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Role of sodium in intracellular calcium elevation and leukotriene B_4 formation by receptor-mediated activation of human neutrophils

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Dedicated to the memory of Professor Simonetta Nicosia.

Abstract

The role of Na^+ and Na^+ exchangers in intracellular Ca^{2+} elevation and leukotriene B_4 (LTBs) formation was investigated in granulocyte macrophage colony-stimulating factor (GM-CSF)-primed, fMLP-stimulated human neutrophils. Isotonic substitution of extracellular Na^+ with *N*-methyl-D-glucamine$^+$ ($NMDG^+$) resulted in over 85% inhibition of the LTBs generation observed (from 14.1 ± 0.9 pmol/10^6 neutrophils to 1.7 ± 1.0 pmol/10^6 neutrophils at 0.3 μM fMLP). Isotonic substitution of Na^+ with $NMDG^+$ also induced a significant inhibition of fMLP-induced rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) (from 2.17- to 0.78-fold increase over basal levels). Pretreatment with an inhibitor of the Na^+/Ca^{2+} exchanger (benzamil) did not inhibit either $[Ca^{2+}]_i$ rise or LTBs production, indicating that the observed effects of extracellular Na^+-deprivation were unrelated to the Na^+/Ca^{2+} exchanger in receptor-mediated Ca^{2+} influx, as previously hypothesized. LTBs production by thapsigargin-activated neutrophils was not affected by Na^+ depletion, but was totally abolished in the presence of EGTA, suggesting that store depletion-driven extracellular Ca^{2+} influx is required for leukotriene synthesis and that this process is independent of Na^+-deprivation. Exposure to Na^+-free medium for the time of GM-CSF priming led to a significant decrease of intracellular pH values, suggesting a role of the Na^+/H^+ exchanger in intracellular Na^+ depletion. Reducing the time of Na^+-deprivation totally reversed the observed effect on LTBs production, resulting in enhanced, rather than inhibited, formation of LTBs. These results indicate that LTBs generation and $[Ca^{2+}]_i$ rise in human neutrophils primed by GM-CSF and stimulated with fMLP is dependent on intracellular Na^+ concentration, and, at variance with previously published results, unrelated to the Ca^{2+} influx through the Na^+/Ca^{2+} exchanger.

Keywords: Calcium ion; Sodium ion; Leukotriene B_4; Neutrophils

1. Introduction

Human neutrophils play an important role in inflammation; they are attracted to the inflamed tissues and have the capacity to release inflammatory mediators, such as lysosomal enzymes (acid hydrolases and neutral proteinases), arachidonic acid (AA) metabolites, and toxic oxygen derivatives [1–4]. An *in vitro* model of neutrophil activation is represented by stimulation with the synthetic chemotactic peptide fMLP, whose structure resembles that of bacterial peptides [5]; fMLP interacts with a membrane receptor and triggers a number of biochemical events, such as G-protein-mediated phospholipase C activation, which in turn generates diacylglycerol and inositol 1,4,5-triphosphate (IP_3), increase in $[Ca^{2+}]_i$ and O_2^- generation [6–9].

Elevation of $[Ca^{2+}]_i$, due to mobilization of Ca^{2+} from intracellular stores and influx from extracellular medium, represents a critical step in leukotriene (LT) synthesis [10]. This biochemical event is necessary, but not sufficient to

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✱ Professor Simonetta Nicosia passed away during the course of this research.

Abbreviations: LTBs, leukotriene B_4 + 20-OH-LTB_4 + 20-COOH-LTB_4; $NMDG^+$, *N*-methyl-D-glucamine$^+$; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; GM-CSF, granulocyte macrophage colony-stimulating factor; 5-LO, 5-lipoxygenase.

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allow formation of substantial amounts of LTs: soluble agonists, such as fMLP, which are able to elevate $[Ca^{2+}]_i$ and determine Ca^{2+} influx in human neutrophils, do not induce synthesis of LTs *per se* [11]. However, priming with GM-CSF prior to fMLP stimulation leads to the formation of substantial amounts of LTB_4 and its metabolites 20-COOH-LTB_4 and 20-OH-LTB_4 (LTBs) [12–14], possibly through an increase in AA availability [15].

It is recognized that phospholipase A_2 (PLA_2; 3.1.1.4) and 5-lipoxygenase (5-LO; EC 1.13.11.34) are two key enzymes involved in LT synthesis. The release of AA from membrane phospholipids may depend on the activation of a PLA_2, some isoforms of which require Ca^{2+} for their activity [16]. Free AA is then metabolized to LTB_4 by 5-LO, which is also dependent on Ca^{2+} for translocation and activity [17]. In human neutrophils, Na^+/Ca^{2+} exchange has been proposed as an important mechanism regulating Ca^{2+} influx upon receptor-mediated cell activation [18].

Results obtained by Wood and Gillespie [19] have shown that Na^+ facilitates IP_3-induced Ca^{2+} release from intracellular stores, and may therefore play a regulatory role in Ca^{2+} elevation and LT synthesis.

In light of these evidences, we studied the role of Na^+ and Na^+ exchangers in receptor-mediated $[Ca^{2+}]_i$ rise and LT synthesis in human neutrophils.

