Mediation of Calcium-Independent Contraction in Trabecular Meshwork through Protein Kinase C and Rho-A

Hagen Thieme,1,2 Michael Nuskovski,1 Jens Uwe Nass,1 Uwe Pleyer,3 Olaf Strauss,1 and Michael Wiederhold1

PURPOSE. Inhibition of protein kinase C (PKC) and rho-kinase (ROCK) may represent a new way of influencing outflow facility through isolated relaxation of the trabecular meshwork (TM). This work was performed to investigate the existence of calcium-independent contraction in this smooth-muscle-like tissue and its modulation by targeting the rho-guanosine triphosphatase (GTPase)-mediated pathway.

METHODS. Isometric tension measurements of bovine TM and ciliary muscle (CM) were performed. Intra- and extracellular calcium buffering was accomplished with EGTA and 1,2-bis(2-aminophenoxy)-ethane-N,N,N′,N′′-tetra-acetic acid tetraakis/acetoxymethyl ester (BAPTA-AM) followed by stimulation of PKC with phorbol ester (PMA) or 4α-phorbol. Calcium-independent contraction was blocked using the highly specific ROCK inhibitor Y-27632. Western blot analysis and immunoprecipitation was performed using human TM cells.

RESULTS. In TM, carbachol induced partial contraction under conditions of extracellular calcium depletion (22.1% ± 2.3% versus 100%, n = 9). The membrane-permeable calcium chelator BAPTA-AM completely blocked this response (1.1% ± 1.4% versus 100%, n = 9). When calcium was completely blocked, PMA induced contraction in TM (16.7% ± 5.9% versus 100%, n = 9) but not in CM (1.8% ± 2.5% versus 100%, n = 6). The inactive PMA analogue 4α-phorbol did not induce contraction, indicating that activation of PKC is involved in this contractile response. The ROCK inhibitor Y-27632 completely blocked the calcium-independent PMA-induced contraction in TM. Western blot analysis and immunoprecipitation revealed the expression of the rho-A protein in human TM cells.

CONCLUSIONS. The data indicate that contrary to CM, the TM features calcium-independent contractile mechanisms linked to rho-A and PKC isoforms that do not require calcium for activation. ROCK inhibitors may allow specific modulation of the TM to enhance outflow facility, thus lowering intraocular pressure. (Invest Ophthalmol Vis Sci. 2000;41:4240–4246)

Treatment of glaucoma focuses on one of the major risk factors: intraocular pressure (IOP).1 The trabecular meshwork (TM) has smooth-muscle-like properties and is actively involved in aqueous humor dynamics through contractile mechanisms.2 Therefore, the TM is regarded as one of the major targets for the development of new antiglaucoma drugs.3–5 Smooth-muscle–relaxing substances are known to increase outflow rates and may simultaneously be beneficial in improving ocular hemodynamics around the optic nerve head. The ideal pressure-lowering drug should therefore be useful in coregulating both pathophysiological conditions, i.e., IOP and microcirculation.1,6 By relaxing the TM, such a compound would ideally lower IOP without affecting the CM, circumventing side effects such as accommodative changes or miosis.

A possible group of compounds fulfilling these needs are the inhibitors of protein kinase C (PKC). Recently, a role for PKC in the regulation of smooth muscle contraction has been postulated.7–9 PKC inhibitors such as H7 or chelerythrine are known to induce relaxation in TM without affecting the CM.10,11 Additionally, PKC inhibitors have been used successfully to lower IOP in an animal model.5 Recently, we were able to show that PKC isoforms show different tissue distribution in both human and bovine TM and CM and that contractility in these tissues is differently regulated by various PKC agonists and antagonists.11 One particular PKC isoform (PKC-ε) is highly expressed in TM and is known for its involvement in the regulation of calcium-independent contraction in various smooth muscle preparations.8,11,12 This form of contractility is primarily based on pharmacomechanical coupling events rather than calcium influx from the extracellular space or release of calcium from intracellular stores. It has been suggested that the small guanosine triphosphatase (GTPase) rho-A may be a target protein for PKC-ε, and their interaction may contribute to the regulation of calcium-independent contractility.13,14

Received March 31, 2000; revised August 29, 2000; accepted September 9, 2000.

