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Ovalbumin sensitization of guinea-pigs reduces fMLP-induced calcium signal in alveolar macrophages

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Abstract

In this study we analyzed the N-formyl-Met-Leu-Phe (fMLP)-induced calcium signal in alveolar macrophages (AM) isolated from ovalbumin-sensitized (OA-sensitized AM) and naive (naive AM) guinea-pigs. Guinea-pigs were sensitized by subcutaneous injection of OA and AM were isolated by bronchoalveolar lavage 6 weeks thereafter. On the following day, we measured in resting and fMLP-stimulated cells: intracellular calcium concentration by fura-2 imaging analysis, forskolin-induced cyclic AMP production and superoxide dismutase inhibitable superoxide anion release of adherent AM. Resting calcium was 82±5.0 nM (n=217) and 144±9.3 nM (n=213, $P<0.001$) in naive and OA-sensitized AM respectively. fMLP ($10^{-11}-10^{-7}$M) induced a dose-dependent calcium increase, 10^{-8}M being the maximal effective dose in both naive and OA-sensitized AM. However, at all doses tested, this fMLP effect was lower in OA-sensitized than in naive AM. While in resting condition 10^{-5}M forskolin increased cyclic AMP both in naive and OA-sensitized AM, in fMPL-stimulated AM forskolin was effective only in OA-sensitized AM. Superoxide anion release measured 10 min after fMLP stimulus was higher in naive than in sensitized AM. These data suggest that the fMLP-induced intracellular signal is different in OA-sensitized AM compared to naive cells.

Keywords: Airway hyperreactivity; Bronchoalveolar lavage; Intracellular calcium; Cyclic AMP; Superoxide anions; Pertussis toxin; Forskolin

Introduction

Alveolar macrophages (AM) represent the prevalent cell population in bronchoalveolar lavage (BAL) and play a pivotal role in pulmonary inflammatory process such as asthma [1,2].

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AM possess the serpentine formyl-methionyl-leucyl-phenylalanine (fMLP) receptor (FPR) [3]. The FPR activation induces an intracellular calcium increase. This calcium rise is mainly dependent on the stimulation of the pertussis toxin (PTX)-sensitive Gi2/Gi3 βγ subunit [4,5,6,7] and α16-containing Gq [8,9]. Indeed, as described by Stickle et al., in quin-2 loaded AM isolated from naive guinea-pigs [10] the formylated peptide formyl norleucyl leucyl phenylalanine (FNLLP) rises intracellular calcium. Moreover, the Gi2/Gi3 alpha subunit of FPR inhibits adenylyl cyclase (AC) [11,12].

The FPR activation also induces superoxide anion (O_2^-) production, by stimulation of NADPH oxidase [13]. Although the calcium increase can influence O_2^- production, the regulation of O_2^- production does not appear to be correlated only with the calcium increase. Rather, in a study of the relationship between calcium and O_2^- production after FPR activation, Watson and co-authors [14] demonstrated that the initial burst of O_2^- production in human neutrophils is calcium dependent, whereas the late, long lasting phase is prevalently sustained by protein kinase C (PKC) activity as appears by inhibiting PKC with staurosporine. Other intracellular signaling routes contribute to the activation of NADPH oxidase after stimulation with fMLP [15,16,17]. Since FPR activation has been extensively studied, cellular responses obtained by FPR occupancy may serve to investigate cell functions in animal models of airway hyperresponsiveness. Among animal models used for study of airway inflammatory pathologies, the guinea-pig is widely used both as normal animals (naive) and after active sensitization (sensitized). Although many reports investigate the responsiveness of AM isolated from sensitized guinea-pigs, no extensive studies have yet been performed to characterize intracellular calcium regulation in sensitized AM compared to naive animals. We therefore studied the fMLP-induced intracellular calcium signal in relationship to superoxide anion production and forskolin-mediated activation of AC in AM isolated from sensitized as compared to those from naive guinea-pigs.

Methods

This investigation conforms to the rules for the care and use of laboratory animals of the European Community.

Sensitization of guinea-pigs

Male guinea-pigs (300–400g) were actively sensitized by s.c. injection of 0.5% ovalbumin (OA) [18]. Animals (500–600 g body weight) were used 4–6 weeks later.