2. Materials and methods

2.1. Materials

Amiloride, BCECF-acetoxymethylether (AM), benzamil, choline chloride, EDTA, EGTA, fMLP, HEPES, $NiCl_2$, $NMDG^+$, and thapsigargin were purchased from Sigma Chemicals Co; absolute methanol from Pharmacia-Carlo Erba; dextrane from Sifra; DMSO from Fisher Scientific Co; Fura2-AM, Fluo3-AM, and pluronic F-127 from Molecular Probes Inc.; GM-CSF from R&D Systems; LTB_4, 20-COOH-LTB_4, 20-OH-LTB_4, and PGB_2 from Cayman Chemicals; Lymphoprep from Nycomed; PAF from Bachem; HPLC solvents and Triton X-100 from Merck; May-Grunwald and Giemsa stains and Turk liquid were supplied by the hospital pharmacy (Policlinico).

2.2. Incubation media

The standard medium used in this study was a HEPES-buffered solution with the following composition: 140 mM NaCl, 5 mM KCl, 5.6 mM glucose, 5 mM HEPES (HEPES-Na). To evaluate the effect of varying external Na^+ concentrations, isotonic solutions were prepared by substituting Na^+ with $NMDG^+$ (HEPES-NMDG) or $choline^+$ (HEPES-choline). The pH of the solutions was brought to 7.4 with HCl.

2.3. Blood collection and neutrophil preparation

Peripheral venous blood was obtained by venipuncture of the antecubital vein of normal adult subjects aged 22–40 years, who had not taken medications for at least 1 week. Informed consent was obtained from each subject before the blood collection. Neutrophil suspensions were prepared by dextrane sedimentation and separation on Lymphoprep (density 1.077 g/mL) at room temperature (30 min, 700 *g*) [20]. Pellet was subjected to erythrocyte lysis by gently resuspending in 5 mL of a NaCl solution (0.2%, w/v) and, after a 2-min incubation, was further diluted with 5 mL of a balancing solution (NaCl 1.6%, w/v; sucrose 0.2%, w/v) at 4°. Cell suspension was centrifuged for 10 min at 300 *g* at room temperature and then resuspended in HEPES-Na. When erythrocyte lysis was considered insufficient, the procedure was repeated. After resuspending, neutrophils were stained with Turk liquid, and counted in a Fuchs-Rosenthal chamber (Walter Schrenk) with a light microscope (magnification 250×). Differential cell count was performed by preparing cytospin slides (100 μL with 2×10^4 to 4×10^4 cells per slide; centrifugation at 180 *g* for 10 min in a Shandon cytocentrifuge; Shandon Southern Ltd), and staining by May-Grunwald Giemsa. Neutrophil yield was above 96%.

Neutrophil concentration was brought to 2×10^7 mL by adjusting the buffer volume. Neutrophils were then divided into aliquots, centrifuged at 300 *g* for 10 min at 20°, and resuspended in HEPES-Na or HEPES-NMDG or HEPES-choline.

2.4. Eicosanoid production by neutrophil preparations and HPLC analysis

Neutrophil suspension (0.5 mL) was added to prewarmed tubes (37°) containing 200 pM GM-CSF in buffer. After incubation for 30 min at 37°, $CaCl_2$ (final concentration 1.8 mM), $MgCl_2$ (final concentration 0.5 mM), and fMLP (concentrations ranging from 0.1 nM to 1 μM) were added, so that the final volume in each tube was 1 mL. In selected experiments, platelet-activating factor (PAF, 0.1 μM) or thapsigargin (0.1 μM) were used as neutrophil stimulators after priming with GM-CSF. When modulation of LTBs production was assessed, benzamil (0.05–50 μM), $NiCl_2$ (0.05–5 mM), EGTA (3 mM), or amiloride (0.1–1000 μM) were added 3 min before fMLP. In selected experiments, in order to minimize the loss of intracellular Na^+ resulting from prolonged exposure to a Na^+-free buffer, neutrophils were primed in HEPES-Na for 30 min at 37°, quickly centrifuged (5 min, 280 *g*), resuspended in either HEPES-NMDG or HEPES-Na (prewarmed), and challenged with fMLP. After a further incubation for 10 min, 1 mL absolute methanol, containing the HPLC internal standard PGB_2 (25 ng), was added to each tube, and 5-LO metabolites analyzed as previously described [21]. Quantitation was

carried out on positively identified peaks only, using their HPLC peak areas relative to that of PGB_2 at 280 nm, and calculated from the responses of standard compounds. LTB_4 production was expressed as the sum of all LTB_4 metabolites, namely 20-COOH-LTB_4, 20-OH-LTB_4, and LTB_4 (LTBs).

2.5. Measurement of $[Ca^{2+}]_i$

Neutrophils, at a concentration of 2.5×10^6 to 3.5×10^6 cells/mL in HEPES-Na, were loaded with Fura2-AM (1 μM) or Fluo3-AM (2 μM) or the vehicle (DMSO 0.1%) in the presence of 0.006% pluronic F-127 (a nonionic detergent which prevents microprecipitation of the dye in aqueous solution) for 15 min at 30° in the dark. The cells were then centrifuged for 10 min at 300 *g* and resuspended in HEPES-Na. Before fluorescence assay, neutrophil aliquots were centrifuged again, resuspended at 106 cells/mL in either HEPES-Na, HEPES-NMDG, or HEPES-choline, and incubated with 200 pmol GM-CSF for 30 min. $MgCl_2$ (0.5 mM) and $CaCl_2$ (1 mM) were added to the neutrophil suspension, which was challenged as described for eicosanoid production.

