The Effects of Protein Kinase C on Trabecular Meshwork and Ciliary Muscle Contractility

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Purpose. The possible role of protein kinase C (PKC) inhibitors in novel pressure-lowering drugs is currently under investigation. To gain further insight into regulation of contractility by PKC in trabecular meshwork (TM) and ciliary muscle (CM), the effects of various PKC inhibitors and activators were tested.

Methods. Isometric tension measurements of bovine TM and CM strips were performed. PKC was stimulated by phorbol ester and by the diacylglycerol analogue diC_8. PKC blockade was accomplished using H7 and myristoilated PKC substrate (mPKC). Western blot analysis was used to identify specific PKC isoforms in human trabecular meshwork (HTM), human ciliary muscle (HCM), and bovine TM and CM.

Results. In tissues precontracted by carbachol PKC antagonist H7 led to a relaxation of TM (25 ± 7.2 versus 100%; $n = 8$) with no effect on CM. mPKC substrate selectively blocks PKC. This substance led to relaxation of TM (32.8 ± 7.4 versus 100%, $n = 7$), whereas CM was not affected. PMA at concentrations of 10^{-6} M led to a slow contraction of both tissues that was more marked in TM. DiC_8 and 4α-phorbol had no effect on contractility. Western blot analysis revealed expression of calcium-dependent PKC-α and calcium-independent PKC-ϵ isoforms in HTM and HCM. PKC-ϵ expression was more pronounced in HTM than in HCM. Similar PKC isoform expression was found in native bovine tissue.

Conclusions. PKC isoforms show different tissue distributions in human and bovine TM and CM. Contractility differences exist in both tissues in response to PKC antagonists and agonists. The data indicate that PKC may be involved in regulation of aqueous humor outflow by the TM. Thus, inhibition of PKC may represent a new way of influencing outflow facility through isolated relaxation of TM. (*Invest Ophthalmol Vis Sci.* 1999;40:3254–3261)

The mechanisms that lead to the development of primary open-angle glaucoma are poorly understood. Several lines of evidence indicate that outflow resistance through the trabecular meshwork (TM) is increased, thus leading to an elevation of intraocular pressure (IOP).[1] Treatment today still focuses on lowering IOP, either by increasing outflow or reducing secretion of aqueous humor. It is widely accepted that the TM displays properties of a passive filter with pores that can be altered in size and shape by the action of the inserting ciliary muscle (CM), thus changing the rate of outflow.[1–3] However, several studies have shown that in addition to this mechanism the TM itself features smooth muscle–like properties and is actively involved in aqueous humor dynamics. It has been shown that substances that relax TM lead to increased outflow rates in the model of the perfused anterior chamber with CM detachment. On the contrary, agents that lead to TM contraction may result in an increase of IOP by lowering outflow rates.[4–7] It has been shown recently that TM and CM show significant differences in the way in which they regulate contractility.[7,8] This is of importance because pressure-lowering drugs affecting both tissues are prone to having ophthalmologic side effects. The ideal pressure-regulating drug should thus lead to isolated relaxation of TM without affecting CM contractility bypassing adverse side effects such as miosis.

Protein kinase C (PKC) is a family of serine-threonine kinases implicated in intracellular signaling events triggered in response to a large variety of agonists.[9,10] Currently, 10 mammalian PKC isoforms have been identified. They are divided into three groups: the calcium-dependent (α, βI, βII, and γ), the calcium-independent (δ, ϵ, η, and θ), and the atypical isoforms (ζ and λ). PKC isoforms are involved in diverse cellular functions, including differentiation, growth control, tumor promotion, and cell death.[11,12] In recent years, a role for specific PKC isoforms in the regulation of smooth muscle contraction has been postulated.[13–15] We have shown that inhibition of PKC with chelerythrine leads to relaxation of TM while leaving CM unaffected.[8] Interestingly, PKC inhibitors have been tested recently as new antiglaucoma agents in an animal model.[16,17] In addition, specific isoforms associated