Isolation of AM from naive and OA-sensitized guinea-pigs

AM were isolated from OA-sensitized guinea-pigs (OA-sensitized AM) and age-matched guinea-pigs (naive AM) as described [18, 19]. Anesthetized guinea-pigs (Pentothal, 80 mg kg^{-1}) were tracheoctomized and 5 ml aliquots of PBS were injected. The collected fluid was centrifuged (700 g for 10 min) and, after erythrocyte hypotonic lysis, AM were resuspended in RPMI 1640 (10% fetal bovine serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) and plated on round glass coverslips (25 mm diameter) at a density of 200,000/coverslip for calcium measurement or 1,000,000/well (6 multiwells) for superoxide and cyclic AMP determination. AM were washed after 2 h and used 24 h after planting in order to promote cell ad-

hesion. This isolation procedure yielded a percentage of adherent macrophages more than 95% in both naive and OA-sensitized guinea-pigs.

Imaging analysis of intracellular calcium concentration ([Ca^{2+}]i)

Coverslip adherent AM were loaded with 4 μM fura-2AM, 0.02% Pluronic F [20] (Molecular Probes, Eugene, OR, USA) for 45 min at room temperature in Hepes/ $NaHCO_3$ medium (composition mM: 140 NaCl, 2.9 KCl, 0.9 $MgCl_2$, 0.5 NaH_2PO_4, 12 $NaHCO_3$, 10 mM glucose, 10 mM Hepes, 1.5 $CaCl_2$, adjusted to pH 7.4 with 1 N NaOH).

Fura-2-loaded AM were visualized using a 40× magnification objective of an inverted fluorescence microscope (Nikon Diaphot, Japan). Fluorescence images were collected with an intensified charge-coupled device ISIS-M extended video camera (Photonic Science, U.K.) by means of an image Analysis System "Magiscan" (Applied Imaging, New Castle, U.K.) equipped with a videotape recorder [21].

For quantification of [Ca^{2+}]i, following-in-time images obtained at 340 and 380 nm excitation, emission 510 nm were digitalized by an analogical/digital converter (256 × 256 pixels) and rationed on a pixel-by-pixel basis. A ratio image was obtained every 800 ms. Calibration curves were performed using ionomycin (Calbiochem) and ethylenebis tetraacetic acid (EGTA, Aldrich-Chemi, Steinheim, Germany). After measuring [Ca^{2+}]i in resting condition, fMLP was administered and continuously present during the experiment as indicated.

Experiments in the absence of extracellular calcium ([Ca^{2+}]out) were performed in nominally Ca^{2+}-free medium in which 0.5 mM ethylenediaminetetraacetic acid (EDTA) was added.

All data were exported in ASCII file format and elaborated for graphic presentation using MicroCal Origin® (2.8 version).

PTX preincubation

Naive and OA-sensitized AM were preincubated for 4 h with 1 μg/ml of PTX, washed and used either for [Ca^{2+}]i determination or superoxide anion (O_2^-) production assay.

Cyclic AMP determination

After preincubation (10 min, 37 C°) with Hepes/$NaHCO_3$ buffer containing 100 μM isobutylmethylxanthine (IBMX), AM were incubated with or without 10^{-5} M forskolin, a direct activator of AC [22] either in resting condition or after stimulation with 10^{-8} M fMLP. The reaction was stopped after 10 min with 70% ice-cold ethanol. Cyclic AMP was determined in dried alcoholic extract using a commercial [^{125}I] cyclic AMP RIA Kit (Amersham International) as specified by the manufacturer.

Superoxide anion assay

O_2^- production was evaluated by superoxide dismutase (SOD) inhibitable reduction of ferricytochrome C [19] after a 10 min incubation in resting conditions and after stimulation with 10^{-8}M fMLP. In order to evaluate fMLP-induced O_2^- production, values obtained in resting conditions under different treatments were subtracted.

