# Effect of leukotriene $B_4$, prostaglandin $E_2$ and arachidonic acid on cytosolic-free calcium in human neutrophils

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Changes in cytosolic free calcium $[Ca^{2+}]_i$ and release of beta-glucuronidase in response to leukotriene $B_4$ ($LTB_4$) were measured in intact neutrophils loaded with the fluorescent $Ca^{2+}$ indicator, quin 2. $LTB_4$ ($10^{-10}$ M or higher) caused a rapid rise in $[Ca^{2+}]_i$ due to influx from the extracellular medium and release from intracellular pools as well as enzyme release. $PGE_2$ (3 $\mu$M) did not alter $[Ca^{2+}]_i$ whereas arachidonic acid (10 $\mu$M) raised $[Ca^{2+}]_i$. Pretreatment of cells with the chemotactic peptide FMLP inhibited the subsequent rise of $[Ca^{2+}]_i$ induced by $LTB_4$. Since chemotactic peptides activate the lipoxygenase pathway of arachidonic acid metabolism, it may be speculated that endogenous $LTB_4$ generation is involved in neutrophil activation.

*Leukotriene $B_4$* *Prostaglandin* *Cytosolic free calcium* *Human neutrophil* *Quin 2*

## 1. INTRODUCTION

The ability of lipoxygenase products of arachidonic acid to initiate or amplify the functional responsiveness of polymorphonuclear leukocytes (PMN) has been the subject of active investigation [1]. Among a number of compounds generated by this pathway, leukotriene $B_4$ ($LTB_4$) is the most potent on a molar basis. $LTB_4$ has been shown to be a potent chemotactic, aggregating and degranulating factor in human PMN at concentrations of the order of $10^{-8}$ M [1,2]. The mechanism by which $LTB_4$ stimulates neutrophils is still unclear though it has been suggested on the basis of in vitro experiments using arsenazo-loaded liposomes [3] that $LTB_4$ may increase cytosolic free, $[Ca^{2+}]_i$ by acting as a Ca ionophore. In the past, measurements of $[Ca^{2+}]_i$ in small cells has been extremely difficult. Recently this has become possible by the use of a new calcium indicator, quin2, which can be introduced into intact neutrophils by means of its intracellularly trappable non-polar ester quin2 AM [4]. Using this technique we have investigated the quantitative and causal relationship between $[Ca^{2+}]_i$ changes and cell function in neutrophils treated with the chemotactic peptide FMLP [5]. Recently also a preliminary report using quin2 as a qualitative probe of $[Ca^{2+}]_i$ has shown that a rise of $[Ca^{2+}]_i$ is a common event in neutrophil activation by a number of chemotactic substances including $LTB_4$ [6].

A quantitative analysis of the effects of selected compounds of the cyclooxygenase and lipooxygenase pathway on the levels of free cytosolic calcium in human PMN forms the basis of this report.

## 2. MATERIALS AND METHODS

Quin2 AM was purchased from Lancaster Synthesis Ltd. $LTB_4$ was kindly provided by P. Borgeat (University of Laval, Saint-Foy, Quebec), $PGE_2$, $PGF_{2\alpha}$ were gifts from J. Pike (Upjohn Laboratories), arachidonic acid was purchased from Sigma. These 4 latter compounds were diluted in absolute ethanol. In the experiments the



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final ethanol concentration was 0.1%, a concentration which did not by itself alter $[Ca^{2+}]_i$.

PMN were separated by dextran sedimentation, density gradient centrifugation in Ficoll-Hypaque (Pharmacia Fine Chemicals) and hypotonic lysis as in [7]. Purified PMN (>97% pure) were resuspended in a medium ('calcium medium') containing 138 mM NaCl, 6 mM KCl, 1.2 mM $P_i$, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, 5 mM $NaHCO_3$, 20 mM Hepes (pH 7.4) at 37°C. For experiments in 'calcium free medium' ($[Ca^{2+}]_o = 10^{-9}$ M) PMN were suspended in the above medium without calcium containing 1 mM EGTA. Degranulation of primary granules was assessed by incubating $5 \times 10^6$ PMN · $ml^{-1}$ in one of the above media in the presence of 5 $\mu$g/ml cytochalasin B at 37°C followed by addition of a stimulus and a further incubation of 5 min. $\beta$-Glucuronidase and LDH in the supernatant were measured as in [8]. Quin2 loading and calibration of quin2 as a function of $[Ca^{2+}]_i$ was performed essentially as in [4] and [5]. Excitation and emission wavelengths were 339 ± 5 and 492 ± 10, respectively, as in [4]. Since in the absence of cytochalasin B the changes in autofluorescence are negligible, the actual tracings with no corrections are shown here. Results are given as means ± SD.

