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Uptake of Arachidonic Acid into Membrane Phospholipids: Effect on Chloride Transport Across Cornea

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Summary. We demonstrate that arachidonic acid (AA) stimulation of chloride transport across frog cornea is mediated via two independent pathways: (1) stimulation of prostaglandins and cAMP synthesis, and (2) a direct physical change in the membrane produced by substitution of different phospholipid acyl chains. AA is well known as a precursor in the synthesis of prostaglandins, which have been shown to stimulate cAMP synthesis and chloride transport in frog cornea. We show that frog cornea can convert exogenous AA to PGE_2, but that in the presence of 10^{-5} M indomethacin both the conversion to PGE_2 and stimulation of cAMP are completely blocked. However, with indomethacin the action of AA to stimulate chloride transport (as measured by SCC) remains, but peak height of the response is reduced to 57% of that found when AA alone is given. Similarly, we show that propranolol completely blocks cAMP stimulation, but stimulation of SCC is reduced to 45% of the original response. Therefore, cAMP appears to be responsible for roughly half of the observed stimulation in SCC. By gas chromatographic analysis we show that significant quantities of AA can rapidly substitute into membrane phospholipids of corneal epithelium and L929 cells following the addition of AA to the medium. Modification of membrane phospholipid structure can affect membrane viscosity, membrane-bound enzyme activity, and the distribution and lateral mobility of integral proteins. It seems likely that such alterations in the properties of the membrane may modulate the rate of chloride transport, and this may constitute the second mechanism. Upon addition of AA, both mechanisms appear to stimulate chloride transport simultaneously, and are apparently additive. We show that prolonged exposure to AA results in a large incorporation of AA into phospholipid and consequently, a perturbation in the ratio of unsaturated to saturated fatty acids. We also find evidence of a compensatory cellular mechanism that alters the ratio of endogenously synthesized fatty acids and tends to reduce the membrane-perturbing effect of AA.

Key Words chloride transport · fatty acids · arachidonic acid · phospholipids · cAMP · prostaglandins · corneal epithelium

Introduction

We have previously reported the phenomenon that highly unsaturated fatty acids are able to stimulate chloride transport across isolated frog cornea (Schaeffer & Zadunaisky, 1979). Arachidonic acid (AA), with four unsaturations, was found to exert the greatest effect; other fatty acids with fewer unsaturations such as linolenic, linoleic and myristoleic acids were also stimulatory, but to a lesser extent. It was also noted that the corneal response to fatty acid additions was rapid in onset, occurring approximately 1 min after addition of fatty acids to the corneal bathing medium. Measurements of the rate of patching of membrane proteins indicate that corneal epithelial cell membranes treated with unsaturated fatty acids have a lower viscosity, or, are more 'fluid' (Schaeffer & Zadunaisky, 1979; Zadunaisky, Schaeffer & Cherksey, 1980). We initiated this study to further explore the way fatty acids modulate such membrane-mediated cell functions as chloride transport. In this paper we report the results of treating frog corneas and L929 cells with exogenous AA. Assays for cAMP and PGE_2 synthesis were done, and specific inhibitors such as propranolol and indomethacin were used to probe the mechanism of AA action in corneas.

We also report data on incorporation of [^{14}C] AA into phospholipid, as well as gas chromatography data showing changes that occur in phospholipid acyl chain composition with AA treatment.

Materials and Methods

Measurement of Short-Circuit Current (SCC) Across Frog Cornea

SCC, which is proportional to the net ion transport across the cornea, was measured as described previously (Zadunaisky, 1966), by mounting freshly excised corneas, obtained from *Rana catesbeiana*, in Ussing-type chambers. Potential difference (PD) and SCC were measured with dual automatic voltage-clamp units, model DC-200 (Biomedical Engineering) and a Varian model 9176 dual pen recorder. Drugs or fatty acids to be tested were added to both halves of the chamber, and desired concentrations were achieved by a rapid mixing of the Ringer's bathing solution.

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Measurement of cAMP

Paired corneal buttons were dissected and placed in Ringer's solution for 1 hr to equilibrate. Corneas were then transferred to fresh Ringer's containing 5 mM theophylline, and drugs to be tested were added to only one of the paired corneas. Corneas were incubated for 20 min at 25 °C to allow for accumulation of cAMP. The reaction was stopped by placing corneas in 1% HNO_3 at 90 °C. Corneas were homogenized for 10 min, the homogenate neutralized with $NaHCO_3$ and then centrifuged. The supernatant was assayed for cAMP using a competitive protein binding assay (Amersham), and the pellet was dissolved in 2 M NaOH and assayed for protein by the method of Lowry, Rosebrough, Farr and Randall (1951). Recovery of cAMP was 87% as shown by use of internal standards. Results are expressed as pmol cAMP per mg protein.

Assay for Prostaglandins (PG)

Paired corneas were dissected and incubated in Ringer's for 30 min to allow for stabilization. Experimental corneas were then incubated for 30 min in indomethacin (10^{-5} M); control corneas received no inhibitor. AA (10^{-4} M) labeled with [^{14}C] AA [0.5 μCi] (New England Nuclear) was added to each cornea and incubated for another 30 min. Corneas were then acidified with citric acid and extracted twice with chloroform. The combined solvent phase was evaporated to dryness under a stream of nitrogen, taken up in chloroform/methanol (2:1), and spotted onto silica gel TLC sheets (Eastman Chromagram). Sheets were developed in an iso-octane/ethyl acetate/acetic acid/water (30:66:12:60) solvent system. Prostaglandin standards (Sigma) were co-chromatographed for identification of prostaglandins in the sample and were visualized in iodine vapor. Prostaglandin spots in the sample were detected by autoradiography by overlaying the TLC plate with Kodak NS-5T X-ray film for 72 hr. Quantitation of radio-labeled spots identified as prostaglandins was done by scintillation counting.

