Propagation of Ciliary Smooth Muscle Cells In Vitro and Effects of Prostaglandin F$_{2\alpha}$ on Calcium Efflux

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The effect of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) on calcium efflux from ciliary smooth muscle cells was studied. Ciliary smooth muscle cells, cultured from human ciliary muscle explants, retained the morphologic and immunologic characteristics of smooth muscle cells. High concentrations (to $10^{-6}$ mol/l) of PGF$_{2\alpha}$ were associated with a dose-dependent increase of calcium ($^{45}$Ca) efflux, whereas at concentrations lower than $10^{-8}$ mol/l there was little or no $^{45}$Ca efflux. Our in vitro data are inconsistent with the experimental hypothesis that PGF$_{2\alpha}$ at pharmacologic concentrations relaxes ciliary muscle with a consequent increase in uveoscleral outflow. Invest Ophthalmol Vis Sci 33:2679-2686, 1992.

Topically administered prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) reduces intraocular pressure significantly in normal subjects$^1$-4 and primary open-angle glaucoma patients$^5$ by increasing uveoscleral outflow facility. However, the precise action by which PGF$_{2\alpha}$ increases uveoscleral drainage has not been elucidated. Two possible mechanisms have been suggested. Crawford and Kaufman$^6$ have hypothesized that there is relaxation of part or parts of the ciliary muscle with resulting increased uveoscleral outflow facility. Luetjen-Drecoll and Tamm$^7$ have suggested that increased uveoscleral outflow facility may be related to loss of extracellular material from among ciliary muscle bundles.

In other biological systems, PGF$_{2\alpha}$ consistently increases smooth muscle cell contractility. In rat aorta smooth muscle cells, PGF$_{2\alpha}$ at concentrations from $1.4 \times 10^{-8}$ to $1.4 \times 10^{-5}$ mol/l causes a dose-dependent increase of cytosolic free calcium concentration.$^8$ In hen uterine smooth muscle cells, there is a dose-dependent increase of calcium ($^{45}$Ca) efflux with PGF$_{2\alpha}$ concentrations ranging between $10^{-10}$ and $10^{-6}$ mol/l.$^9$ Both of these effects, an increase in cytosolic free calcium concentration and increase in calcium efflux, are known to be associated with smooth muscle contraction.$^{10}$ Hence, these data suggest that PGF$_{2\alpha}$ causes a dose-dependent contraction of smooth muscle cells, not relaxation, as proposed in the ciliary muscle. To investigate this possible inconsistency, we examined the effect of PGF$_{2\alpha}$ on $^{45}$Ca efflux in cultured smooth muscle cells from human ciliary muscle.

Materials and Methods

Cell Culture

Human ciliary smooth muscle cells were obtained from the pars plana portion of the ciliary body of a 30-year-old postmortem eye enucleated 2 hr after the donor’s death. The female donor had no known eye disease. To obtain a ciliary muscle explant, the eye was bisected between the ora serrata and equator, and the anterior segment was placed in a dish with corneal epithelium down. Under a dissecting microscope, the lens was removed and the iris was disinserted. The ciliary body was gently removed from the sclera and divided into segments by radial incision. The pars plicata of each segment was excised, leaving a portion of the pars plana. Epithelial layers then were separated from the ciliary muscle region of the ciliary body. The ciliary muscle explant was placed on the bottom of 24-well plate (Corning Glass Works, Corning, NY), which was filled with Dulbecco’s modified Eagle’s medium and F12 nutrient mixture (University of California San Diego Cell Culture Facility) supplemented with 10% fetal bovine serum (J R Scientific, Woodland, CA), 20 mmol/l L-glutamine, and antibiotics (penicillin-streptomycin 100 U/ml, gentamycin 10 μg/ml, amphotericin B 1.2 μg/ml). The plate was incubated at 37°C in a humidified atmosphere of 95% air:5% O$_2$. Confluent cells were trypsinized and subcultured at a ratio of 1 to 3. The culture medium was changed every 2 to 3 days. The cultures reached confluency after 7 to 10 days and then were employed for $^{45}$Ca efflux experiments. For our studies, cells in the
fourth passage were subcultured and grown on 6-well plates (Corning).