From the 1Institut für Klinische Physiologie, and 2Augenklinik Universitätsklinikum Benjamin Franklin, Freie Universität Berlin; and 3Klinik und Poliklinik für Augenheilkunde, Charité, Campus Virchow Klinikum, Humboldt Universität Berlin, Germany.

Submitted for publication May 3, 2000; revised August 9, 2000; accepted August 29, 2000.

Commercial relationships policy: N.

Corresponding author: Hagen Thieme, Institut für Klinische Physiologie, Universitätsklinikum Benjamin Franklin, Freie Universität Berlin, Hindenburgdamm 30, 12203 Berlin, Germany, thieme@ukbf.fu-berlin.de
In this study calcium-independent contraction existed exclusively in the TM, and this PKC-dependent modulation of contractility was specifically blocked by rho-A kinase inhibitor Y-27632. Based on these findings, we suggest a possible modulation of IOP through substances such as Y-27632 through relaxation of the TM. First evidence for such pressure-reducing properties in an animal model has been presented recently.\textsuperscript{15}

METHODS

Contractility Measurements

Enucleated bovine eyes were obtained from a slaughterhouse and placed on ice. Small TM and CM strips were dissected carefully from bovine eyes according to methods described in detail previously.\textsuperscript{16} TM strips were prepared in a circular direction, and meridional CM strips were excised perpendicular to the circular ciliary body. Isolated strips of 2 to 4 mm length and 0.5 mm width were allowed to rest under control conditions for at least 1 hour before administration of various compounds. Only strips showing a stable basic contractility were used for the experiments.

The effects of agents on contractility were measured isometrically with a custom-made force length transducer system, as described.\textsuperscript{10} Tissue strip contractions were expressed relative to the response obtained with a maximally effective carbachol concentration (10\textsuperscript{\textminus 6} M), which was tested in each tissue strip as a control. To determine the activity of a compound, the agent was added to the tissues at basal tension. The chamber solutions were kept at a stable temperature (37°C) and pH. The ionic concentrations (in millimolar) of Ringer’s solution were: 151 Na\textsuperscript{+}, 5 K\textsuperscript{+}, 1.7 Ca\textsuperscript{2+}, 0.9 Mg\textsuperscript{2+}, 131 Cl\textsuperscript{−}, 0.9 SO\textsubscript{4}^{2−}, 1 H\textsubscript{2}PO\textsubscript{4}\textsuperscript{−}, 28 HCO\textsubscript{3}\textsuperscript{−}, and 5 glucose. Calcium-free solutions were buffered with EGTA (10\textsuperscript{−5} M) and/or BAPTA-AM (3 × 10\textsuperscript{−5} M). All solutions were gassed with 5% CO\textsubscript{2} in air, which resulted in a pH of 7.4.

Human TM Cell Cultures

Human TM cells were isolated by methods based on those of Grierson et al.\textsuperscript{17} and Siegren et al.\textsuperscript{18} with tissue obtained from donor eyes (Department of Ophthalmology, Charité, Campus Rudolf Virchow, Berlin). Tenets of the Declaration of Helsinki were followed, informed consent was obtained, and institutional human experimentation committee approval was granted for the studies. In brief, TM strips were carefully dissected under microscopic view. A fine wire probe (0.5 mm) was used to cannulate Schlemm’s canal, thus aiding visualization of the TM. The strips were placed under glass coverslips in 35-mm plates and were incubated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all cell culture material was obtained from Biochrom, Berlin, Germany). The cells were maintained in a 95% air-5% CO\textsubscript{2} atmosphere at 37°C and were passaged by the trypsin-EGTA method after having reached confluence. Only well-characterized human TM cells from early passage (passages 3–7) were used for Western blot and immunoprecipitation experiments. Histologic characterization was performed by Elke Lütjen-Drecoll, (Department of Anatomy, Universität Erlangen, Nürnberg, Germany) and showed typical immunostaining as described previously.\textsuperscript{19}