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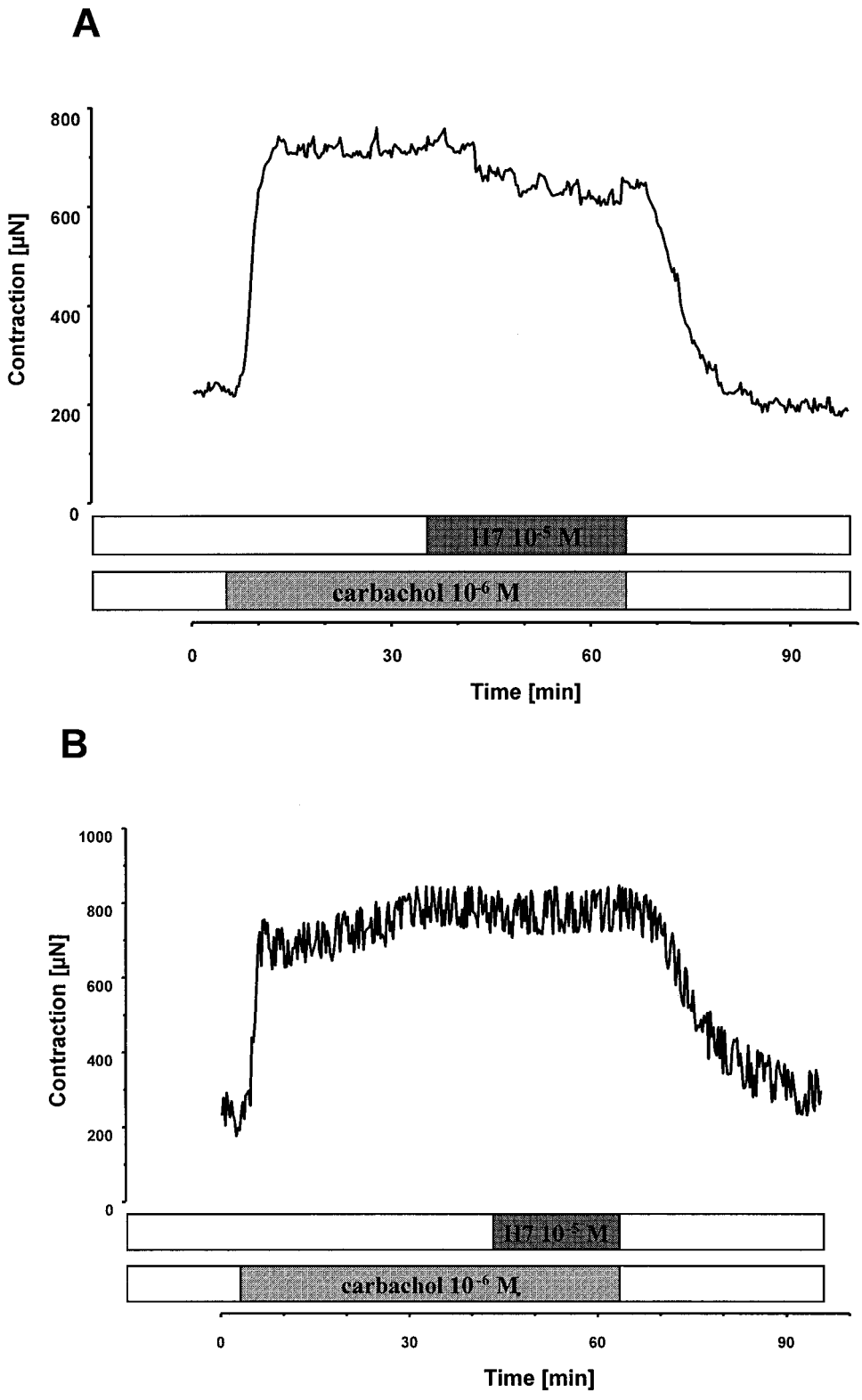

FIGURE 1. Original recordings of isometric force developed in isolated strips of bovine TM (**A**) and CM (**B**) strips. After a carbachol-induced contraction, PKC inhibitor H7 led to relaxation only in the TM strip.

with smooth muscle contraction show a different distribution pattern in TM and CM.

In this study, we were able to demonstrate significant functional differences between TM and CM with respect to PKC inhibition and activation by measuring contractile force directly. Furthermore, expression of specific PKC isoforms predominantly associated with smooth muscle contractility were identified for the first time in human and bovine TM and CM cells using western blot analysis. Thus, tissue-dependent differences in PKC-mediated contractility and relaxation suggest a possible involvement of specific PKC isoforms.

METHODS

Measurement of Contractility

Enucleated bovine eyes were obtained from the local abattoir and placed on ice. Small TM and CM strips were carefully dissected according to procedures described previously.[18] Briefly, after excision of the iris, only TM and CM tissue remained on the corneoscleral segment. TM strips were prepared in a circular direction, and meridional CM strips were excised perpendicularly to the circular ciliary body. The isolated strips were approximately 2 to 4 mm long and 0.5 mm wide. The effects of agents on TM and CM tension were obtained after the tissues had been allowed to rest under control conditions for at least 1 hour. The chamber solution with the isolated strips was maintained at constant temperature and stable pH. Only strips showing a stable tone were used for experiments. Isometric contractions were expressed relative to the response obtained with a maximally effective carbachol concentration 10^{-6} M, which was tested in each tissue strip as a control. To determine the activity of a compound, it was added either to the precontracted tissues or given to tissues of basal tension.

