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# Mechanism for Leukotriene $C_4$ Stimulation of Chloride Transport in Cornea

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**Summary.** The leukotriene, $LTC_4$, exerts a stimulatory effect on chloride transport in the frog cornea. In the work described here, the mechanism of action of $LTC_4$ to stimulate chloride transport was studied.

In corneas pretreated with indomethacin, the effect of $LTC_4$ was abolished, suggesting the involvement of cyclo-oxygenase products in the response. Incubation of corneas with $LTC_4$ resulted in a significant stimulation in $PGE_2$ synthesis, as determined by TLC-autoradiography and radioimmunoassay. In addition, $LTC_4$ was found to stimulate cAMP synthesis in the cornea, and this stimulation was blocked with indomethacin. $PGE_2$ was previously shown by us to be the dominant cyclo-oxygenase product formed in the frog cornea, and is capable of stimulating cAMP and chloride transport. We suggest that $LTC_4$ stimulation of chloride transport is mediated via activation of the cyclo-oxygenase pathway, resulting in enhanced $PGE_2$ synthesis. Elevated $PGE_2$ levels induce cAMP synthesis, and ultimately, the stimulation of chloride transport. Further, the activation of cyclo-oxygenase was found to be dependent on phospholipase $A_2$ activity. This was shown by the inhibition of the $LTC_4$ effect in the presence of quinacrine. Similarly, inhibition of the $LTC_4$ effect in the presence of trifluoperazine suggests that cyclo-oxygenase activation by $LTC_4$ may be mediated via calmodulin. We have previously demonstrated that the frog cornea has the biosynthetic capacity to produce $LTC_4$. Therefore $LTC_4$ may function as an endogenous regulator of chloride transport in this tissue.

**Key Words** leukotriene · cornea · chloride transport · arachidonic acid

## Introduction

We have previously reported the phenomenon that leukotriene $C_4$ ($LTC_4$) is able to stimulate chloride transport across the isolated frog cornea [14, 16]. We have also previously demonstrated that the frog cornea has the capacity to synthesize $PGE_2$, as well as leukotrienes $LTB_4$ and $LTC_4$, and other metabolites representing both branches (cyclo-oxygenase and lipoxygenase) of the "arachidonic acid cascade." The leukotrienes represent a group of potent arachidonic acid oxygenation products that have recently been shown to have important regulatory roles in cell function. It is known, for example, that $LTB_4$ has the ability to stimulate random migration (chemokinesis) and directed migration (chemotaxis) of neutrophils. It is also a potent stimulus of neutrophil adherence, aggregation, and lysosomal degradation [7]. Leukotriene $C_4$ is a vasoactive compound, that has been shown to produce constriction of arterioles, exudation, and increased vascular permeability in postcapillary venules as well as biphasic changes of arterial blood pressure. It is also a potent smooth muscle contractile principle [7, 8]. Because of the potential biological significance of $LTC_4$ as an endogenous regulator of chloride transport, we initiated this study to further explore the mechanism of action of $LTC_4$ to stimulate chloride transport.

## Materials and Methods

### MEASUREMENT OF CHLORIDE TRANSPORT

Chloride ions are transported by the frog cornea from the aqueous (endothelium) to tear (epithelium) side by a pump located in the epithelium [11, 19].

The methodology for measuring chloride transport is well established, and has been described in detail elsewhere [15, 18]. Briefly, freshly dissected corneas were mounted in a modified Ussing-type chamber, with Ringer's solution bathing both corneal surfaces. Measurements of the short-circuit current (SCC) which is nearly proportional to the net ion transport and potential difference (PD) were made using a dual automatic voltage-clamp unit. Substances to be tested for their effect on chloride transport were added directly to the Ringer's solution in microliter aliquots to either the endothelial side alone or to both corneal surfaces. Dilution of test substances occurs rapidly in the chamber with the aid of bubble-lift circulation of the Ringer's solution. It was previously determined that leukotriene ($LTC_4$) stimulates chloride transport only when added to the endothelial side of the chamber [14]. Therefore, in the present study, only endothelial