## 3. RESULTS AND DISCUSSION

When quin2-loaded human PMN are exposed to $LTB_4$ there is a rise in free cytosolic calcium which is maximal at $10^{-8}$ M $LTB_4$ followed by a slow return to basal levels (fig.1A). The rise in $[Ca^{2+}]_i$ is due to influx from the extracellular medium and in part to release from intracellular pools as demonstrated by fig.1B in cells incubated in a medium containing $10^{-9}$ M $Ca^{2+}$. Under these conditions, though calcium influx from the extracellular medium is ruled out, $LTB_4$ is still capable of raising $[Ca^{2+}]_i$, albeit to a much smaller extent. It is theoretically possible, therefore, that calcium influx could act as a trigger of calcium release from intracellular pools. $LTB_4$ is a potent degranulating agent [1]. In non-loaded cells (in the presence of cytochalasin B), $LTB_4$ ($10^{-7}$ M) causes

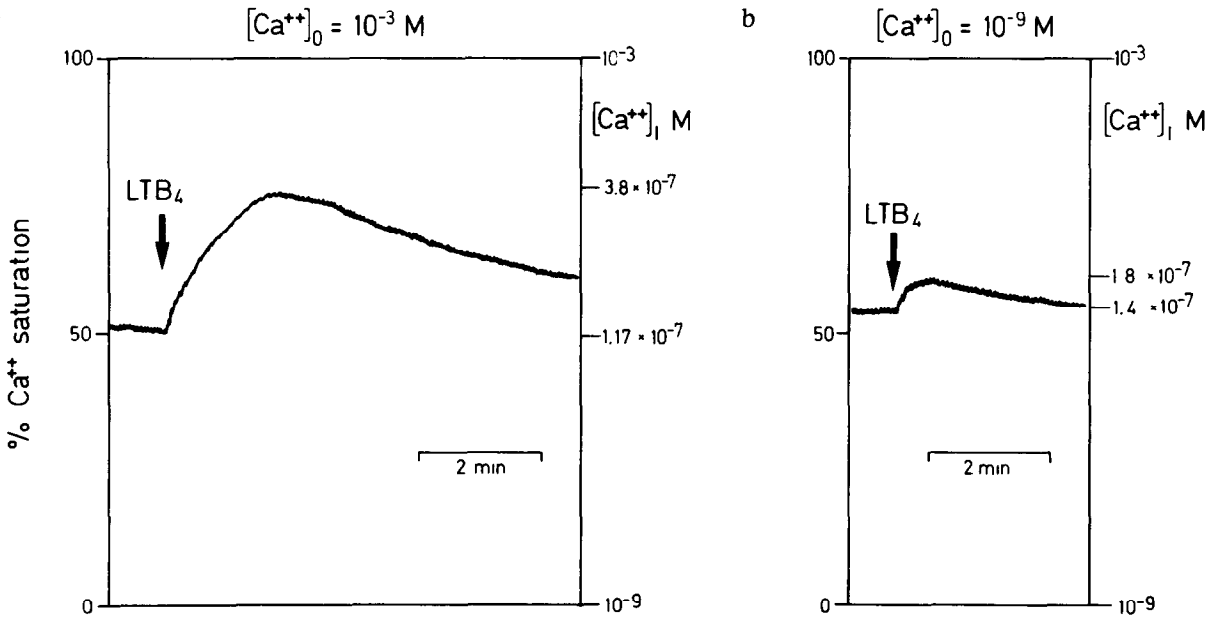


Fig.1. Effect of $LTB_4$ on the levels of free cytosolic calcium in human neutrophils, in calcium (PCa = 3) or calcium free (PCa = 9) medium. $LTB_4$ concentration, $10^{-8}$ M; Quin2 concentration, 0.8 nmol/$10^6$ PMN. Donor no.1. It should be noted that although for a given batch of cells the results were very reproducible, variations among different donors at different days were noted. In 6 experiments at different days with 3 different donors $LTB_4$ raised $[Ca^{2+}]_i$ from 140 ± 18 nM (mean ± SD) (range 115–166 nM) to 694 ± 308 nM (range 364–117 nM) with an intracellular quin2 of 0.64 ± 0.18 nmol/$10^6$ PMN.

the secretion of 37 ± 2% ($n$ = 3) of total $\beta$-glucuronidase content in calcium medium *vs* 34 ± 3.7% in calcium free medium. In neutrophils loaded with quin2 (0.39 nmol/$10^6$ PMN), $LTB_4$ ($10^{-7}$ M) causes secretion of 37 ± 2% ($n$ = 3) of total $\beta$-glucuronidase content in calcium medium *vs* 16.9 ± 1.8% in calcium free medium. Thus, loading the cells with quin2 when calcium influx is not operative decreased the degree of degranulation induced by $LTB_4$ probably due to the introduction of a calcium buffer in the cytoplasm of neutrophils. A detailed analysis