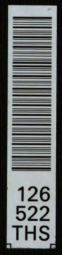
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THE EFFECTS OF CYCLIC NUCLEOTIDE MODULATORS ON NEUTROPHIL SHAPE CHANGE

By

Stephen L. Spielbauer

A THESIS

Submitted to
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ABSTRACT

THE EFFECTS OF CYCLIC NUCLEOTIDE MODULATORS ON NEUTROPHIL SHAPE CHANGE

By

Stephen L. Spielbauer

Shape change is an early part of the neutrophil chemotactic response. Chemotaxis and shape change are affected by cyclic nucleotide levels which are subject to pharmacological modification. The objective of this study was to investigate the effects of various agents associated with altered cyclic nucleotide levels on shape change in isolated neutrophils. N-formyl-methionyl-leucyl-phenylalanine or arachidonic acid were used to induce shape change. Isoproterenol and theophylline, which are agents known to increase adenosine 3`,5`-monophosphate (cAMP) were used to inhibit shape change. Guanosine 3`,5`-monophosphate (cGMP) and agents which increase cGMP like serotonin, phenylephrine and carbachol were evaluated as shape change enhancers. Shape change was evaluated by scoring wet preparations of fixed cell suspensions for altered neutrophil shapes. Agents which elevate cAMP inhibited shape change. No agent tested enhanced shape change at 2 minutes. Enhancement was not tested for at later times.

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LIST OF ABBREVIATIONS

ascorbic acid	L-ascorbate
cAMP	adenosine 3`,5`-monophosphate or cyclic-AMP
cGMP	guanosine 3`,5`-monophosphate
carbachol	carbamylcholine
EGTA	ethyleneglycol-bis-glycol-tetraacetic acid
f actin	actin microfilaments
FMLP	N-formly-methionly-leucyl-phenylalanine
FMP	N-formly-methionly-phenylalanine
g actin	actin monomers
Iso	isoproterenol
MLCK	myosin light chain kinase
MT	microtubules
PBS	phosphate buffered saline
serotonin	5-hydroxytryptamine
Theo	theophylline
Untreat	untreated
uropod	tail like knob
volex	6 percent hetastarch
W-7	N-(6-aminohexyl)-5-chloro-1-napthaline sulfonamide

INTRODUCTION

Neutrophil function can be tested in a variety of ways. The traditional chemotaxis assay simulates the *in vivo* neutrophil response. In this test isolated neutrophils move through a filter towards a chemoattractant. The chemotactic response is related to the distance the cells travel in the filter. This process is complex which sometimes makes interpretation of the results difficult. Simpler tests like shape change assays, which focus on a single aspect of neutrophil function, have been developed to eliminate some of the complexity of the chemotactic process.

Increased adherence and shape change are early overlapping events which are part of the chemotactic process. Under normal circumstances adherence changes begin before the cells change shape. However, if the experiments are conducted quickly using dilute suspensions of cells, surface contact and adherence effects can be minimized. Relatively low sustained concentrations of chemoattractants transform the resting cells from a round to a long polarized form without initiating other processes important to chemotaxis. Shape change, which can occur and be reversed in 1 to 2 minutes, can also be tested in whole blood samples. Although this simplifies the procedure, chemoattractant-

albumin binding and activation of platelets or complement complicate interpretation of the results.

Cyclic nucleotides can affect monocyte or neutrophil chemotaxis and monocyte shape change. Neutrophil shape change is probably also influenced by altering cyclic nucleotide levels. These altered levels are obtained by treating the cells with various agents. This study was designed to see if neutrophil shape change would be influenced by these agents and to compare these results with the corresponding results obtained for monocytes by others (8).

LITERATURE REVIEW

Shape Change

Shape change in neutrophils has been recognized for some time (2). Circulating neutrophils were seen as spherical and as these cells encountered some stimulating factor, they adhered to the vessel walls. If the stimulant persisted, the cells changed shape and moved to an interface between endothelial cells lining the vessel. They then migrated into the tissues via the process of diapedesis (3,4).

Initial studies on neutrophil shape change were performed on protein or gelatin coated surfaces. It was later shown that neutrophil shape change also occurred in suspension, free of surface contact (5,6). Shape change in suspension has been performed with isolated neutrophils, whole blood neutrophils and monocytes (5,7,8). Stimulated neutrophils changed through a continuum of shapes from round to elongated as shown in Figure 1. These morphologic alterations result from several overlapping mechanisms which are divided into four topics (1) the initiation of shape change, (2) calcium flux as a positive effector of shape change, (3) adenosine 3`,5`-monophosphate (cyclic-AMP or cAMP) flux as a negative effector of shape change and (4) the resulting cytoplasmic movement. The following discussion will cover these four topics including specific information about neutrophils.

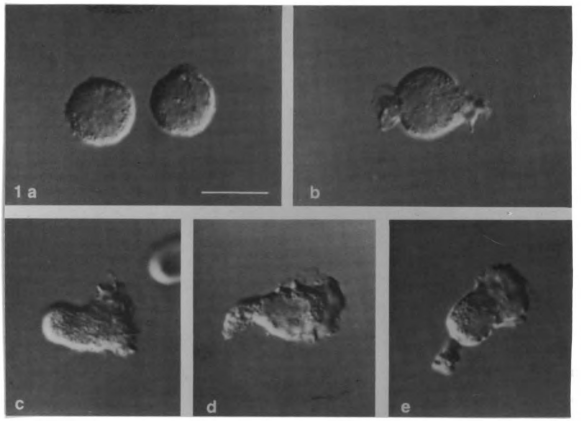

Figure 1

Figure 1. Change in Shape of Neutrophils (x1600)

Change in shape of neutrophils (x1600) exposed to a chemotactic factor for 2 minutes. 1a represents round cells b is ruffled c is a bipolar cell d and e are uropod cells (5).

