Prolonged elevation of intracellular cyclic AMP levels in U937 cells increases the number of receptors for and the responses to formyl-methionyl-leucylphenylalanine, independently of the differentiation process

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The effects of elevated levels of cyclic AMP induced by cholera toxin (CTx) were investigated on the differentiated promyelomonocytic cell line U937. After CTx treatment, the initial inhibition of the oxidative burst induced by *N*-formylmethionyl-leucyl-phenylalanine (FMLP) was followed by a progressive increase over 20 h, resulting in 4–6-fold potentiation of the initial burst. Various cyclic-AMP-elevating agents produced similar potentiation of the FMLP- or C5a-induced oxidative burst, but the phorbol 12-myristate 13-acetate-induced oxidative burst was not affected by CTx pretreatment of cells. Furthermore, the increase in arachidonate release and intracellular Ca^{2+} triggered by FMLP were amplified after CTx treatment. ADP-ribosylation

of $G_i\alpha$ subunits catalysed by pertussis toxin was slightly increased after CTx treatment, despite similar immunoreactivity of the α subunit of G_i2 . FMLP binding sites present in CTx-treated membranes were 3–6 times more abundant than in control membranes. Expression of mRNAs encoding the FMLP receptor and one of its related receptors were enhanced after CTx treatment of both differentiated and undifferentiated U937 cells. In parallel, after undifferentiated cells were treated with CTx, they were able to increase intracellular Ca^{2+} , but not the oxidative burst, in response to FMLP. These data demonstrate that CTx, by increasing cyclic AMP, enhances the expression of chemotactic receptors independently of U937 cell differentiation.

INTRODUCTION

Binding of bacterial formyl-peptides, such as *N*-formyl-methionyl-leucylphenylalanine (FMLP) or C5a anaphylatoxin, to specific human leucocyte receptors (e.g. monocytes or neutrophils) evokes many responses *in vivo* or *in vitro* that determine the roles of these compounds in inflammatory processes [1,2]. The responses of phagocytic cells include chemotaxis, exocytosis of lysosomal contents and generation of highly reactive oxygen intermediates (ROI). FMLP responses are mediated by a recently cloned [3] seven-transmembrane-domain receptor and pertussis toxin (PTx)-sensitive G_i proteins (G_i2 and/or G_i3), whose $\beta\gamma$ -dimer was shown to activate phospholipase $C\beta_2$, thus triggering the production of inositol phosphates and diacylglycerol [4]. FMLP generation of superoxide ions can be inhibited by increasing the intracellular concentration of cyclic AMP via β -adrenergic stimulation [5], by dibutyl cyclic AMP (dbcAMP) in guinea-pig neutrophils [6], and by cyclic AMP in electropermeabilized neutrophils [7,8]. High levels of cyclic AMP inhibit phospholipase A_2 activity, partly due to phosphorylation of several substrates by protein kinase A.

It is difficult to conduct studies on the regulation of FMLP and other chemotactic receptors in human monocytes or macrophages, mainly because of donor variability and heterogeneity of cellular activation states. Cell lines such as HL-60, THP-1 and U937 have therefore been used in most studies. These proliferative cells can acquire characteristic properties of granulocytes or macrophages by differentiation under the actions of pharmacological drugs or physiological agents. For instance, phorbol-ester-differentiated HL-60 cells acquire markers of matured monocytes, but without expressing the formyl-peptide receptor

(FPR). HL-60 cells differentiated by dimethyl sulphoxide or dbcAMP exhibit functional receptors for chemotactic stimuli, along with neutrophil-like markers.