Statistical methods

For each experiment, at least 5 different AM preparations were used. Values are presented as means ± s.e.mean of analyzed cells. Statistical comparisons between data groups were performed using Student's t test (paired and independent); dose-response curves were also analyzed by one-way ANOVA and linear regression analysis. A P value of 0.05 was considered significant.

fMLP (formyl MET-LEU-PHE), EDTA disodium salt, penicillin, streptomycin, PTX, IBMX, forskolin, SOD and ferricytochrome C were purchased from Sigma-Aldrich s.r.l. (Milan, Italy). RPMI 1640 medium and fetal bovine serum were obtained from Gibco BRL-Life Technologies (Milan, Italy). Cell culture plastic supports were purchased from Costar (Corning Costar Co., Costar Italia, Milan, Italy). All other reagents were of analytical grade.

Results

Resting intracellular calcium concentration

Resting $[Ca^{2+}]i$ was 82±5.0 nM (n=217) and 144±9.3 nM (n=213, $P<0.001$) in naive and OA-sensitized AM respectively.

Effect of fMLP on $[Ca^{2+}]i$

fMLP induced a dose-dependent increase in $[Ca^{2+}]i$ in both naive and OA-sensitized AM, the maximal effective dose being 10^{-8} M (Fig. 1). As shown, the dose-response curve obtained in OA-sensitized AM was shifted to the right, showing that at all doses tested, the cell response was lower than that obtained in naive AM ($F=6.3831$, $P< 0.03$, ANOVA). The time course of the calcium increase was further investigated in naive and sensitized AM at the maximal fMLP dose.

In naive AM, 10^{-8}M fMLP induced a $[Ca^{2+}]i$ increase characterized by a steep, early phase lasting a few seconds (5–10 s). Thereafter, in several cells a slower rising phase until a calcium peak of high intensity occurred. Other cells showed a constant increase in $[Ca^{2+}]i$ until the maximum. Then the $[Ca^{2+}]i$ decreased to a sustained plateau. During this plateau phase, high frequency, high intensity calcium oscillations were observed. In some cases, peaks superimposed on the plateau phase were higher than those observed during the earlier phases (Fig. 2). This kinetic characteristic of the calcium increase is in line with the dual G-protein coupling of FPR.

In OA-sensitized AM the fMLP-induced $[Ca^{2+}]i$ increase was also characterized by a steep, early phase lasting a few seconds (5–10 s), but the maximal $[Ca^{2+}]i$ peak was lower and calcium oscillations were less intense and less frequent than in naive cells (Fig. 3). Indeed, calcium oscillations superimposed on the plateau phase were of lower intensity than the first peak.

In the absence of $[Ca^{2+}]$out, resting $[Ca^{2+}]i$ was slightly decreased both in naive AM (63±5.5 nM, n = 45) and OA-sensitized AM (129±11.8 nM, n = 54, $P<0.01$ vs. naïve, resting). In naive AM the calcium peak induced by 10^{-8} M fMLP was reduced by 78% (Δ $[Ca^{2+}]i$ 563±63.8 nM) and the plateau phase as well as calcium oscillations disappeared. In OA-sensitized AM, the calcium peak was similarly reduced, by 74% (Δ $[Ca^{2+}]i$, 333±35.9