Fatty Acid Supplementation into Cell Cultures and Corneal Epithelium

L929 cell cultures were grown to confluency in Dulbeccos minimum essential medium (MEM) plus 5% fetal calf serum (Flow). AA stock solution in methanol (100 mg/ml) was stored frozen under nitrogen. Aliquots of stock solution were added directly to the tissue culture medium while mixing to make a final concentration of 10^{-4} M AA. Cell monolayers were then incubated at 37 °C for the times required in each experiment.

Fatty acid supplementations into frog cornea for gas chromatography studies were done by incubating whole frog eyes in Ringer's solution containing 10^{-4} M AA for 5 min and 1 hr. Eyes were washed three times in fresh Ringer's, and corneal epithelium was scraped and collected using a sharp scalpel.

Time Course of Arachidonic Acid Incorporation

L929 cell monolayers were grown to confluency and washed twice with phosphate-buffered saline (PBS). At time zero, 10 ml of fresh complete medium containing 10^{-4} M AA labeled with [^{14}C] AA (0.1 μCi) was added to each flask (Falcon) containing 1×10^7 cells. Flasks were incubated at 37 °C for 2, 30 and 120 min, and the medium decanted and saved. After two washings with PBS, monolayers were scraped, centrifuged, washed three times, and assayed for AA incorporation by TLC as described below.

Lipid Analysis

The lipids of whole cells were extracted as described by Bligh and Dyer (1959). Washed cell pellets were covered with methanol and sealed under nitrogen. The samples were then sonicated and incubated for 1 hr at 55 °C. Chloroform was added to give a chloroform/methanol ratio of 2:1, followed by sonication. The solvent phase was removed and the pellet extracted a second time. Combined extracts were mixed with distilled water, the two phases separated, and the solvent layer removed and evaporated under nitrogen. To separate phospholipids from other lipid classes, concentrated extract was spotted onto silica gel G thin-layer chromatography (TLC) plates (Supelco), and developed in anhydrous ether/petroleum ether (20:80). In this system phospholipids remain at the origin, while neutral lipids and free fatty acids migrate away from the origin. We tested this system by spotting TLC plates with varying concentrations of a mixture of dipalmitoyl phosphatidylcholine, AA and [^{14}C] AA. Successive 0.5 cm scrapings were assayed for AA by scintillation counting. We found that free fatty acid always migrates in a broad band, completely separating from the origin. In addition, gas chromatography of material scraped from the origin revealed only palmitic acid, and no AA, indicating that free fatty acids do not exchange into phospholipids during the procedure.

Samples for assay by gas chromatography were scraped from the origin into chloroform-methanol, centrifuged, and the solvent phase removed and evaporated to dryness under a stream of nitrogen. The phospholipid sample was taken up in benzene and acyl chains esterified using boron trifluoride-methanol (BF3) reagent (Supelco). BF3 reagent was added to the sample in benzene and incubated at 96 °C for 3 min. After adding water to stop the reaction, it was mixed and centrifuged. The benzene layer was evaporated to dryness and taken up in hexane for gas chromatography.

Esterified fatty acid samples were analyzed by gas chromatography using a Varian 3700 gas chromatograph equipped for operation with capillary columns. A WCOT type glass capillary column with FFAP liquid phase, 15 m × 0.25 mm ID, was used isothermally at 180 °C. The injector and FID detector were maintained at 230 and 300 °C, respectively. Nitrogen carrier gas column flow rate was adjusted to 1 ml/min with a bubble flowmeter, and the split ratio was 100:1. Fatty acid peaks were identified by comparison of retention times with authentic standards (Supelco, NuChek Prep). Peaks were quantitated by generating a correction factor for each fatty acid peak with commercially available quantitative standard mixtures, and multiplying the factor by peak height. Multiplying peak height by peak retention time also yielded essentially the same results. Data are reported in terms of weight percent composition of principal phospholipid fatty acids.

Results

Stimulation of Cl^- Transport with AA and its Partial Inhibition with Indomethacin and Adrenergic Antagonists

Frog corneas were dissected, placed in Ussing-type chambers and the SCC was allowed to stabilize. Figure 1 (upper trace) shows a typical stimulation in SCC after addition of 10^{-4} M AA to the bathing medium. Such stimulation occurs immediately following the addition, with an average increase over

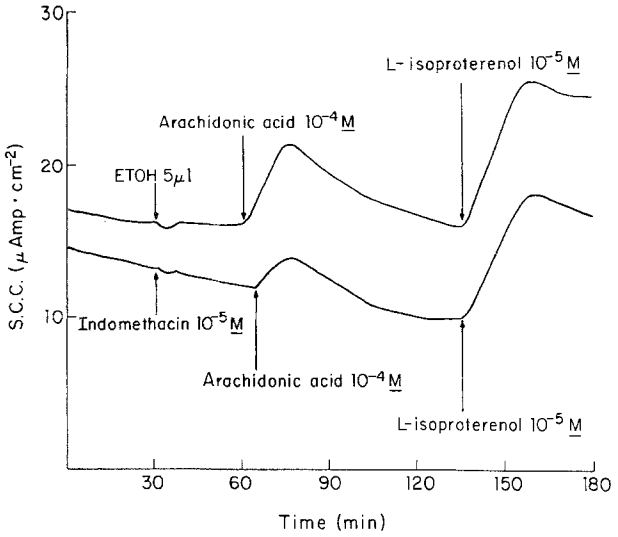

Fig. 1. Frog corneas were mounted in Ussing-type chambers and SCC was measured as described in Materials and Methods. Upper trace shows typical stimulation in SCC following addition of AA (10^{-4} M) and isoproterenol (10^{-5} M). Cornea in lower trace was pretreated for 30 min with indomethacin (10^{-5} M) and then given AA and isoproterenol as in the control cornea above. Following indomethacin pretreatment, the magnitude of the SCC response to AA is reduced to 57% of the control response, while the response to isoproterenol remains unchanged. Magnitude of response is calculated by dividing SCC at peak height by baseline SCC

