Figure 5, C–H. Tip cells are seen extending into the peripheral retina in both wild-type and Clic4^{-/-} eyes (Figure 5, C and D). Occasional tip cells are seen that appear to contain large intracellular vacuoles. These structures are more easily found in Clic4^{-/-} retinas (Figure 5D). Multivacuolated structures very reminiscent of the structures observed in the Matrigel plugs can be found immediately behind the front of the expanding vascular tree (Figure 5, E–H). Although such structures are found in wild-type retinas (Figure 5, E and G), they appear much more abundantly and profusely hypertuated in Clic4^{-/-} eyes (Figure 5, F and H). These structures are most prominent in a focal plane slightly above that of the vascular tree itself. Figure 5, G and H, shows two images of the identical field taken at two different focal planes. The left image of each pair is in the...
optimal focal plane of the major vascular network. The image to the right of each pair shows the same focal plane focused ~20 μm higher. Although some multivacuolated structures are visible in the focal plane of the vascular network, more abundant, profusely hypervacuolated structures are prominent in the higher plane. These structures are dramatically more prominent in the Clic4−/− retinas (Figure 5H) than in the wild type (Figure 5G).

Images of retinas from 7-day-old mice are shown in Figure 6, A and B. At day 7, the primary vascular plexus has spread to cover almost the entire surface of the retina and has begun to mature. Both wild-type and Clic4−/− retinas appear approximately comparable, although, the leading edge of the vascular network has reached the end of the retina in the wild-type mice, whereas the vascular network of the Clic4−/− mice tend to be a little delayed and have not quite reached the periphery of the retina. As a measure of the extent of vascular development, the radius of the developing vascular network as a fraction of the radius of the entire retina was determined. Among wild-type retinas, the radial fraction was 0.962 ± 0.008 (n = 7). Among Clic4−/− retinas, the radial fraction was 0.857 ± 0.013 (n = 7), the difference being highly significant (P < 0.00005).

Images of fully mature retinas from 21-day mice are shown in Figure 6, C and D. The overall patterns appear quite similar between wild-type (Figure 6C) and Clic4−/− mice (Figure 6D). Density of the vascular tree was quantified using the angiogenesis module of Metamorph software (Molecular Devices, Sunnyvale, CA). Fraction of the entire area that was comprised of vessels, length of vessels per area, and number of branches per area were determined and averaged among the retinas from each group (Table 1), revealing that the retinal vascular tree in fully developed Clic4−/− mice is significantly less dense and complex than that of wild type.

Oxygen toxicity is a stimulus of neangiogenesis in retinas and is thought to be one cause of the human disease, retinopathy of prematurity. We subjected Clic4−/− and wild-type mice to an oxygen-induced retinopathy model in which 7-day-old mice are exposed to 75% oxygen for 5 days followed by recovery for 5 days in normal atmosphere. Central retinal capillaries are obliterated during the high constant oxygen exposure followed by intense angiogenesis induced by the relative hypoxia after return to room air. The images in Figure 6, A and B, demonstrate that the retinal vasculature is not dramatically different between wild-type and Clic4−/− mice on day 7 at the onset of the hyperoxygen exposure period. Images of retinas from wild-type and Clic4−/− mice on day 12 immediately after oxygen exposure are shown in Figure 7, A and B. As is typical for this model of vascular injury, there has been obliteration of the central capillaries with preservation of peripheral vessels. The extent of vascular obliteration is approximately comparable be-
between wild-type and Clic4−/− retinas. Representative images of retinas on day 17 after recovery from oxygen exposure are shown in Figure 7, C and D. The wild-type mice show a profuse hypervascularity including the typical tufts of preretinal vascular growth into the overlying vitreous humor (prominent in Figure 6C, lower panel). This vascular proliferation is dramatically attenuated in the Clic4−/− retinas. The obvious difference in vascular density is born out by the quantitative analysis shown in Table 1, revealing marked attenuation of vascular proliferation in the Clic4−/− background.

### Table 1. Retinal Vascular Density

<table>
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<tr>
<th>Condition</th>
<th>Percent area covered</th>
<th>Vessel length (nm/μm²)</th>
<th>Branch points per 1000 μm²</th>
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<tr>
<td>Native vessels</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Wild-type, n = 9</td>
<td>32.5 ± 1.26</td>
<td>43.6 ± 2.1</td>
<td>1.8 ± 0.12</td>
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<td>CLIC4−/−, n = 8</td>
<td>21.2 ± 0.63</td>
<td>31.5 ± 0.83</td>
<td>1.3 ± 0.05</td>
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<tr>
<td>P value</td>
<td>3 × 10−6</td>
<td>0.00014</td>
<td>0.00079</td>
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<tr>
<td>After oxygen stress</td>
<td></td>
<td></td>
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<tr>
<td>Wild-type, n = 7</td>
<td>58.8 ± 0.79</td>
<td>56.9 ± 0.73</td>
<td>1.97 ± 0.06</td>
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<tr>
<td>CLIC4−/−, n = 8</td>
<td>32.4 ± 1.01</td>
<td>38.4 ± 0.94</td>
<td>1.41 ± 0.04</td>
</tr>
<tr>
<td>P value</td>
<td>3.7 × 10−11</td>
<td>2.2 × 10−9</td>
<td>1.9 × 10−6</td>
</tr>
</tbody>
</table>

