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NO/cGMP Pathway Activation and Membrane Potential Depolarization in Pig Ciliary Epithelium

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Purpose. To investigate whether in isolated porcine ciliary processes, stimulation of the nitric oxide (NO)-guanylate cyclase (GC)-3′,5′-cyclic guanosine monophosphate (cGMP) pathway modulates ciliary epithelial transmembrane potential.

Methods. Changes in transmembrane potential induced by the two NO donors, sodium nitroprusside (SNP; 100 μM) and S-nitroso-N-acetyl-penicillamine (SNAP; 100 μM), or by the cGMP-analogue 8-*para*-chlorophenylthioguanosine-3′,5′-cyclic guanosine monophosphate (8-pCPT-cGMP; 100 μM) were measured with microelectrodes in the presence or in the absence of the GC-inhibitor 1-H-(1,2,4)oxadiazole(4,3-α)quinoxalin-1-1 (ODQ; 10 μM). The effect of 8-pCPT-cGMP was also assessed in the presence of the anion channel inhibitors niflumic acid (100 μM), diisothiocyanatostilbene-2,2′ disulfonic acid (DIDS; 1 mM), anthracene-9-carboxylic acid (9-AC; 1 mM), or the K^+ channel blocker tetraethylammonium chloride (TEA; 10 mM). cGMP production was measured by immunoassay.

Results. Significant membrane depolarizations ($P < 0.05$–0.001; $n = 5$–8) were induced by SNP (6 ± 1 mV; mean ± SEM), SNAP (8 ± 1 mV), or 8-pCPT-cGMP (13 ± 1 mV). In presence of ODQ, the effect of SNP and SNAP were significantly inhibited (-2 ± 0 mV and 0 ± 0 mV, respectively; $P < 0.05$; $n = 5$–6), but not depolarizations elicited by 8-pCPT-cGMP. These were prevented ($P < 0.05$–0.01; $n = 5$) by niflumic acid (1 ± 1 mV), DIDS (1 ± 1 mV), or 9-AC (5 ± 1 mV), but not by TEA (12 ± 2 mV). The increase in cGMP production induced by SNP (9.5-fold) was inhibited by ODQ ($P < 0.001$; $n = 6$).

Conclusions. Activation of the NO-GC-cGMP pathway modulates epithelial transmembrane potential in isolated porcine ciliary processes. (*Invest Ophthalmol Vis Sci.* 2000;41:1759–1763)

In the eye, aqueous humor is essentially produced by the epithelium of the ciliary processes.[1] Ionic currents across the ciliary epithelium (with a secondary passive transport of water) are a major driving force for the formation of aqueous humor in ciliary processes.[1] Changes in transmembrane ionic currents can induce changes in transmembrane potential that can be measured with microelectrodes. In the process of aqueous formation, it has been suggested that, among other ions, the anion chloride is an important rate-limiting factor.[1]

Nitric oxide (NO), a cellular mediator that can activate the enzyme guanylate cyclase (GC) and thus increase 3′,5′-cyclic guanosine monophosphate (cGMP) production (i.e., the NO-GC-cGMP pathway), has been implicated in transepithelial fluid transport (gut, trachea).[2–4] It has been reported, for example, that NO and cGMP can increase rat gastric mucus production,[2] or that NO can stimulate rat colon mucosa anionic (chloride) secretion.[3]

In this study, we investigated whether in isolated porcine ciliary processes activation of the NO-GC-cGMP pathway modulates ciliary epithelial transmembrane potential, and, if such a modulation occurs, whether anion channel inhibitors can modulate this effect.

Methods

Tissue Preparation

In adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, porcine eyes were obtained from an abattoir. Under a stereo microscope (M38; Wild, Heerbrugg, Switzerland), ciliary processes were dissected in cold modified Krebs–Ringer HEPES solution (in mM: Na^+ 140, K^+ 5.2, Cl^- 117.5, Ca^{2+} 2.5, $H_2PO_4^-$ 1.2, HCO_3^- 30, HEPES 20, glucose 10; pH 7.40). For electrophysiological experiments, radial wedges containing four to six ciliary processes were isolated (3–4 mm anterior to the pars plana) and with the epithelium facing upward pinned to the bottom of a 200-μl recording chamber coated with Sylgard (Dow Corning, Ithaca, NY). Preparations were then superfused with modified Krebs–Ringer HEPES solution (1500 μl/min, 95% O_2-5% CO_2, 37°C) and left quiescent for at least 20 minutes before any recording was performed. For cGMP immunoassay, ciliary processes were placed in a 24-well plate, covered with 700 μl Hanks' balanced salt solution (37°C), and left quiescent for 60 minutes before exposure to different drugs.