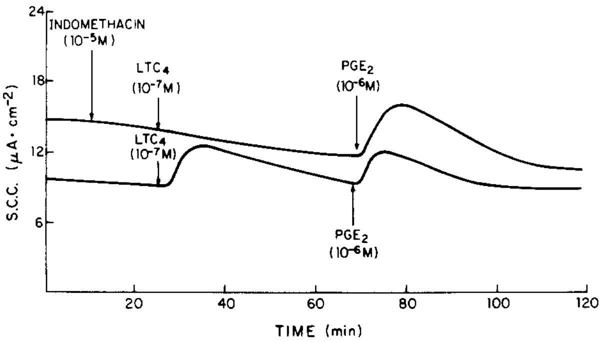

**Fig. 1.** Response of SCC to $LTC_4$ in frog corneal pairs. Cornea in lower trace shows typical stimulation of SCC in response to endothelial side application of $LTC_4$, followed by typical response to $PGE_2$. Cornea in upper trace was first treated with indomethacin and then given $LTC_4$ and $PGE_2$. In this case the SCC response to $LTC_4$ is abolished, but the response to $PGE_2$ remains

additions of $LTC_4$ were made. The various inhibitors, however, were applied to both corneal surfaces. Indomethacin was added 15 min prior to the addition of $LTC_4$. However, the inhibitors, quinacrine and trifluoperazine (TFP), were added 30 min before the addition of $LTC_4$ to allow for the restabilization of the SCC at a new lower baseline level. The addition of appropriate solvent blanks to the chamber indicated that no significant change in the SCC or PD occurred with any of the solvents used.

### Thin-Layer Chromatography (TLC)

Corneal samples for TLC were freshly obtained, hemisected, and the resulting corneal halves from each cornea were divided into control and test samples. Eight corneal halves were utilized for each TLC sample. All samples were incubated in 0.5 ml Ringer's solution together with $^{14}C(U)$-arachidonic acid (0.5 μCi, sp act, 390 mCi/mmol, New England Nuclear, Boston, MA).

The incubation was carried out for 1 hr at room temperature with gentle agitation, in order to achieve loading of the labeled arachidonic acid into membrane phospholipids. "Preloaded" corneal halves were then washed by transferring three times to fresh Ringer's solution, in order to remove unbound labeled arachidonic acid. Test corneas then had either $LTC_4$ ($10^{-7}$ M) or calcium ionophore A23187 ($2 \times 10^{-6}$ M) added to the Ringer's solution, while paired control corneas were incubated only in Ringer's solution. This additional incubation was carried out for 30 min with gentle agitation, to allow for biosynthesis of the various eicosanoids from the labeled arachidonic acid precursor.

At the end of 30 min, all sample tubes were placed in an ice bath, briefly homogenized with a Polytron homogenizer, and acidified to pH 3.0 with citric acid. Samples were extracted twice with chloroform, and then concentrated by evaporation under a stream of nitrogen gas. The concentrated chloroform extracts were spotted onto silica gel G Chromagram sheets (Eastman Kodak, Rochester, N.Y.) under a stream of nitrogen gas, and run in a solvent system composed of the organic phase of iso-octane/ethyl-acetate/acetic acid/$H_2O$ (30 : 66 : 12 : 60) [6, 17]. The dried chromatographs were sprayed with the autoradiography enhancer, Enhance® (New England Nuclear), and then exposed to X-Ray film (DEF-5, Kodak, Rochester, N.Y.) for 72–96 hr at −20°C. Developed autoradiographs were superimposed over the chromatograph sheets in order to locate eicosanoid bands for subsequent cutting and scintillation counting. Mean scintillation counts from five experiments were used to calculate the recovery of six different eicosanoids in terms of nanograms of each eicosanoid per cornea. Eicosanoid standards were routinely cochromatographed with samples, and visualized either by the use of iodine vapor, or the use of fluorescent chromatograph sheets, while tritiated standards were detected by autoradiography.

The possibility of band formation due to auto-oxidation of arachidonic acid was ruled out by incubating boiled corneas with $^{14}C(U)$-arachidonic acid, and also the incubation of blanks containing only $^{14}C(U)$-arachidonic acid, under conditions identical to the test samples [16]. The absence of radiolabeled bands in these controls indicated that the bands observed in our test samples were the result of enzymatic processes, and not auto-oxidation.

### Radioimmunoassay for cAMP and $PGE_2$

Corneas were dissected and incubated in Ringer's for 30 min to allow for equilibration. Assays for cAMP and $PGE_2$ were done on corneal pairs following the addition of $LTC_4$ ($10^{-7}$ M) to one cornea of each pair. The cAMP assay was carried out following the procedure previously described [13], utilizing a competitive protein binding assay kit TRK 432 (Amersham). The assay for $PGE_2$ was performed on unextracted Ringer's samples following a 30-min incubation, as described elsewhere [16], and utilized the Amersham RIA kit for bicyclic $PGE_2$ (TRK 800).