Values were determined for each retina, then averaged to yield the means. Values reported are means ± standard error of mean. P values determined with Student’s t-test.

### Endothelial Tubulogenesis in Culture

Endothelial cells cultured in a three-dimensional matrix can proceed through tubulogenesis with formation and fusion of intracellular vacuoles culminating in multicellular tubes. To determine whether the angiogenesis defect associated with absence of CLIC4 could be detected in cultured cells, primary cultures of mouse heart endothelial cells were prepared and cellular vacuolization/tubulogenesis in fibrin gels was studied as described. Typical appearance of toluidine blue-stained cells in fibrin gels are shown in Figure 8. Immediately after plating, both the wild-type (Figure 8A) and Clic4−/− (Figure 8B) cells have a simple round ap-

![Figure 7](image1.png)

**Figure 7.** Retinal vascular response to oxygen toxicity. Representative composite low-power images of lectin-stained retinal whole mounts. A and B: Twelve-day-old wild-type (A) and Clic4−/− (B) after 5 days of high (75%) oxygen exposure. C and D: Seventeen-day-old wild-type (C) and Clic4−/− (D) after 5 days of 75% oxygen exposure (upper) and subsequent 5 days of recovery in room air (lower).

![Figure 8](image2.png)

**Figure 8.** Vacuolization of endothelial cells in fibrin gels. Primary cultures of mouse heart endothelial cells were induced to undergo tubulogenesis in fibrin gels and stained with toluidine blue. A and B: Wild-type (A) and Clic4−/− (B) cells immediately after plating. Note homogenous populations of small round nonvacuolated cells in both. C: Wild-type cells after 16 hours of culture. Small nonvacuolated cells and cells with a single large vacuole or multiple vacuoles are all present. D: Clic4−/− cells after 16 hours of culture. Fewer cells are vacuolated and multivacuolated cells are more prominent. E: Wild-type cells after 16 hours in the presence of 200 μmol/L IAA-94. F: Wild-type cells after 16 hours in the presence of 100 μmol/L bafilomycin. Scale bar = 50 μm.
pearance with no obvious intracellular compartment. Throughout several hours, cells acquire large intracellular vacuoles. Most vacuolated cells have one large vacuole occupying almost the entire intracellular space. Other cells accumulate numerous large vacuoles. Typical images of cells after 16 hours of culture are shown for wild-type (Figure 8C) and Clic4−/− (Figure 8D) cells. Because the appearance of the wild-type cells becomes extremely heterogeneous as vacuolization progresses, it is difficult to make generalizations about differences in the appearance based on the few cells seen in a single microscopic field. Some cultures were also grown in the presence of the CLIC inhibitor, IAA-94, at 200 μmol/L, or in the presence of the proton ATPase inhibitor, bafilomycin A1 at 100 μmol/L. Images of toluidine blue-stained cultures at 16 hours in the presence of these inhibitors are shown in Figure 8, E and F, respectively.

Fibrin gel cultures were fixed and stained with toluidine blue at various times and scored for vacuolization,16,17 plotted in Figure 9A. All cells in all focal planes in randomly selected microscope fields were scored; at least 400 cells were scored for each data point. The data shown are the average of three experiments using independent primary cell cultures. Clearly wild-type cells vacuolated more rapidly and completely than CLIC4−/− cells. Vacuolization was inhibited by either IAA-94 or, most dramatically, by bafilomycin.

In addition to overall vacuolization, toluidine blue-stained cultures were also scored for presence of multivacuolization, defined as three or more vacuoles per cell. The fraction of vacuolated cells that were multivacuolated by this definition is shown in Figure 9B. At all time points, the Clic4−/− cultures had a greater degree of multivacuolation than did the wild-type cultures. The cells grown in the presence of the either inhibitor also had a tendency toward multivacuolization, although the differences did not reach statistical significance at all time points.