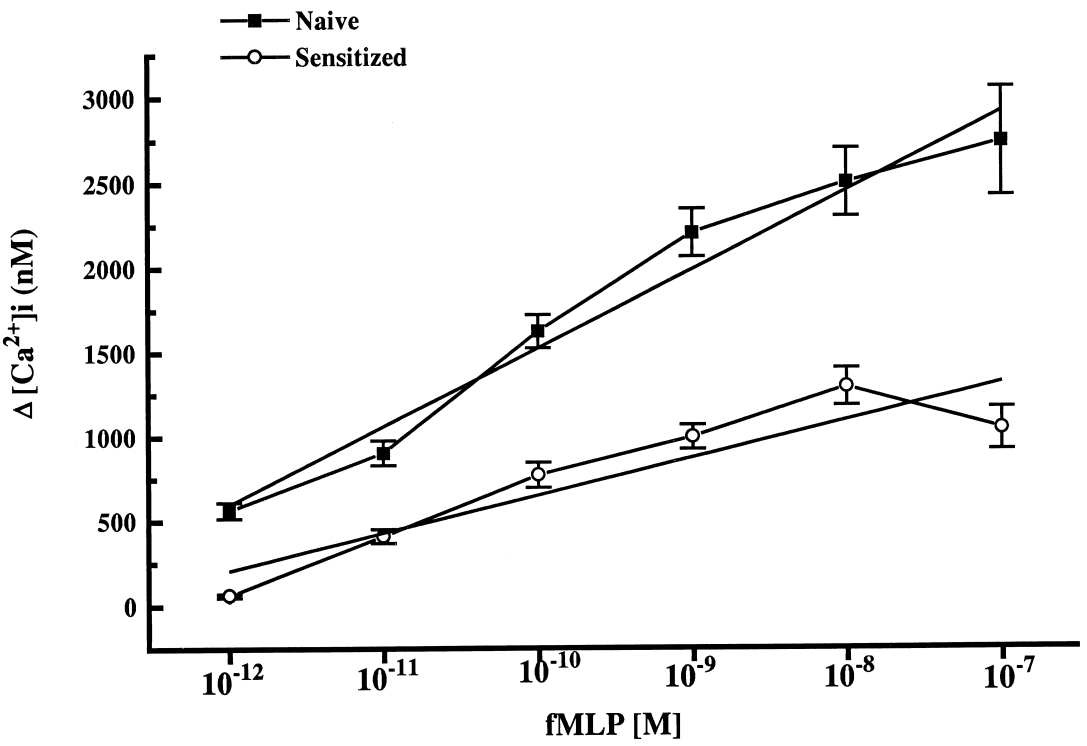

Fig. 1. Dose-response curve of fMLP in naive and OA-sensitized AM. Points are means ± SEM of at least 50 cells obtained in 5 separate experiments.

nM) and plateau phase and calcium oscillations were also absent. Also the preincubation with 3 μM econazole strongly reduced the fMLP-induced calcium oscillations (Fig. 4) both in naïve and OA-sensitized AM. Moreover, when both naive and OA-sensitized AM were preincubated with 30 nM thapsigargin, 10^{-8} M fMLP was ineffective (Fig. 4).

These data indicate that in both naive and sensitized AM the calcium increase is mediated by an intracellular calcium pool discharge and calcium influx, accounting for the plateau phase and calcium oscillation. The calcium influx seems to be mediated trough store-operated calcium channels (SOCC), since the SOCC blocker econazole [23] totally suppressed calcium oscillations.

Effect of PTX on fMLP-induced calcium signal

In both naive and OA-sensitized AM, preincubation with the Gi-protein blocker PTX [24] strongly modified the 10^{-8} M fMLP-induced calcium signal.

The first peak was decreased and oscillations disappeared. PTX incubation, however, reduced the fMLP-induced $[Ca^{2+}]$i increase in OA-sensitized AM less than in naive AM. In naive AM, preincubation with PTX reduced the calcium peak by 62%, while in OA-sensitized AM PTX preincubation reduced it only by about 37%. It should be noted that after PTX preincubation, the time course and intensity of the calcium signal were similar in naive and sensitized AM (Fig. 5). The PTX-insensitive calcium increase is likely to be dependent on activation of the PTX-insensitive Gq/16α protein [7,8].