## Results

### Effects of Indomethacin on $LTC_4$ Stimulation of $Cl^-$ Transport

Indomethacin, a well known inhibitor of the cyclooxygenase pathway, was applied to both sides of the Ussing-type chamber at a final concentration of $10^{-5}$ M. A simultaneously run control chamber received no indomethacin. Fifteen minutes later, $LTC_4$ ($10^{-7}$ M) was applied to the endothelial side of both chambers.

The trace in Fig. 1 (lower) shows a typical $LTC_4$-induced stimulation of SCC, whereas in the indomethacin pretreated cornea (upper) the $LTC_4$ response is completely blocked. Table 1 shows a significant increase in both SCC and PD in control corneas ($n = 6$) treated with $LTC_4$, while indomethacin pretreated corneas ($n = 6$) yielded no significant change in either of these parameters. $PGE_2$ ($10^{-6}$ M) was subsequently added to both sides of the chamber, in both the test and control corneas, as a test of corneal responsiveness to a known stimulator of SCC [1]. A typical stimulation of SCC was observed for each cornea (Fig. 1, Table 1), thus confirming the test cornea's capacity to respond.

**Table 1.** The effects of cyclo-oxygenase inhibition by indomethacin, on the ability of $LTC_4$ and $PGE_2$ to affect the electrical properties of isolated frog cornea in Ussing-type chambers[a]

| Experiment | SCC ($\mu A \cdot cm^{-2}$) | PD (mV) | R ($\Omega \cdot cm^2$) | Time to maximum effect (min) |
|---|---|---|---|---|
| Control ($n = 6$) | 14.87 ± 2.18 | 24.08 ± 2.43 | 1790 ± 259 | 10 |
| $LTC_4$ | 20.34 ± 2.06 | 26.78 ± 2.36 | 1379 ± 165 | |
| Change (%) | +36.8 (0.001) | +11.2 (0.005) | −23.0 (0.01) | |
| Control ($n = 4$) | 14.93 ± 3.46 | 23.65 ± 4.37 | 1752 ± 368 | 9 |
| $PGE_2$ | 20.37 ± 3.02 | 25.85 ± 3.86 | 1323 ± 202 | |
| Change (%) | +36.4 (0.005) | +7.2 (NS) | −24.5 (NS) | |
| Indomethacin ($n = 6$) | 18.25 ± 2.75 | 26.0 ± 1.88 | 1615 ± 276 | — |
| $LTC_4$ | 18.25 ± 2.75 | 25.82 ± 1.92 | 1600 ± 283 | |
| Change (%) | 0 (NS) | −0.7 (NS) | −0.9 (NS) | |
| Indomethacin ($n = 4$) | 17.81 ± 3.08 | 24.83 ± 2.12 | 1609 ± 454 | 9 |
| $PGE_2$ | 25.64 ± 3.56 | 27.15 ± 2.21 | 1151 ± 251 | |
| Change (%) | +44.0 (0.005) | +9.3 (NS) | −28.5 (NS) | |

[a] Results expressed as the mean ± SE. Final drug concentrations were: indomethacin, $10^{-5}$ M; $PGE_2$, $10^{-6}$ M; $LTC_4$, $10^{-7}$ M. Level of significance ($P$) based on Student's $t$ test. NS = no significant difference.

## EFFECTS OF QUINACRINE ON $LTC_4$ STIMULATION OF $Cl^-$ TRANSPORT

Quinacrine, an inhibitor of phospholipase $A_2$ activity, is known to prevent the release of arachidonic acid from membrane phospholipid stores, thereby blocking eicosanoid synthesis. Quinacrine ($5 \times 10^{-5}$ M) was added to the Ringer's solution bathing isolated test corneas mounted in Ussing-type chambers, but not added to control corneas. The addition of quinacrine caused an immediate decrease in SCC and PD which stabilized approximately 15 min later.

$LTC_4$ ($10^{-7}$ M) was added to both test and control corneas approximately 30 min after quinacrine addition. In corneas preincubated with quinacrine, the $LTC_4$ stimulation of SCC and PD was completely blocked (Fig. 2, Table 2); however, the subsequent addition of $PGE_2$ ($10^{-6}$ M) elicited a stimulation of the SCC, indicating that the cornea was capable of responding to a known SCC stimulus. Control corneas yielded typical stimulations of SCC and PD with the sequential addition of $LTC_4$ and $PGE_2$ (Fig. 2, Table 2).