Cell Cultures

Human TM and CM cells were isolated by methods based on those of Flügel et al.[19] Human eyes were obtained from multiorgan donors who were identified with respect to age, gender, and time of death. History of glaucoma was ruled out by screening the donors' history sheets. Donor ages were 23, 26, 35, 41, 47, 51, and 65 years. Tenets of the Declaration of Helsinki were followed, informed consent was obtained from donors of eyes, and institutional human experimentation committee approval was granted for the studies. Briefly, TM strips were isolated under microscopic view using a fine wire probe (0.5 mm). The canal of Schlemm was probed to help visualization of TM, thus aiding dissection. The strips were placed under coverslips in 35-mm plates to prevent floating and facilitate attachment without pharmacologic aids. Small CM strips were dissected from the outer part of the muscle and were placed under coverslips. TM and CM cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 20% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (all cell culture material from Biochrom, Berlin, Germany). Cells were maintained in a 95% air- 5% CO_2 atmosphere at 37°C and were passaged using the trypsin–EGTA method. Only well-characterized, early-passage, normal human trabecular meshwork (HTM) and human ciliary meshwork (HCM) cells from passages three through eight were used for studying PKC isoform distribution. Histologic characterization was performed by Elke Lütjen–Drecoll, (Department of Anatomy Universität Erlangen, Nürnberg, Germany) and showed typical immunostaining, as described previously.[19]

Whole-Cell Lysates, Gel Electrophoresis, and Western Blot Analysis

The cells were kept in fetal calf serum–free DMEM overnight to rule out PKC upregulation. Confluent cell monolayers were placed on ice and washed three times with ice-cold phosphate-buffered saline (PBS) containing 0.1% sodium orthovanadate. Cells were then scraped and lysed in lysis buffer A (1% NP40, 20 mM Tris [pH 8.80], 137 mM NaCl, 10% glycerol) containing protease inhibitors (Complete, Protease; Boehringer–Mannheim, Mannheim, Germany). After brief homogenization (Polytron homogenizer; Kinematic, Lucerne, Switzerland) whole-cell lysates were centrifuged at 14,000 rpm for 5 minutes at 4°C. The supernatant was subjected to protein measurement and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Whole-cell lysates and prestained molecular weight markers were heated at 95°C for 5 minutes and were then separated electrophoretically by SDS-PAGE using 7.5% polyacrylamide. Equal amounts of protein were loaded in each lane of the gels (20, 25, or 30 μg total protein) and electrophoresed for 1 hour at 150 V in electrophoresis cells (Mini-Protean; Bio-Rad, Life Science Group; Richmond, CA). The proteins were transferred electrophoretically to nitrocellulose filters (Polyscreen, NEN Life Science Products, Boston, MA) for 1 hour at 100 V. The blots were blocked in PBS containing 1%

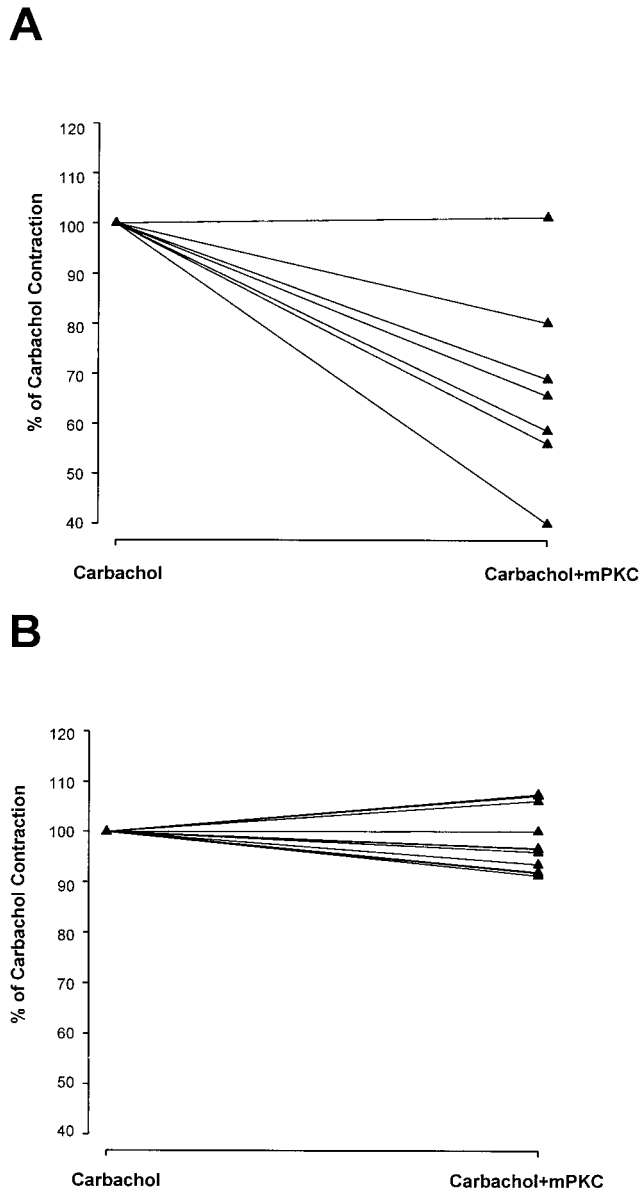

FIGURE 2. Effects obtained with mPKC substrate. Six of seven bovine TM strips showed relaxation (**A**). Bovine CM (**B**) was not affected.