Besides their effects on cell proliferation and cell differentiation, high levels of cyclic AMP have been shown to modulate the expression of several receptor genes. Down-regulation of the β -adrenergic receptor [9] and platelet-activating-factor receptor [10] have thus been observed at transcriptional and post-transcriptional levels. FPR regulation appears rather complex, and no clear responsive elements have been characterized [11]. Indeed, the C5a-receptor gene, FPR gene and two closely related genes encoding orphan receptors (identified as FPRH2 [12], FPRL2 [13] and FPR2 [14] for the first one and FPRH1 [12], RFP [15] and FPRL1 [16] for the second) have been reported to map to chromosome 19 [17] and form a chemotactic-receptor cluster. Modulation of FPR-mRNA expression by cyclic-AMP-elevating agents was observed on HL-60 cells in the early phase of granulocytic differentiation [18]. However, it could not be clearly determined whether this effect was solely due to an increase in cyclic AMP or whether it was part of the differentiation process.

The aim of the present study was therefore to clarify the effects of elevated levels of cyclic AMP on macrophages, since it inhibits the oxidative response induced by FMLP, and potentially activates the expression of the gene encoding the FMLP chemotactic receptor. We thus used human leukaemic U937 cells which were differentiated by a combination of 1,25-dihydroxyvitamin D_3 (VD) and retinoic acid (RA). These physiological agents have been shown [19] to induce cellular differentiation, leading to the expression of the monocytic/macrophagic markers CD14, CD68, CD11b. Cholera toxin (CTx) was used as an intracellular cyclic-

Abbreviations used: FMLP, *N*-formyl-methionyl-leucylphenylalanine; C5a, fifth fraction of complement anaphylatoxin; FPR, formyl-peptide receptor (Swiss-prot accession no. P21462); FPR1 or FPR2 (Swiss-prot P25089 and P25090 respectively), formyl-peptide-related receptors (cloned orphan receptors homologous to FMLP receptors); RA, retinoic acid; VD, 1,25-dihydroxyvitamin D_3 ; CTx, cholera toxin; ROI, reactive oxygen intermediates; dbcAMP, dibutyl cyclic AMP; IBMX, 3-isobutyl-1-methylxanthine; PTx, pertussis toxin; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; PMA, phorbol 12-myristate 13-acetate.

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AMP-elevating agent since, by ADP-ribosylation of the α subunit of the heterotrimeric G_s protein, the toxin is able to sustain elevated adenylate cyclase activity [20]. We demonstrate that a prolonged high level of intracellular cyclic AMP rapidly reverses the initial inhibitory action on the FMLP-induced oxidative burst through an increase in the number of FMLP receptors.

MATERIALS AND METHODS

Materials and chemicals

The cell line U937 was originally obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Culture media, i.e. RPMI 1640, L-glutamine, fetal-calf serum and PBS, were obtained from Gibco BRL (France). RA and CTx were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and VD was generously given by C. Damais (INSERM U-313, France). PTx was purchased from List Biological Laboratories (Campbell, CA, U.S.A.), Fura-2/AM was obtained from Molecular Probes (Eugene, OR, U.S.A.), and ionomycin from Calbiochem (San Diego, CA, U.S.A.). Radiolabelled compounds, i.e. [3 H]arachidonic acid, [3 H]FMLP, [3 H]adenine and [32 P]NAD $^+$, were purchased from New England Nuclear-DuPont (Boston, MA, U.S.A.).

Culture, differentiation and treatment of U937 cells

U937 cells were routinely grown in RPMI 1640 supplemented with 10% heat-inactivated fetal-calf serum and 2 mM L-glutamine, in the absence of antibiotics. Cells were cultured in a humidified atmosphere (5% CO $_2$), at a cell density of $(0.2-1) \times 10^6$ cells/ml. Cells in the exponential growth phase, at 2×10^5 cells/ml, were differentiated for 72 h with a combination of RA and VD at 100 nM each. After scraping, cells were centrifuged and incubated for 24 h at $(1-2) \times 10^6$ cells/ml in fresh medium alone or with CTx at 1 ng/ml, except when otherwise stated. Cells were routinely tested and found to be free of mycoplasma by 4,6-diamino-2 phenylindole (DAPI) fluorescence.