$[Ca^{2+}]_i$ was monitored by dual-excitation wavelength fluorimetry using a Perkin-Elmer fluorescence spectrometer LS 50B. The cell suspension (1 mL), stirred with a Teflon-coated magnetic bar, was kept at 30°, and fluorescence was recorded at 340 and 380 nm excitation and 505 emission (Fura2). When neutrophils were loaded with Fluo3, excitation was set at 505 nm and emission at 530 nm.

To evaluate $[Ca^{2+}]_i$ from fluorescence recording, the calibration was performed as follows: 0.2 mM $MnCl_2$ and 0.5 mM CaDTPA were added to estimate Fura2 or Fluo3 leakage; to obtain the minimal fluorescence of the system, EGTA (10 mM), Tris (120 mM), and Triton X-100 (0.2%) were added sequentially. Finally, $CaCl_2$ (20 mM) was added to record the maximal fluorescence of the system. $[Ca^{2+}]_i$ was obtained from the observed fluorescence according to Grynkiewicz *et al.* [22], after subtracting the autofluorescence and leakage values from the fluorescence values at the respective wavelength.

2.6. Evaluation of intracellular pH

Neutrophils, at a concentration of 2.5×10^6 to 3.5×10^6 cells/mL in HEPES-Na, were loaded with BCECF (2.5 μM) for 40 min at 30°. After centrifugation at 300 *g* for 30 min, room temperature, cells were resuspended in HEPES-Na or HEPES-NMDG at the final concentration of 2×10^6 cells/mL. Excitation and emission were set at 503 and 540 nm, respectively.

2.7. Statistical analysis

Results are expressed as mean ± SEM of N experiments. One-way ANOVA and Student's *t* tests for paired or unpaired data, as appropriate, were used to analyze data. The level of significance considered was less than 5% ($P < 0.05$).

3. Results

3.1. Effect of extracellular Na^+-deprivation

Stimulation of human neutrophils with fMLP (0.3 μM) alone did not bring about formation of measurable amounts of LTBs. In agreement with previously published data [23], preincubation with 200 pM GM-CSF was required in order to achieve LTBs production. 20-COOH-LTB_4 represented the major metabolite, accounting for 60–80% of the total LTB_4 metabolites detected, 20-OH-LTB_4 and LTB_4 being the remaining metabolites [24].

In human neutrophils at 10^7 cells/mL, primed with 200 pM GM-CSF, the stimulation with fMLP determined a concentration-dependent LTBs formation that was maximal at concentrations higher than 0.1 μM (14.2 ± 0.35 pmol/10^6 neutrophils at 0.3 μM).

Removal of Na^+ from extracellular medium during both priming and incubation, and its isotonic substitution with $NMDG^+$ resulted in over 85% inhibition of the LTBs production observed (14.1 ± 0.9 pmol/10^6 neutrophils in HEPES-Na and 1.7 ± 1.0 pmol/10^6 neutrophils in HEPES-NMDG; N = 4). The same results were obtained when extracellular Na^+ was substituted with $choline^+$ (16.80 ± 7.40 pmol/10^6 neutrophils in HEPES-Na and below the detection limit of the HPLC method, 0.5 pmol/10^6 neutrophils, in HEPES-choline; N = 4). The effect of $[Na^+]_o$ on LTBs formation appeared to be concentration-dependent: in fact, the increase in $[Na^+]_o$ from 0 to 140 mM was associated with a progressive restoration of LTBs generation (Fig. 1).

A similar behavior was observed when primed neutrophils were stimulated with PAF (0.1 μM). LTBs production was greatly enhanced when neutrophils were primed with 200 pmol GM-CSF before stimulation with PAF (5.2 ± 3.55 pmol/10^6 neutrophils vs. 53.4 ± 13.29 pmol/10^6 neutrophils in absence or presence of priming, respectively; N = 5). Na^+-deprivation led to a marked decrease in LTBs production (<0.5 and 16.6 ± 9.9 pmol/10^6 neutrophils in absence or presence of priming, respectively; N = 5).

Neutrophil stimulation with 1 μM fMLP caused a significant rise in $[Ca^{2+}]_i$. Priming with 200 pM GM-CSF did not significantly affect either basal $[Ca^{2+}]_i$ levels or the fMLP-stimulated rise in the ion concentration. Evaluation of the effect of Na^+ removal on $[Ca^{2+}]_i$ values in primed neutrophils showed that the absence of extracellular Na^+ did not affect $[Ca^{2+}]_i$ basal levels, but caused a significant decrease in fMLP-induced $[Ca^{2+}]_i$ rise (Fig. 2A and B).

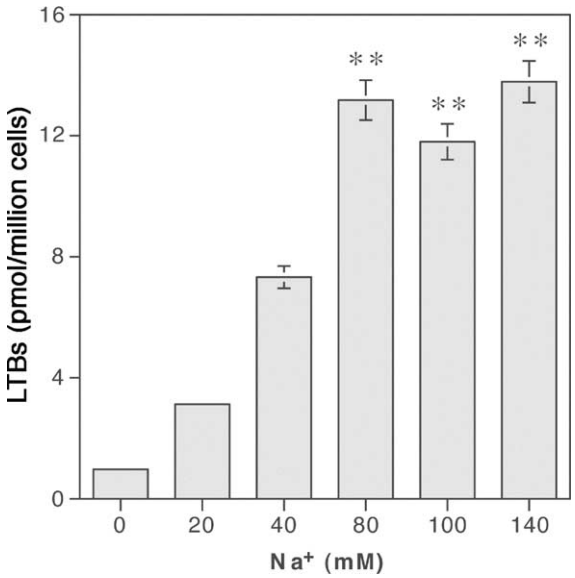

Fig. 1. Effect of extracellular Na^+ concentration ($[Na^+]_o$) on LTBs production by GM-CSF-primed neutrophils. Neutrophil preparations (10^7 cells/mL) suspended in HEPES buffer, where Na^+ was substituted isotonically with different concentration of $NMDG^+$, were primed with GM-CSF (200 pmol, 30 min) and stimulated with 0.3 μM fMLP. Supernatants were analyzed by RP-HPLC as described in Section 2. Results are expressed as mean ± SEM of four experiments. $^{**}P < 0.01$.