Western Blot Analysis and Immunoprecipitation

Western blot analysis was performed as previously described in detail.\textsuperscript{11} Confluent human TM cell monolayers were washed three times with ice cold phosphate-buffered saline (PBS)-Tween, scraped, and lysed in buffer A (1% NP40, 20 mM Tris [pH 8.80], 137 mM NaCl, and 10% glycerol) containing protease inhibitors (Complete; Protease, Boehringer–Mannheim, Mannheim, Germany). After a precentrifugation step (14,000 rpm for 5 minutes at 4°C), the whole cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 25 μg protein per lane, 7.5%-gel) and immunoblotting. Nitrocellulose filter membranes (Polyscreen; NEN Life Science Products, Boston, MA) were blocked in 5% bovine serum albumin (BSA) for 2 hours at room temperature and consequently incubated overnight with rho-A primary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) diluted in 2% BSA-PBS (1:2000). The blots were visualized using a peroxidase-conjugated secondary antibody (Dianova; Jackson Immunoresearch, West Grove, PA) diluted in PBS-Tween (1:20,000; 1 hour at room temperature) and a chemiluminescence kit (ECL, Amersham, Amersham, UK) according to manufacturers instructions. The images were digitalized using an image analyzer (Fujifilm; LAS 1000; Fuji, Tokyo, Japan) and software (Aida 2.1; Raytest, Berlin, Germany).

For immunoprecipitation, protein A bound primary antibody (prewashed Sepharose A beads incubated with antibody for 1 hour at 4°C) was incubated with precleared whole-cell lysate overnight under gentle rotation at 4°C. After several washing steps (six times with lysis buffer A), the beads were centrifuged (2000 rpm for 2 minutes) and boiled in 50 μl 1× Laemmli buffer for 5 minutes. The supernatant was subjected to reducing SDS-PAGE, immunoblot assay, and signal detection, performed as described earlier. Blocking peptide was used in fivefold excess to primary antibody during the immunoprecipitation to verify specificity of the protein bands.

Reagents

The following reagents were used for contractility measurements: 4α-phorbol, (RBI; Sigma, Deisenhofen, Germany); phorbol-12-myristate 13-acetate (Biomol, Hamburg, Germany); 1,2-bis(2-aminophenoxy)-ethane-N,N,N’,N’-tetra-acetic acid tetrakis(acetoxymethyl ester; BAPTA-AM; Sigma); (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane-carboxamide dihydrochloride, monohydrate (Y-27632) was kindly supplied by Yoshitomi Pharmaceutical, Osaka, Japan. All other chemicals were of analytical grade and were purchased from Sigma.

Statistical Analysis

The results of contractility measurements were expressed as mean values ± SEM. Statistical analysis was performed using analysis of variance and Student’s t-test for paired observations (% changes versus carbachol-contracted tissues). The unpaired Student’s t-test was used for comparison of values in TM and CM. Significance was assumed when \( P < 0.05 \). The number (n) refers to the number of experiments. Western blot and immunoprecipitation experiments were repeated at least three times, with cells from individual primary cultures showing identical results.
**RESULTS**

**Effects of Calcium Removal on Carbachol-Induced Contraction**

Calcium-independent contraction in TM strips was accomplished under two different conditions. As has been shown before,16 muscarinic agonists led to a contraction in TM (Fig. 1) after adjustment of baseline tension to 100 to 200 μN. First, extracellular calcium buffering was accomplished by exchanging the bath solution with nominal calcium-free solution buffered with 10^{-5} M EGTA. Under these conditions, carbachol still contracted TM tissue strips to 22% (Figs. 1, 2) of the level obtainable in the presence of calcium (22.1% ± 2.3% versus 100% precontracted tissue with carbachol 10^{-6} M; n = 9; P < 0.01 versus zero contraction). In a second step, additional intracellular calcium buffering with the membrane-permeable compound BAPTA-AM (5 × 10^{-6} M) completely blocked the carbachol-induced contraction indicating the effectiveness of BAPTA-AM in buffering intracellular calcium (generated force expressed in micronewtons).

**Effects of 4α-Phorbol on Contractility**

4α-Phorbol is a biologically inactive analogue of PMA, serving as negative control. The experiments were performed with TM only, because only this tissue showed a development of contractile force under calcium-independent conditions. The PMA analogue had no effect on baseline tension in TM (Fig. 5, summarized data: 0.25% ± 0.42% versus 100%, n = 4, P > 0.62 versus zero contraction).

**Effects of Y-27632 on Contractility**

The pyridine derivative Y-27632 is known for its highly specific inhibition of rho-kinase (ROCK). After the TM strips were preincubated with Y-27632 for 30 minutes (10^{-6} M), PMA (10^{-6} M) was applied. Again extra- and intracellular calcium depletion was performed. Under these conditions PMA did not induce a contractile response in TM (Fig. 6).