Respiratory burst

The oxidative burst was assayed by luminol-dependent chemiluminescence in an automatic luminescence analyser (Lumicon, Hamilton, Switzerland), by using 0.95 ml of PBS containing about 5×10^6 cells and luminol at 5 μ g/ml. Stimulations were started with 50 μ l of each triggering agent, and chemiluminescence was routinely recorded for 10 min. The oxidative response induced by phorbol 12-myristate 13-acetate (PMA; 100 ng/ml) was also assessed by measuring the superoxide dismutase-inhibitable reduction of cytochrome *c* as previously described [21].

Release of arachidonic acid in U937 cells

Approx. $(1.75-2) \times 10^6$ cells/ml were treated with CTx, as described above, or left untreated as controls. After 12 h incubation, phospholipid labelling was carried out for another 12 h with 0.4 μ Ci/ml [3 H]arachidonic acid (sp. radioactivity 218.2 Ci/mmol). At the end of the radioactive labelling, 10% of the total added precursor was incorporated. Cells were scraped, extensively washed with PBS (containing 1.3 mM MgCl $_2$, 0.3% BSA and 0.1% glucose), resuspended at 5×10^6 cells/ml, supplemented with 40 μ M arachidonic acid and kept on ice. Assays were started by adding 200 μ l of the packed labelled cell suspension to 200 μ l of supplemented PBS at 37 °C, containing either ionomycin or FMLP. Reactions were stopped by 10-fold dilution with ice-cold TED buffer, containing 20 mM Tris/HCl, pH 8.0,

1 mM EDTA and 1 mM dithiothreitol, followed by centrifugation for 15 min at 600 *g* and 4 °C. Samples (1 ml) of supernatant were counted for radioactivity.

Measurements of cytosolic Ca $^{2+}$ concentration ([Ca $^{2+}$])

After CTx treatment, as described above, scraping and washing, RA/VD-differentiated U937 cells (2×10^6 cells/ml) were loaded with 2.5 μ M fura-2 AM for 30–45 min at 37 °C in fresh RPMI 1640. After two washes, cells were resuspended in PBS and stored on ice until used. Fluorescence measurements were performed with $(2.5-5) \times 10^5$ cells in a 2 ml cuvette at 37 °C, under magnetic stirring in a spectrofluorimeter (Hitachi), thus enabling almost simultaneous measurement at two excitation wavelengths, λ_{exc} 340 nm and 380 nm; λ_{em} 540 nm. Ca $^{2+}$ was analysed as previously described [22].

SDS/PAGE, Immunoblotting and ADP-ribosylation

Membranes from CTx-treated or control cells were obtained after cell lysis at 4 °C in lysis buffer (50 mM Tris/HCl, pH 7.5, 3 mM EDTA, 5 μ g/ml soybean trypsin inhibitor), followed by centrifugation at 4000 *g* for 25 min at 4 °C. Proteins (25–75 μ g/lane) were either ADP-ribosylated or not *in vitro* by 100 ng/ml PTx in the presence of [32 P]NAD $^+$ as described elsewhere [23], before separation by SDS/PAGE. Proteins were electrotransferred by a semi-dry blotting technique on to poly(vinylidene difluoride) membranes (Immobilon-P; Millipore). Immunoblotting was performed on membranes not ADP-ribosylated *in vitro*, by using a polyclonal affinity-purified α_2 C-terminus-directed antiserum. Labelling of the immune complex was carried out with 125 I-Protein A, followed by overnight autoradiography without an intensifying screen.

[3 H]FMLP binding and Scatchard analysis

Membranes were prepared as follows at 4 °C. After cell lysis, the supernatant from a first centrifugation at 1000 *g* was centrifuged at 40000 *g* for 25 min. Samples (35 μ g) of these membranes were incubated for 30 min with tritiated ligand before filtration on to GF/C filters (Whatman). Non-specific binding was determined in the presence of 0.1 mM FMLP and never exceeded 10% of total binding. Scatchard analysis was done by linear regression.