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Electrophysiology

Transmembrane potentials in the ciliary epithelium were measured using microelectrodes pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) filled with 3 M KCl (30–90 MΩ) and referenced to an Ag-AgCl ground electrode in the bath. Signals were amplified (Cyto 721; World Precision Instruments), AD converted (MacLab 2e; ADInstruments, Castle Hill, Australia) and stored on a computer (Macintosh Powerbook 1400; Apple Computers, Cupertino, CA). Signal analysis was performed off-line. Criteria for acceptance of a recording were a sharp change of potential at penetration and withdrawal of the microelectrode from the tissue and a stable baseline membrane potential recording below −55 mV.

Experimental Protocols

Once a stable baseline membrane potential recording was obtained, the preparations were exposed a first time (first exposure) to different drugs: 100 μM of the NO donor sodium nitroprusside (SNP), 100 μM of another NO donor S-nitroso-*N*-acetyl-penicillamine (SNAP), or 100 μM 8-*para*-chlorophenylthioguanosine-3′,5′-cyclic guanosine monophosphate (8-pCPT-cGMP), a stable, membrane-permeable analogue of cGMP. After a washout period of 30 minutes, the same preparations were exposed a second time (second exposure) to the same drugs, in the absence or presence of 10 μM 1-H-(1,2,4)oxadiazole(4,3-α)quinoxalin-1-1 (ODQ), a specific GC inhibitor. Incubation time of ODQ before the second exposure to drugs was 5 minutes.

In another set of experiments, in a similar manner, the preparations were exposed a first time (first exposure) to 100 μM 8-pCPT-cGMP, and then after a washout period of 30 minutes, the same preparations were exposed a second time (second exposure) to the same drug in the presence or absence of one of the anion channel inhibitors, such as 100 μM niflumic acid, 1 mM diisothiocyanatostilbene-2,2′ disulfonic acid (DIDS), 1 mM anthracene-9-carboxylic acid (9-AC), or 10 mM of the nonspecific K^+ channel inhibitor tetraethylammonium chloride (TEA). Before the second exposure, preparations were incubated for 5 minutes with the different inhibitors.

Cellular Lucifer Yellow Staining

To confirm that the recording corresponded to the measurement of a ciliary epithelial transmembrane potential, some experiments were conducted with microelectrodes, with the tips filled with 5% lucifer yellow dilithium dye and backfilled with 150 mM LiCl. At the end of the experiment, the dye was iontophoretically injected into the cell by applying hyperpolarizing pulses of direct current (3–5 nA, 0.5 seconds' duration at 1 Hz) for at least 2 minutes (HSE Stimulator II; Hugo Sachs Eletronik, March–Hugstetten, Germany). Afterward, the tissues were fixed with 4% paraformaldehyde in phosphate-buffered solution and the injected cells identified and photographed with a fluorescence microscope (Diaphot; Nikon, Kogaku, Japan; 450–490-nm excitation wavelength; Fig. 1A). The portion of the tissue containing the injected cells was then embedded in Epon without osmification and sectioned. The lucifer yellow immunoreactive sites were localized on thin sections (60 nm) by the protein A gold citrate technique using an anti-lucifer yellow antiserum (Molecular Probes, Eugene, OR) and an electron microscope (EM 10; Carl Zeiss, Oberkochen, Germany; Fig. 1B).