of the effect of intracellular quin2 on the functional responses of PMN is currently being investigated. It has been claimed that $LTB_4$ functions simply as a Ca ionophore [3]. The present findings are not entirely consistent with this conclusion because calcium ionophores such as A23187 and ionomycin, in calcium free medium, although capable of releasing $Ca^{2+}$ from stores, and elevating $[Ca^{2+}]_i$ to an extent similar to that of chemotactic peptides, are unable to stimulate primary granule exocytosis [5]. Therefore in addition to raising $[Ca^{2+}]_i$, $LTB_4$ must have some other effect critical for stimulation of exocytosis. This conclusion is further supported by the observation that human PMN possess specific $LTB_4$ receptors [9].

Fig.2 indicates that the $LTB_4$-induced rise of $[Ca^{2+}]_i$ has some characteristics of a receptor-triggered event:

(i) The dose-dependence curve is very steep: at $10^{-11}$ M $LTB_4$ there is practically no response whereas at $10^{-10}$ M a nearly maximal response can be elicited.

(ii) If the cells are then exposed to the same concentration of $LTB_4$ there is no further rise in $[Ca^{2+}]_i$. The absence of response to the same concentration of the stimulant might be ascribed to a deactivation of the cells. However, increasing $LTB_4$ concentration by one order of magnitude generates a maximal $[Ca^{2+}]_i$ rise suggesting that adaptation had occurred to the new level of stimulus [10].

Adaptation of the cells to local changes in stimulus concentration could be the mechanism by which neutrophils maintain responsiveness to a chemoattractant gradient as they continually migrate towards its source [10].

The dose-response characteristics of $[Ca^{2+}]_i$ elevation and $\beta$-glucuronidase release, were not identical. Thus, while the $[Ca^{2+}]_i$ response to $LTB_4$ was almost maximal at $10^{-10}$ M, the corresponding degranulation response was 8% and was progressively increased to 37% at $10^{-7}$ M $LTB_4$. This observation further supports the hypothesis that in order to stimulate exocytosis, chemotactic peptides