1 Initiation of Shape Change

Neutrophils have several non-hormonal receptors including one for N-formyl-methionyl-phenylalanine (FMP) (8). When appropriate levels of FMP were added to the cell suspensions, receptor filling and shape change occurred in 2 minutes or less for isolated neutrophils. Whole blood neutrophils by comparison, showed a slow shape change response which could result from plasma proteins binding FMP. In both environments the shape change response was maintained through 45 minutes if FMP remained with the cells. If FMP were removed from the receptors on isolated neutrophils by dilution or addition of a competitive inhibitor, shape change reversed in about 30 seconds (Smith, Unpublished). N-formyl-methionyl-leucyl-phenylalanine (FMLP) has properties like FMP. Pure FMLP became available after FMP and, since it is a better agonist, was used as its replacement in later experiments (8). Both FMLP or FMP bound to neutrophil receptors and induced shape change. These filled receptors altered the neutrophil membrane and eventually stimulated calcium influx into the cells by the processes described below. Shape change has also been initiated by methods which bypass the filling of membrane receptors.

2 Calcium Flux

When cells are activated calcium enters from outside the cell or from "storage pools" within the cell.

Mitochondria, endoplasmic reticulum and the inner surface of the cell membrane are possible storage pools (9). Calcium bound to the inner surface of the cell membrane may be released by chemical disturbances such as the filling of chemotactic peptide or immune complex receptors. Extrinsic calcium enters the cytosol with the aid of a lipid or phospholipid ionophore.

Two membrane processes have been investigated for their role in the transport of calcium across cell membranes. Both begin with receptor filling and a change in membrane structure. Subsequent steps differ but both processes eventually release arachidonic acid. This release is inhibited by cAMP in platelets (10). From this point on these membrane processes are the same. The common second phase of these processes begins as cyclooxygenase or lipoxygenase, in conjunction with other enzymes, convert arachidonic acid into various prostaglandins, thromboxanes, leukotrienes and hydrotetraenoic acids. Leukotriene B_4 and thromboxane A_2 have been suggested as calcium ionophores (11). Arachidonic acid and some of its products may simply enhance calcium activity (12).

In the first of these membrane processes a chemical cycle begins as phosphatidylinositol, incorporated in the membrane, is converted to phosphatidylinositol 4,5-diphosphate. This is split into diacylglycerol and inositol trisphosphate. Phosphatidic acid, a product of

diacylglycerol, combines with inositol-1-phosphate from several sources to produce phosphatidylinositol in the membrane and complete the cycle. Phospholipase A_2 and possibly phospholipase C are able to convert diacylglycerol, an intermediates from this cycle, to arachidonic acid (13). The common second phase, discussed above, leads to ionophore production. Inositol triphosphate and phosphatidic acid, may also be ionophores (14,15).

The second membrane process suggests that membrane stimulation induces multiple methylations of phosphatidylethanolamine in the inner membrane and a concomitant transfer to the outside of the product, phosphatidylcholine. This "transmethylation" results in local changes in membrane viscosity and permeability. Phospholipase A_2, which is calcium dependent, is then exposed to increased calcium in these local fluid areas. This exposure produces arachidonic acid from phosphatidylcholine. As before, the common second phase produces ionophores from arachidonic acid production (16,17). It has been reported that both phosphatidylinositol and transmethylation pathways are functional in human neutrophils but that transmethylation is not the major source of arachidonic acid (15,13).

Calcium flux can be regulated in ways that bypass the initial membrane changes discussed above by directly controlling phospholipase A_2 activity. Mellitin and phorbol

esters are phospholipase A_2 activators, while mepacrine and tetracaine are inhibitors (16). In rat leukocytes and rabbit neutrophils, an endogenous inhibitor of phospholipase A_2 is produced by the glucocorticoids hydrocortisone, prednisolone and dexamethasone. This endogenous inhibitor is transcribed and translated from the cell`s DNA which explains the time delay associated with the action of these glucocorticoids (18). This is a common mode of action for steroid hormomes (19). In the presence of resting levels of calcium diacylglycerol, a precursor to arachidonic acid, activates protein kinase C. This kinase mimics the calcium-calmodulin complex and also induces the cell to change shape (14,12). Granulocytes have an active transport system for removing calcium from the cytosol (20).

The release of storage pool calcium is important to neutrophil shape change because extracellular calcium is not needed for neutrophil shape change in response to FMP or FMLP (5)(Hollers unpublished data). This could mean that the increase in intracellular calcium needed for shape change is small and may not require ionophore production. Membrane changes that release calcium from their inner surface could be sufficient.