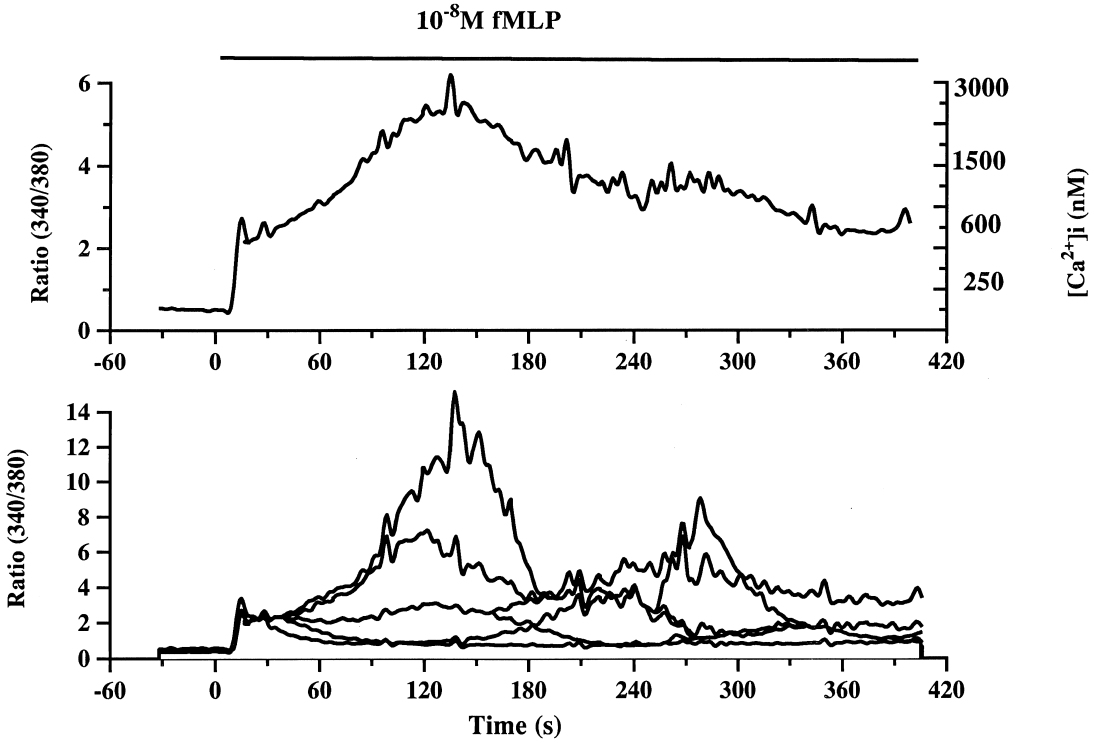

Fig. 2. Typical time-course of 10^{-8} M fMLP-induced calcium signal on the fluorescent ratio (340/380 nm, left axis) and intracellular calcium concentration (right axis) in naive AM. Bottom : each curve is the single response measured in an individual cell. Top: mean time course of analyzed cells.

Cyclic AMP

In resting conditions cyclic AMP content was very similar in naive and OA-sensitized AM. Forskolin administration (10^{-5} M) induced a significant increase in cyclic AMP content, being forskolin effect more pronounced in naive than OA-sensitized AM. Cyclic AMP content was only slightly increased by 10^{-8} M fMLP stimulation (not significant). Whereas in naive AM fMLP pretreatment inhibited the forskolin-induced cyclic AMP increase, in OA-sensitized AM, fMLP pretreatment did not interfere with forskolin-induced cyclic AMP increase. Indeed, in naive AM forskolin-induced cyclic AMP increase was significantly reduced in fMPL-stimulated cells compared to resting conditions (0.57 ± 0.090 pmol/10^6 cells in fMLP-stimulated versus 0.8 ± 0.12 pmol/10^6 cells in resting AM, $P<0.05$), while it was not modified in OA-sensitized AM (0.42 ± 0.089 pmol/10^6 cells in fMLP-stimulated versus 0.47 ± 0.086 pmol/10^6 cells in resting AM).

Superoxide anion production

Since O_2^- radicals are a typical ROS produced by monocyte/macrophage cells after stimulation with fMLP, we tested the release of O_2^- radicals in control conditions and after preincubation with PTX. At rest (fMLP unstimulated cells), the O_2^- release was slightly smaller in