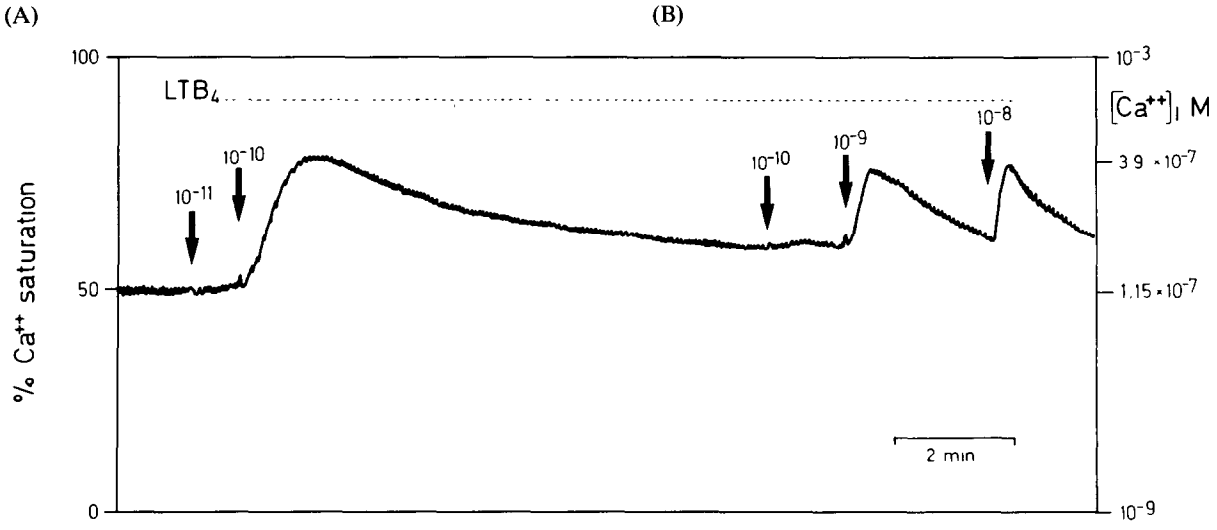


Fig.2. Effect of sequential addition of $LTB_4$ on $[Ca^{2+}]_i$ levels of free cytosolic calcium in human neutrophils. $LTB_4$ concentrations are indicated in the figure in mol/l. Cells from the same batch as used in fig.1. The quantitative rises in $[Ca^{2+}]_i$ induced by various $LTB_4$ concentrations were similar, whether $LTB_4$ was added sequentially as shown in the figure or added to different cuvettes (not shown).

[5] and leukotrienes generate at least another signal and that the two signals can be differently modulated by ligand concentration. In contrast to various products of the lipooxygenase pathway which activate neutrophil function, several stable prostaglandins, products of the cyclooxygenase pathway, have been shown to be weak inhibitors of neutrophil functions [11]. Fig.3A indicates that $PGE_2$ (3 $\mu$M) alters neither $[Ca^{2+}]_i$, nor the $[Ca^{2+}]_i$ rise induced by $LTB_4$ and formyl–methionyl–