Cyclic AMP accumulation assay

Intracellular cyclic AMP was measured by the prelabelling technique, as described previously [24]. Cells were treated with CTx at 1 ng/ml for 24 h and incubated for another 2 h with 2 μ Ci/ml [3 H]adenine. Cells were then washed twice in RPMI and resuspended in PBS containing 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX) before stimulation with isoprenaline (1 μ M) for 5 min. The reaction was stopped by addition of 5% trichloroacetic acid and 250 μ M of both cyclic AMP and ATP. Particulate fractions were discarded by centrifugation at 5000 *g* for 5 min. Cyclic [3 H]AMP and [3 H]ATP present in the supernatant were separated by sequential chromatography [25].

Semi-quantitative measurements of abundance of the various FPR mRNAs by reverse-transcriptase PCR

mRNAs from U937 cells were isolated by using the Pharmacia RNA purification kit according to the manufacturer's instructions. cDNAs were synthesized with 200 units of Superscript II reverse transcriptase (Gibco) and 10 μ Ci of [α - 32 P]dCTP, with 5 μ g of total mRNA as template. Reverse-transcription products were quantified by filtration after precipitation of 2 μ l of the

samples with 5% trichloroacetic acid and diluted to 1 ng/ μ l with diethyl pyrocarbonate-treated water. FPR- and FPRII-mRNA levels were quantified by cDNA-based PCR using different primers chosen in coding regions of the genes: 5'-CCATGTTG-ACGGTGAGAGGC-3' and 5'-AGGGCACTTGTCACA-TCCAC-3' for FPR, 5'-CCAGAGGGATTATCCGGTTTG-3' and 5'-TGGCAGCCGTGTCATTAGTTG-3' for FPRII. Amplifications were done at various number of cycles in the presence of [α - 32 P]dCTP. In each amplification cycle, 8 μ l of sample was analysed by PAGE and, after ethidium bromide staining, radiolabel incorporation in the PCR product was determined after excision of each corresponding band (FPR 280 bp, FPRII 413 bp, β 2-microglobulin 268 bp). The values of the counts were plotted on a log scale versus the number of amplification cycles. Values were then normalized with those obtained for the β 2-microglobulin amplification product.

RESULTS AND DISCUSSION

U937 cells were treated with a combination of RA and VD for cell differentiation, to become monocytic or macrophagic. Cells were then responsive to various extracellular signals such as FMLP, which induces production of ROI through activation of the NADPH oxidase complex. Indirect analysis, by luminol-dependent chemiluminescence, of superoxide generation revealed that production was maximal about 60 s after the addition of FMLP and then returned to the basal level within 2 min (Figure 1). When cells were pretreated with CTx for 4 h, followed by extensive washing, the time course of FMLP-induced superoxide production remained unaltered. However, the amplitude of the response varied with time (Figure 1). In the first 4 h after pretreatment, the recorded signal was progressively decreased to a minimum value of about 50% of the control response. The mean FMLP-induced oxidative burst obtained on control cells was $(1.02 \pm 0.26) \times 10^5$ c.p.m. Thereafter the FMLP response gradually increased, returning to its control level after 10 h of treatment, and finally resulting in 4-fold potentiation of the original response, to $(4.33 \pm 0.64) \times 10^5$ c.p.m. after 24 h.

The initial inhibitory phase was expected, since it is well documented that, with many leucocytic cells, elevated cyclic AMP levels decrease or prevent activation of neutrophils [5,7,26], guinea-pig macrophages [27] or HL-60 cells [8]. In contrast, the

Figure 1 Effect of CTx treatment on FMLP-induced oxidative burst in U937 cells

Cells were differentiated and treated as described in the Materials and methods section, and oxidative burst was induced by FMLP (1 μ M) after different treatment times with 1 ng/ml cholera holotoxin. The resulting chemiluminescence was expressed as c.p.m. The graphs show the results of a single experiment which was reproduced three times with similar results.