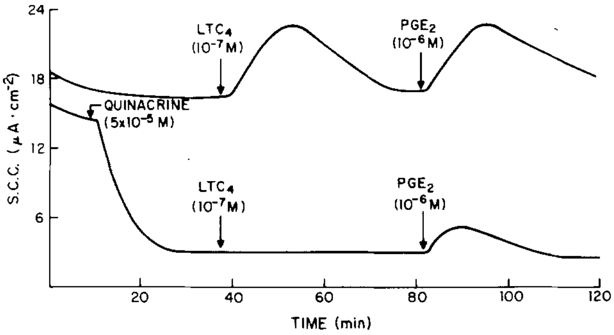

**Fig. 2.** Cornea in lower trace was first treated with quinacrine, an inhibitor of phospholipase $A_2$ activity. Subsequently, this cornea fails to respond to $LTC_4$. In the upper trace, the untreated control cornea shows a typical response to $LTC_4$. Application of $PGE_2$ stimulates the SCC in both corneas

## EFFECTS OF TRIFLUOPERAZINE (TFP) ON $LTC_4$ STIMULATION OF $Cl^-$ TRANSPORT

TFP is known to inhibit the action of calmodulin, the calcium-binding protein. Calmodulin has been shown to play a role in many calcium-dependent intracellular events, such as the activation of phospholipase $A_2$. Inhibition of this enzyme blocks the release of arachidonic acid from phospholipid stores in the cell membrane, thus depriving the cell of the necessary precursor of eicosanoid synthesis. TFP ($10^{-4}$ M) was added to the Ringer's solution bathing corneas mounted in Ussing-type chambers. No TFP was given to control corneas. In corneas given TFP, the SCC and PD were observed to decrease almost immediately, and thereafter to stabilize at a new lower baseline. The addition of $LTC_4$ ($10^{-7}$ M) 30 min later resulted in the typical stimulation of SCC and PD in control corneas; however, in corneas pretreated with TFP, the stimulation was completely blocked (Fig. 3, Table 3). Subsequent addition of $PGE_2$ ($10^{-6}$ M) elicited a typical stimulation of SCC in both treated and control corneas.

**Table 2.** The effects of phospholipase $A_2$ inhibition by quinacrine, on the ability of $LTC_4$ and $PGE_2$ to affect the electrical properties of isolated frog cornea in Ussing-type chambers[a]

| Experiment | SCC ($\mu A \cdot cm^{-2}$) | PD (mV) | R ($\Omega \cdot cm^2$) | Time to maximum effect (min) |
|---|---|---|---|---|
| Control ($n = 5$) | 17.52 ± 1.84 | 23.84 ± 2.04 | 1368 ± 198 | 11 |
| $LTC_4$ | 23.26 ± 1.40 | 25.82 ± 1.88 | 1137 ± 138 | |
| Change (%) | +32.8 (0.001) | +8.3 (0.02) | −16.9 (NS) | |
| Control ($n = 5$) | 18.75 ± 1.49 | 23.54 ± 1.71 | 1286 ± 170 | 10 |
| $PGE_2$ | 25.93 ± 2.25 | 25.20 ± 1.59 | 931 ± 161 | |
| Change (%) | +38.3 (0.005) | +7.1 (0.005) | −27.6 (0.01) | |
| Quinacrine ($n = 5$) | 2.38 ± 0.33 | 4.82 ± 0.96 | 1942 ± 418 | — |
| $LTC_4$ | 2.40 ± 0.32 | 4.50 + 1.02 | 2021 ± 444 | |
| Change (%) | 0.8 (NS) | −6.6 (NS) | −4.1 (NS) | |
| Quinacrine ($n = 5$) | 2.52 ± .44 | 5.21 ± 0.90 | 2256 ± 434 | |
| $PGE_2$ | 4.62 ± .58 | 5.28 ± 0.51 | 1234 ± 217 | 11 |
| Change (%) | +83.3 (0.005) | +1.3 (NS) | −45.3 (NS) | |

[a] Results expressed as the mean ± SE. Final drug concentrations were: quinacrine, $5 \times 10^{-5}$ M; $PGE_2$, $10^{-6}$ M; $LTC_4$, $10^{-7}$ M. Level of significance ($P$) based on Student's $t$ test. NS = no significant difference.