Calmodulin

Many processes are regulated by changing calcium concentration. Calmodulin promotes the activity of calcium by acting as an intracellular receptor for calcium. For

example, calcium or calmodulin alone had little effect on in vitro microtubule assembly. However, when calcium and calmodulin were combined, microtubule assembly was prevented (21). The cell`s calmodulin content remains relatively constant. Changing calcium concentration and binding regulates these cellular mechanisms (22). Calmodulin is often associated with membranes and contractile filaments. It binds 4 calcium ions per molecule and is a common "conserved" protein found in plants and animals. As binding between calcium and calmodulin occurs there is a conformational change and the active site of the molecule is exposed. Phenothiazine, an antipsychotic, and the N-(6-aminohexy)-5-chloro-1-napthaline sulfonamide (W-7) group of compounds are selective inhibitors of calmodulin that compete for the active site (23). They are inhibitors of the respiratory burst and degranulation in neutrophils (23,24,25,26). Calcium-calmodulin is involved in many cellular mechanisms such as prostaglandin metabolism, mitosis, guanosine 3`,5`-monophoshpate (cGMP) flux, cAMP flux, actin-myosin interaction, actin and microtubule structure and calcium flux across the cell membrane (19,27,28,29). The calcium-calmodulin complex regulates several of the enzymes involved in controlling cAMP and cGMP levels and serves as part of the link between calcium and the cyclic nucleotides.

Changing calcium concentrations from 10^{-7} to 10^{-4} M in neutrophils induces many of the responses associated with chemotaxis and shape change. Calmodulin, as a receptor, is responsible for making calcium so versatile.

3 Cyclic AMP Flux

It has been shown that increased levels of cAMP have an inhibitory effect on neutrophils, platelets and monocytes (1,19,29,30,31). Cyclic AMP has a high energy bond and is stable in the cytosol. Adenylate cyclase produces cAMP while phosphodiesterases hydrolyze cAMP to AMP. The neutrophil adenylate cyclase complex is activated by beta agonists like isoproterenol. Phosphodiesterase activity is blocked by theophylline or caffeine resulting in elevated cAMP (29,30,31,32,33,34). Phosphodiesterase activity is promoted by calcium-calmodulin resulting in lower cAMP levels (10).

4 Cytoplasmic Movement

The three structural proteins involved in physical neutrophil shape change are actin, myosin and tubulin. These monomers assemble to form large "active" structures. Actin forms crosslinked microfilaments, which are responsible for transient bulging structures near the cell surface. Myosin forms bundles which apply traction to microfilaments. Tubulin forms microtubules, which give structure to the cell core and may transport lysosomes toward the outside. The monomers are in equilibrium with the respective large "active" structures. These events will be discussed.

Actin Microfilaments

Actin monomers (g-actin) form actin microfilaments (f-actin). If these microfilaments become long and abundant, they will be crosslinked by actin-binding protein. This crosslinked mesh forms a solid bulge. The transition from g-actin and short microfilaments to long microfilaments is controlled by acumentin, profilin and gelsolin, the last of which is calcium dependent. Acumentin, independent of calcium, blocks the slow growing end of a microfilament and gelsolin blocks the fast growing end but only if the calcium concentration is greater than 10^{-7} M. Profilin sequesters g-actin. If calcium is less than 10^{-7} M, gelsolin frees the fast growing end of the microfilaments which lengthen and are crosslinked (14). Mesh formation depends on low calcium levels. Other factors may be involved but are not understood.

The crosslinked mesh forms near the plasma membrane of the neutrophil and excludes cell organelles (35). As neutrophils change shape, the uniform layer of actin becomes structured in 2 areas inducing a tail-like knob (uropod) and a broad ruffled structure, see Figure 1 (e). If local membrane calcium pumps become activated, intracellular calcium levels drop and actin mesh formation is enhanced (20). It is the actin mesh that is primarily responsible for shape change.

Myosin

Myosin is the energy transducing part of the system. It compresses and moves parts of the mesh along by pulling on actin microfilaments. Some myosin is trapped in the gel at formation and contributes to its compression when myosin is activated (36). Myosin bundles draw actin filaments from liquid areas of the cell to areas where the crosslinked mesh is forming (14).

The calcium-calmodulin complex promotes contraction of myosin by indirectly phosphorylating it with an active myosin light chain kinase (MLCK). In the presence of 10^{-7} to 10^{-5} M calcium, the calcium-calmodulin complex binds and activates MLCK. Active MLCK induces myosin contraction and movement. Cyclic AMP-dependent protein kinase and cAMP inactivate MLCK through phosphorylation. In this state MLCK cannot complex with calcium-calmodulin. These events are in equilibrium. If high calcium levels exist, myosin contraction will occur. However, cAMP can reverse the contraction in the presence of high calcium levels by shifting the equilibrium (37). Details of this relationship have been worked out in platelets (38,39).

Recent information indicates other factors released directly by membrane activation are involved here. Protein kinase C, activated by diacylglycerol in the presence of less than 10^{-7} M calcium, can also phosphorylate the light chain of myosin. Arachidonic acid and other related lipids

are able to enhance the binding of calcium-calmodulin to MLCK. Some of the suspected calcium ionophores may be simply enhancers of calcium activity (12).

Calcium-calmodulin and cAMP are involved in control of neutrophil movement. In neutrophils ingesting zymosan particles, cAMP and a cAMP-dependent protein kinase are present in the area of the pseudopods (40). This area was observed by others to contain actin mesh and myosin (36). In resting neutrophils, no concentrated areas of cAMP or cAMP-dependent kinase were seen (40). This indicates that in neutrophils with active pseudopods, cAMP and the kinase are exerting some control over myosin activation. Myosin bundles could be collecting actin filaments from areas of higher calcium to the base of the low calcium actin bulge where they will be crosslinked into the bulge structure (20). The degree of myosin activation involved in shape change is unknown.