Table 1 Effect of cyclic-AMP-elevating agents on the FMLP-, C5a- and PMA-induced oxidative burst

U937 cells were differentiated and treated for 24 h with agents listed in the second column. Oxidative bursts were triggered by the addition of 1 μ M FMLP, 1 nM C5a or 100 ng/ml PMA and luminol-dependent chemiluminescence was measured. The results of three independent experiments are expressed as means $\pm$ S.E.M. of the potentiation obtained in cells with the triggers alone, or after pretreatment with CTx, forskolin, dbcAMP or IBMX.

Triggering agent	Cell treatment	Potentiation (fold)
FMLP	(1 μ M)CTx (1 ng/ml)	2.93 $\pm$ 1.0
	dbcAMP (3 mM)	2.49 $\pm$ 0.9
	Forskolin (10 μ M)	3.81 $\pm$ 1.8
	IBMX (300 μ M)	1.84 $\pm$ 0.2
C5a (1 nM)	CTx (1 ng/ml)	2.26 $\pm$ 0.1
	dbcAMP (3 mM)	3.56 $\pm$ 1.7
	Forskolin (10 μ M)	4.72 $\pm$ 0.5
	IBMX (300 μ M)	2.90 $\pm$ 0.9
PMA (100 ng/ml)	CTx (1 ng/ml)	1.07 $\pm$ 0.11

potentiation phase leading to an increased FMLP response was unexpected. This potentiation after CTx pretreatment rose dose-dependently with 0.01–1 ng/ml CTx (results not shown). The continuous presence of the toxin at 1 ng/ml in the incubation medium resulted in magnified responses, similar to those obtained when cells were washed after 4 h pretreatment. The following experiments described in this study were carried out in the continuous presence of toxin in the incubation medium at 1 ng/ml.

To determine whether this potentiating effect of CTx at 1 ng/ml was solely due to elevated intracellular cyclic AMP levels, cells were treated with other agents known to increase intracellular cyclic AMP levels. Table 1 indicates that treatments with dbcAMP, forskolin or the phosphodiesterase inhibitor IBMX significantly increased production of the superoxide ion induced by FMLP. Comparable effects were observed when treated cells were stimulated with C5a [28], suggesting similar modulation of both chemotactic peptide responses to elevated cyclic AMP. In contrast, the superoxide anion production triggered by PMA remained unchanged after CTx treatment. This result, obtained with the luminol-dependent chemiluminescence technique (Table 1), was confirmed by using the cytochrome *c* method (results not shown). This implied that CTx treatment did not significantly affect the NADPH oxidase components or the myeloperoxidase activity involved in the sensitive chemiluminescence method. Long-term elevation of cyclic AMP thus seemed to affect the chemotactic-receptor signalling pathway upstream from components responsible for the oxidative response.

The effects of CTx on arachidonate release were then investigated on RA/VD-differentiated U937 cells, since cyclic AMP was found to exert an inhibitory effect on permeabilized HL-60 by inhibiting formyl-peptide-stimulated phospholipase A_2 . FMLP induced concentration dependent arachidonate release in both control and CTx-treated cells, but at 100 nM FMLP the maximal level reached was 3.5-fold higher in CTx-treated cells than in control cells (Figure 2). This enhanced release of arachidonate in treated cells cannot be attributed to an increased quantity of phospholipase A_2 , since basal arachidonate release and ionomycin-induced release ($5.29 \pm 0.49\%$ in control, versus $4.31 \pm 0.28\%$ in CTx-treated cells) were not significantly enhanced by the toxin treatment. Potentiation of the arachidonate response thus appeared to be due to higher mobilization or activation of phospholipase A_2 .