Characterization of Ciliary Muscle Cell Cultures

Ciliary muscle cells in culture were identified by their pattern of growth, ultrastructural features, and immunocytochemical staining characteristics. As the cells became confluent, they grew in a hill-and-valley pattern, as seen under phase contrast microscopy (Fig. 1). Fourth passage cells grown directly onto dishes for transmission microscopy showed ultrastructural characteristics of smooth muscle cells, such as myofilaments and associated dense bodies, plasma-membrane vesicles, basal lamina, polysomes, mitochondria, and rough endoplasmic reticulum (Fig. 2).

To ascertain whether the smooth muscle cells were from the ciliary muscle or from periendothelial cells, including capillary pericytes or larger vessel-associated smooth muscle cells within the ciliary body, the expression of desmin was examined. Desmin is an intermediate filament subtype found in different concentrations in smooth muscle cells and pericytes. Initially, careful comparison was made regarding the amount of desmin in these cell types within cryostat sections of the ciliary body. To identify blood vessels, the sections also were stained with an antibody against von Willebrand's factor, which is found only within vascular endothelial cells. Then these results were compared with cell cultures stained using the dilution and staining conditions used for the sections.

Immunocytochemistry

To investigate desmin expression, 2 μm-thick frozen sections of fresh ciliary body tissue were cut with the Instrumedics Frozen Sectioning Aid (Instrumedics, Inc., Teaneck, NJ) fitted to a Tissue Tek II cryostat (Miles Laboratories, Inc., Naperville, IL). Sections were fixed by freeze substitution in acetone at −20°C and labelled with antibodies against desmin and against von Willebrand's factor (mouse monoclonal antihuman desmin clone D33 and polyclonal rabbit antihuman von Willebrand's factor; Dako Corp., Carpintera, CA). The presence of these antibodies was demonstrated with a commercial kit in which biotinylated goat antimouse IgG is demonstrated using alkaline phosphatase and swine antirabbit IgG is demon-

Fig. 1. Topographic view of confluent fourth passage ciliary smooth muscle cells. Note prominent hill (arrow) of interwined, multilayered cells and organization of the surrounding monolayer of parallel cells, the so-called "valley" (open arrow). (Toluidine blue, X224.)
Fig. 2. Electron micrograph of fourth passage ciliary smooth muscle cells. Note myofilaments (MF) and associated dense bodies (DB), plasmalemmal vesicles (PV), mitochondria (M), and polysomes (P) (Original magnification ×48,500.)

Measurements of 45Ca Efflux

45Ca efflux from cells cultured in a 6-well plate was measured as described by Dorn et al. Briefly, 10^−4 mol/l PGF2α (Sigma, St. Louis, MO) stock solution was diluted to an appropriate concentration and added to the 35 mm dish of a 6-well plate and allowed to incubate for 2.5 min. Confluent cells were rinsed twice with serum-free culture medium and incubated in 1 ml of serum-free medium containing 5 μCi of 45CaCl2 (Amersham, Arlington Heights, IL) for 1.5 hr at 37°C. Uptake of 45Ca reached equilibrium by 30 min. Prior to the 45Ca efflux experiments, 35 mm dishes were rinsed twice with phosphate buffered saline containing 1 mmol/l CaCl2 and 1 mmol/l MgCl2 (experimental buffer). For measurement of 45Ca efflux, 1 ml aliquots of experimental buffer was added to each 35 mm dish of cells and exchanged at 2.5 min intervals for 30 min. Desaturation was commenced simultaneously in six 35 mm dishes of 45Ca-labelled cells. The amount of 45Ca released from cells during each time interval was measured by liquid scintillation counting. Results are expressed as counts per minute/culture or as percentage increase of 45Ca efflux, which was estimated as follows: Percent 45Ca efflux = (Y/X - 1) × 100, where X = amount of released radioactivity during 5 min just before the addition of agonist and Y = amount of released radioactivity during 5 min just after the addition of agonist. Data were subjected to one-way analysis of variance. Differences of P less than .05 were considered significant.

Results

Ciliary Muscle Cell Culture

With antidesmin at 1:100 dilution, ciliary muscle and vascular smooth muscle in the ciliary body were
stained intensely. At 1:500 dilution, ciliary muscle stained with moderate intensity (Fig. 3). Lightly red-stained (desmin) smooth muscle cells were seen associated with a large iris artery. Brown-stained (von Willebrand's factor) vessels within the central ciliary body stroma only rarely had lightly red-stained cells in close association. However, in the subepithelial stroma, there were many small intensely red-stained cells that often appeared in close association with brown-stained capillaries. Their position within the ciliary body tissue and their close association with capillaries suggest these small red-stained cells are pericytes. These results also indicate that ciliary smooth muscle contains less desmin than pericytes and that vascular smooth muscle cells associated with ciliary arterioles, although similar in desmin content to stromal smooth muscle, are relatively rare.