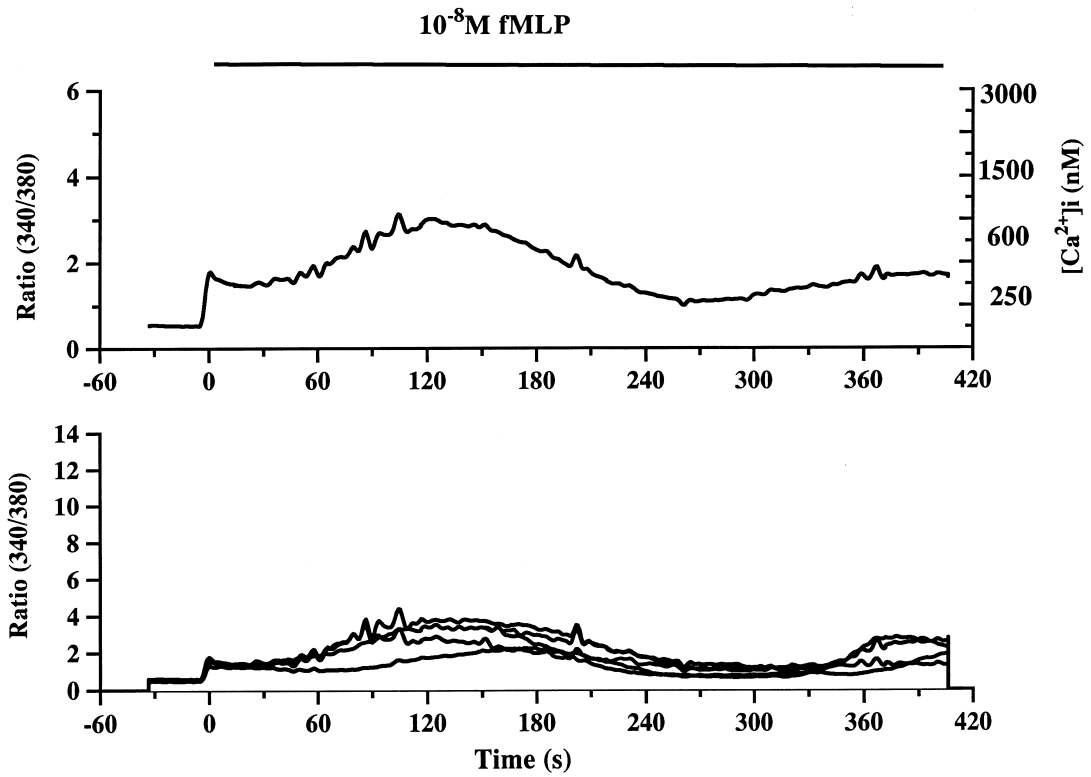

Fig. 3. Typical time-course of 10^{-8} M fMLP-induced calcium signal on the fluorescent ratio (340/380 nm, left axis) and intracellular calcium concentration (right axis) in OA-sensitized AM. Bottom: each curve is the single response measured in an individual cell. Top: mean time course of analyzed cells.

naive (not significant) than in OA-sensitized AM independently from the experimental conditions (0.26 ± 0.153 and 0.34 ± 0.138 nmol $\times$ 10^6 cells in control condition; 0.22 ± 0.086 and 0.25 ± 0.086 nmol $\times$ 10^6 cells in PTX-treated naïve- and OA-sensitized AM respectively). The administration of 10^{-8}M fMLP induced a strong increase in O_2^- production ($P<0.001$ vs. resting in both naive and OA-sensitized AM), O_2^- production in OA-sensitized AM being significantly lower than in naive AM (Fig. 6). Preincubation with PTX strongly decreased O_2^- production. It should be noted that after PTX preincubation, O_2^- production was similar in both naive and OA-sensitized AM.

Discussion

Our study demonstrates for the first time that in OA-sensitized AM $[Ca^{2+}]$i behavior is altered as compared to naive AM. In OA-sensitized AM, the resting calcium is higher than that measured in naive cells, suggesting that OA-sensitized cells are in such an "activated condition" before agonist administration. On the other hand, the fMLP-induced calcium increase in OA-sensitized AM is lower compared to naive AM as demonstrated by the shift to the right of the dose-response curve. Also a remarkable difference in the calcium signal is the low fre-