Microtubules

Microtubules (MT) are a structural component of neutrophils and play some role in shape change, secretion of granules and phagolysosome formation. In resting neutrophils fewer MT are seen than in neutrophils that have changed shape. Microtubules assemble at one end and are disassembled at the other. The rate of the turnover increases with increased calcium. The core of an activated neutrophil contains MT while the outer actin bulges do not (41,42).

This segregation of assembled components could be due to differing calcium concentrations.

Colchicine, a plant alkaloid, blocks the assembly of MT. If colchicine is added to round neutrophils they change shape as areas of actin mesh appear and the MT are reduced. Treatment with colchicine also inhibits granule secretion (42). The details of MT function are not clear.

Microtubules, crosslinked microfilaments and myosin each play some role in the process of cytoplasmic movement that results in shape change. Microfilaments seem to be the most visible component in the early stages of this process. Many questions remain unanswered.

In summary, neutrophil shape change is generally initiated by filling some surface receptor which induces calcium concentrations to increase to 10^{-5}M. Calcium binds to calmodulin which allows actin, myosin and tubulin to assemble more completely and change the shape of the neutrophil. This process can be altered at several points by beta agonists, lipids, drugs, ions and other agents.

METHODS AND RESULTS

Neutrophil Isolation

Sterile Dulbecco's phosphate buffered saline (PBS), containing 0.1 % glucose, was prepared in the laboratory. Ficoll and hypaque, obtained from Sterling-Winthrop Labs, New York, NY 10016, were used to prepare a ficoll-hypaque cushion (43). Six percent hetastarch (volex) was used to enhance sedimentation, McGaw Laboratories, Ann Arbor, MI 48108. All reagents used were brought to room temperature. Five milliliters of citrated blood, diluted with 5 ml of PBS in disposable glass tubes, were layered over 4 ml of Ficoll-Hypaque and centrifuged in a 20 C centrifuge at 400 G for 20 minutes. The cells, suspended in Ficoll-Hypaque, were transferred to a serum protein-coated tube and diluted to twice the original volume with PBS. A total of 1.7 ml of volex was added. The tubes were undisturbed for 30 minutes to permit cells to sediment. The supernatant was removed to another serum-coated tube and washed with large amounts of PBS using a 15 minute 200 G spin for each wash. The cells were suspended in 1 ml of PBS and counted. The concentration of neutrophils was adjusted to 10^7 per ml with PBS and the cells stored at 5 C for up to 5 hours. This preparation resulted in a mixture of 5 % platelets, 62 % red blood cells and 33 % neutrophils. Cell viability was assessed during the experiments.

Serum Coated Tubes

Pre-cleaned disposable glass tubes were selected to avoid chemical contamination. Tubes were coated with a 1:1 dilution of human serum to prevent attachment of the cells to the glass surface and were kept moist until use.

Assessment of Shape Change

Suspensions of $5X10^5$ to $1X10^6$ neutrophils per ml were used in experiments to assess shape change. Shape change was preserved to allow measurement by cell fixation with 1 % glutaraldehyde. This was done by diluting the tube contents with cold 2 % glutaraldehyde and holding at 5 C for 30 minutes. One hundred neutrophils from each suspension were classified according to cell shape by observing fixed wet mounts at a magnification of 1000X using a phase contrast microscope. For analysis the shape change spectrum was divided into 4 cell shapes labeled round, ruffled, bipolar and uropod (Figure 1). The percentage of neutrophils in the bipolar and uropod categories were added together. This result was labeled % shape change.

For example:

round	ruffled	bipolar		uropod		% Shape Change
5%	23%	42%	+	30%	=	72%

Unpublished data showed that if neutrophils in suspension and in a non-round shape were killed before fixation, they would quickly become round. All neutrophils classed as ruffled, bipolar and uropod were viable during the test;

hence, in the above example, 95 % were viable. To induce shape change means to produce or bring forth some % shape change, for example 72 %. To enhance shape change means to add to some baseline % shape change, for example > 72 %. To inhibit shape change means to restrict the baseline % shape change to some lower value, for example < 72 %.

N-formyl-methionyl-leucyl-phenylalanine (FMLP) or arachidonic acid (Sigma Chemical Company, St. Louis, MO 63178) were used to induce shape change. Theophylline (ICN Biochemicals Cleveland, OH 48812) and isoproterenol were used to inhibit shape change. Phenylephrine, 5-hydroxytryptamine (serotonin), cGMP, L-ascorbate (ascorbic acid), carbamycholine (carbachol), acetylcholine, succinyl choline and D-tubocuraine (Sigma Chemical) were agents thought to enhance shape change.

Statistical Procedure

Most of the experiments used a randomized block design with 2 or 4 blocks (r=number of blocks). The first and second experiments were factorial in design which allows testing for an interaction between factors. The factors were theophylline and isoproterenol. In the other experiments, treatments consisting of inhibitors or enhancers of shape change were evaluated. Analysis of variance testing for significant differences was done by using the F-test at the 95 % level of significance. In some instances where the null hypothesis was rejected, Tukey`s method for multiple

comparisons was used to determine which means were different from the others. The original data, being expressed as a percentage, were put through an arcsin square root of the percentage transformation to eliminate skewed distributions near 0 or 100 %. The experiments in which serotonin was the enhancer were analyzed using multivariate analysis of variance from the statistical package for the social sciences. Other analysis of variance was calculated using the Apple version of VisiCalc (44,45,46,47).