baseline SCC of 40.9%. Potential difference (PD) is similarly stimulated, and follows a similar time course. The lower trace shows a cornea pretreated for 30 min with indomethacin (10^{-5} M), a dosage that inhibits prostaglandin synthesis (*see below*). Addition of 10^{-4} M AA results in an average stimulation in SCC of 23.3% over baseline. With indomethacin present, the mean peak height in eight experiments is 43% less than in paired control corneas treated only with AA (Table 1).

Experiments with alpha and beta adrenergic antagonists were done on corneal pairs, with only one cornea receiving antagonist; both corneas were given AA and then epinephrine after re-establishing a new baseline. Degree of stimulation was calculated by taking the SCC at peak response as a percent of baseline SCC. Suppression of current due to antagonists was calculated for each corneal pair by comparing experimental with control corneas.

The results (Table 2 and Fig. 2) clearly show that when both alpha and beta receptors are blocked with 10^{-5} M phentolamine and propranolol, the response to AA is 79% suppressed; and as expected, the epinephrine response is completely eliminated. Corneas that were either alpha or beta blocked showed significant suppression in their response to both AA and epinephrine. However, the suppression of the AA response is greater with a blocked beta receptor; while suppression of the epinephrine response is greater with a blocked alpha receptor.

Table 1. Effect of indomethacin on subsequent stimulation of SCC with arachidonic acid in frog cornea pairs

Exp. No.	Cornea A	Cornea B
	Arachidonic acid (10^{-4} M)	Indomethacin (10^{-5} M) + Arachidonic acid (10^{-4} M)
	(percent increase in SCC)	
1	32.2	26.9
2	48.5	8.8
3	40.0	47.6
4	30.0	3.5
5	16.2	29.1
6	100.0	33.3
7	45.5	22.2
8	14.5	15.3
	40.9 ± 9.5[a]	23.3 ± 5.0 (43% inhibition)

[a] Mean ± SEM.

Table 2. Effect of adrenergic antagonists on stimulation of SCC by arachidonic acid and epinephrine in frog cornea pairs

	Arachidonic acid	Epinephrine
	(percent increase in SCC ± SEM)	
1. Phentolamine (10^{-5}) ($n=8$)	59.3 ± 10.3	2.3 ± 0.94
	92.8 ± 12.1 (control)	51.2 ± 9.1 (control)
	36.1% suppression	95.5% suppression
2. Propranolol (10^{-5}) ($n=7$)	25.7 ± 1.9	26.8 ± 3.0
	56.6 ± 2.6 (control)	53.8 ± 3.9 (control)
	54.6% suppression	50.2% suppression
3. Phentolamine + propranolol (10^{-5}) ($n=7$)	11.1 ± 2.0	1.3
	53.0 ± 7.0 (control)	58.2 ± 10.5 (control)
	79.1% suppression	97.8% suppression

Control corneas were given AA (3×10^{-4} M), and after returning to a stable baseline, they were given epinephrine (10^{-6} M). Experimental corneas of each pair were pretreated with antagonists as indicated for 30 min, followed by AA and epinephrine.

Stimulation of cAMP Synthesis with AA and its Inhibition with Indomethacin and Propranolol

cAMP assays were done on paired corneas following the addition of fatty acids to one cornea of

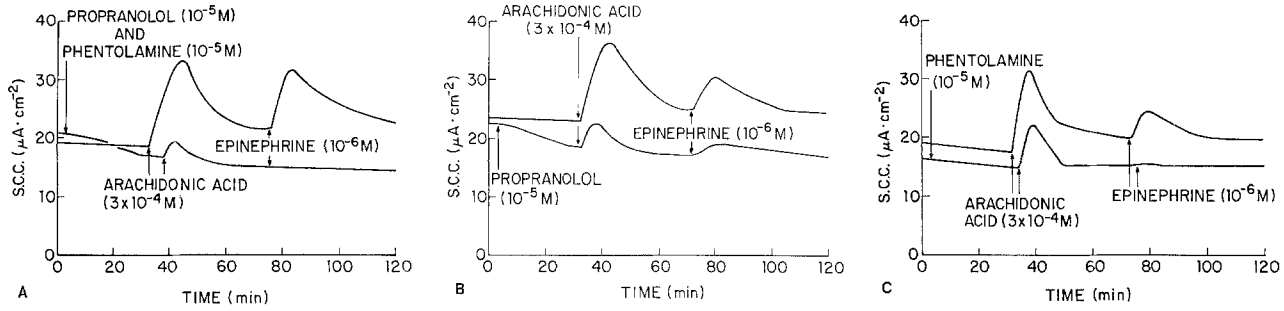

Fig. 2. Traces show stimulation in SCC with AA and epinephrine in frog cornea following pretreatment with adrenergic blocking agents. (*A*) The combined action of alpha and beta blockers reduce the SCC response to AA by 79%, and the response to epinephrine is completely suppressed. (*B*) Propranolol pretreatment alone supresses the SCC response to AA and epinephrine by 50%. (*C*) Phentolamine pretreatment alone suppresses the SCC response to AA by 36% and to epinephrine by 95%