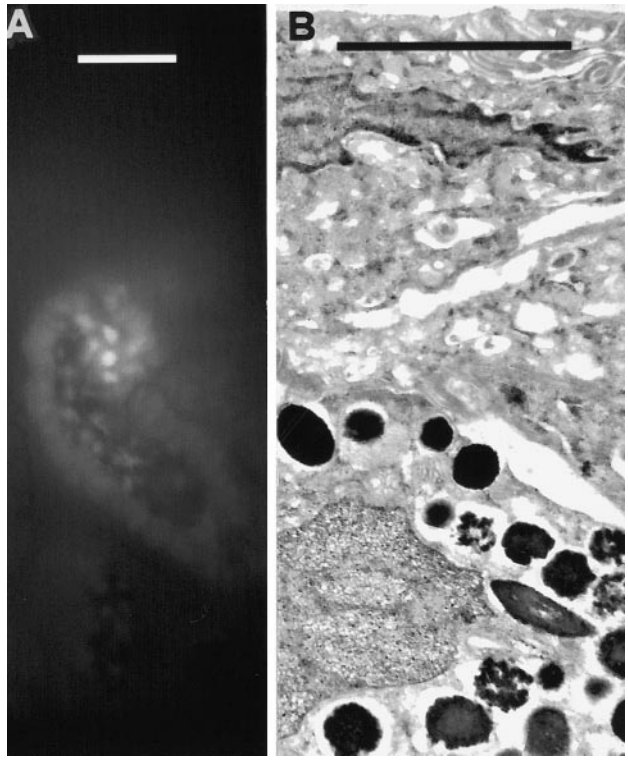

Figure 1. Fluorescence and electron microscopy of porcine ciliary processes after injection of lucifer yellow dilithium dye through a microelectrode used to record ciliary epithelium transmembrane potential. (**A**) Cells stained with lucifer yellow show a bright fluorescence. After a single injection of lucifer yellow, the dye could be seen in adjacent cells. (**B**) Electron microscopy of the portion of the tissue where the dye was previously seen by fluorescence microscopy. Lucifer yellow immunoreactive sites were localized by the protein A gold citrate technique using an anti-lucifer yellow antiserum. The *small black particles* of the immunogold staining can be seen in both nonpigmented (*top*) and pigmented ciliary epithelial cells (*bottom*), pigmented cells characterized by *large round* melanin inclusions. Bar, (**A**) 50 μm; (**B**) 5 μm.

cGMP Immunoassay

After incubation with the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 0.5 mM, 37°C, 30 minutes) tissues were exposed for 5 minutes to SNP (100 μM) in the presence or in the absence of ODQ (10 μM; incubation time 5 minutes). Then, tissues were rapidly frozen (liquid nitrogen) and stored at −70°C. For cGMP measurements (triplicate), each sample was homogenized (4°C) in the presence of 0.5 ml ice-cold 6% trichloroacetic acid and centrifuged (2000*g*, 20 minutes, 4°C). The supernatant was removed, extracted four times with water-saturated ethyl ether, and evaporated with a centrifuge vacuum pump (40°C). The cGMP content was measured with an enzyme immunoassay kit (Amersham, Buckinghamshire, UK). The amount of proteins was measured by colorimetric reaction (Bio-Rad, Glattbrugg, Switzerland) after the pellet was dissolved in NaOH (0.1 N).

Drugs

Sodium nitroprusside, SNAP, ODQ, DIDS, niflumic acid, and 9AC were purchased from Fluka Chemie (Glattbrugg, Switzerland), 8-pCPT-cGMP from BioLog Life Science Institute (Bre-

men, Germany), and lucifer yellow, dimethyl sulfoxide (DMSO), osmium, paraformaldehyde, anti-lucifer yellow antiserum, isobutyl-methylxanthine, and tetraethylammonium chloride from Sigma (Buchs, Switzerland). Stock solutions were prepared with DMSO for DIDS and 9-AC (organ chamber's final DMSO concentration $< 0.2\%$) and with bidistilled water for 8-pCPT-cGMP. All other drugs were prepared fresh daily in distilled water solutions.

Statistical Analysis

Membrane potential recordings are expressed in millivolts or in seconds (δt_{max} is time to reach maximal depolarization), and cGMP measurements in picomoles per milligram of protein. Results are given as means ± SEM with n corresponding to the number of eyes studied (one preparation per eye). Data were analyzed using a multivariate ANOVA (Holme's correction), with $P < 0.05$ considered to be significant.

Results

Membrane Potential Measurements in Ciliary Epithelium

On penetration of the preparations with the microelectrode, the electrical signal showed a rapid deflection before stabilizing at a level of -63.7 ± 0.8 mV ($n = 50$). In preparations in which lucifer yellow was injected through the recording microelectrode, the injected fluorescent dye could be seen in the superficial layer of the preparations over a relatively large area of the tissue (Fig. 1A). Furthermore, by electron microscopy, immunogold staining against lucifer yellow could be observed in both pigmented and nonpigmented ciliary epithelial cells (Fig. 1B). These observations are in agreement with previous reports indicating that nonpigmented and pigmented layers of the ciliary epithelium function as a syncytium (between and within the layers)[1] and further indicate that, in our experiments, the first electrical deflection recorded on penetration of the tissue by the microelectrode corresponds to the measurement of a ciliary epithelium transmembrane potential.