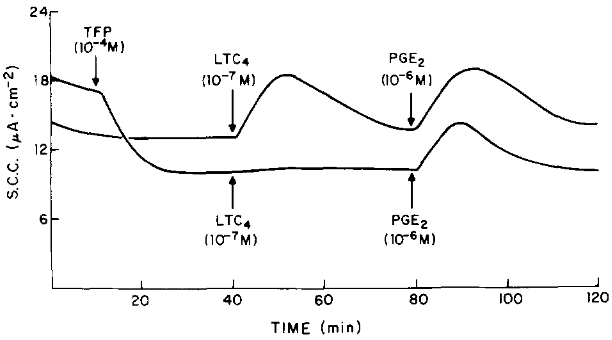

**Fig. 3.** Cornea in lower trace was first treated with trifluoperazine (TFP), an inhibitor of calmodulin. When the SCC restabilized, the application of $LTC_4$ failed to elicit a response, whereas the untreated control cornea shows a typical response to $LTC_4$. Application of $PGE_2$ stimulates the SCC in both corneas

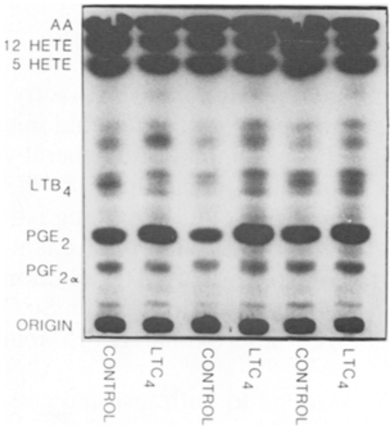

**Fig. 4.** Shown, is a series of three paired corneal preparations, treated as described in Table 4, and then run on TLC as described in Materials and Methods. The resulting TLC autoradiograph clearly shows the enhanced synthesis of $PGE_2$ in $LTC_4$-treated corneas, compared to untreated paired controls

## $LTC_4$ STIMULATION OF $PGE_2$ BIOSYNTHESIS

Experiments were performed in which we tested the effects of $LTC_4$ on eicosanoid biosynthesis in the isolated bullfrog cornea. Corneas were 'preloaded' with $^{14}C$(U)-arachidonic acid and then incubated with either $LTC_4$ or calcium ionophore A23187 (an activator of eicosanoid synthesis), as described in Materials and Methods. Paired control corneas received no $LTC_4$ or ionophore. Corneal samples were assayed for eicosanoid synthesis by TLC-autoradiography and the scintillation counting of bands. In addition, $PGE_2$ biosynthesis was monitored by means of radioimmunoassay in order to verify differences in $PGE_2$ levels obtained by TLC.

Figure 4 shows the recovery of five different $^{14}C$-labeled eicosanoids from three replicate sets of paired corneal samples. In each set, it can be observed that in the samples incubated with $LTC_4$ the $PGE_2$ bands are larger, indicating that more $PGE_2$ is present. In addition, scintillation counts of radioactivity in these bands confirm this observation. The data appear in Table 4, which shows mean eicosanoid synthesis in five sets of corneal samples, in terms of nanograms of each eicosanoid per cornea. In corneas treated with $LTC_4$, it can be seen that of the six eicosanoids identified, only $PGE_2$ shows a statistically significant increase over untreated controls. $LTC_4$ elicited nearly a threefold increase in

**Table 3.** The effects of inhibiting calmodulin (and $Ca^{2+}$ mobilization) by TFP (trifluoperazine), on the ability of $LTC_4$ and $PGE_2$ to affect the electrical properties of isolated frog cornea in Ussing-type chambers[a]

| Experiment | SCC ($\mu A \cdot cm^{-2}$) | PD (mV) | R ($\Omega \cdot cm^2$) | Time to maximum effect (min) |
|---|---|---|---|---|
| Control ($n = 5$) | 12.75 ± 0.89 | 25.42 ± 2.22 | 1973 ± 120 | |
| $LTC_4$ | 16.94 ± 1.03 | 27.02 ± 2.20 | 1606 ± 116 | 9 |
| Change (%) | +32.9 (0.005) | +6.3 (0.02) | −18.6 (0.001) | |
| Control ($n = 5$) | 12.85 ± 0.92 | 23.52 ± 1.57 | 1905 ± 251 | |
| $PGE_2$ | 18.87 ± 1.18 | 27.3 ± 3.21 | 1446 ± 137 | 10 |
| Change (%) | +46.8 (0.02) | −14.9 (NS) | −24.1 (NS) | |
| TFP ($n = 5$) | 9.36 ± 0.79 | 19.1 ± 1.35 | 2126 ± 272 | |
| $LTC_4$ | 9.82 ± 0.88 | 19.3 ± 1.36 | 2063 ± 270 | — |
| Change (%) | +4.9 (NS) | +1.0 (NS) | −3.0 (NS) | |
| TFP ($n = 4$) | 10.14 ± 1.44 | 24.78 ± 2.16 | 2890 ± 672 | |
| $PGE_2$ | 13.76 ± 1.78 | 26.78 ± 2.30 | 2257 ± 521 | 10 |
| Change (%) | +35.7 (0.005) | +8.07(NS) | −21.9 (NS) | |

[a] Results are expressed as the mean ± SE Final drug concentrations were: TFP, $10^{-4}$ M; $PGE_2$, $10^{-6}$ M; $LTC_4$, $10^{-7}$ M. Significance, ($P$) based on Student's $t$ test. NS = no significant difference.