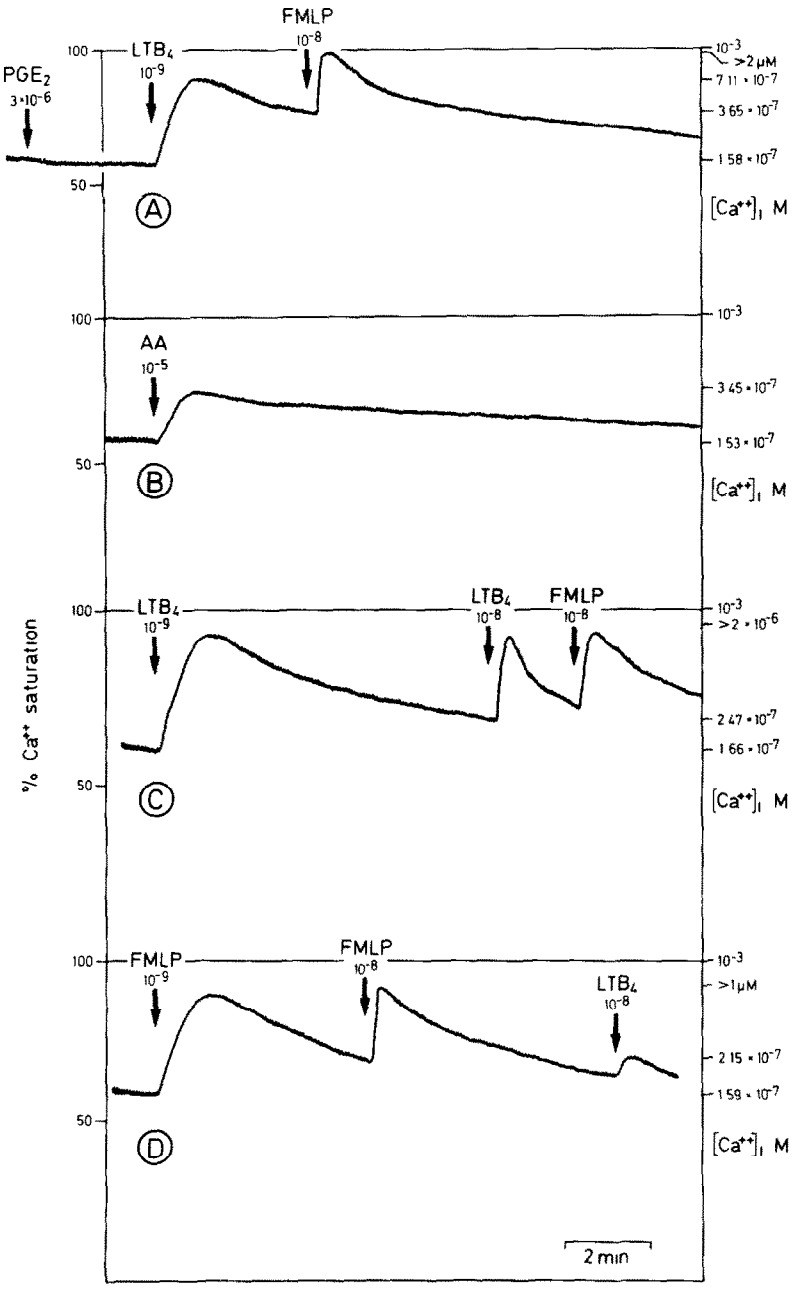


Fig.3. Effect of $PGE_2$, arachidonic acid and FMLP on $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ transients induced by $LTB_4$. Concentration of various reagents is indicated in the figure in mol/l. Quin2 concentration, 0.4 nmol/$10^6$ PMN. Donor no.2.

leucyl–phenylalanine (FMLP). Similar results were obtained with $PGF_{2\alpha}$ at equal concentrations (not shown). By contrast, arachidonic acid, the precursor of both cyclooxygenase and lipooxygenase products, is able to raise cytosolic free calcium (fig.3B), though the concentration used was much higher and its effect smaller than that of $LTB_4$.

Several studies using $^{45}Ca$ fluxes in rabbit neutrophils have suggested that pretreatment of PMN with the chemotactic peptide, FMLP, alters the subsequent response to $LTB_4$ stimulation [12]. Fig.3C,D indicate such a response pattern of $[Ca^{2+}]_i$ transients. In several experiments FMLP induced similar rises in $[Ca^{2+}]_i$ with slightly different kinetics, whether added before or after equimolar concentrations of $LTB_4$. In contrast, if $LTB_4$ was added after FMLP, a marked inhibition of the $LTB_4$-induced $[Ca^{2+}]_i$ rise was observed, whether added shortly or some minutes after FMLP.

A number of explanations for this sequential inhibition can be suggested: competition for binding, competition for calcium pools and/or inhibitory signals, generated by FMLP. Another possibility is that $LTB_4$ produced by the stimulation of arachidonic acid metabolism induced by FMLP desensitized the cells to exogenous $LTB_4$. The fact that FMLP activates the lipoxygenase pathway of arachidonic acid metabolism [1] raises the important question of whether all, or part, of the $[Ca^{2+}]_i$ rise induced by FMLP is due to $LTB_4$ production.

In conclusion, the present findings demonstrate that one of the earliest events upon interaction of $LTB_4$ with human neutrophils, is a rise in cytosolic free calcium. Although this rise is probably critical for neutrophil activation by $LTB_4$ it is not by itself sufficient to explain all the functional responses of PMN to this leukotriene as well as to other stimuli. A general picture thus emerges for a number of stimuli in various cell-types where cell activation requires other intracellular messengers to be generated, in addition to $Ca^{2+}$ and cyclic nucleotides, in order to explain cell responses to receptor-mediated activation; e.g., protein kinase C, activated by phosphotidylinositol breakdown products, has been suggested to be a key step in the physiological response of cells to extracellular stimuli [14].

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