FMLP Dose For Optimal Inhibition

Neutrophils were treated with isoproterenol ($1X10^{-4}$ M), theophylline ($1X10^{-3}$ M), the combination of theophylline at $1X10^{-3}$ M plus isoproterenol at $1X10^{-4}$ M (T+I) or were untreated for 2 minutes. The neutrophils were then combined with FMLP concentrations ranging from $1X10^{-10}$ M to $5X10^{-9}$ M in the presence of isoproterenol, theophylline, (T+I) or PBS (untreated) for 5 minutes. Percent shape change was measured and recorded. See Table 1. Instances where neutrophils were combined with $1X10^{-9}$ M FMLP have r=4. Other instances have r=2. All following experiments have r=4 and were performed at 37 C unless otherwise indicated.

Results

Isoproterenol caused no significant inhibition. Significant inhibition of shape change by theophylline occurred at several FMLP concentrations including the optimal concentration of $1X10^{-9}$M FMLP.

Table 1 FMLP Dose for Optimal Inhibition[a]

(FMLP)	0	$1X10^{-10}$	$3X10^{-10}$	$6X10^{-10}$	$1X10^{-9}$	$5X10^{-9}$
	----------% Shape Change----------					
(Untreat)	4	9	22	55	77	96
(Theo)	3	2	5	31[b]	54[b]	94
(T+I)	2	2	14	34[c]	44[c]	88[c]
(Iso)	4	8	25	52	78	98

[a]N-formyl-methionyl-leucyl-phenyalanine (FMLP) Dose for Maximum Inhibition Different doses of FMLP are used to induce the % shape change seen in the table. Theophylline and Isoproterenol were tested as inhibitors of % shape change at different doses of FMLP.

(FMLP) N-formyl-methionyl-leucyl-phenyalanine at various molar concentrations

(Untreat) Untreated with an inhibitor

(Theo) Theophylline at $1X10^{-3}$ M

(T+I) The combination of theophylline plus isoproterenol at the concentrations used when these inhibitors were used alone.

(Iso) Isoproterenol at $1X10^{-4}$ M

[b]In these instances the % shape change was significantly different from the corresponding untreated % shape change. Significant differences indicate differences at the 95 % level of significance.

[c]In these instances the % shape change was significantly different from the corresponding untreated % shape change. Isoproterenol did not interact with theophylline.

Secondary Inhibition of Shape Change Over Time

Neutrophil percent shape change levels were known to be relatively stable for about 30 minutes or more for FMLP and FMP (Hollers, unpublished data). In this experiment neutrophils were combined with $1X10^{-9}$ M FMLP for 1 minute and 50 seconds and then treated with isoproterenol ($1X10^{-4}$ M), theophylline ($1X10^{-3}$ M), (T+I) or were untreated. Portions for % shape change evaluation were taken at 0, 1, 2, 3, 4, 5, 7, 10, 15 and 20 minutes. See Figure 2.

Results

Theophylline was able to significantly inhibit the shape change induced by FMLP from 3 through 20 minutes. Isoproterenol caused a significant interaction with theophylline at 4 minutes and caused significant inhibition when present alone at 4 minutes.

Drug Prior to FMLP Inhibition and Inhibition of Arachidonic Acid Shape Change Over Time

Time studies were done on neutrophils which were treated with theophylline ($1X10^{-3}$ M), (T+I) or were untreated for 2 minutes before combination with FMLP ($1X10^{-9}$ M) or arachidonic acid ($5X10^{-7}$ M) at 0 time. Portions were taken at -10 seconds, 1, 2, 3, 4 and 5 minutes. This was done to compare arachidonic acid with FMLP as a shape change inducing agent. See Table 2 and Figure 3.

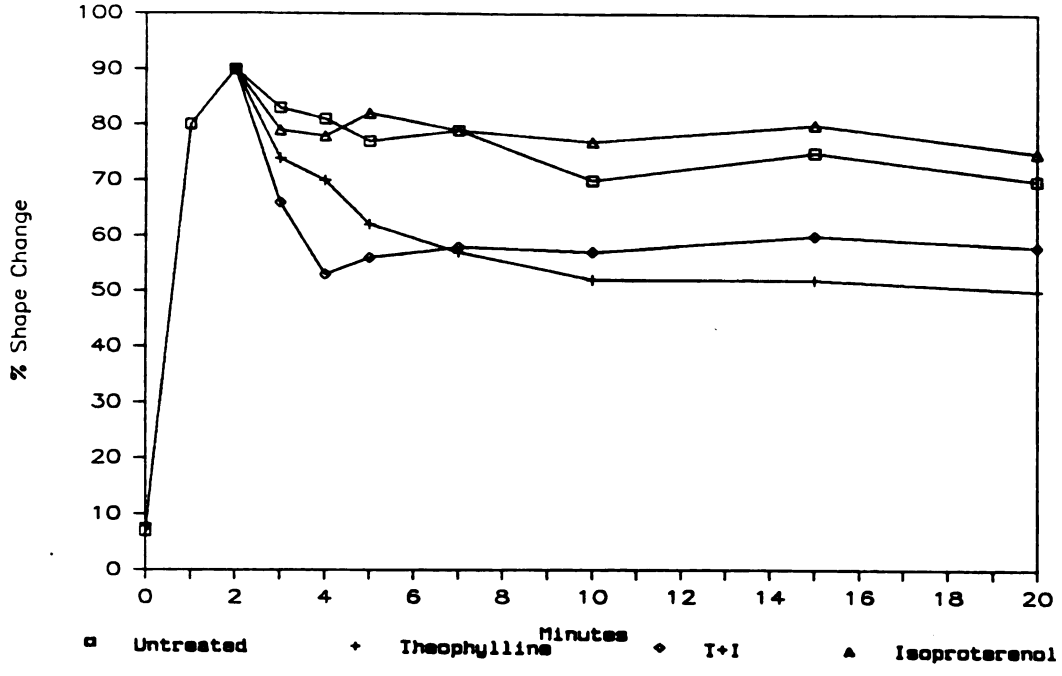

Figure 2

Figure 2. Secondary Inhibition of Shape Change Over Time.