**Table 4.** Effects of $LTC_4$ and ionophore A23187 on eicosanoid synthesis in frog cornea

| Metabolite | Control | $LTC_4$ | Control | A23187 |
|---|---|---|---|---|
| $PGF_{2a}$ | 0.126 ± 0.033 | 0.127 ± 0.027 | 0.103 ± 0.006 | 0.149 ± 0.038 |
| $TXB_2$ | 0.035 ± 0.004 | 0.054 ± 0.014 | 0.031 ± 0.001 | 0.083 ± 0.027 |
| $PGE_2$ | 0.428 ± 0.075 | 1.205 ± 0.111[a] | 0.454 ± 0.008 | 1.309 ± 0.031[a] |
| $LTB_4$ | 0.119 ± 0.014 | 0.144 ± 0.013 | 0.089 ± 0.002 | 0.142 ± 0.011[a] |
| 5 HETE | 0.451 ± 0.034 | 0.489 ± 0.031 | 0.192 ± 0.013 | 0.342 ± 0.041[a] |
| 12 HETE | 0.681 ± 0.106 | 0.677 ± 0.059 | 0.358 ± 0.054 | 0.605 ± 0.070 |

Metabolites expressed as ng per cornea. $N = 5$ in each case. Corneas were preloaded with $^{14}C(U)$-AA for 1 hr and then washed in Ringer's three times to remove free AA. $LTC_4$ ($10^{-7}$ M) or ionophore A23187 ($2 \times 10^{-6}$ M) was added to test corneas, and corneas were incubated for an additional 30 min to allow for eicosanoid synthesis. Samples were then cooled to 0°C, homogenized, and extracted with chloroform. Extracts were concentrated by evaporation under nitrogen, and run on TLC. Metabolite bands were visualized by autoradiography and quantitated by scintillation counting.

[a] Denotes a significant increase over control: $P < 0.02$, based on 2-tailed Student's $t$ test.

the synthesis of $PGE_2$, as determined by TLC autoradiography. Table 4 also shows that calcium ionophore enhances eicosanoid synthesis via both the cyclo-oxygenase and lipoxygenase pathways. In contrast, the effect of $LTC_4$ is limited to the cyclooxygenase pathway.

Verification by radioimmunoassay of changes in $PGE_2$ synthesis showed that control samples ($n = 7$) contained 5.00 ± 0.46 ng of $PGE_2$ per cornea, while test samples ($n = 7$) treated with $LTC_4$ contained 7.59 ± 0.45 ng of $PGE_2$ per cornea ($P < 0.005$ based on Student's $t$ test).

### $LTC_4$ Stimulation of cAMP

Preincubation of corneas with $LTC_4$ ($10^{-7}$ M) results in the marked potentiation of cAMP levels, as illustrated in Fig. 5. When corneas were treated with both indomethacin ($2 \times 10^{-5}$ M) and $LTC_4$, no stimulation of cAMP was observed, and cAMP levels were identical to those found in untreated control corneas.

Indomethacin treatment alone resulted in a marked reduction of cAMP, indicating that cyclooxygenase activity is apparently required for maintenance of control levels of cAMP. In corneas pretreated with $PGE_2$ ($10^{-6}$ M), a very large stimulation in cAMP was observed, confirming previous findings [13].

## Discussion

Our initial observations that $LTC_4$ exerts a stimulatory effect on chloride transport in the frog cornea,

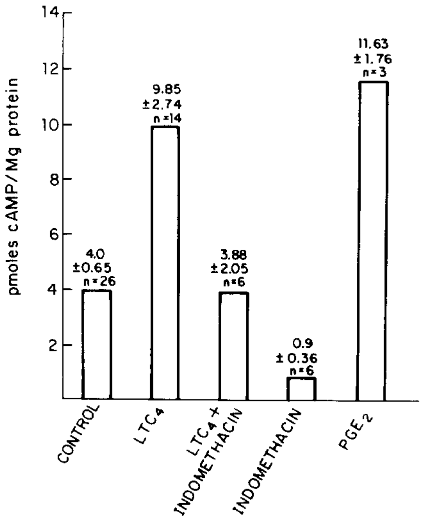

**Fig. 5.** The effects of treatment of corneas with $LTC_4$ ($10^{-7}$ M), indomethacin ($2 \times 10^{-5}$ M) or $PGE_2$ ($10^{-6}$ M) on cAMP levels. Both $LTC_4$ and $PGE_2$ stimulate cAMP synthesis. $LTC_4$ stimulation of cAMP is abolished in the presence of indomethacin. Indomethacin alone reduces cAMP to below control levels. Assays for cAMP were performed as previously described [13]