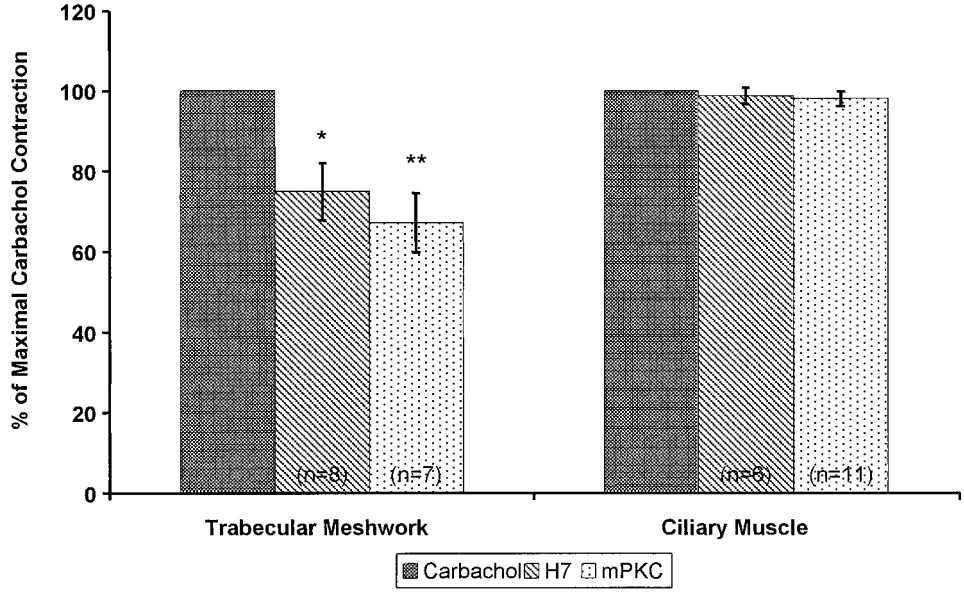

FIGURE 3. Summary of data obtained with H7 and mPKC. Both compounds showed significant relaxation of TM only (*$P < 0.05$; **$P < 0.01$).

bovine serum albumin and 0.025% sodium azide for 2 hours at room temperature. The same solution was used to dilute the primary antibodies (1:2000). The blots were probed with anti-PKC antibodies (Gibco, Life Technologies, Grand Island, NY) at 4°C overnight on a rocking platform. Antibody was added accordingly to three- to fourfold excess of blocking peptide (Gibco) diluted in the same buffer. After washing (three times; 10 minutes each) in PBS-Tween, the blots were incubated with a 1:10,000 dilution of peroxidase-conjugate secondary antibody (Dianova; Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. After final washing (three times; 10 minutes each in PBS-Tween), the blots were visualized using a chemiluminescence kit (ECL, Amersham, Amersham, UK) according to the manufacturer's instructions. Each experiment was performed at least three times and showed identical results. Densitometry was performed using an image analyzer (Fujifilm; LAS 1000; Fuji, Tokyo, Japan) and software (Aida 2.0; Raytest, Berlin, Germany).

Bovine Protein Preparation

Bovine TM and CM strips were dissected as mentioned and described before[18] and placed on ice. Approximately 250 μg of total dissected protein was transferred into 500 μl lysis buffer A. The whole cell lysate was further homogenized by 20 strokes in the homogenizer and then subjected to three freeze and thaw steps. The lysate was centrifuged at 14,000 rpm for 5 minutes at 4°C and the supernatant saved. An equal amount of protein was loaded onto each gel and SDS-PAGE performed as previously described. The antibodies, solutions, and detection method were identical with those used for the human cell lysates.

Reagents

The following reagents were used for contraction experiments: 1-(5-iosquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H7), 1,2-dioctanoyl-*sn*-glycerol (diC_8), and 4α-phorbol, (RBI, Sigma, Deisenhofen, Germany); mPKC substrate (Calbiochem–Novabiochem, La Jolla, CA); and phorbol-12-myristate 13-acetate (Biomol, Hamburg, Germany). All other chemicals were of analytical grade and were purchased from Sigma.

Statistical Analysis

The results of contractility measurements were expressed as SEM. Statistical analysis was performed using analysis of variance and Student's *t*-test for paired observations (percentage change versus carbachol-contracted tissues). The unpaired Student's *t*-test was used for comparison between TM and CM. Significance was assumed when $P < 0.05$. The number (n) refers to the number of experiments. Western blot experiments were performed at least three times.

RESULTS

Inhibition of Serine-Threonine Kinase C

H7 is a potent inhibitor of various kinases and inhibits PKC in a nonselective manner.[20] After adjustment of baseline tension to 100 to 200 μN, carbachol at a concentration of 10^{-6} M was used to induce contraction of both TM and CM. H7 led to relaxation of TM (in precontracted tissue, 100%–75% contraction, i.e., 25.0% ± 7.2% relaxation; $n = 8$, $P < 0.05$), but had no effect on CM (in precontracted tissue, 100%–98.8%, i.e., 1.2 ± 2.1% relaxation; $n = 6$; Fig. 1).

mPKC substrate interacts in a highly specific way with the catalytic fragment of PKC, thereby leading to its inhibition.[21] In our experiments, at concentrations of 10^{-6} M, this compound had a relaxing effect only on TM (32.8 ± 7.4% versus 100%; $n = 7$; $P < 0.01$), whereas CM was not affected (1.8% ± 1.9% versus 100%; $n = 11$; Fig. 2). Figure 3 summarizes the data obtained with both PKC inhibitors.