To examine whether the increase in $[Ca^{2+}]_i$, essential for translocation of the phospholipase A_2 [29], would also be affected

Figure 2 Effect of CTx on FMLP- and ionomycin-induced release of $[^3\text{H}]$ arachidonate

Cells were treated as described in the Materials and methods section and grown in the presence of $[^3\text{H}]$ arachidonic acid at $0.4 \mu\text{Ci}/\text{ml}$ for 12 h. Cells were then challenged for 15 min with various concentrations of FMLP ($\circ$, $\bullet$) or $10 \mu\text{M}$ ionomycin (Δ , $\blacktriangle$) in control ($\circ$, Δ) or CTx-treated cells ($\bullet$, $\blacktriangle$). Basal release of $[^3\text{H}]$ arachidonate represented 4% of the incorporated labelled precursor. Released $[^3\text{H}]$ arachidonate was determined in duplicate. The results are expressed as percentages of the incorporated precursor, and represent means $\pm$ S.E.M. of four separate experiments. Statistics by Student's *t* test: significantly different from untreated cells; **P* < 0.005; ***P* < 0.001.

Figure 3 FMLP-activated $[\text{Ca}^{2+}]_i$ variations in CTx-treated cells

Fura-2-loaded cells (10^6) were diluted with 1.5 ml of PBS and incubated at 37°C . After stabilization of baseline fluorescence, $1 \mu\text{M}$ FMLP was added to cells treated with different concentrations of CTx. Traces are from one experiment representative of four separate experiments.

after CTx treatment, FMLP-induced increase in $[\text{Ca}^{2+}]_i$ was studied with the fluorescent probe Fura-2 (Figure 3). Ca^{2+} levels in resting cells were not significantly modified by CTx treatment, but FMLP-induced increases in $[\text{Ca}^{2+}]_i$ rose from $79 \pm 11 \text{ nM}$ in control cells to $684 \pm 79 \text{ nM}$ in treated cells. An enhanced increase in $[\text{Ca}^{2+}]_i$ was detected after CTx treatment at concentrations as low as $10 \text{ pg}/\text{ml}$. It has been reported that, in rat T-cells, the B oligomer of the CTx can alone induce (at $10 \mu\text{g}/\text{ml}$) an intracellular increase in Ca^{2+} , triggering proliferation [30]. To determine whether this was the case in differentiated U937 cells, we also treated cells with B oligomer at concentrations ranging from $100 \text{ pg}/\text{ml}$ to $100 \text{ ng}/\text{ml}$. A potentiating effect on the FMLP-

Figure 4 Immunoblot and PTx-catalysed ADP-ribosylation of CTx-treated cells

U937 membranes treated *in vivo* with CTx at different indicated times were revealed with an anti- α_2 C-terminus-directed antibody, or were subjected to ADP-ribosylation *in vitro* in the presence of $100 \text{ ng}/\text{ml}$ dithiothreitol-activated PTx and $[^{32}\text{P}]\text{NAD}^+$. Similar results were obtained in three separate experiments.

Figure 5 Effect of CTx treatment on FMLP-binding on U937 cell membranes

Membranes of CTx-treated cells ($\bullet$) or control cells ($\circ$) were incubated at 25°C . Saturation curves were plotted and the Scatchard analysis of $[^3\text{H}]$ FMLP binding on membranes presented here shows one representative experiment. Parameters (K_d and B_{max}) were determined graphically for each experiment where total binding was assayed in triplicate.

induced $[\text{Ca}^{2+}]_i$ increase was observed, but this occurred at a 100-fold higher concentration than with CTx holotoxin (results not shown). The fact that the B oligomer was much less active than the holotoxin was not in favour of a direct action of the B oligomer, but suggested that its effect was due to holotoxin contamination of the preparation.