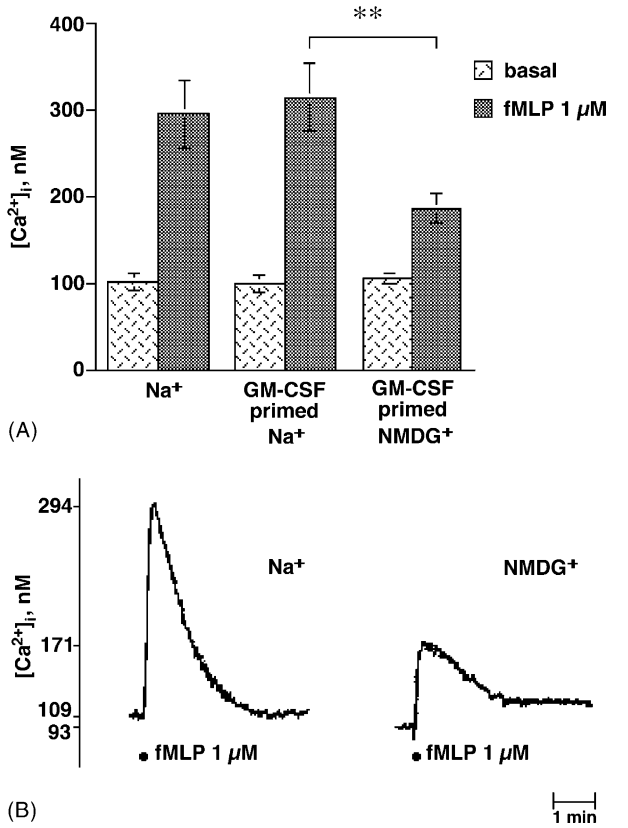

Fig. 2. Effect of GM-CSF priming and Na^+-deprivation on fMLP-induced $[Ca^{2+}]_i$ rise in neutrophils. Fura2-loaded neutrophils (10^6 cells/mL), suspended in HEPES-Na or HEPES-NMDG, and treated with 200 pmol GM-CSF or vehicle for 30 min, were challenged with 1 μM fMLP (panel A). Representative tracings of the effect of extracellular Na^+ on fMLP-induced $[Ca^{2+}]_i$ elevations in GM-CSF-primed neutrophils (panel B). $[Ca^{2+}]_i$ was monitored by dual-excitation wavelength fluorimetry. Results are expressed as mean ± SEM of seven different experiments. $^{**}P < 0.01$.

3.2. Effects of drugs interfering with intracellular and transmembrane Ca^{2+} movements

Pretreatment with benzamil, an inhibitor of the Na^+/Ca^{2+} exchanger [25], did not inhibit LTBs production; on the contrary, a significant increase in fMLP-induced 5-LO metabolite production was observed at the highest concentration tested (50 μM) (Fig. 3A).

In order to evaluate the relative importance of extracellular vs. intracellular sources of Ca^{2+} with respect to fMLP-induced production of LTs, we tested the effects of either Ni^{2+}, a Ca^{2+} channel blocker, or EGTA, an extracellular Ca^{2+} chelator. The results showed that challenge in the presence of increasing concentrations of Ni^{2+} (0.5–5 mM) resulted in a dose-dependent inhibition of LTBs formation (Fig. 3B). Similarly, challenge in the presence of 2 mM EGTA totally abolished the synthesis of 5-LO metabolites (<0.5 pmol/10^6 neutrophils).

Thapsigargin (0.1 μM), which blocks the Ca^{2+}-ATPases that promote Ca^{2+} uptake into intracellular organelles, therefore, producing a slow $[Ca^{2+}]_i$ elevation (Fig. 4A), caused a significant formation of LTBs, that was not affected by extracellular Na^+-deprivation (Fig. 4B). In order to determine if the production observed upon thapsigargin challenge was related to extracellular Ca^{2+} influx, the same experiments were carried out in the presence of excess extracellular EGTA, resulting in a complete suppression of LT formation to levels below the detection limit of the analytical method (<0.5 pmol/10^6 neutrophils) either in the presence or absence of extracellular Na^+.

Analysis of $[Ca^{2+}]_i$ changes in the presence of benzamil was carried out using the fluorescent dye Fluo3, given the strong interference of benzamil with calibration substances using the excitation and emission wavelengths of Fura2. Preincubation for 3 min with this inhibitor of Na^+/Ca^{2+} exchanger, at the concentration of 50 μM, did not significantly affect the immediate fMLP-induced $[Ca^{2+}]_i$ rise (Fig. 5), but, in agreement with the observed increase in LTBs production, it yielded a significantly elevated $[Ca^{2+}]_i$ level at 4 min after fMLP (Fig. 5, bars).