**FIGURE 1.** Original recording of isometric force developed in a bovine trabecular meshwork strip under calcium-free conditions. After a carbachol (10^{-6} M)-induced peak, extracellular calcium depletion was accomplished by addition of 10^{-5} M EGTA (Ca^{2+}-free indicates EGTA in calcium-free solution). Carbachol induced a small contraction without reaching a plateau through release of calcium from the intracellular stores. The membrane-permeable calcium chelator BAPTA-AM (5 × 10^{-6} M) completely blocked the carbachol-induced contraction indicating the effectiveness of BAPTA-AM in buffering intracellular calcium (generated force expressed in micronewtons).

**FIGURE 2.** Summary of contractility data obtained with TM strips under both extra- and intracellular calcium depletion (EGTA 10^{-5} M) in calcium-free solution, plus addition of BAPTA AM (5 × 10^{-6} M). The number of experiments is shown in brackets above the bars (**P < 0.001).

**FIGURE 3.** Original trace showing calcium-independent contraction in a bovine TM strip after application of PMA (10^{-6} M). Calcium-free conditions were achieved by using 10^{-5} M EGTA (Ca^{2+}-free) and 5 × 10^{-6} M BAPTA-AM and were identical with those shown in Figure 1.
rized in Figure 7 (0.98% ± 1.4% versus 100%, \( n = 6 \), \( P < 0.51 \) versus zero contraction). Therefore, this compound blocked the PKC-associated, calcium-independent contraction observed with PMA in the TM.

**Western Blot Analysis and Immunoprecipitation**

Human TM cell cultures from individual primary cultures were screened for the expression of rho-A on the protein level. Whole-cell lysate of human TM cells clearly displayed a signal at approximately 21 kDa in the Western blot analysis that resembled the rho-A protein. To detect the amplification of the signal, whole-cell lysate was immunoprecipitated with rho-A antibody, as described in the Methods section. A signal of the same molecular size could be amplified and specifically blocked with blocking peptide. The strong 80-kDa bands result from nonspecific signals caused by the antibodies forming complexes in the immunoprecipitation process. These signals were not present in the Western blot analysis (Fig. 8).

**DISCUSSION**

In this study, in contrast to the CM, TM was able to contract under calcium-independent conditions. The TM features smooth-muscle–like properties and is actively involved in aqueous humor outflow, in the sense that its contraction decreases outflow, whereas relaxation increases this parameter. Because the conventional outflow through the TM accounts for 80% to 90% of the total aqueous humor outflow rate, knowledge about the modulation of contractility in this tissue is important. Smooth-muscle–relaxing substances appear to be suitable candidates for glaucoma therapy with the goal of reducing IOP through TM relaxation without adverse effects on accommodation or pupil diameter, by circumventing the CM and by possibly improving retinal hemodynamics through relaxation of vascular smooth muscle cells or pericytes, i.e., vasodilation.
Smooth muscle contractility is regulated by cytosolic calcium concentration, either by calcium influx through voltage- and receptor-operated membrane channels or by release of calcium from intracellular calcium stores. There are similar mechanisms in the TM. In recent years it has become obvious that smooth muscle cell contractility is not only regulated by changes of intracellular calcium and electromechanical coupling. Other important signaling mechanisms such as membrane potential-independent, pharmacomechanical coupling events seem to trigger the tonic (slow) phase of smooth muscle contractility and are referred to as calcium-independent mechanisms. Calcium-independent contractility has been investigated in various smooth muscle preparations. The involvement of PKC isoforms that are activated in a calcium-independent fashion (namely PKC-), as well as participation of the small GTPase rho-A in responses linked to myosin light-chain kinase phosphorylation and dephosphorylation, has been postulated by many investigators.

It has been shown before that part of the carbachol-induced contraction in TM is still present under conditions in which all extracellular calcium has been removed with calcium buffers. It is of interest that CM did not contract under these conditions. The data indicate that this contractile force in TM relies on the release of calcium from intracellular stores. BAPTA-AM is a membrane-permeable intracellular calcium chelator that leads to a complete depletion of cytosolic calcium levels in TM strips exposed to extracellularly calcium-free environment. Under these conditions the carbachol-induced contraction was completely absent. That part of TM contractility appeared to be regulated in a different way than CM indicates that the development of TM-specific pharmacologic agents for the regulation of contractility should be possible.