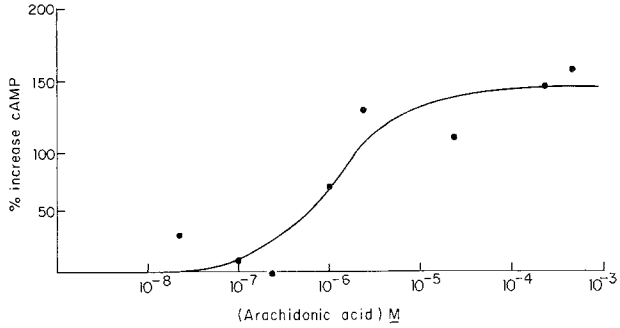

Fig. 3. Stimulation of cAMP levels in frog cornea by AA at various concentrations, with maximal stimulation at 10^{-4} M AA. cAMP assays were performed as described in Materials and Methods. Increases in cAMP are calculated by dividing pmol cAMP in the AA-treated cornea by pmol cAMP in the untreated control in each experiment

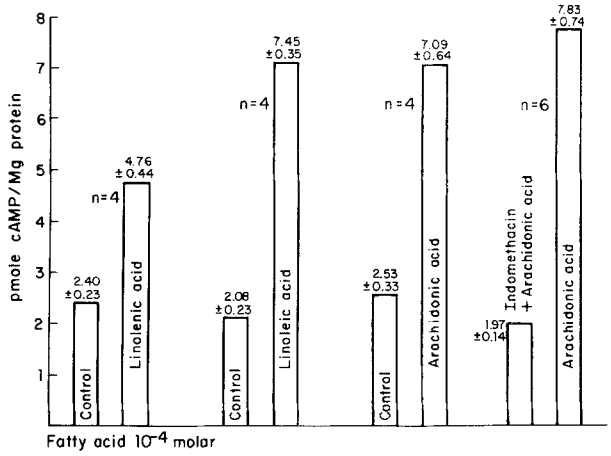

Fig. 4. In addition to AA, other long-chain unsaturated fatty acids have a stimulatory effect on cAMP levels in frog cornea. Pretreatment of corneas with indomethacin (10^{-5} M) can be seen to completely block the increase in cAMP induced by AA. Mean ± SEM is shown

each pair. A dose-dependent increase in cAMP was found in response to AA over a concentration range of 10^{-7} to 10^{-4} M (Fig. 3), with a maximal increase to over 7 pmol cAMP/mg protein in corneas treated with 10^{-4} M AA as compared with normal control levels of 2 pmol cAMP. Linoleic and linolenic acids at 10^{-4} M also induced similar increases in cAMP levels (Fig. 4).

cAMP assays were then repeated, but PGE_2 synthesis was first inhibited with indomethacin, in order to determine whether cAMP levels were increasing as a result of the conversion of AA to PGE_2. Corneas treated with indomethacin (10^{-5} M) and then AA showed a mean cAMP level of 2.0 pmol, a value comparable to untreated control corneas. Corneas treated only with AA showed a stimulation in cAMP levels to 7.8 pmol (Fig. 4). This shows that the stimulation of cAMP synthesis with AA is mediated via prostaglandin synthesis.

Corneas were also assayed for cAMP following propranolol or phentolamine pre-incubation and AA treatment. The stimulation of cAMP synthesis that is normally found following AA treatment was completely eliminated with propranolol. With phentolamine pretreatment, the cAMP response to AA is half the normal response (Figs. 4 and 5). Each inhibitor appears to suppress cAMP to below the normal basal level, and both inhibitors combined further suppress the basal cAMP level (Fig. 5).

Inhibition of Prostaglandin Synthesis with Indomethacin

Assays for PG synthesis were done in order to show (1) whether PG synthesis results from supplementation with AA, (2) whether such PG synthesis can be blocked by conventional cyclo-oxygenase inhibitors, and (3) whether in the presence of a

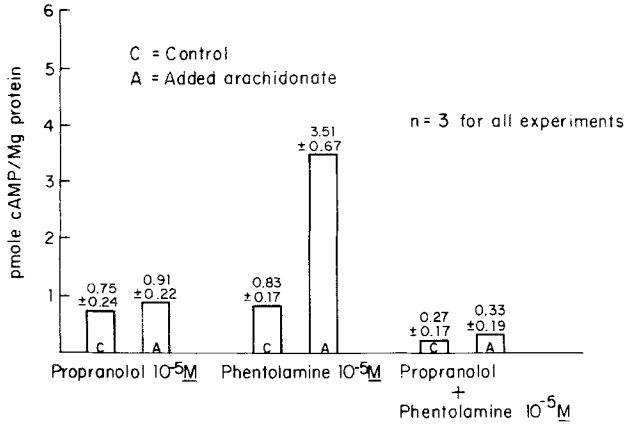

Fig. 5. Both corneas of each pair were preincubated with the indicated inhibitors for 30 min; one cornea of each pair was then given AA (10^{-4} M). Stimulation in cAMP levels was completely blocked by propranolol and partially blocked by phentolamine. Mean ± SEM is shown