When the Ca^{2+} channel blocker Ni^{2+} (5 mM) was added 1 min before fMLP challenge, a significant reduction in $[Ca^{2+}]_i$ levels was observed (from 314.4 ± 39.1 nM to 186.6 ± 18.6 nM in controls and Ni^{2+}-treated samples, respectively; N = 7, $P < 0.05$).

3.3. Effects of inhibition of the Na^+/H^+ exchanger on LTBs production and $[Ca^{2+}]_i$ elevation

Pretreatment with amiloride, a reported inhibitor of Na^+/H^+ exchanger [32], did not cause inhibition of LTBs

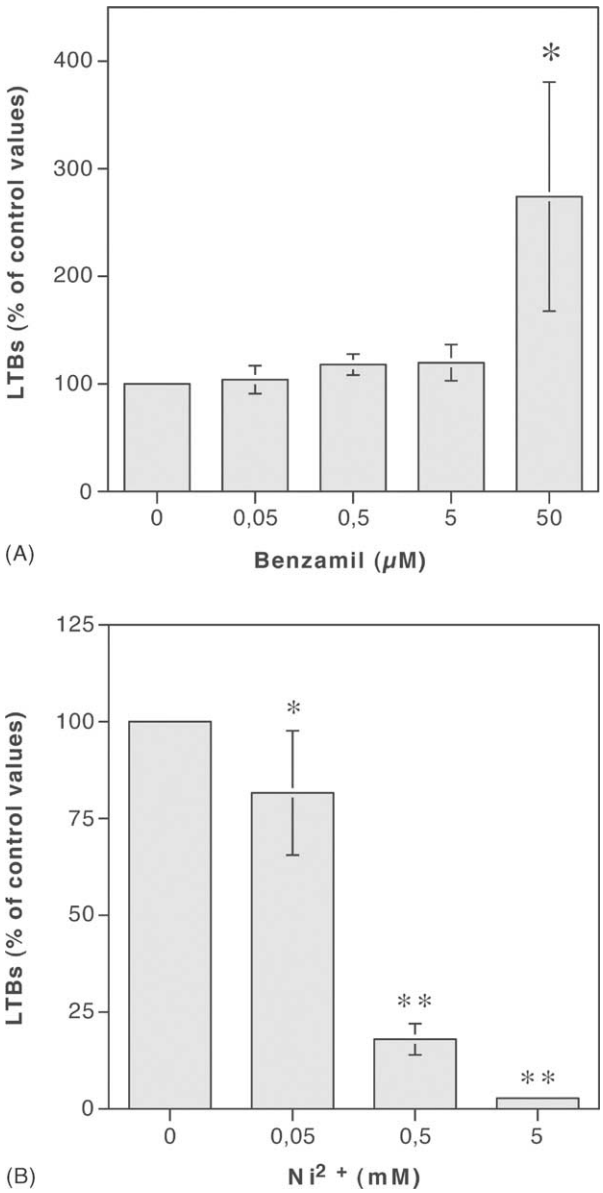

Fig. 3. Effects of the Na^+/Ca^{2+} exchanger inhibitor benzamil (panel A) and of the Ca^{2+} channel blocker Ni^{2+} (panel B) on LTBs formation by GM-CSF-primed neutrophils. Neutrophil preparations (10^7 cells/mL) were challenged with 0.3 μM fMLP and supernatants analyzed by RP-HPLC. Results are expressed as % of control LTBs production and represent the mean ± SEM of four experiments. LTBs production in controls (100%) was 12.2 ± 1.7 pmol/10^6 neutrophils and 25.3 ± 4.3 pmol/10^6 neutrophils in experiments with benzamil and Ni^{2+}, respectively. $^{**}P < 0.01$, $^{*}P < 0.05$.

production in neutrophils at any of the concentration tested (Fig. 6).

3.4. Effect of Na^+-deprivation on intracellular pH

Neutrophil resuspended in HEPES-NMDG for GM-CSF priming showed a significantly lower intracellular pH at the end of the priming and prior to fMLP challenge, when compared to neutrophils resuspended in HEPES-Na buffer, as indicated by lower fluorescence values observed in BCECF-loaded samples (334 ± 50 vs. 501 ± 15 arbitrary unit, HEPES-NMDG and HEPES-Na, respectively; N = 4, $P < 0.05$).

3.5. Effect of short-term Na^+-deprivation

In order to minimize the intracellular Na^+ depletion, resulting from prolonged exposure to a Na^+-free buffer, we performed experiments where neutrophils were primed in the presence of Na^+ for 30 min at 37°, quickly centrifuged (5 min, 280 *g*), resuspended in either HEPES-NMDG or HEPES-Na, and challenged with fMLP for 10 min. Under these experimental conditions, the production of LTBs from Na-deprived neutrophils resulted significantly higher, rather than lower, than that observed in control cells (Fig. 7), a behavior very similar to that observed in the presence of the Na^+/Ca^{2+} exchanger inhibitor benzamil (50 μM).