Growth of ciliary muscle cells from the 59-year-old postmortem eye was similar to that in the 30-year-old eye. Third passage cells were processed for immunocytochemical demonstration of smooth muscle actin and desmin. Phase image photomicrograph of negative control (without primary serum) cultures show the spindle-shaped nature of these cells (Fig. 4A). The nonspecific staining in these control cultures visualized with bright field optics was low (Fig. 4B). Cells in cultures prepared with antisera to smooth muscle actin antibodies displayed uniformly positive staining (Fig. 4C).
Fig. 4. Immunocytochemical localization of smooth muscle actin and desmin in third passage ciliary smooth muscle cells. Phase image (A) of negative control culture shows confluent monolayer of spindle-shaped cells. Bright field image (B) of same field as in (A) shows faint outlines of unstained cells. Bright field image of culture stained with antismooth muscle actin antibodies (C) shows uniform staining of virtually all cells in the culture. Bright field image of culture stained with antidesmin antibodies at 1:500 dilution (D) shows that most cells did not stain, whereas a few scattered cells exhibit various levels of positive staining (Original magnification ×430.)
When prepared with antidesmin antibodies at 1:500 dilution, approximately 5% of the cells exhibited various levels of positive staining, whereas the remaining cells did not stain (Fig. 4D).

Calcium Efflux Studies

$^{45}$Ca efflux from the ciliary smooth muscle cells resulted in a double exponential decay curve (Fig. 5). PGF$_{2\alpha}$ was added to the experimental buffer medium at 17.5 min, and the resulting changes in $^{45}$Ca efflux affected the high affinity, slowly equilibrating calcium pool. When the cells preloaded with $^{45}$Ca were exposed to PGF$_{2\alpha}$ from $10^{-10}$ to $10^{-8}$ mol/l, there was no significant change in $^{45}$Ca efflux. This indicates that ciliary smooth muscle cells were uncontracted. However, at the concentration from $10^{-8}$ to $10^{-6}$ mol/l, a significant dose-dependent efflux of $^{45}$Ca was observed ($P < .0001$) (Fig. 6). These findings suggest that ciliary smooth muscle cells were contracted.

Next, we investigated the effect of successive additions of $10^{-6}$ mol/l PGF$_{2\alpha}$ on $^{45}$Ca efflux in experimental buffer and calcium-free solution, which was obtained by omitting CaCl$_2$ from the experimental buffer. A first addition of $10^{-6}$ mol/l PGF$_{2\alpha}$ caused a reduced magnitude of $^{45}$Ca efflux in response to a subsequent addition of $10^{-6}$ mol/l PGF$_{2\alpha}$ in the calcium-free solution, in which calcium influx across the cell membrane was negligible. However, in experimental buffer, an increase in $^{45}$Ca efflux was observed after a second addition of $10^{-6}$ mol/l PGF$_{2\alpha}$, but the extent of increase was less than that after a first treatment with the same dose of PGF$_{2\alpha}$ (Fig. 7). This suggests that PGF$_{2\alpha}$ elicits calcium mobilization from an intracellular store in ciliary muscle cells.

Discussion

An important consideration in evaluating the results from this study is the identity of the cells within the cultures. Several cell types are observed in small slices of ciliary muscle tissue, including vascular endothelial cells, pericytes, and vascular smooth muscle as well as ciliary smooth muscle cells (ciliary epithelium was removed before the explant was plated). The overall spindle shape of the cells plus the hill and valley pattern of growth is consistent with previously described smooth muscle cultures. This observation was supported by our transmission electron microscopic observations of the cultured cells, which revealed cytoplasmic structures characteristic of smooth muscle cells. Loose cobblestone patches of
cells characteristic of endothelial cells were not observed within the cultures.