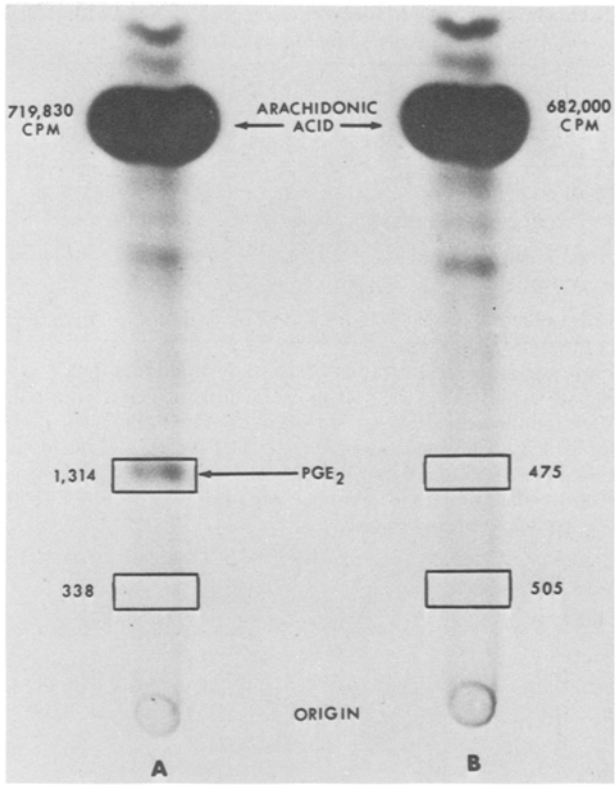

Fig. 6. TLC autoradiograph of paired frog corneas. Cornea A was treated with (^{14}C) AA as described in Materials and Methods, and shows the presence of a band identified as PGE_2, demonstrating its synthesis from AA. Cornea B was pretreated with indomethacin (10^{-5} M), then given (^{14}C) AA, and demonstrates the complete inhibition of PGE_2 synthesis. Unidentified bands near AA represent metabolites and autooxidation products of AA. Scintillation counts of delineated areas are indicated

demonstrated cyclo-oxygenase inhibition, AA can still stimulate chloride transport across frog cornea, as well as cAMP levels.

PGE_2 synthesis in frog cornea, and its subsequent inhibition in the presence of the cyclo-oxygenase inhibitor, indomethacin, was demonstrated by means of TLC autoradiography. Paired corneas were treated with the same concentration of AA and indomethacin as in chamber experiments, except that [^{14}C] AA was added in addition to cold AA. Incubation times were also identical. A typical autoradiograph can be seen in Fig. 6. The control cornea 'A' was given [^{14}C] AA+AA (10^{-4} M), and then incubated for 30 min. This cornea shows the presence of a band identified as PGE_2, thus demonstrating that frog cornea normally has the capacity to synthesize PGE_2 from exogenously supplied AA within 30 min. PGE_2 also seems to be the major prostaglandin present in frog cornea. Other prostaglandins were not detected by these methods. The experimental cornea 'B' was pretreated with indomethacin for 30 min, then given [^{14}C] AA+AA (10^{-4} M). The autoradiograph shows complete absence of the PGE_2 band, indicating that indomethacin at 10^{-5} M completely inhibits PGE_2 synthesis. In addition, scintillation counting of the PGE_2 band and the identical region in the indomethacin-treated cornea, confirmed the complete inhibition of PGE_2 synthesis.

Aspirin (5×10^{-4} M), another cyclo-oxygenase inhibitor, was tested in this system, and was also found to completely block PGE_2 synthesis. However, two other inhibitors of cyclo-oxygenase and lipoxygenase activity were tested: eicosatetraynoic acid (ETYA) and nordihydroguaiaretic acid (NDGA), and at 10^{-5} M these did not inhibit PGE_2 synthesis in our system. Although ETYA is generally considered to be a specific inhibitor of PG synthesis, others have also reported on the ineffectiveness of ETYA as an inhibitor of PG synthesis (Smolen & Weissmann, 1980). This illustrates the importance of testing the effectiveness of cyclo-oxygenase inhibitors in each system, and of not assuming that PG synthesis has been blocked.

Time Course of AA Incorporation into L929 Cell Monolayers

L929 cells were used as a convenient model system for following the incorporation of [^{14}C] AA. Confluent cell monolayers were overlayed with [^{14}C] AA in complete medium and incubated for the appropriate times. The lipid extract from whole cells was run in a TLC system which facilitated

Table 3. Time course of incorporation of [^{14}C] arachidonic acid into L929 cell monolayers in complete medium

	CPM		
	2 min	30 min	120 min
Medium above cells and combined washings	95,114	82,411	65,318
Arachidonic acid bound to phospholipids	1,012	4,070	7,416
Free arachidonic acid	97	3,463	9,952

Fatty acid supplementation consisted of adding [^{14}C] AA (0.1 μCi) plus AA (10^{-4} M) to each flask of cells containing 10 ml Dulbeccos MEM + 5% FCS. At selected times, cells were scraped, washed, and the lipids extracted as detailed in Materials and Methods. Lipid extract was run in a TLC solvent mixture that permitted complete separation of free AA from AA bound to phospholipid.

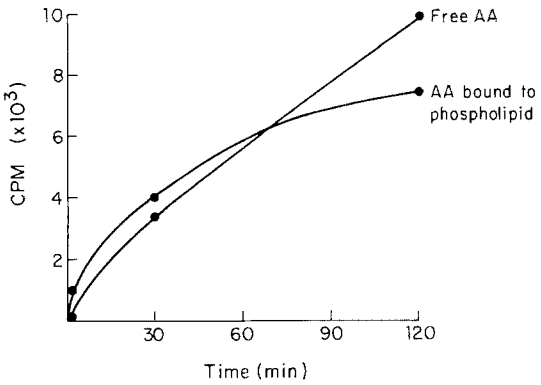

Fig. 7. Time course of incorporation of (^{14}C) AA into L929 cell monolayers, showing the rate of AA binding to phospholipid, and also uptake as free AA. Plot of data shown in Table 3

complete separation of phospholipids from free fatty acids. The two regions were scraped and quantitated by scintillation counting. As early as 2 min after its addition to the cell medium, a rapid and significant incorporation of AA into membrane phospholipid was observed. At the same time, only a trace of free AA was found in the whole cell extract (Table 3). That such brief exposures to AA resulted in significant phospholipid incorporation, supports the premise of a large membrane-mediated component in the AA stimulation of SCC. With longer incubations, uptake of free AA (presumably into cytoplasm), proceeds at a more rapid pace than does incorporation into phospholipids, although at the end of 30 min there is still slightly more AA bound to phospholipid than free within the cells. After 2 hr, incorporation into phospholipids appears to be saturating, while free AA uptake continues at the same rate (Fig. 7).