Stimulation of Serine-Threonine Kinase C

PMA and the membrane-permeable diacylglycerol (DAG) analogue diC_8 were used to stimulate membrane-bound PKC. Administration of PMA (10^{-6} M) at baseline tension led to a slow, dose-dependent contraction both in TM (9% ± 2.5% versus 0%; $n = 6$; $P < 0.05$) and CM (5.9% ± 1.2% versus 0%;

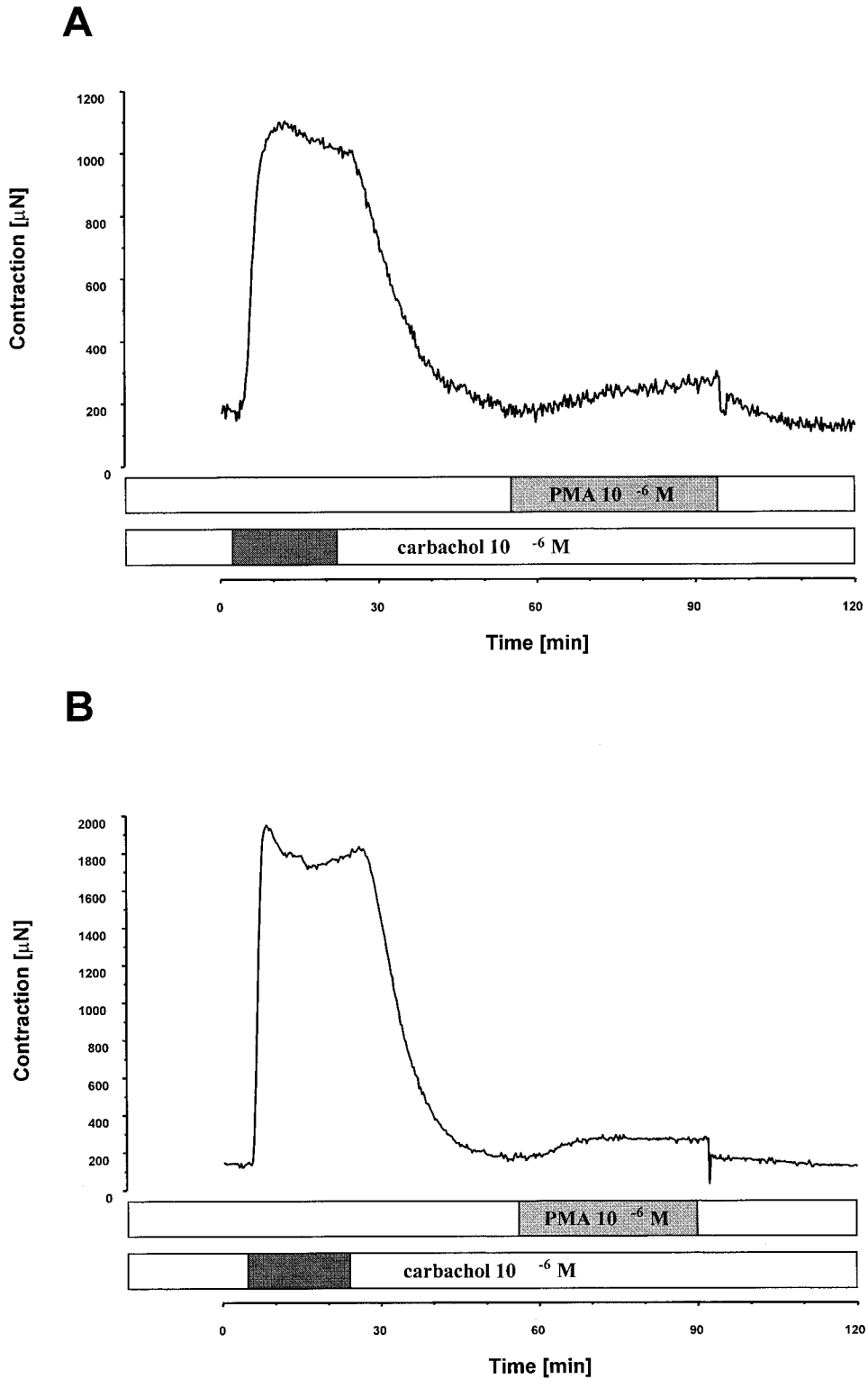

FIGURE 4. Original recordings illustrating the slow, sustained contraction occurring in both tissues after administration of PMA (10^{-6} M) after an initial carbachol-induced peak. (**A**) bovine TM; (**B**) bovine CM.

$n = 7$; $P > 0.01$; Fig. 4). 4α-Phorbol (10^{-6} M) a biologically inactive PMA analogue did not show any changes of baseline tension ($n = 6$, data not shown). diC_8 (10^{-5} M) had no effect on baseline tension in both tissues ($n = 8$, data not shown).