4. Discussion

In the present study, we investigated the role of Na^+ and Na^+ exchangers in intracellular Ca^{2+} elevation and LTBs formation in human neutrophils primed with GM-CSF and stimulated with fMLP. Removal of extracellular Na^+ and its isosmotic substitution with $NMDG^+$ or $choline^+$ resulted in over 85% inhibition of fMLP-induced LTBs production, extending previous observation obtained using the Ca^{2+} ionophore A23187 by Osaki *et al.* [26]. The effect of Na^+ was dependent on its concentration in the extracellular medium, since a progressive increase in $[Na^+]_o$ from 0 to 140 mM was associated with a restoration of LTBs production. Maximum production was achieved at $[Na^+]_o$ similar to the concentration observed intracellularly (60 mM) in neutrophils after stimulation with fMLP [27].

Measurement of $[Ca^{2+}]_i$ in resting and stimulated neutrophils, after priming with GM-CSF, revealed that Na^+ removal from extracellular medium, which presumably causes intracellular Na^+ depletion, caused a significant decrease in fMLP-induced $[Ca^{2+}]_i$ rise. Previous results obtained by Simchowitz and coworkers in human neutrophils showed that Na^+ removal from the extracellular medium leads to a complete abrogation of $^{45}Ca^{2+}$ influx in resting cells [18] and to the inhibition of cellular activation, namely, superoxide production following fMLP stimulation [28]. Additional studies showed that fMLP challenge causes activation of the Na^+/Ca^{2+} exchanger, as indicated by the inhibitory effect of benzamil (a reported inhibitor of Na^+/Ca^{2+} exchanger) [25] on the fMLP-induced increase in Fura2-fluorescence [29]. Based on these observations, the group of Simchowitz and coworkers have hypothesized that, in human neutrophils, fMLP challenge activates the Na^+/Ca^{2+} exchanger to operate in reverse conditions (i.e. importing Ca^{2+} and exporting Na^+), thus

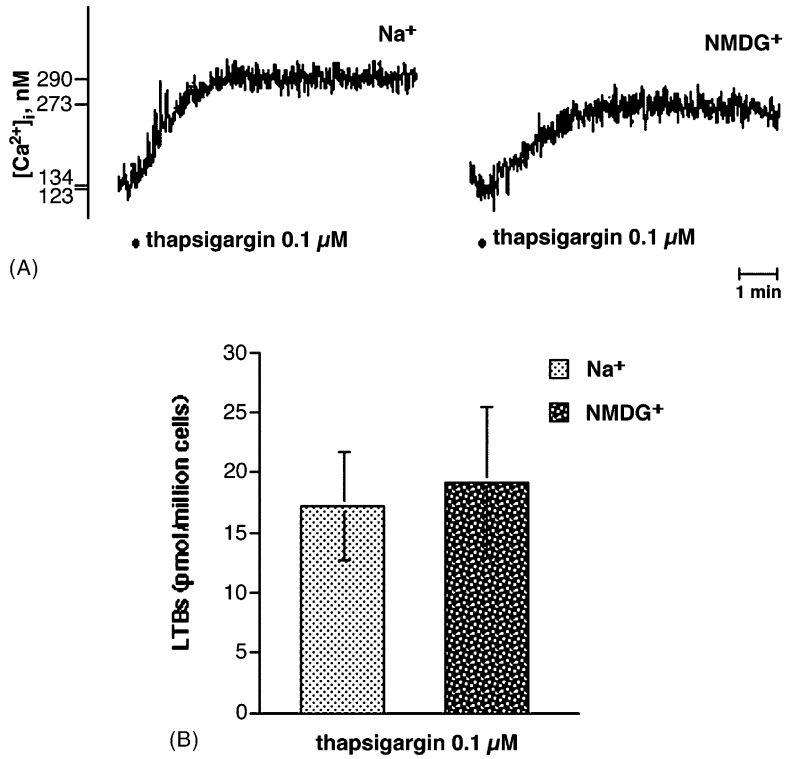

Fig. 4. Effect of Na^+-deprivation on $[Ca^{2+}]_i$ elevation and LTBs formation induced by 0.1 μM thapsigargin in GM-CSF-primed neutrophils. Representative tracings of Fura2-loaded neutrophils (10^6 cells/mL) suspended in HEPES-Na or HEPES-NMDG (panel A). $[Ca^{2+}]_i$ was monitored by dual-excitation wavelength fluorimetry. After challenge, LTBs formation by neutrophil preparations (10^6 cells/mL) was analyzed by RP-HPLC (panel B). Results are expressed as mean ± SEM of four experiments.

contributing to the receptor-mediated activation of Ca^{2+} influx [18,29]. In striking opposition with this hypothesis and the results obtained by Simchowitz [29], pretreatment with benzamil did not inhibit LTBs production by fMLP-stimulated neutrophils; on the contrary, a significant increase was observed at the highest concentration tested. This observation was confirmed by the results of $[Ca^{2+}]_i$ studies, which clearly showed the lack of significant inhibitory effect of benzamil on fMLP-stimulated $[Ca^{2+}]_i$ rise, while a significant enhancement of $[Ca^{2+}]_i$ was measured 4 min after fMLP challenge. Taken together, these findings are in contrast with the potential role of the Na^+/Ca^{2+} exchanger in fMLP-induced $[Ca^{2+}]_i$ elevation and suggest that in human neutrophils the exchanger operates predominantly in the forward mode, i.e. to drive Ca^{2+} out of the cells using the inwardly directed Na^+ electrochemical gradient. The discrepancy between our findings and those of Dale and Simchowitz [29] can be explained by the fact that, in our hands, benzamil gave a strong fluorescent signal, which interfered with calibration of Fura2-loaded neutrophils and made a quantitative evaluation of $[Ca^{2+}]_i$ impossible. For this reason, we overcame the problem using Fluo3, thus avoiding potential artifacts.