and that $LTB_4$ inhibits the process [14, 16], suggested to us that these leukotrienes may form the basis of a control mechanism for the regulation of chloride transport. The additional finding that the frog cornea is capable of synthesizing both $LTC_4$ and $LTB_4$ [16] lends further support to this hypothesis. In the work described here, we have studied the mechanism of action of $LTC_4$ to stimulate chloride transport. Our finding, that in corneas pretreated with indomethacin the effect of $LTC_4$ is completely blocked, suggested the involvement of cyclo-oxygenase products in this response. Since we previously showed that $PGE_2$ is the principal cyclo-oxygenase product formed by the frog cornea [14], and that $PGE_2$ can stimulate chloride transport [1], it seemed likely that $LTC_4$ might act by stimulating the synthesis of $PGE_2$. Indeed, in the present study we demonstrate via two different methods (TLC-autoradiography and radioimmunoassay) that incubation of frog corneas with $LTC_4$ results in a significant stimulation in $PGE_2$ synthesis. It was also previously shown that $PGE_2$ can stimulate cAMP in the frog cornea [13]. This finding was confirmed in the present study. In addition, we show here that $LTC_4$ is also capable of stimulating cAMP synthesis.

When $LTC_4$ is applied to corneas pretreated with indomethacin, no stimulation in cAMP is observed, suggesting that indomethacin blocks the synthesis of $PGE_2$, and therefore the stimulation of cAMP is prevented. Taken together, these results imply that the sequence of events leading to $LTC_4$ stimulation of chloride transport is mediated via the activation of the cyclo-oxygenase pathway, which leads to the enhanced synthesis of the principal cyclo-oxygenase metabolite found in frog cornea, $PGE_2$. Elevated $PGE_2$ levels then result in the increased synthesis of cAMP, which ultimately leads to the stimulation of chloride transport. Interestingly, we also show that indomethacin treatment alone reduces cAMP to below control levels, suggesting that a basal level of $PGE_2$ synthesis is normally present for maintenance of optimal cAMP and chloride transport levels. (*See* schematic diagram, Fig. 6.)

We then went on to another series of experiments designed to shed light on the mode of action of $LTC_4$ to activate the cyclo-oxygenase pathway. Two inhibitors were used in these experiments. Since an increase in the amount of free arachidonic acid can activate the cyclo-oxygenase pathway, we utilized quinacrine as a tool to prevent the release of arachidonic acid bound to membrane phospholipids. Quinacrine acts by inhibiting the phospholipase $A_2$ that is required to cleave the fatty acid moiety from the phospholipid molecule. If $LTC_4$ activation of cyclo-oxygenase is dependent on phospholipase $A_2$ activity, then in the presence of quinacrine we would expect to see an inhibition of the effect of $LTC_4$ to stimulate chloride transport. This, in fact, is what we observe. The stimulation of SCC and PD by $LTC_4$ is completely blocked in quinacrine-treated corneas, indicating that the response is dependent on phospholipase $A_2$ activity. Interestingly, quinacrine alone caused a large decrease in baseline SCC and PD levels, indicating that a basal level of phospholipase $A_2$ activity is both typical and necessary for maintenance of optimal levels of chloride transport. The subsequent addition of $PGE_2$ to quinacrine-treated corneas resulted in a typical stimulation response in SCC, confirming that the quinacrine block of $LTC_4$ action is specific at the level of phospholipase $A_2$, and has no effect on the $PGE_2$-cAMP-chloride transport sequence beyond that level.

The second inhibitor, TFP, inhibits the action of calmodulin, and therefore inhibits many calcium-dependent cellular events. Since phospholipase $A_2$ is known to be calcium sensitive, the possibility existed that TFP might inhibit phospholipase $A_2$ and thereby block the release of free arachidonic acid, thus blocking eicosanoid synthesis. We observed that TFP causes an immediate decrease in baseline SCC and PD, similar to that observed with quinacrine, suggesting that phospholipase $A_2$ activity was inhibited by the block to calmodulin. Subsequent application of $LTC_4$ to TFP-treated corneas resulted in no stimulation of SCC or PD, further suggesting that calcium plays a role in the regulation of chloride transport and, more specifically, that $LTC_4$ activation of cyclo-oxygenase is mediated via calmodulin. When $PGE_2$ was given to the same TFP-treated corneas, a typical stimulation of SCC was observed, suggesting that the TFP block to $LTC_4$ action exists at the level of phospholipase $A_2$, and that the $PGE_2$-cAMP-chloride transport pathway remains functional in the presence of TFP. However, since TFP may not be specific to calmodulin at $10^{-4}$ M, the involvement of calmodulin must be regarded as tentative.