Shape change caused by $1X10^{-9}$ M N-formyl-methionyl-leucyl-phenyalanine (FMLP) was tested for inhibition by theophylline, isoproterenol, and theophylline plus isoproterenol (T+I) 2 minutes after shape change began. The average of 4 values as % shape change were plotted against time through 20 minutes.

Theophylline significantly inhibited the shape change induced by FMLP from 3 through 20 minutes. Isoproterenol caused a significant interaction with theophylline at 4 minutes and caused significant inhibition when present alone at 4 minutes. Significant differences indicate differences at the 95 % level of significance.

Table 2 Drug Prior to FMLP Inhibition[a]

(Time)	0	1	2	3	4	5
	----------% Shape Change----------					
(Untreat)	3	72	83	74	66	67
(Theo)	3[b]	59	71	59[b]	55	48
(T+I)	3[c]	54[c,d]	57[c,d]	51[c,d]	47[d]	42[c,d]

[a]Drug Prior to N-formyl-methionyl-leucyl-phenyalanine (FMLP) Inhibition The inhibitors theophylline or theophylline plus isoproterenol were introduced prior to induction of shape change by FMLP. Percent shape change was recorded through 5 minutes after the addition of FMLP.

(Time) Time in minutes

(Untreat) Untreated with an inhibitor

(Theo) Theophylline at $1X10^{-3}$ M

(T+I) The combination of isoproterenol at $1X10^{-4}$ M plus theophylline at the concentration used above.

[b]In these instances the % shape change was not significantly different from the corresponding untreated % shape change. Significant differences indicate differences at the 95 % level of significance.

[c]In these instances the % shape change was not significantly different from the corresponding theophylline % shape change.

[d]In these instances the % shape change was significantly different from the corresponding untreated % shape change.

Figure 3. Inhibition of Arachidonic Acid Shape Change.

The first 5 minutes

Neutrophils were exposed to $5X10^{-7}$ M arachidonic acid 2 minutes after treatment with the inhibitors theophylline or theophylline plus isoproterenol (T+I). The untreated neutrophils were not combined with an inhibitor before exposure to arachidonic acid. The average of 4 values as % shape change were plotted against time through 5 minutes.

Theophylline caused significant inhibition only at 3 minutes. The combination (T+I) caused significant inhibition from 1 to 4 minutes. Theophylline was significantly different from (T+I) at 2 minutes. Untreated shape change and inhibition were maximal at 2 to 3 minutes and decreased by 5 minutes. Significant differences indicate differences at the 95 % level of significance.

The second 5 minutes

At 5 minutes a second dose of arachidonic acid was added to neutrophils inhibited with theophylline or (T+I). A portion of the untreated neutrophils were exposed to a second dose of arachidonic acid at 5 minutes and are labeled double untreated. The remaining untreated neutrophils were not altered by a second exposure to arachidonic acid and are labeled untreated. The average of 4 values as % shape change were plotted against time from 5 through 10 minutes.

At 6, 7 and 8 minutes, double untreated neutrophils were significantly different from untreated neutrophils. Treated neutrophils did not differ significantly from the untreated neutrophils. The inhibiting drugs were more effective during the second dose of arachidonic acid. The double untreated % shape change response was transient, like the untreated response in the first 5 minutes.