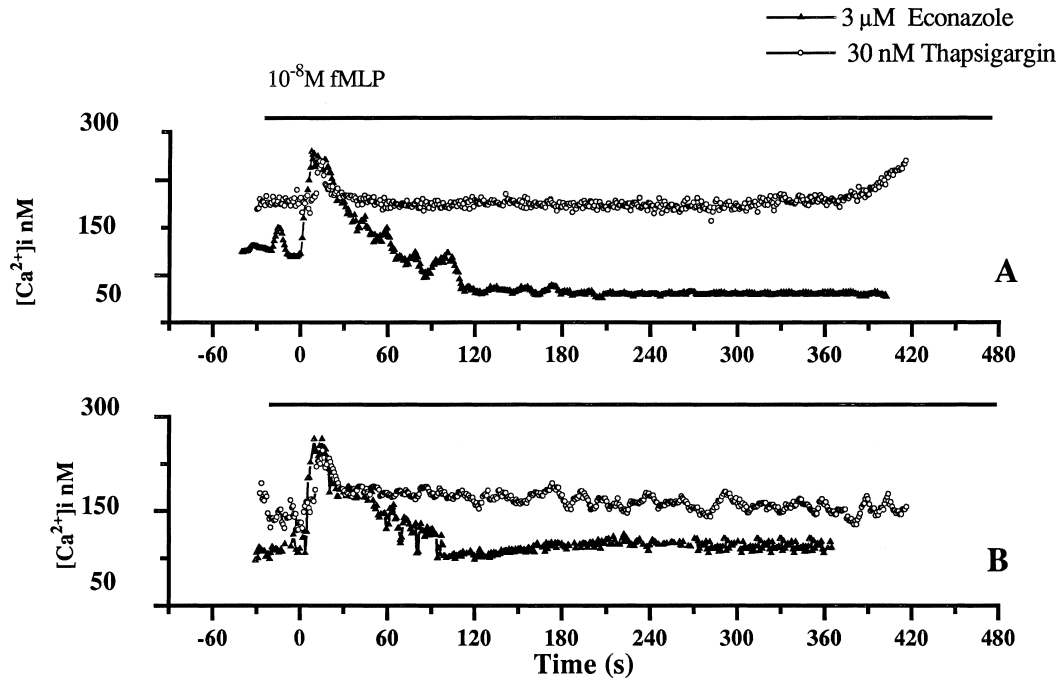

Fig. 4. Typical time-course of 10^{-8} M fMLP-induced calcium signal on intracellular calcium concentration in either thapsigargin- or econazole-pretreated naive (panel A) and OA-sensitized (panel B) AM. Each curve is the mean of at least 12 analyzed cells.

quency, smooth amplitude of calcium oscillations in OA-sensitized AM compared to the high frequency, high amplitude in naive AM. This may imply a different IP_3 generation by FPR activation and/or a different behavior in the refilling mechanisms of intracellular calcium stores. Further research will clarify this point.

Experiments performed in PTX-preincubated AM demonstrate that the block of Gi protein

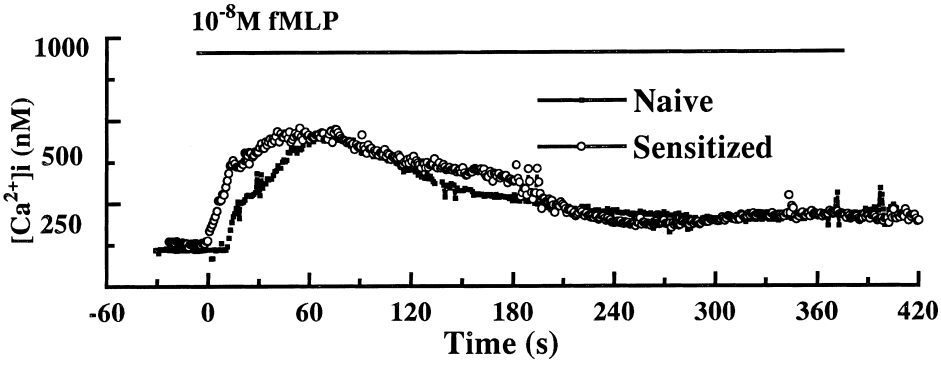

Fig. 5. Typical time-course of 10^{-8} M fMLP-induced calcium signal on intracellular calcium concentration in PTX-pretreated naive and OA-sensitized AM. Each curve is the mean of at least 12 analyzed cells.

Table 1
Forskolin effect on cyclic AMP content of naive and OA-sensitized AM in resting and 10^{-8}M fMLP-stimulated cell

	Cyclic AMP content (pmol/10^6 cells)			
	Resting AM		fMLP-stimulated AM	
	Control	10^{-5}M Forskolin	Control	10^{-5}M Forskolin
Naive AM	0.61±0.068	1.41±0.157*	0.85±0.128	1.42±0.235
OA-sensitized	0.63±0.058	1.10±0.127*	0.88±0.108	1.30±0.175*

Values are means±SEM of 7 experiments performed in triplicate.* P<0.05 vs control, paired Student's t test.

strongly reduces the fMLP-induced calcium signal in naïve AM, whereas this reduction is less intense in OA-sensitized AM. Indeed after PTX preincubation, the calcium signal is similar in naive and sensitized AM. A decreased functional Gi-coupled FPR in sensitized AM may justify this reduced PTX responsiveness.