Table 4. Change in fatty acid composition of phospholipids of frog corneal epithelium supplemented with arachidonic acid

Fatty acid	Fatty acid content (percent of total phospholipid fatty acid)		
	Normal control (n=4)	Incubation time in arachidonic acid	
		5 min (n=2)	60 min (n=2)
12:0	1.7	0.2	0.1
14:0	3.6	0.7	0.6
14:1	1.3	0.4	0.2
16:0	17.8	7.4	7.7
16:1	12.4	10.7	10.6
18:0	7.5	5.6	4.5
18:1	33.3	43.7	26.9
18:2	5.5	5.1	6.0
18:3	0.85	2.0	1.0
Unknown	9.1	10.1	0.5
20.0	1.1	2.1	1.4
20:1	1.6	2.2	1.6
20.4	4.3	8.8	38.6

Whole eyes were incubated in Ringer's containing arachidonic acid (10^{-4} M). Fatty acid composition of the phospholipids of corneal epithelial scrapings were determined as described in Materials and Methods.

Fatty Acid Composition of Control and AA Supplemented Frog Corneas

Whole excised frog eyes were incubated for appropriate times in 10^{-4} M AA in Ringer's solution in order to achieve fatty acid substitution. Following the incubation period the corneal epithelium was scraped and prepared for analysis by gas chromatography as described earlier. Since each eye yields such a small quantity of tissue, scrapings from six eyes were pooled for each analysis.

Table 4 shows the phospholipid fatty acid composition in frog corneal epithelium before and after AA substitution. The data show that AA (20:4) normally accounts for 4.3% of phospholipid fatty acids, and that after 5 min of incubation in the AA-Ringer's medium, the % AA is approximately doubled. After 1 hr of incubation, AA accounts for 38.6% of the phospholipid fatty acid, and has become the predominant fatty acid. The rapid substitution of AA into phospholipids of corneal epithelium is compatible with our findings above of its similarly rapid incorporation into phospholipids of L929 cells.

Fatty Acid Composition of Control and AA Supplemented L929 Cells

L929 cell monolayers were incubated for times ranging from 2 min to 48 hr in complete medium

Table 5. Time course of change in fatty acid composition of phospholipids of L929 cells supplemented with arachidonic acid[a]

Fatty acid	Fatty acid content (percent of total phospholipid fatty acid)								
	Normal control	Incubation time in arachidonic acid							
		2 min	10 min	30 min	2 hr	5 hr	15 hr	24 hr	48 hr
12:0[b]	0.22	0.08	0.25	0.19	0.06	0.25	0.13	0.13	0.04
14:0	0.44	0.37	0.49	0.32	0.30	0.37	0.35	0.31	0.33
14:1	4.5	3.1	3.7	5.0	3.5	4.4	5.0	3.7	5.1
16:0	8.1	6.2	8.1	6.9	6.7	6.7	8.0	5.9	8.1
16:1	3.3	3.2	3.9	2.9	2.2	2.3	2.0	1.7	1.5
17:0	1.0	0.89	0.81	0.66	0.9	0.57	0.60	0.53	0.90
Unknown	5.7	5.0	4.5	6.1	4.4	5.4	5.9	3.9	1.8
Unknown	4.7	3.6	3.4	5.0	4.8	3.8	4.9	4.1	2.0
18:0	16.8	20.0	16.8	10.4	13.0	11.6	13.3	10.2	21.5
18:1	44.0	44.4	43.5	38.4	39.0	36.2	27.3	23.6	18.4
18:2	0.88	0.92	1.2	0.87	0.52	0.77	1.0	0.39	0.64
18:3	0.57	0.61	0.70	1.0	0.54	0.40	0.20	0.27	0.32
20:0	2.4	1.8	2.6	2.1	1.1	1.3	0.40	1.1	0.59
20:1	2.2	2.7	2.3	1.6	1.5	2.0	1.4	1.1	0.72
20:4	4.1	6.7	6.9	18.4	22.0	22.0	28.5	42.2	41.1
Percent unsaturated fatty acids	69.9	70.2	70.1	79.3	78.5	77.3	76.2	80.8	71.6

[a] Each number represents the mean of at least three determinations. L929 cell monolayers were grown to confluency and arachidonic acid (10^{-4} M) was added to the complete medium and incubated at 37 °C for the times indicated. Cells were scraped and the lipids extracted and prepared as described. Fatty acid methyl esters were assayed by gas chromatography.

[b] Fatty acid nomenclature: Number of carbons/number of double bonds.

supplemented with 10^{-4} M AA. The phospholipid fatty acid spectrum is shown in Table 5 for normal control cells as well as for cells incubated in AA for each time interval. The data shows that AA % composition is normally 4.1%, very similar to that found in frog corneal epithelium (4.3%). The AA % composition increases rapidly during the first 24 hr of incubation in supplemented medium, at which time incorporation into phospholipid appears to level off at approximately 40% (Fig. 8). In contrast, fatty acid substitution into corneal epithelium proceeds at a more rapid rate, and this level of incorporation is reached after only 1 hr of incubation. There is virtually no further change in the AA % composition during the second 24-hr period. While AA is rapidly substituting into phospholipids during the first 24 hr, the ratio of unsaturated/saturated fatty acid increases from 69.9% to approximately 80%. This increase is due primarily to the increased % of AA. However, during the second 24-hr period when no further AA substitution is occurring, the ratio of unsaturated/saturated fatty acid returns to almost its original value in spite of a continued 40% AA composition.