HTM and HCM Western Blot Analysis

Seven individual preparations of human trabecular meshwork (HTM) and human ciliary muscle (HCM) cell cultures were screened for PKC isoforms. Antibodies specific for six different PKC isoforms were used. Rat brain cell lysate was used as a positive control. Blocking peptide was used in cases in which unspecific bands showed up after detection with chemiluminescence. Figure 5 demonstrates a positive signal for PKC-α at 80 kDa, which was similar in both tissue types. The Ca^{2+}-independent isoform PKC-ϵ was clearly displayed as a signal at 90 kDa in HTM, with a significantly weaker band in HCM (Fig.

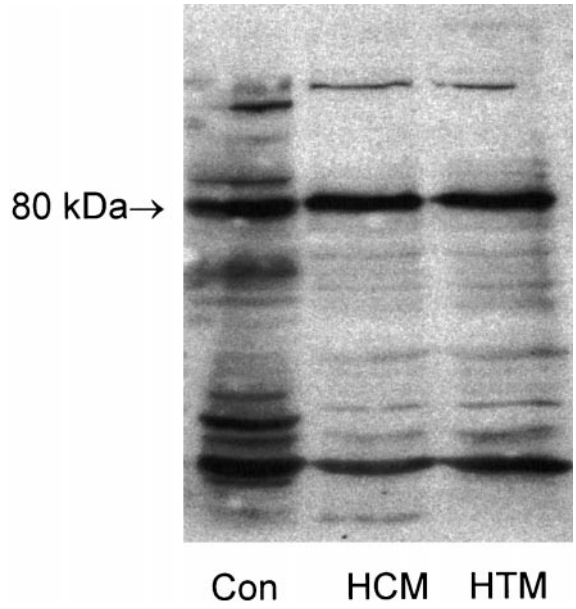

FIGURE 5. Western blot analysis using whole-cell lysates of HTM and HCM cell cultures blotted with anti-PKC-α antibody. A band at approximately 82 kDa is marked in rat brain control (Con) as well as in HCM and HTM. Equal amounts of protein were loaded in *lanes HCM* and *HTM*. The experiment was repeated three times with identical results.

6). Screening for other smooth muscle–associated PKC isoforms (β, γ, δ and ζ) showed no detectable bands when compared with rat brain control lysate (data not shown).

Bovine Tissue Western Blot Analysis

Bovine tissue strips were separated and were used either for contraction measurements or were subjected to whole-cell lysis and subsequent western blot analysis. Screening for PKC-α and -ϵ isoforms showed the same tissue-dependent results as in human tissue. Figure 7 shows a representative experiment. The higher expression of PKC-ϵ was verified using densitometry analysis. The mean results of six densitometry experiments are shown in Figure 8.

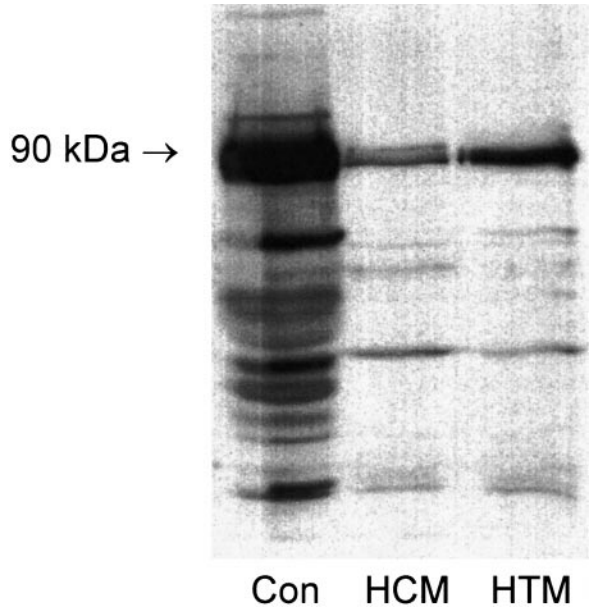

FIGURE 6. Whole-cell lysates blotted with anti-PKC-ϵ antibody. Equal amounts (25 μg) of protein were loaded into *lanes HCM* and *HTM*. A band estimated at 90 kDa showed up in all specimens, including rat brain control (Con). The signal in HTM was considerably stronger than in HCM. A representative of three individual experiments with identical findings is shown.

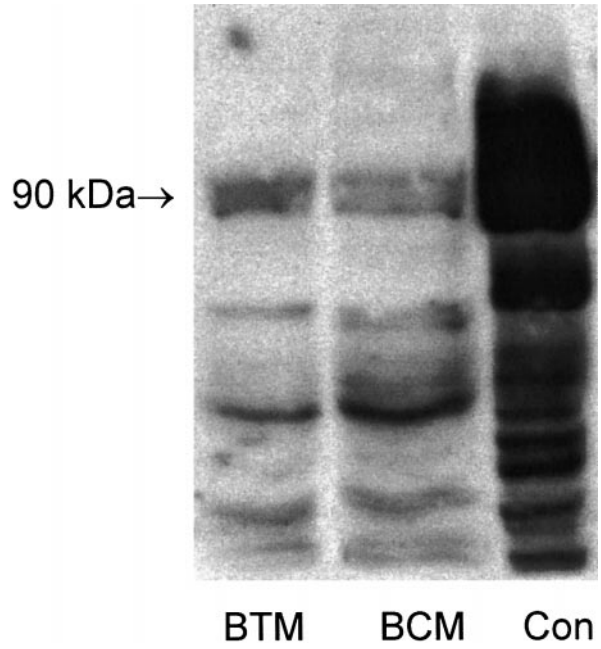

FIGURE 7. Western blot analysis of native bovine tissue whole-cell lysates using anti-PKC-ϵ antibody. Equal amounts of protein (25 μg) were loaded into *lanes BTM* (bovine TM) and *BCM* (bovine CM). A signal of 90 kDa showed up in all specimens, including rat brain control (Con). The experiment was performed three times with identical results.