SNP- and SNAP-Induced Membrane Depolarization

A significant depolarization of the ciliary epithelium baseline transmembrane potential was observed after exposure of the preparations to the NO donors SNP (6 ± 1 mV, $\delta t_{max} = 180 \pm 19$ seconds, $P < 0.05$) or SNAP (8 ± 1 mV, $\delta t_{max} = 98 \pm 21$ seconds, $P < 0.05$; Fig. 2A; Table 1). This effect was reversible (after washout of the drugs) and reproducible (30 minutes after washout, preparations were exposed a second time to the drugs), although the second depolarization in response to SNP ($81\% \pm 8\%$, $n = 8$; $P = 0.09$) or SNAP ($73\% \pm 11\%$, $n = 5$; $P = 0.17$) was slightly lower than the one observed after the first exposure. In presence of the specific GC inhibitor ODQ, the depolarization observed after the second exposure to SNP or SNAP was totally abolished (Fig. 2A, Table 1). A small hyperpolarization (-2 mV) could even be observed after SNP exposure in the presence of ODQ. Thus, these results indicate that drugs such as SNP or SNAP can induce a membrane potential depolarization through the activation of GC in porcine ciliary epithelium.

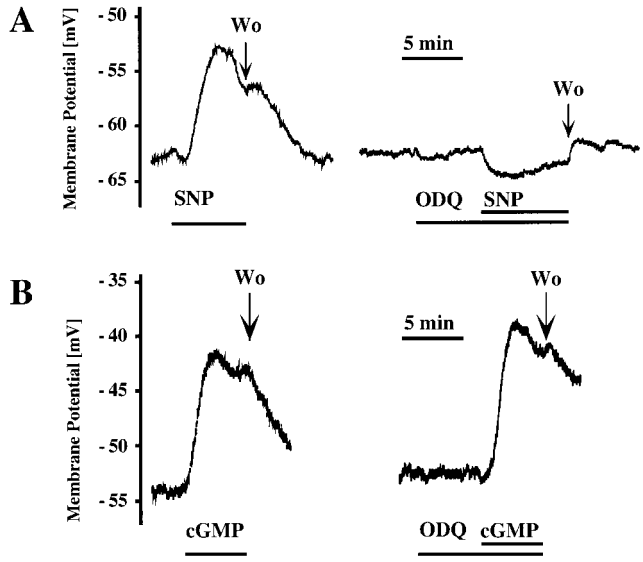

Figure 2. Original recording of the transmembrane potential obtained after penetration of the epithelium of an isolated porcine ciliary process with a microelectrode. (**A**) The NO donor SNP (100 μM) evoked a depolarization that could be prevented by the guanylate cyclase inhibitor ODQ (10 μM). (**B**) The cGMP analogue 8-pCPT-cGMP (100 μM) also induced depolarization. However, in contrast to SNP, the depolarization evoked by cGMP was not inhibited by ODQ. Wo: washout.

8-pCPT-cGMP–Induced Membrane Depolarization

Exposure to the cGMP analog, 8-pCPT-cGMP, evoked a significant depolarization (13 ± 1 mV, $\delta t_{max} = 153 \pm 14$ seconds, $P < 0.001$) of the ciliary epithelium baseline transmembrane potential (Fig. 2A, Table 1). A depolarization that again was reversible (after washout) as well as reproducible (30 minutes after washout), although slightly attenuated in comparison with the one induced after the first exposure to 8-pCPT-cGMP ($82\% \pm 8\%$, $n = 8$; $P = 0.06$). In contrast to SNP or SNAP, the GC inhibitor ODQ had no effect on the depolarization evoked by the second exposure to 8-pCPT-cGMP (Fig. 2B, Table 1). These results indicate that in porcine ciliary epithelium the cGMP analogue 8-pCPT-cGMP induces a depolarization of the transmembrane potential that is not affected by the GC inhibitor ODQ.