Ca^{2+} involvement in the LT biosynthetic pathway mainly relies on the Ca^{2+}-dependence of both 5-LO and cytosolic PLA_2 ($cPLA_2$). It is indeed known that a ligand–receptor interaction that results in $[Ca^{2+}]_i$ increase may induce LT synthesis in neutrophils. However, a rise in $[Ca^{2+}]_i$ is not sufficient *per se* to trigger LT synthesis, and influx of Ca^{2+} across the plasma membrane is required to obtain LTBs production [30,31], unless calcium-independent activation of 5-LO is induced using cellular stress together with micromolar concentrations of exogenous AA [32]. It has been shown that the influx of Ca^{2+} across the plasma membrane correlates with $cPLA_2$ phosphorylation, resulting in increased activity and enhanced AA availability. Three main mechanisms have been implicated in transmembrane Ca^{2+} movements in human neutrophils: receptor-operated Ca^{2+} channels [7], store-dependent Ca^{2+} channels [33,34], and a Na^+/Ca^{2+} exchanger [18]. In light of the results obtained with benzamil, we verified the potential contribution of extracellular Ca^{2+} to fMLP-stimulated LTBs formation. In agreement with previous reports, we found that extracellular Ca^{2+} is required for LTB_4 production following fMLP activation, since chelation of Ca^{2+} by EGTA totally abolished LTB_4 formation. Furthermore the use of the Ca^{2+} channel blocker Ni^{2+} exerted a dose-dependent inhibitory effect on 5-LO metabolite production, supporting the mandatory role of Ca^{2+} influx toward LT biosynthesis under our experimental conditions.

Treatment of human neutrophils with thapsigargin, a tumor-promoting agent inhibiting microsomal Ca^{2+}-ATPases that favor cytoplasmic Ca^{2+} reuptake into storage

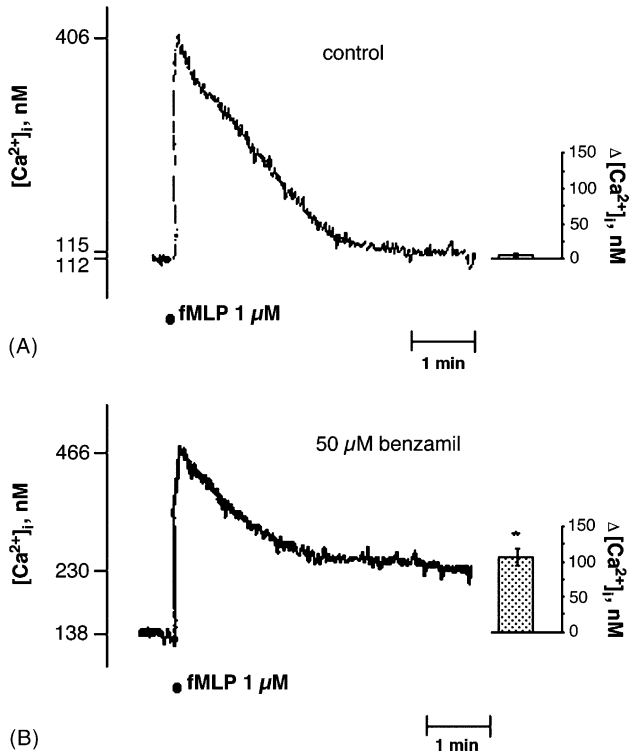

Fig. 5. Effect of the Na^+/Ca^{2+} exchanger inhibitor benzamil (50 μM) on fMLP-induced $[Ca^{2+}]_i$ elevations in GM-CSF-primed neutrophils. Fluo3-loaded neutrophils (10^6 cells/mL) were suspended in HEPES-Na, pre-incubated for 3 min with vehicle (panel A) or 50 μM benzamil (panel B), and then challenged with 1 μM fMLP. $[Ca^{2+}]_i$ was monitored by single-wavelength fluorimetry. Right insets represent $\Delta[Ca^{2+}]_i$ elevation at 4 min over the basal levels. Results are expressed as mean ± SEM of four experiments. $^*P < 0.05$.

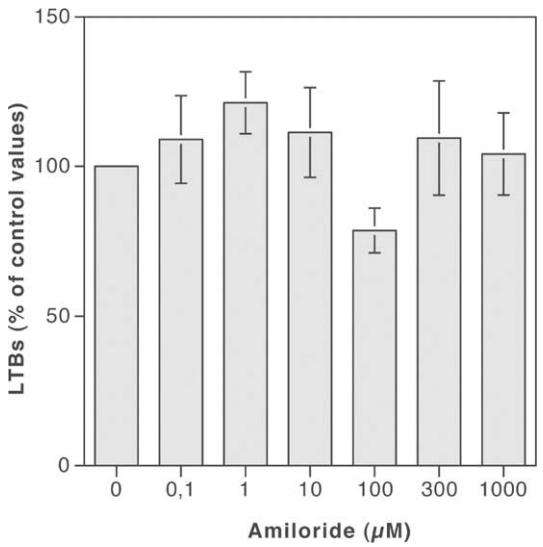

Fig. 6. Effects of the Na^+/H^+ exchanger inhibitor amiloride on LTBs formation by GM-CSF-primed neutrophils suspended in HEPES-Na. Neutrophil preparations (10^7 cells/mL) were challenged with 0.3 μM fMLP and supernatants analyzed by RP-HPLC. Results are expressed as % of control LTBs production and represent the mean ± SEM. of four experiments. 100% was 22.7 ± 6.7 pmol/10^6 neutrophils.