Our studies into the effects of $LTC_4$ on eicosanoid synthesis clearly indicate that, of the six metabolites studied, only $PGE_2$ synthesis is significantly stimulated by $LTC_4$. We suggest that the reason for this is that $LTC_4$ affects only cyclo-oxygenase pathway synthesis, thus explaining why $LTB_4$, 5-HETE and 12-HETE levels are unchanged.

While $PGF_{2\alpha}$ and $TXB_2$ are cyclo-oxygenase pathway metabolites, their presence in the cornea at extremely low levels may account for the apparent lack of stimulation of these metabolites by $LTC_4$. Although the numbers do show a small stimulation in these two metabolites with $LTC_4$, it is not statistically significant.

Experiments were also done on the effects of calcium ionophore on eicosanoid synthesis in cornea, because calcium ionophore is known to generally stimulate both the cyclo-oxygenase and lipoxygenase pathways of eicosanoid synthesis in many different tissues. Not surprisingly, we found that calcium ionophore stimulates both pathways in the frog cornea as well. We found a statistically significant increase in $PGE_2$, similar to that found with $LTC_4$. In addition, $LTB_4$ and 5-HETE (both lipoxygenase pathway metabolites) showed a statistically significant increase. These results demonstrate that a general stimulation of eicosanoid synthesis is possible in frog cornea, and also confirm that the $LTC_4$ stimulation is limited only to the cyclo-oxygenase branch of the arachidonic acid cascade.

Previous reports have appeared in the literature in which the relationship between leukotriene action and cyclo-oxygenase products is described. Samhoun and Piper [10, 12] found that the actions of leukotrienes were mediated via formation of cyclo-oxygenase products in guinea-pig isolated perfused lung and parenchymal strips. Leukotriene-induced contractions were inhibited with indomethacin, and also with inhibitors of thromboxane synthetase and phospholipase $A_2$, suggesting that leukotriene action is mediated via generation of thromboxane $A_2$ in this tissue. Dahlen (4) found that the bronchoconstrictor action of $LTC_4$ in the guinea pig depends upon two separate mechanisms: a direct mechanism, and a secondary mechanism in which the release of cyclo-oxygenase products is involved

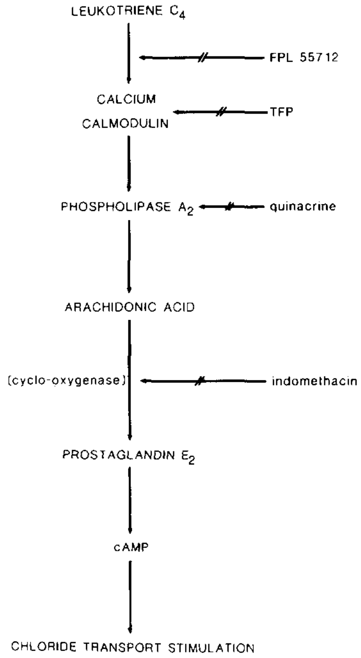

**Fig. 6.** Schematic diagram showing the proposed mechanism of action of $LTC_4$ in the frog cornea

Other interactions between cyclo-oxygenase and lipoxygenase have also been described. For example, Engineer et al. [5] noted that production of SRS-A (a naturally occurring mixture of leukotrienes) was markedly potentiated in the presence of cyclo-oxygenase inhibitors. This raises the possibility that prostaglandins may be involved in a type of feedback regulation of leukotriene synthesis. Such a relationship, if found also in the cornea, would have implications for the homeostatic regulation of chloride transport in the cornea.

The present sudy contributes to the clarification of basic mechanisms regulating chloride transport in the cornea. However, the potential for therapeutic applications also exists. Many different investigators have recently reported finding elevated levels of leukotrienes during ocular inflammation [2, 3, 9]. An increase in leukotriene levels may cause further pathological changes, in addition to the primary inflammatory condition. Because of their effect on chloride transport in the cornea, the leukotriene increase induced by inflammation may result in pathological alterations in corneal hydration, and excessive corneal edema, and may lead to tissue damage. Specific pharmacologic control of the arachidonic acid cascade may ultimately allow us to restore leukotriene levels to normal during inflammation and thus may lead to the avoidance both of tissue damage associated with inflammatory conditions and of the side effects associated with the use of steroids.

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