The dose-response curve for CTx on FMLP-induced elevation of $[\text{Ca}^{2+}]_i$ parallels that of the FMLP-induced oxidative burst (results not shown). These matching responses suggested that the two potentiating effects of 24 h CTx treatment were derived from the same signalling modification. It probably occurred at the receptor-coupling level, before the increase in $[\text{Ca}^{2+}]_i$ and phospholipase A_2 activation. FPR is coupled to PTx-sensitive G_{i2} or G_{i3} [31]. Figure 4 revealed no modified immunoreactivity towards α_2 (α subunit of G_{i2}) over the course of the 24 h CTx treatment of U937 cells, indicating that there was no increase in the α_2 content in the cells. However, ADP-ribosylation of both α_2 and α_3 substrates catalysed by PTx was progressively enhanced during CTx treatment. A similar facilitated effect on PTx-catalysed ADP-ribosylation of α_1 subunits has been shown to occur on guinea-pig alveolar macrophages [27], detected after only 4 h of CTx treatment. In that case, however, no changes in FMLP- or platelet-activating-factor-induced stimulation of phospholipase A_2 were detected. The exact nature of the molecular modifications produced by the CTx treatment has not yet been elucidated, but the α_i modification might only affect a

Table 2 Effect of CTx treatment on cyclic AMP accumulation and formyl-peptide mRNAs in undifferentiated and differentiated U937 cells

Cyclic AMP formation was induced (or not) by isoprenaline (1 μ M) on either undifferentiated or RA/VD-differentiated cells. Accumulation of cyclic [3 H]AMP in control or CTx treated cells (1 ng/ml) was measured after 10 min in the presence of 0.5 mM IBMX. Data are expressed in c.p.m. and represent means $\pm$ S.E.M. of quadruplicate determinations in one experiment out of three performed independently. Semi-quantitative PCR analysis of formyl-peptide mRNA transcription was determined, including [α - 32 P]dCTP, as described in the Materials and methods section. Relative transcription levels were evaluated with 2 ng of the four cDNAs with each condition: undifferentiated, CTx-treated undifferentiated, differentiated and differentiated U937 cells treated with CTx. Amplifications were carried out in parallel with the three pairs of primers (FPR, FPRII and β_2 -microglobulin). The results are expressed for each type of receptor as percentages of the maximum gene-transcription activity obtained for differentiated U937 cells after CTx treatment. The results of the two experiments are presented.

	Cells			
	Undifferentiated		Differentiated	
	0	CTx	0	CTx
Cyclic AMP level				
Basal	498 $\pm$ 73	1559 $\pm$ 36	427 $\pm$ 21	1875 $\pm$ 71
+ Isoprenaline (1 μ M)	5517 $\pm$ 197	5401 $\pm$ 214	6689 $\pm$ 148	5411 $\pm$ 261
Receptors cDNA (%)				
FPR: Expt. 1	12.8	65.8	50.7	100
Expt. 2	20.0	95.9	53.3	100
FPRII: Expt. 1	44.9	100	34.9	100
Expt. 2	29.9	81.5	25.1	100

pool of the α_1 population not involved in FMLP-induced responses. To gain further insight into possible modifications of the FMLP signalling pathway induced by CTx treatment, we carried out binding studies with [3 H]FMLP on purified plasma membranes, from control or CTx-treated U937 cells. Scatchard-plot analysis (Figure 5) revealed no significant changes in the affinity of FMLP for binding sites in the control and treated cells (K_d values 0.4–1.5 nM for control cells and 0.3–0.9 nM for treated cells). Interestingly, the number of binding sites was strongly enhanced by CTx treatment (3.6–6.7-fold). These results are similar to those obtained previously by Banno et al. [32] on an osteoclast-like cell line, where CTx enhanced one of the two bradykinin-receptor subtypes present in this type of cell. In contrast, vasopressin and angiotensin receptors have been shown to be down-regulated by CTx. To determine whether the CTx treatment would also affect receptors coupled to G_i proteins, the increased intracellular cyclic AMP induced by stimulation of the β -adrenergic receptor was assessed in differentiated or undifferentiated cells (Table 2). In control U937 cells, after addition of the β -adrenergic agonist isoprenaline, substantial accumulation of cyclic AMP was noted in both differentiated and undifferentiated cells (about 5% of the radioactive ATP pool). This indicated that the β -adrenergic-receptor transduction pathway leading to adenylate cyclase activation was fully functional in undifferentiated cells. As expected, when cells were treated for 24 h with CTx, basal cyclic AMP production was enhanced at least 3-fold. This high level of cyclic AMP did not affect the response to isoprenaline, regardless of whether or not cells were differentiated with RA/VD. Elevation of cyclic AMP concentrations, induced by the CTx treatment, therefore did not affect β -adrenergic responsiveness [33], but it dramatically affected FMLP-induced responses by increasing the number of agonist-binding sites.