The PKC family of isoforms consists of three groups: the calcium-dependent (, , and ), the calcium-independent (, , , and ), and the atypical isoforms ( and ). We have shown recently that the TM, contrary to the CM, features a high expression of the PKC-isoform. This PKC-isoform has been linked to calcium-independent contraction in various smooth muscle preparations. The involvement of PKC- in the calcium-independent contraction of the TM presented in this study seems likely, because the PMA-induced contractility was completely absent in CM. That the biologically inactive form of PMA 4-phorbol failed to induce contraction suggests a PMA-induced effect highly specific for PKC. Focusing on PKC regulation appears to be important, because inhibitors of this enzyme have been used successfully in an animal model to lower IOP. The main mode of action of these compounds seems to be the initiation of relaxation of the TM or by the modulation of actin microfilaments. It is believed by some investigators that PKC inhibition ultimately leads to disruption of actin filaments and thereby alters the organization of cell–cell and cell–extracellular matrix adhesions in TM. In our experiments, however, the main action of PKC blockers was the initiation of relaxation. This observation is further supported by findings of other groups investigating PKC in different smooth muscle tissues. It has been shown that activation of PKC enhances contraction by inhibiting myosin phosphatase directly.

The need for the modulation of more specific signaling pathways is justified, because most inhibitors of PKC are rather nonspecific, acting on a wide variety of intracellular enzymes. Currently, only the PKC- isoform can be inhibited through an orally effective antagonist. However, PKC- was not detected when bovine and human TM and CM tissues were screened for smooth-muscle-associated isoforms of PKC.
A promising target for a highly specific modulation of calcium-independent contraction in smooth muscle tissues appears to be the small GTPase rho-A and its kinase, ROCK. The active, GTP-bound form of rho-A activates a serine-threonine kinase, ROCK, which in turn phosphorylates myosin light-chain phosphatase (MLCP). This results in inhibition of MLCP and in increased myosin phosphorylation—i.e., contraction. The identification of this pathway is greatly facilitated by the use of the highly selective ROCK inhibitor Y-27632. This pyridine derivative has been used successfully in inhibiting smooth muscle contraction and in reducing blood pressure in hypertensive rats without affecting blood pressure in normotensive animals. Our experiments clearly show that specific inhibition of the rho-A/ROCK pathway blocks calcium-independent contraction initiated through activation of PKC. Furthermore, the rho-A protein was detected in human TM cells by Western blot and immunoprecipitation analysis. Some investigators suggest the PKC-mediated inhibition or activation of MLCP to be an independent pathway of rho-A’s effects on contractility. Contrary to this, our results suggest an interaction of PKC-ε and the small GTPase rho-A in the modulatory pathway influencing TM contractility. In our experiments, contractility was measured in intact tissue strips rather than permeabilized smooth muscle preparations as performed by other groups, which may explain the varying results. In addition, the variable findings may also be the result of differences in the tissue types or cells investigated. Further experiments are needed to clarify the downstream effector proteins of rho-A in the TM and the effects of Y-27632 on modulation of TM contractility. The IOP-lowering properties of Y-27632 have been demonstrated recently in an animal model, where it was administered intracameral, intravitreally, and topically.

In summary, we have shown that in TM contractility is partly regulated in a unique way that is independent of extracellular calcium and not present in the CM. In addition to the established ways of initiating contractile force through calcium-dependent mechanisms, the TM presents an alternate route leading to contraction that involves pharmacomechanical coupling events. Furthermore, this calcium-independent contraction is most probably modulated by PKC-ε, which does not require calcium for its activation, and rho-A. Both proteins are strongly expressed in the human TM, suggesting that this smooth-muscle-like tissue may be influenced by highly specific compounds such as Y-27632. Modulating TM contractility downstream of PKC with specific inhibitors of rho-A seems more promising than nonspecific inhibition of a broad selection of PKC isoforms. The data in this study indicate that the ROCK inhibitor Y-27632 may have beneficial effects on IOP in primates.

Acknowledgments

The authors thank Marianne Boxberger, Helga Höffken, and Karin Oberländer for technical support.

References