Also experiments performed with forskolin indicate a decreased functional Gi-coupled FPR in OA-sensitized AM. A functional coupling of Gi protein to FPR would decrease the forskolin-induced cyclic AMP increase, since the activation of FPR inhibits AC trough pertussis sensitive Gi2/Gi3 proteins [11,12].

Indeed, the stimulatory forskolin effect is lost in fMLP-stimulated naive AM, whereas it is still effective in sensitized AM. These data further suggest a different expression of functional Gi-coupled FPR in OA-sensitized AM. Also an enhanced AC activity can explain our data. An enhanced AC responsiveness in sensitized AM has also been demonstrated in whole AM stimulated by β-adrenergic drugs [25] and in AM membrane preparations after stimulation with β-adrenergic agents and PGE2 [26].

An altered regulation of cyclic AMP can influence calcium signal. Indeed, the Gβγ-dependent

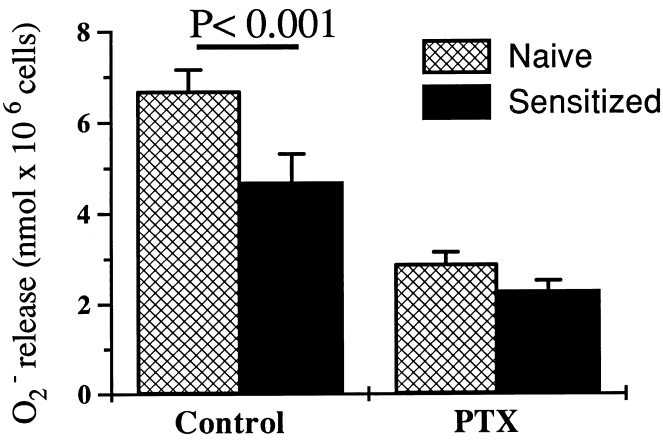

Fig. 6. 10^{-8} M fMLP-induced superoxide ion production in naive and OA-sensitized AM in control conditions and in cells preincubated with PTX for 4 h. Values are the mean (± SEM) of 7 different preparations. ** P< 0.01, independent Student's t test.

activation of PLCβ2 is inhibited by specific PKA-mediated phosphorylation [27]. On the other hand, the PTX-independent Gq-pathway would be only marginally affected by an altered cyclic AMP regulation. Our data show that only the PTX-dependent signal is modified in OA-sensitized AM. Indeed, in PTX-treated cells, the fMLP-induced calcium signal is similar in naïve and OA-sensitized AM, whereas in control conditions OA-sensitized AM show a decreased fMLP responsiveness.

Cyclic AMP can also reduce O_2^- production in macrophages [28]. fMLP-induced O_2^- production is mediated by the calcium increase in the early phase of NADPH oxidase activation [14], while in the late phases of O_2^- production, PKC-dependent NADPH oxidase activation can supply the limited calcium regulation. Therefore, the reduced O_2^- production in OA-sensitized AM after a short time of fMLP incubation, reflects the lower calcium signal in OA-sensitized compared to naive AM. The different incubation time can explain the discrepancy between this result and previous work showing similar O_2^- production in naive and sensitized AM after 1 hour of fMLP stimulation [19].

PTX preincubation strongly reduces O_2^- production in both naive and OA-sensitized AM, further supporting a prominent role of Gi in FPR activation. This reduction is similar in OA-sensitized and naive AM. As shown for calcium increase, O_2^- production after PTX preincubation is no longer different in naive and sensitized AM. Moreover, an increase in cyclic AMP can also reduce O_2^- production in macrophages [28].

In conclusion, our study demonstrates that OA-sensitized versus naive AM have different behavior in calcium regulation in response to FPR activation. These decreased responsiveness can influence functional characteristics of OA-sensitized AM.

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