Cultures were immunostained for CLIC4 and images collected by confocal microscopy as shown in Figure 10. Paired DIC and immunofluorescence images are shown. CLIC4 co-localizes with the membranes surrounding the large vacuoles in wild-type cells (Figure 10A). Similar vacuolar structures are found in Clic4−/− cells, but as expected these are less common and there is no staining for CLIC4 (Figure 10B). The appearance of wild-type cells grown in the presence of IAA-94 (not shown) was indistinguishable from cells without the drug except that vacuolated cells were less common. Typical images of wild-type cells grown in the presence of bafilomycin are shown in Figure 10C. Some vacuolated cells are present that are similar to cells in the absence of bafilomycin (Figure 10C, left panel). In addition, some larger cells accumulate numerous small vacuoles that stain only weakly for CLIC4 (Figure 10C, right panel). Cells of this appearance are not seen in the absence of bafilomycin. Cells like the one in Figure 10C, right panel, would probably not have been perceived as multivacuolated with toluidine blue stain because the vacuoles are so small.

**Endothelial Vacuolar Acidification**

One way chloride channels may function in membrane traffic is by supporting acidification by the electrogenic V-H-ATPase, but whether vacuoles along the intracellular tubulogenic pathway acidify has not been reported. We measured pH of endothelial vacuoles directly. Large vacuoles of tubulogenic endothelial cells have been noted to load with extracellular macromolecules.17,25,27,28 We plated endothelial cells in fibrin gels that had been supplemented with a ratiometric pH indicator, fluorescein-tetramethylrhodamine dextran.29–31 The fluorescence of fluorescein is strongly pH sensitive, quenching at lower pH within the pH range 7.5 to 5.0. The fluorescence of rhodamine is relatively insensitive to pH in

![Figure 9](image-url)
that same range. Therefore, the ratio of fluorescein to rhodamine fluorescence can be used to determine the pH of the environment in which dextran conjugated with both fluorescein and tetramethylrhodamine resides. After 8 to 12 hours of culture, cells were assessed under confocal microscopy. Typical vacuolated wild-type cells loaded with dextran are shown in Figure 11, A–C, and a typical dextran-loaded Clic4−/− cell is shown in Figure 11D. The absence of fluorescence in the cytoplasm indicates the dextran has been excluded from the cell as expected. Punctate fluorescent compartments in the cells are consistent with the endosomal/lysosomal pathway, which is expected to load with an extracellular fluid phase marker. The large vacuoles show variable loading with dextran: some vacuoles are efficiently loaded (examples in Figure 11, A–D), some contain dextran at lower concentration than in the extracellular space (see vacuoles to the right in Figure 11B), and some appear not to be loaded with dextran at all (Figure 11C). This variability of loading was observed with both wild-type and Clic4−/− cells. Paired fluorescence images were obtained with excitation at 488 or 543 nm and with emission detected using a 560- to 590-nm band pass filter. The fluorescence intensity ratio was determined over labeled intracellular compartments. A standard curve for the relationship between pH and fluorescence intensity ratio in our system is shown in Figure 11E. A two-point standard curve was generated for each fibrin gel and the pH of each intracellular compartment determined. The large vacuoles (minimum greater than 4 μm diameter; most vacuoles analyzed were greater than 10 μm) and the small endosomal/lysosomal compartments (less than 2 μm diameter) were analyzed separately. The experiment was performed three times and results pooled.

In wild-type cells, the average pH of the large vacuoles was 6.75 ± 0.034 (n = 145) and of the small vesicles was 5.43 ± 0.033 (n = 146). In Clic4−/− cells, the average pH
of large vacuoles was 7.06 ± 0.036 ($n = 112$) and the pH of the small vesicles was 5.42 ± 0.031 ($n = 108$). The difference between the pH of the large vacuoles of wild-type and \textit{Clic4}−/− cells is highly significant ($P < 10^{-8}$) whereas the difference between the pH of the small vesicles is not significant ($P = 0.75$). Thus, large vacuoles along the endothelial tubulogenesis pathway acidify and this acidification is attenuated in \textit{Clic4}−/− cells. In contrast, lysosomal/endosomal acidification is preserved in \textit{Clic4}−/− cells.

**Discussion**

These experiments lead to several novel conclusions. First, \textit{Clic4}−/− mice are viable and have a minimal phenotype in unstressed conditions, but show increased embryonic lethality (~50%), have defective spontaneous development of blood vessels in the retina with decreased density of retinal vessels in the adult, and show attenuated response to two different models of induced angiogenesis \textit{in vivo}. Second, both the Matrigel and the day 4 retina staining reveal an accumulation of multivacuolated cells in the \textit{Clic4}−/− mice that are reminiscent of the multivacuolated secretory cells of the \textit{C. elegans} CLIC mutant, suggesting they may represent unfused vacuolar precursors of the mature intracellular tube. Third, \textit{CLIC4} is present in the membranes of wild-type endothelial vacuoles. Fourth, \textit{Clic4}−/− endothelial cells demonstrate impaired vacuolization that is reproduced in the wild-type cells by a CLIC inhibitor. Fifth, endothelial vacuoles acidify and vacuolization is inhibited by a vH-ATPase inhibitor, indicating that acidification is important for tubulogenesis. Sixth, endothelial cells from \textit{Clic4}−/− mice demonstrate impaired acidification of their large intracellular vacuoles whereas acidification of the small endosomal/lysosomal compartments is intact. Taken together, these data support the hypothesis that the selective failure to acidify the vacuoles along the tubulogenic pathway is the mechanism by which absence of CLIC4 impairs angiogenesis.