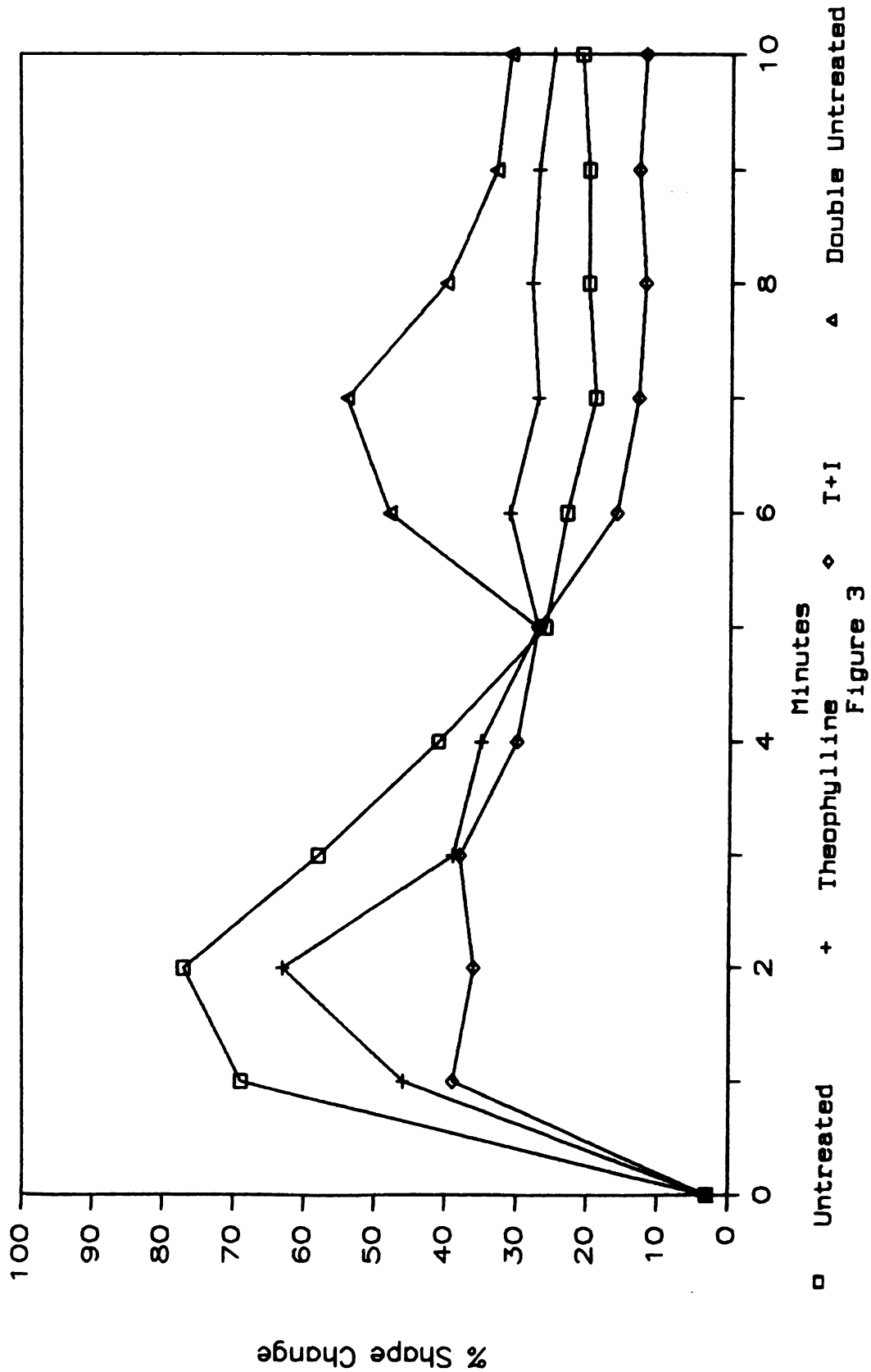
% Shape Change
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
Minutes
□ Untreated
+ Theophylline
◇ T+I
△ Double Untreated

Figure 3

Results

When shape change was induced by FMLP, both theophylline and (T+I) generally caused significant inhibition at all times. When arachidonic acid induced shape change, theophylline caused significant inhibition only at 3 minutes. The combination of theophylline plus isoproterenol caused significant inhibition from 1 to 4 minutes. Theophylline inhibition was significantly different from (T+I) inhibition at 2 minutes. When arachidonic acid induced shape change, both induction and inhibition were maximal at 2 to 3 minutes and decreased by 5 minutes.

Inhibition of Subsequent Arachidonic Acid Dose

This experiment was designed to determine whether neutrophils combined with arachidonic acid for 5 minutes, as described above, were completely refractory to a second treatment with arachidonic acid. This was done by adding more arachidonic acid at the 5 minute time to neutrophils that had encountered arachidonic acid in the previous experiment. The concentration of arachidonic acid was $5X10^{-7}$ M at 0 time and was increased to $1.9X10^{-6}$ M at 5 minutes. This was based on the assumption that the arachidonic acid concentration did not change from 0 to 5 minutes. No attempt was made to quantitate arachidonic acid. Untreated neutrophils that had been exposed to one dose of arachidonic acid in the first 5 minutes were divided into 2 untreated groups during the second 5 minutes. One group (double

untreated) was given a second dose of arachidonic acid. The other group (untreated) was not given a second arachidonic acid dose and served as a baseline for comparison. Groups treated with inhibiting drugs also received a second dose of arachidonic acid at 5 minutes. Portions taken in the second 5 minutes were labeled with respect to 0 time and were taken at 6, 7, 8, 9 and 10 minutes. See Figure 3.

Results

At 6, 7 and 8 minutes, the double untreated neutrophils were significantly different from the untreated neutrophils. The treated neutrophils did not differ significantly from the untreated neutrophils. When treated with inhibiting drugs, the neutrophils failed to change shape when combined with the second dose of arachidonic acid. The second shape change response induced by arachidonic acid was transient.

Serotonin Enhancement of FMLP Shape Change and Serotonin Enhancement of Arachidonic Acid Shape Change

Neutrophils were treated with PBS (untreated) or with serotonin at a final concentration of $1X10^{-5}$ M for 2 minutes. Concentrations of FMLP ranging from 0 to $5X10^{-9}$ M were then combined with these neutrophils for 2 minutes before measurements were taken. This was repeated substituting arachidonic acid ranging from 0 to $1X10^{-6}$ M for 1 minute before measurement, in place of the range of FMLP concentrations.

Results

These data were submitted to a multivariate anaylsis of variance which indicated that serotonin treated and untreated neutrophils were not significantly different.