DISCUSSION

This article presents for the first time the differences in PKC-related contraction and relaxation between TM and CM. These functional tissue characteristics are probably linked to different PKC isoform expression in TM and CM. Our data support the theory that PKC inhibition alters the outflow pathway through inhibition of TM contractility.

Contraction of TM by PKC Activators

PKC was first implicated in the regulation of smooth muscle contractility after the observation that phorbol esters (PMAs) induce slowly developing, sustained contraction.[13,14,22] PKC occurs in at least four calcium-dependent (α, βI, βII, and γ) and four calcium-independent (δ, ϵ, ζ, and η) isoforms. Both classes of isoforms have been linked to the regulation of smooth muscle contractility. So far, only the α, β, ϵ, and ζ isoforms have been identified in smooth muscle tissue.[13] New concepts on aqueous humor outflow state that TM has smooth muscle–like properties and is actively involved in regulating outflow resistance.[2,6,7,18,23] Thus, TM contains contractile filaments and displays electrophysiological properties of smooth muscle cells.[5,24] Recently, we were able to demonstrate a functional

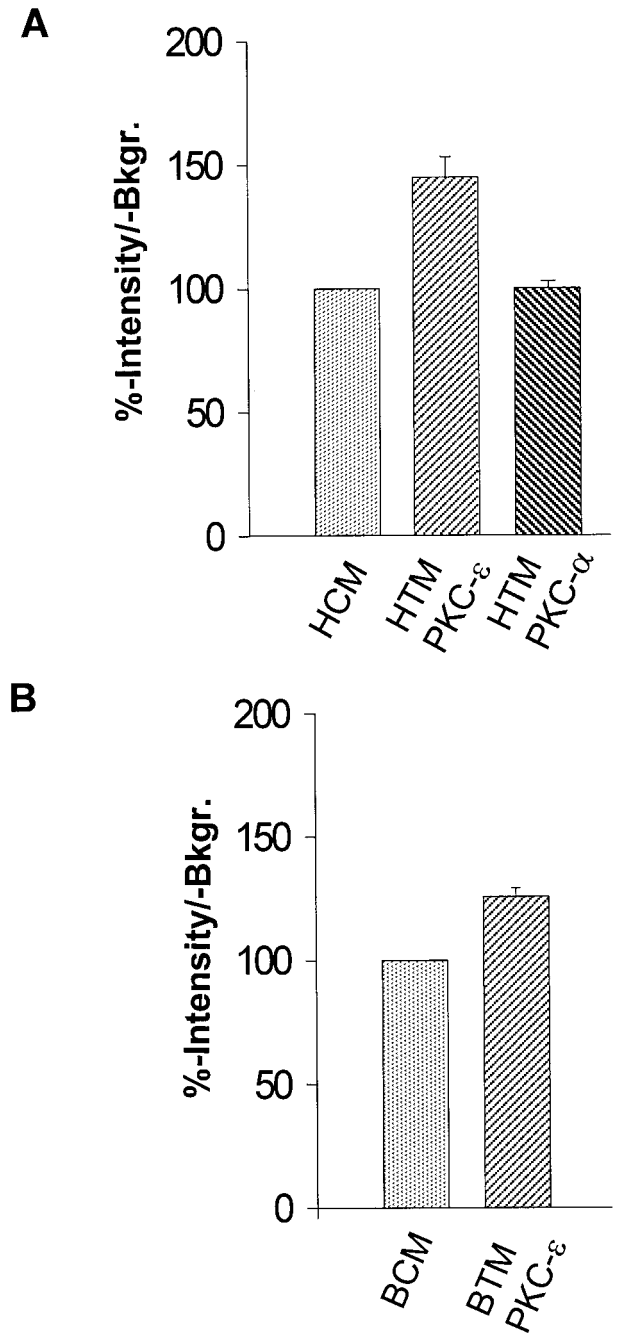

FIGURE 8. Summary of densitometry measurements. (**A**) Signals of HCM were set at 100% and compared with HTM probed with anti-PKC-ε or anti-PKC-α antibodies, respectively. A total of six individual experiments are shown (mean ± SEM). (**B**) Bovine tissue showed similar results (only results for PKC-ε shown). $P < 0.05$ for PKC-ε expression in TM compared with CM.

antagonism in response to activation and inhibition of various protein kinases.[8]

The effects of specific PKC activators and inhibitors previously used as pressure-lowering substances in an animal model have not been tested before in intact TM and CM strips. The effects of PKC activators were tested measuring contractile force on baseline tension directly. PMA led to a slow contraction in TM and CM that was considerably weaker when tissues were precontracted by carbachol through a G-protein–linked receptor. The absence of effect of 4α-phorbol suggests that the PMA-induced contraction is mediated through PKC.