The cultures were further characterized by comparing the relative expression of desmin in tissue sections to the cells in culture. Although the expression of desmin in a pericyte population in vivo can vary, this approach is valid because those pericytes that do express desmin continue this expression when placed in vitro. In the present study, vascular pericytes in the ciliary body were found to stain intensely with anti-desmin antibodies, whereas ciliary muscle cells were found to stain only lightly. Under identical staining conditions, very few of the cells in the ciliary muscle cultures stained with the same antidesmin antibodies. These results suggest that the cultures contained very few pericytes. If the primary antibody was more concentrated, the difference in staining intensity between these two cell types was not as apparent by visual examination. Although the present results do not determine whether the small portion of desmin-positive cells in the cultures are pericytes or ciliary smooth muscle cells, they suggest that the cultures contain at least 95% ciliary smooth muscle cells.

The morphologic and immunocytochemical results cannot rule out the possibility that some smooth muscle cells from arterioles and possibly from large venules may be present in the cultures. During the dissection, most of the subciliary epithelial vasculature was removed with the epithelium. However, it is likely that a small portion remained with the muscle tissue. Unless the vascular smooth muscle cells grew considerably more rapidly than the ciliary smooth muscle cells, the vascular smooth muscle cells, if present, would represent only a small fraction of the total cells within the cultures.

When an agonist activates the calcium messenger system in a cell, there is a transient, rather than a sustained, increase in calcium concentration, a sustained increase in the rate of calcium ion influx, and a net efflux of calcium ion from the cell. There are two components in calcium efflux from smooth muscle cells—fast and slow. We examined the effect of PGF on the latter component of calcium efflux from cultured human ciliary smooth muscle cells. The slow component of efflux is thought to represent the compartment of calcium originating from high-affinity binding sites within cells and is considered the fraction of intracellular calcium in equilibrium with activator calcium ions. Ciliary smooth muscle cells demonstrate a dose-related efflux of calcium in response to concentrations ranging from $10^{-8}$ to $10^{-6}$ mol/l PGF, but demonstrate no detectable change in efflux in response to concentrations ranging from $10^{-10}$ to $10^{-8}$ mol/l PGF. The former suggests there is contraction of the cells, and the latter suggests there is little or no effect on the contractile state of the cells. These findings do not indicate relaxation of the cells at these concentrations.

The calcium messenger system has a central role in mediating muscle contraction. There are two branches in this system: calmodulin leads to transient effect, whereas protein kinase C leads to a more sustained cellular response. The rise in the calcium concentration from intracellular or extracellular sources, or both, leads to activation of calmodulin-dependent myosin light-chain kinase. The active kinase catalyzes phosphorylation of a myosin light chain, which permits myosin to interact with actin, causing smooth muscle contraction. However, after this increase, the calcium concentration falls back to its basal level and the content of phosphorylated myosin declines slowly to its resting state even though the muscle remains contracted. During this sustained phase, contraction depends on the efflux of calcium ion, indicating that calcium ion still has a messenger function, despite that its concentration is no longer elevated.

Uveoscleral outflow appears to be highly dependent on the degree of tone in the ciliary muscle. Pilocarpine-induced contraction of the ciliary muscle blocks uveoscleral outflow almost completely, while atropine increases it. Pilocarpine blocks the ocular hypotensive effect of PGF, and simultaneously blocks the effect of PGF on the uveoscleral outflow. Recent morphologic studies show that after 4 to 8 days of PGF treatment, the ciliary muscle has a relaxed appearance and the spaces between the ciliary muscle fiber bundles are enlarged. There also is a loss of extracellular material, which may decrease the resistance to flow and thus increase the uveoscleral outflow. Interestingly, a larger intraocular pressure reduction has been observed after 4–5 days of daily PG treatment than after a single dose. Furthermore, the increase in uveoscleral outflow also is larger after multiple dosing than after a single dose.

Thus, one may speculate that the long-term effects of PG on uveoscleral outflow are, at least partly, caused by structural or metabolic changes, and that the antagonistic effect of pilocarpine upon PGF-induced ocular hypotension might be due to obliteration of intermuscular space that is created by the net effect of PGF on the extracellular material and the ciliary muscle tone. Further investigations are needed to clarify this point. By correlating the dose-response relationship of PGF with contractility and extracellular material loss, more information may be gained concerning the mechanism of the ocular hypotensive effect of PGF.

Key words: calcium efflux, ciliary muscle, glaucoma, prostaglandin F, uveoscleral outflow
Acknowledgments

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References