This is accounted for primarily by a large compensatory increase in stearic acid (18:0) and also a decrease in oleic acid (18:1). These two fatty acids are major components that together comprise 60% of the total phospholipid fatty acid in control cells. Excluding AA from the calculation of % composition, stearic acid increased from 16.8 to 36.5% after 48 hr, while oleic acid decreased from 44 to 31.2%. Both of these changes serve to restore the original unsaturated/saturated fatty acid ratio (Fig. 9). It is also of interest to note that the ratio of unsaturated/saturated fatty acid in control corneal epithelium is 68.4% and is nearly identical to that found in control L929 cell cultures (69.9%).

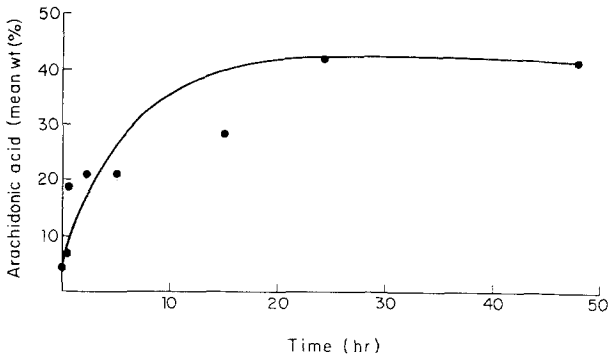

Fig. 8. Rate of incorporation of AA into phospholipid in L929 cell monolayers incubated with AA (10^{-4} M). AA is expressed as a percent of the total phospholipid fatty acid

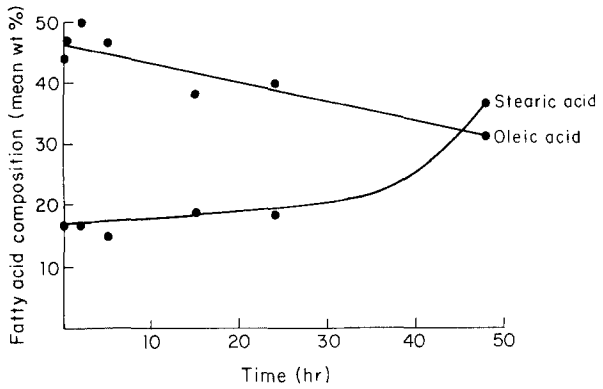

Fig. 9. Change in % composition of stearic and oleic acid in phospholipid of L929 cells that are actively incorporating AA. AA has here been excluded from the calculation of % composition because the increasing % of AA would otherwise skew the % of each fatty acid component

Table 6. Fatty acid composition of fetal calf serum

Fatty acid	Total fatty acid content (percent)	
	Lot a	Lot b
12:0	1.8	2.0
14:0	3.8	4.0
14:1	1.3	1.3
16:0	20.5	18.5
16:1	11.1	14.0
18:0	7.6	7.9
18:1	34.9	39.3
18:2	4.3	2.8
18:3	0.8	0.5
Unknown	10.7	7.4
20:4	3.3	2.2

Another experiment was done in which cell cultures were pretreated with propranolol (10^{-5} M) for 24 hr, followed by AA for 2 hr. The object was to test whether the membrane stabilizing property of propranolol would have any effect on AA incorporation into phospholipids. Fatty acid analysis by gas chromatography revealed no significant differences either in AA incorporation or total fatty acid distribution between propranolol pretreated cells and cells treated only with AA for 2 hr. Thus, AA incorporation into phospholipids proceeds at the same rate in the presence of propranolol.

Fatty Acid Composition of Fetal Calf Serum (FCS)

Since supplementation of cell cultures with fatty acids was done in the presence of 5% FCS it was important to verify that the fatty acid composition of FCS remains uniform from lot to lot. Table 6 shows the fatty acid composition for two lots of FCS. Apparently the fatty acid composition remains reasonably constant from one lot to the next, and therefore no significant variations in fatty acid composition of cell cultures should result.

Discussion

The present experiments were done in order to further elucidate the mechanism of fatty acid stimulation of chloride transport in frog corneal epithelium.

Our findings show that treatment of frog corneas with exogenous AA results in the synthesis of both PGE_2 and cAMP. Blocking the cyclo-oxygenase pathway with indomethacin completely inhibits (to the level of sensitivity of our techniques) the AA stimulation of PGE_2 and the resultant cAMP synthesis. However, with indomethacin pretreatment, we are still able to stimulate chloride transport by exogenous administration of AA, although the stimulation is half that found in control corneas treated only with AA. These findings clearly indicate that the cAMP stimulation induced by AA treatment is completely prostaglandin-mediated. Prostaglandins stimulate adenylate cyclase, which subsequently raises the intracellular level of cAMP, and chloride transport is stimulated concomitantly. In addition, these findings point to the existence of a cAMP-independent mechanism for the AA stimulation of chloride transport. Since indomethacin completely inhibits cAMP, yet the AA stimulation of chloride transport is only 50% inhibited, other factors in addition to cAMP must be involved.

We have found that AA incorporation into cell phospholipids is significant after only 2 min of exposure to exogenous AA. It is a possibility that the physical change in the membrane that results from AA incorporation can affect chloride transport directly. Since SCC is stimulated within the same rapid time frame following AA addition, changes in membrane phospholipid acyl chains may induce a change in SCC via a cAMP-independent mechanism. Perhaps the alteration in membrane lipid composition may directly affect the activity of membrane-bound proteins involved with ion transport.