DAG is an intracellular lipid second messenger displaying direct interaction with membrane-bound PKC, thereby leading to its activation. It serves as the physiological activator for calcium-dependent and calcium-independent isoforms. Its metabolism had been extensively researched in aortic and single vascular smooth muscle cells.[25] Naturally occurring DAG with long-chain fatty acids is not taken up by intact cells. Enzymatic hyperpermeabilization of the smooth muscle cells has been performed by other groups as an experimental approach to measure contractility. This setup was inadequate in our experimental setup in which contraction was measured in intact tissue strips. Therefore, we used diC_8 for our experiments, because it is cell permeable and can activate PKC pathways in intact cells.[25] Whereas PMA treatment led to contraction in TM and CM, diC_8 had no effect on baseline tension in both tissues. Interpreting these results is difficult. Contradictory effects have been observed when comparing responses of PMAs with those generated by DAG analogues.[26] Whereas DAG and DAG analogues are rapidly metabolized by DAG lipase and DAG kinase after entering the cytosolic compartment, PMA can exist in intact cells for several hours after administration. This may explain the absence of contractile response in the experiments.

As has been shown before, part of the contractility of TM is independent of extracellular Ca^{2+}. Under similar conditions, the contractile response of CM was completely abolished.[27] PKC-ε is an isoform that does not require Ca^{2+} for activation and is highly expressed in TM. DAG therefore may lead to activation of PKC-α in both TM and CM but not to initiation of contractile force.

Effects of PKC Inhibition on Contractility

As has been shown before, selective blocking of PKC by chelerythrine and NPC 15437 led to relaxation in TM but not in CM. In this study, the underlying reasons for these tissue differences could not be illuminated.[8] H7 is a protein kinase inhibitor that has also been used for studying PKC. The ability of H7 to inhibit PKC is nonselective when compared with compounds such as chelerythrine or NPC 15437.[20,28,29] Recently, it has been suggested that this substance has the potential to lower IOP and has been used successfully in an animal model.[16,17] In our experiments, the substance led to isolated relaxation only in TM and had no effect on CM. H7's ability to influence TM contractility may explain the pressure-lowering effects of this compound. It has been shown that substances that lead to TM relaxation result in enhanced outflow rates.[4] However, in addition to the effects on PKC, H7 has been reported to have inhibitory effects on PKA, tyrosine kinases, and calmodulin kinases, suggesting that modulation of additional signal transduction pathways may be involved in the effects induced by H7. We used mPKC substrate, an *N*-myristoilated oligopeptide analogue that is known to interact directly with the catalytic fragment of free PKC, leading to highly specific inhibition of the enzyme.[21] Again, the results were comparable to the effects of the nonspecific compound H7. All but one of the TM tissue strips tested showed relaxation, whereas CM was not affected.

PKC-α and -ϵ Isoforms in HTM and HCM

This is the first study demonstrating the presence of PKC-ϵ and -α isoforms in HTM and HCM cells. PKC isoforms associated with smooth muscle contractility have not been evaluated before in these tissues. There is increasing evidence suggesting that Ca^{2+}-independent mechanisms may be involved in the slow component of contractile response in smooth muscle cells.[13,30] It has been suggested that Ca^{2+}-independent PKC isoforms may be involved in the ability of smooth muscle cells to contract under Ca^{2+}-free conditions.[30] Interestingly, PKC-ϵ, an isoform known for its association with smooth muscle contraction through Ca^{2+}-independent mechanisms, is more pronounced in TM than in CM. PKC-α is equally detectable in both TM and CM. It seems unlikely that differences in contractile response after PKC stimulation and inhibition are linked to the PKC-α isoform. There is strong evidence to suggest that contractility may be linked to PKC-ϵ, which is Ca^{2+}-independent and is strongly expressed both in human and bovine TM. This particular isoform is considered to be a key link between extracellular signaling through receptors and myosin light-chain kinase by many investigators.[11,13,22,30] Data obtained in experiments under extracellular Ca^{2+} depletion suggest Ca^{2+}-independent contraction in TM but not in CM.[5] The generation of contractile force in TM under these experimental conditions may be explicable by activation of PKC-ϵ, the Ca^{2+}-independent PKC isoform that is not highly expressed in CM. The capability for depletion of intracellular Ca^{2+} stores may also be different in both tissues and should be taken into consideration. More studies are necessary to investigate this question.

In summary, we have shown that contractility of TM and CM is differently modulated with respect to PKC activation and inhibition. Contrary to CM, TM features a higher expression of PKC-ϵ, an isoform that is known to be both Ca^{2+}-independent and associated with smooth muscle contraction under Ca^{2+}-free conditions. Thus, PKC isoform-specific inhibition may be a suitable target in influencing TM directly and therefore in modulating ocular outflow. Further studies are needed to elucidate the exact signaling pathways involved in PKC-related contractility or intermediate proteins related to myosin light-chain phosphorylation.

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