FMLP Shape Change Enhancement Screen

A standard level of % shape change was established by combining neutrophils with $5X10^{-10}$ M FMLP for 2 minutes before measurement. Neutrophils were treated with a drug for 3 minutes before FMLP was added. Each drug was tested at 3 different concentrations within a 100-fold range. The drugs used were cGMP, succinyl choline, ascorbic acid, phenylephrine, acetylcholine, D-tubocuraine, carbachol and the combination D-tubocuraine plus acetylcholine. Some of these drugs were dissolved in DMSO. Dimethyl-sulfoxide controls were run with the tests. r=1 for this experiment.

Results

A baseline level of % shape change was established and % shape change resulting from enhancing drugs was compared to the baseline level. This was a one-time experiment in which FMLP was used to induce shape change. None of the drug treatments appeared to enhance shape change, but ascorbic acid did seem to inhibit it. These results were used to screen drugs for inclusion in the next experiment.

Enhancement of Arachidonic Acid Shape Change

The previous format was used to test cGMP, phenylephrine and carbachol as enhancers of % shape change

induced by $5X10^{-8}$ M arachidonic acid. Each drug was tested at 4 concentrations within a 1000-fold range. A baseline level of % shape change was established by combining neutrophils with arachidonic acid in the absence of enhancing drugs. The enhancing drug results were compared to the baseline. r=4 in this experiment.

Results

None of the drugs tested gave results that were significantly different from the baseline % shape change which was 60 %.

Calcium Requirement for Arachidonic Acid Shape Change Over Time

All previous experiments included calcium in the PBS. Some calcium-free PBS containing $1X10^{-6}$ M ethyleneglycol-bis-glycol-tetraacetic acid (EGTA) was prepared. The EGTA was added to bind small amounts of calcium that might be contaminating the calcium-free PBS. After the normal isolation process neutrophils were washed with and stored for 20 minutes in calcium-free (EGTA) PBS. Arachidonic acid dissolved in calcium-free (EGTA) PBS at a final concentration of $1X10^{-7}$ M was combined with neutrophils. Untreated portions used regular PBS. Portions were taken at 0, 1, 2, 3, 4 and 5 minutes. r=1 for this experiment.

Results

The results suggest that arachidonic acid induced shape change does not require calcium, but further testing is needed to confirm this.

DISCUSSION

Each cell preparation had to meet certain shape change guidelines before data could be accepted. First, the unstimulated % shape change had to be 8 % or less. Second, a maximum % shape change of greater than 80 % had to be obtained to insure viability of the cells. Several cell preparations failed to meet an acceptable maximum or minimum % shape change and were rejected.

In the first two experiments isoproterenol alone was an effective inhibitor at only one time. Theophylline was an effective inhibitor and showed an interaction with isoproterenol. The inhibition does not require preincubation with the inhibitor before FMLP is added. These results are consistent with the results from similar chemotactic and shape change studies of monocytes except that monocytes are more responsive to isoproterenol than neutrophils (1).

Arachidonic acid-induced shape change is brief compared to that induced by FMLP. Surface receptors and multiple activation pathways could explain the persistence of FMLP-induced shape change. Theophylline and (T+I) are effective inhibitors of arachidonic acid-induced shape change. The shape change burst can be repeated by increasing the concentration of arachidonic acid. It seems that, if the shape change mechanism and the inhibition mechanism have been primed by one pass, the inhibition is stronger during the second shape change burst. This may be due to a build-up

of cAMP. It appears that, as with FMLP, extracellular calcium is not needed (5). However, both arachidonic acid and FMLP-induced shape change may be inhibited by depleting the intracellular calcium pools (9). Monocyte shape change induced by FMLP is delayed by a calcium-free medium (1). This does not appear to be true for neutrophils.

Monocyte shape change can be induced by a variety of agents that are not effective for neutrophils under similar conditions. These agents are thought to increase calcium and cGMP in the cells. They will also enhance monocyte shape change or chemotaxis induced by FMLP (1). In neutrophils, under similar conditions, shape change enhancement was not seen; however, enhancement of neutrophil chemotaxis was seen. It is assumed that the agents used are able to alter the levels of the cyclic nucleotides as the literature indicates (29,30,31,48). In these experiments, that ability seems to be limited for neutrophils.

Monocytes, activated to similar levels of shape change, are more responsive then neutrophils to agents that alter cyclic nucleotides (1). Neutrophils did not respond to drugs that should increase the shape change level at 2 minutes. However these drugs are effective in neutrophil chemotaxis experiments which can last up to an hour (29,30,31,48). It is not clear why neutrophil shape change was not enhanced by serotonin, carbachol and phenylephrine. These results suggest that neutrophils are less responsive

than monocytes to agents that enhance or inhibit the *in vitro* cell response. It may be possible to alter the time of arrival and relative number of the different cell types at an inflammatory site by using these enhancers or inhibitors.

SUMMARY AND CONCLUSIONS

This study focused on neutrophil shape change and how it is affected by altering the levels of intracellular cAMP and cGMP. Theophylline increased cAMP and inhibited shape change induced by arachidonic acid or FMLP. Within certain time limits isoproterenol, when combined with theophylline, increased the inhibition. Serotonin, phenylephrine and carbachol have been shown to enhance neutrophil and monocyte chemotaxis and monocyte shape change (1,30,31,48). These enhancers are thought to increase cGMP. Serotonin, phenylephrine and carbachol did not enhance neutrophil shape change as expected. These results indicate that neutrophils are less sensitive to agents that elevate cGMP and cAMP than monocytes. It may be possible to use this difference in sensitivity to manipulate the inflammatory response.

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VITAE

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