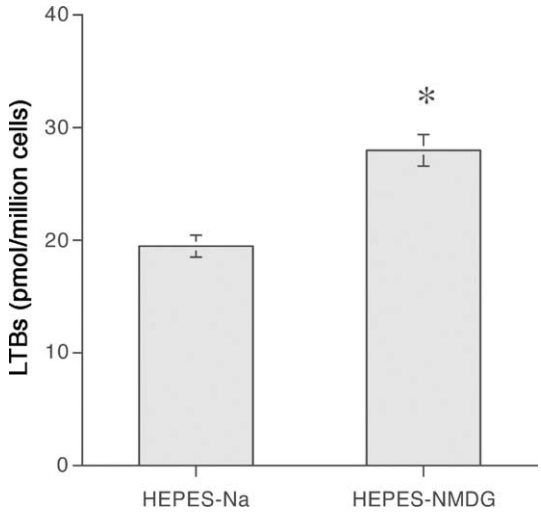

Fig. 7. Effects of short-term Na^+-deprivation on LTBs formation by GM-CSF-primed neutrophils. Neutrophil preparations (10^7 cells/mL) were primed with GM-CSF (200 pmol, 30 min) in HEPES-Na, centrifuged, resuspended in either HEPES-Na or HEPES-NMDG, and challenged with 0.3 μM fMLP. Supernatants were analyzed by RP-HPLC. Results are expressed as mean ± SEM of four experiments. $^*P < 0.005$.

pools, caused a significant production of LT associated with a sustained rise in $[Ca^{2+}]_i$. The observed inhibitory effect of extracellular EGTA indicates that depletion of intracellular Ca^{2+} stores is responsible for LT synthesis in neutrophils only because is able to induce a sustained influx of extracellular Ca^{2+}. The effect of thapsigargin was completely independent from the presence or absence of extracellular Na^+, further suggesting that the effect of Na^+-deprivation was not related to specific mechanisms mediating the influx of extracellular Ca^{2+}, such as the hypothesized Na^+/Ca^{2+} exchanger.

In light of the data obtained by Osaki *et al.* [26] on the relationship between intracellular pH and the production of LTBs in A23187-activated neutrophils, we therefore evaluated intracellular pH modifications in human neutrophils suspended in $NMDG^+$. In resting neutrophils the activity of proton pumps, such as the vacuolar-type H^+-ATPases, is not measurable in the plasma membrane [35], and therefore H^+ entering the cell may accumulate lowering the intracellular pH. We found that prolonged Na^+-deprivation during priming with GM-CSF led to a significant reduction of basal intracellular pH, likely resulting from loss of intracellular Na^+ through the Na^+/H^+ exchanger. Given that Na^+ and K^+ have been shown to facilitate IP_3-induced Ca^{2+} release from intracellular stores [19], in human neutrophils suspended in $NMDG^+$ the decrease of intracellular $[Na^+]$ is likely to affect the effect of IP_3 on Ca^{2+} mobilization from intracellular stores, resulting in the inhibition of fMLP-induced $[Ca^{2+}]_i$ rise and LTBs formation. However, when we tested amiloride, a reported inhibitor of the Na^+/H^+ exchanger, we could not find any significant modification of LTBs formation, at variance with the effective inhibition observed by Osaki *et al.* [26],

using more potent, but not commercially available, analogs of amiloride to modulate A23187-dependent LT formation in human neutrophils. It must be noted that neutrophil activation through a receptor-mediated stimulus, such as fMLP, represents a more sensitive approach, when compared to the Ca^{2+} ionophore A23187, for the evaluation of signal transduction mechanisms.

Limiting the incubation time in Na^+-free medium to a very short interval before fMLP challenge, thus avoiding intracellular Na^+ depletion, did not cause any inhibition of LTBs formation. Rather, the synthesis of LTs was significantly enhanced, as observed with benzamil, the reported inhibitor of the Na^+/Ca^{2+} exchanger. This result clearly suggest that intracellular rather than extracellular concentration of Na^+ represents an important factor in calcium mobilization and synthesis of 5-LO-derived eicosanoids. Furthermore, the increased synthesis of LTBs following challenge of neutrophils in the absence of extracellular Na^+ provides additional evidence that in human neutrophils the Na^+/Ca^{2+} exchanger operates exporting Ca^{2+} and importing Na^+, and not driving Ca^{2+} inside the cells as previously hypothesized and poses a serious challenge to the hypothesis put forward by Simchowitz.

In conclusion, the results of this study demonstrate that prolonged extracellular Na^+-deprivation leads to a significant reduction of receptor-mediated $[Ca^{2+}]_i$ rise in, and LTBs formation by, human neutrophils. At variance with previous results appeared in the literature, this effect is not related to the Na^+/Ca^{2+} exchanger, which operates exporting Ca^{2+} and importing Na^+. Conversely, prolonged extracellular Na^+-deprivation causes cytoplasmic acidification, suggesting intracellular Na^+ depletion through the Na^+/H^+ exchanger. It is therefore possible to hypothesize that subphysiological intracellular Na^+ concentrations affect IP_3-induced Ca^{2+} mobilization from intracellular stores, and LTBs formation in GM-CSF-primed, fMLP-activated human neutrophils.

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