It is possible that the cAMP-independent component of the SCC response to AA may be calcium-mediated. It has been shown that AA and its lipoxygenase pathway products can act as a calcium ionophore (Serhan, Korchak & Weissman, 1980). It has been shown that the ionophore A23187 pro-

duces an immediate stimulation of SCC in frog cornea, yielding an SCC trace similar to that produced by AA (Zadunaisky, Welch & Garretson, 1976; Candia, Montoreano & Podos, 1977). Similarly, AA-induced changes in Ca^{++} flux may stimulate SCC in frog cornea.

That our data show an inhibition of the SCC response to AA in the presence of phentolamine is interesting in this regard. Many alpha-adrenergic responses appear to involve changes in cell calcium (Assimacopoulos-Jeannet, Blackmore & Exton, 1977; Exton, 1980). Since phentolamine, a specific alpha-adrenergic antagonist, partially inhibits the SCC response to AA (Table 2), it is possible that there may be an alpha component to the response.

It has been postulated that a decrease in membrane viscosity should increase the diffusion rate of membrane-bound receptors and enzymes, increasing their rate of interaction, ultimately increasing their activity. We have previously found evidence of the increased rate of diffusion of membrane proteins with unsaturated fatty acids by means of fluorescent antibody patching (Schaeffer & Curtis, 1977). While the present data indicate that AA stimulates cAMP synthesis, the fact that this stimulation can be completely inhibited with indomethacin, shows that the increase in cAMP content is totally prostaglandin-mediated, and is not related to concomitant changes in membrane viscosity. The data therefore do not appear to support the mobile receptor hypothesis (Cuatrecasas, 1974; Rimon, Hanski, Braun & Levitzki, 1978) at least with regard to adenylate cyclase activity. We cannot presently rule out the possibility that other membrane-bound enzymes may be activated. Others have shown, for example, that membrane-bound guanylate cyclase is activated by unsaturated fatty acids, including AA (Wallach & Pastan, 1976; Glass, Frey, Carr & Goldberg, 1977). It is possible that cytoskeletal constraints may prevent adenylate cyclase from diffusing freely, and therefore changes in membrane viscosity would not have an effect on enzyme activity.

Our data indicate that cAMP synthesis is activated by PGE_2 and that this activation is somehow blocked by propranolol. The mechanism of propranolol inhibition here is not clear. However, since propranolol specifically binds the beta-receptor, one possible explanation might involve receptor binding to adenylate cyclase. It has been shown that the beta-receptor and adenylate cyclase normally exist in the membrane as an inactive complex, associated with, and constrained by, cytoskeletal elements (Cherksey, Zadunaisky & Murphy, 1980). According to this hypothesis, agonist binding would destabilize this complex, release the receptor, and thus allow adenylate cyclase to be activated. On the other hand, antagonists such as propranolol, would bind the receptor and stabilize the inactive complex. While in the inactive complex, adenylate cyclase would not be susceptible to PGE_2 activation, and would remain inactive in spite of PGE_2 synthesis within the cell.

In addition, the well-known nonspecific membrane stabilizing (anaesthetic) effect of propranolol might also explain the propranolol inhibition. A more stable membrane could result in the attenuation of adenylate cyclase activity. It is well known that adenylate cyclase activity requires a "fluid" lipid membrane. Membrane stabilization may prevent conformational changes in the enzyme which are necessary for activation. This explanation is consistent with our finding that propranolol alone reduces the basal adenylate cyclase activity.

It has generally been assumed that in order to substitute fatty acids into phospholipids in cell cultures, elaborate procedures are required (Wisnieski, Williams & Fox, 1973). We have found that merely by adding the selected fatty acid to the complete medium, significant incorporation results. In addition to our findings of a very rapid substitution of AA into corneal epithelium and L929 cell phospholipids, our analysis of longer term supplementations shows that AA continues to incorporate into phospholipids and that ultimately it becomes the predominant phospholipid fatty acid. At the end of 24 hr AA % composition reaches its maximum at 40% in L929 cell phospholipids. In corneal epithelium, however, AA substitution appears to proceed much more rapidly and this level of substitution is reached after only 1 hr of incubation.

There is evidence in the literature that various cells types are able to regulate phospholipid fatty acid composition so that membrane viscosity remains constant (Huang, Lorch, Smith & Haug, 1974; Sinensky, 1974; Miller, Hill & Smith, 1976). Although this homeoviscous response is usually thought of as an adaptation mainly of poikilotherms to changes in environmental temperature, its counterpart in mammalian cells may be the effort of such cells to maintain a relatively constant ratio of unsaturated to saturated fatty acids. Previous studies (Ferguson, Glaser, Boyer & Vagelos, 1975) demonstrate that when the growth temperature of mammalian cells in culture is lowered to 28 °C, the unsaturated fatty acid content of phospholipids increases, thus preserving membrane viscosity levels. Some evidence has also been found that supplementation of cells with long-chain un-

saturated fatty acids such as AA alters the ratio of endogenously synthesized fatty acids. Thus, the stearic acid (18:0) content increases after 24 hr, while oleic acid (18:1) decreases (Ferguson et al., 1975; Doi et al., 1978; Mandel, Shimizu, Gill & Clark, 1978). These studies were done on cells grown in serum-free, chemically defined medium. However, our long-term supplementation data for cells grown in the presence of fetal calf serum support these findings. We find that by 15 hr of incubation in AA, oleic acid content begins to decrease. We don't observe a significant increase in stearic acid until 48 hr in our system. By 48 hr, the ratio of unsaturated to saturated fatty acid has nearly returned to control levels in spite of a persisting AA content of 40%. This represents an extraordinary cellular effort to minimize the membrane-perturbing effect of AA and to return membrane viscosity to normal levels. Further studies are underway to determine the effects of long-term membrane perturbations and the homeoviscous response on chloride transport as well as cAMP levels.

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