8-pCPT-cGMP–Induced Depolarization and Anion Channel Inhibitors

The membrane potential depolarization evoked by the cGMP analogue 8-pCPT-cGMP was significantly ($P < 0.05$) inhibited by the three anion channel inhibitors, niflumic acid, DIDS, or 9-AC. In contrast, the nonspecific (cationic) potassium channel blocker TEA had no significant effect on the depolarization induced by 8-pCPT-cGMP (Table 2). These results suggest the involvement of anionic transmembrane currents in the depolarization induced by the cGMP analogue 8-pCPT-cGMP. It must be noted that during the time of incubation with DIDS and 9-AC alone (but not with niflumic acid or TEA) a hyperpo-

TABLE 1. Effect of the Guanylate Cyclase Inhibitor ODQ (10 μM) on the Membrane Potential Depolarization Induced by SNP, SNAP, or 8-pCPT-cGMP in Porcine Ciliary Epithelium

	n	Baseline [mV]	First Exposure [mV]	Baseline [mV]	Second Exposure [mV]
SNP					
Control	8	−61 ± 3	6 ± 1*	−62 ± 2	5 ± 1*
ODQ	6	−67 ± 2	6 ± 1†	−66 ± 2	−2 ± 1§
SNAP					
Control	5	−61 ± 3	8 ± 1*	−60 ± 2	6 ± 1*
ODQ	5	−67 ± 1	7 ± 1*	−65 ± 0	0 ± 0§
8-pCPT-cGMP					
Control	5	−63 ± 2	13 ± 1‡	−66 ± 4	11 ± 1‡
ODQ	5	−64 ± 2	10 ± 2*	−63 ± 2	9 ± 1§

First exposure: agonist alone (100 μM SNP, SNAP, 8-pCPT-cGMP); second exposure: agonist + inhibitor (10 μM ODQ). Data are means ± SEM; *n*: number of ciliary processes studied.

* $P < 0.05$.

† $P \leq 0.01$.

‡ $P < 0.001$: significantly different from baseline (multivariate ANOVA with Holme's correction).

§ $P < 0.05$: significantly different from first exposure (multivariate ANOVA with Holme's correction).

larization of the baseline membrane potential occurred (−9 ± 2 mV and −6 ± 1 mV, respectively; $n = 5$, $P < 0.05$).

SNP-Induced cGMP Production

In comparison with controls (2.1 ± 0.3 fmol/mg protein), a 5-minute exposure to SNP significantly increased the cGMP concentration in isolated porcine ciliary processes (20.0 ± 1.4 fmol/mg protein; $n = 6$, $P < 0.001$). An increase that was significantly blunted by the presence of the GC inhibitor ODQ (9.8 ± 1.6 fmol/mg protein; $n = 6$; $P < 0.001$). These experiments demonstrate that in porcine ciliary processes the NO donor SNP increases cGMP production through the activation of a GC enzyme.

DISCUSSION

The present study indicates that activation of the NO-GC-cGMP pathway induces a membrane potential depolarization of the ciliary epithelium in isolated porcine ciliary processes. Indeed, the NO donors SNP and SNAP evoked a depolarization and SNP an increase in cGMP production that were inhibited by the GC inhibitor ODQ. In contrast, ODQ had no effect on the depolarization elicited by the cGMP analogue 8-pCPT-cGMP (Fig. 2, Table 1). Furthermore, this study suggests that the depolarization evoked by cGMP could reflect the activation of transmembrane anionic currents. Indeed, although anion channel blockers are in general not very specific, niflumic acid, DIDS, and 9-AC were able to inhibit the depolarization evoked by 8-pCPT-cGMP. In contrast, the nonspecific (cationic) potassium channel blocker TEA had no effect on 8-pCPT-cGMP–induced depolarization (Table 2).