We conclude that CLIC4 plays an important role in angiogenesis by supporting intracellular vacuolar fusion and perhaps formation in tubulating endothelial cells at least in part through allowing acidification of the intracellular vacuoles. The absence of CLIC4 impairs ability to proceed with intracellular tubulogenesis and is manifested as an attenuated response to angiogenic challenges accompanied by an accumulation of multivacuolated endothelial intermediates that have been unable to rapidly pass through the tubulogenic program. However, the mildness of the phenotype in mice that survive gestation as well as the fact that any \textit{Clic4}−/− mice are viable, indicates clearly that angiogenesis can occur in the absence of CLIC4.

Functional redundancy among CLIC family members may account for the mildness of the \textit{Clic4}−/− phenotype. Each of the two CLICs encoded by \textit{C. elegans} is able to support tubulogenesis and only cell-specific expression prevents functional complementation \textit{in vivo}. In mammals, CLIC1 is widely expressed in virtually all tissues and cell types and is present in primary cultures of mouse endothelial cells (not shown). Our hypothesis is that functional redundancy between CLIC4 and other CLICs is adequate to allow a fraction of \textit{Clic4}−/− mice to survive through embryogenesis to birth and to allow essentially normal postnatal growth and development in the unstressed laboratory environment. However, when angiogenesis is maximally stimulated, as in the Matrigel or the oxygen-stressed retinal...
experiments, and perhaps during intrauterine development, a defect in angiogenic capacity is unmasked because of inadequate CLIC activity to support this accelerated pace of angiogenesis. The accumulation of cells filled with unfused vesicles indicates that vesicle fusion may be the rate limiting step of angiogenesis in the CLIC family.33–36 Although a necessary role for this process in general intracellular membrane traffic has never been proven. In addition, CLICs have been proposed to be involved in a variety of protein-protein interactions. Identified binding partners for CLICs include a variety of proteins involved in membrane traffic and the cytoskeleton.37–41 Undoubtedly recruitment of components of the membrane fusion machinery and dynamic regulation of the cytoskeleton must play an important roles in endothelial tubulogenesis. Whether CLIC4 contributes through these mechanisms remains to be explored.

Based on the observation that the vacuoles of tubulating endothelial cells can be loaded with extracellular fluid phase markers, it has been suggested that vacuoles along the endothelial tubulogenesis pathway arise from pinocytosis.25,28 However, simple pinocytosis followed by fusion cannot account for our observation that many vacuoles load poorly or not at all when cells are grown continuously in the presence of fluorescent dextran. Because the dextran is homogeneously present in the extracellular space during the entire time of vacuole formation, these nonloaded vacuoles must either have extruded the dextran after pinocytosis (seemingly unlikely), or else they arose from intracellular vesicles that were not the products of endocytosis/pinocytosis and thus did not contain the extracellular fluid phase marker. However, if the vacuoles do not arise from primary pinocytosis, how does that fraction of vacuoles that do load acquire the dextran? Of course, one possibility is that the origin of the vacuoles is heterogeneous, some vacuoles arising from pinocytosis, some from fusion of nonpinocytic vesicles. A second possible mechanism by which extracellular components could get into these vacuoles would be that vacuoles first form from a nonpinocytic mechanism and then transiently fuse with the plasma membrane during their subsequent maturation. This event would be analogous to the final irreversible fusion with the plasma that must occur as the vacuole becomes the extracellular lumen of the new capillary.29 The primary difference being that it would be transient rather than irreversible. This mechanism would also be consistent with our observation that the fraction of large vacuoles that are not loaded with dextran decreases with time of incubation (not shown). Thus, although our data do not firmly identify an alternative pathway, these experiments are clearly inconsistent with the hypothesis that the vacuoles of tubulating endothelial cells arise exclusively from simple pinocytosis.

In conclusion, we show that CLIC4 plays an important role in angiogenesis by supporting acidification of vacuoles along the cell-hollowing tubulogenic pathway. The identification of a chloride channel as a component of the cellular basis for angiogenesis opens new avenues for identifying potential therapeutic agents for diseases in which angiogenesis plays a critical role.

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