There is evidence that activation of the NO-GC-cGMP pathway is involved in the transport of water across the epithelium of different tissue. Indeed, in the rat gastrointestinal tract, activation of this pathway stimulates the production of mucus through an increase in chloride secretion.[2,3] In the human respiratory airway, NO mediates chloride and, thus, water secretion induced by a β-adrenergic receptor agonist.[4]

In porcine ciliary processes, the presence[5] as well as the activity[6] of nitric oxide synthase (the enzyme responsible for NO formation) has also recently been documented. Marked immunostaining against the neuronal isoform of nitric oxide synthase has been observed at the junction between nonpigmented and pigmented ciliary epithelial cells.[5] Furthermore, in isolated ciliary processes, β-adrenoreceptors activation could increase the production of nitrite (a metabolite of NO) through a pathway involving a protein kinase A and a nitric oxide synthase.[6]

It has also been reported that cGMP can modulate short-circuit current across rabbit ciliary epithelium.[7] In this study, when cGMP was applied on the stromal side of the ciliary body, the short-circuit current was increased, whereas when cGMP was applied on the aqueous humor side, the short-circuit current was reduced.[7] In the present study in which the ciliary epithelium membrane potential was measured in porcine ciliary processes (and not short-circuit current across the epithelium), only depolarization, and never hyperpolarization, was observed after 8-pCPT-cGMP exposure. Although there are methodologic differences between the two studies, these observations could reflect heterogeneity between species.

In the present study, although ODQ completely abolished SNP-induced depolarization, it only partially (although significantly) inhibited SNP-induced cGMP production. This apparent discrepancy is likely to reflect differences in experimental protocol. Indeed, values of cGMP production corresponded to the accumulation of the cyclic nucleotide in the presence of the phosphodiesterase inhibitor IBMX over a period of 5 minutes after SNP exposure. In contrast, values of membrane potential corresponded to measurements conducted in the absence of IBMX when the depolarization was maximal after SNP exposure (an effect that lasted for only a few seconds). Therefore, no quantitative correlation can be drawn between these two types of experiments. Nevertheless, the results of the cGMP production clearly illustrate that in porcine ciliary

TABLE 2. Effect of Different Anion Channel Inhibitors on the Membrane Potential Depolarization Induced by 8-pCPT-cGMP (100 μM) in Porcine Ciliary Epithelium

	n	Baseline [mV]	First Exposure [mV]	Baseline [mV]	Second Exposure [mV]
Control	8	−64 ± 2	13 ± 1†	−65 ± 4	11 ± 1†
Niflumic acid 100 μM	5	−63 ± 2	14 ± 2*	−61 ± 2	1 ± 1‡
DIDS 1 mM	5	−66 ± 3	17 ± 2*	−70 ± 1	11 ± 1§
9-AC 1 mM	5	−66 ± 2	10 ± 1†	−69 ± 2	5 ± 1§
TEA 10 mM	5	−75 ± 3	15 ± 2†	−71 ± 2	12 ± 2

Data are mean ± SEM; *n*: number of ciliary processes studied.

* $P < 0.01$.

† $P < 0.001$: significantly different from baseline (multivariate ANOVA with Holme's correction).

‡ $P < 0.05$.

§ $P < 0.01$: significantly different from first exposure (multivariate ANOVA with Holme's correction).

processes an NO donor, such as SNP, is able to increase cGMP production through the activation of GC.

Although the depolarization induced by 8-pCPT-cGMP could be inhibited by three different anion channel blockers, it cannot be concluded that anion channels are involved in the effect observed. Because anion channel inhibitors are known to be very unspecific, the present data can only suggest the possibility that transmembrane anionic currents are involved in the depolarization observed after 8-pCPT-cGMP exposure. That anion channel inhibitors are not specific could also explain why in resting conditions, DIDS and 9-AC alone (but not niflumic acid) induced a hyperpolarization of the basal membrane potential.[8]

The present observation made in porcine ciliary processes showing that activation of the NO-GC-cGMP pathway induces a depolarization of the ciliary epithelium raises the hypothesis that NO may be involved in the process of aqueous humor formation. This hypothesis is supported by a report indicating that shortly after topical application of the NO donor SNP an increase in intraocular pressure can be observed in rabbits, possibly through an increase in aqueous humor flow.[9] This early effect of NO on intraocular pressure seems to be different from its action at the level of the iridocorneal angle where it is known to increase aqueous humor excretion and thus decrease pressure in the eye.[10]

In conclusion, in line with the role played by NO in the transport of water across the epithelium of different organs,[2–4] the present study shows that in isolated porcine ciliary processes the activation of the NO-GC-cGMP pathway induces a ciliary epithelium membrane depolarization, possibly through the activation of anionic currents. This observation provides additional evidence for the hypothesis that NO may modulate aqueous humor production in ciliary processes.[5–7,9]

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