We next decided to determine whether CTx treatment was unmasking cryptic FPR, such as those sequestered in neutrophil secretory vesicles [34], or whether it promoted synthesis of FPR *de novo*. This question was investigated by semi-quantitative

Figure 6 Analysis of the effect of CTx on transcription of mRNA for FPR and FPRII in U937 cells by semi-quantitative PCR analysis

PCR products obtained after 35 cycles of PCR amplification of 2 ng of cDNA from (1) undifferentiated U937 cells, (2) CTx-treated undifferentiated U937 cells, (3) differentiated U937 cells, and (4) differentiated U937 cells treated with CTx. PCR products were separated by agarose-gel electrophoresis and stained with ethidium bromide. (MW, ladder marker, multimers of 123 bp; β_2 , β_2 -microglobulin.)

determination of the FPR mRNA. In this study, we also included the two FPR-related orphan receptors, i.e. FPR1 and FPRII, which respectively share 56% and 69% homology with FPR, and for which no high-affinity ligand has been described. FPRII only exhibited weak affinity for the FMLP ligand (K_d = 400 nM) and was expressed concomitantly with FPR in cell lines such as HL-60 or U937. FPR1 mRNA was only recently identified in human lung RNA by Northern-blot analysis, and on isolated human monocytes [13]. In our study, detection of FPR, FPR1 and FPRII mRNAs was carried out by reverse-transcription PCR performed on four groups of cells: undifferentiated or differentiated U937 cells, with or without CTx treatment for 24 h. No FPR1 mRNAs were detected in any of the four groups of cells. In contrast with results of other laboratories [12,35], we detected low levels of FPR and FPRII mRNAs in undifferentiated cells. The fact that this detection was only observed after more than 30 cycles of sequential amplification might explain why these mRNAs have not been identified previously in undifferentiated cells (Figure 6). The high sensitivity of radioactive PCR allowed direct comparison of mRNA concentrations after normalization of the amplification products with those of β_2 -microglobulin as invariant cellular probe (Table 2). Interestingly, we found that differentiation of the cells by RA/VD led to increased FPR mRNA levels (4-fold in Expt. 1), whereas those for FPRII remained unchanged (Figure 6). CTx treatment of differentiated cells strongly increased mRNA quantities for both FPR (2.1-fold) and FPRII (3.7-fold). Differentiation would thus only increase FPR mRNA, whereas high levels of cyclic AMP would enhance both receptor mRNAs. Surprisingly, increases in FPR and FPRII mRNA were also noted when undifferentiated cells were subjected to the same CTx treatment. This suggests that FPR expression can be independently up-regulated by cyclic AMP.

Figure 7 Effect of CTx on FMLP-induced variations in $[Ca^{2+}]_i$ in undifferentiated U937 cells

FMLP (1 μ M) was added (arrows) to control cells (left) or CTx treated cells (right). Traces shown were recorded as described in Figure 3 and are from one representative experiment out of three performed separately.

To determine whether the appearance of FPR mRNA in undifferentiated cells resulted in the synthesis of functional receptors, we compared the $[Ca^{2+}]_i$ increase induced by FMLP in undifferentiated cells with or without CTx treatment. Although no response was observed with undifferentiated U937 cells, we found that after CTx treatment FMLP induced a transient increase in Ca^{2+} levels of 56.7 ± 2.8 nM (Figure 7), close to that obtained in differentiated cells (79 nM) in the absence of CTx. This indicates that the G_i protein and phospholipase C present in undifferentiated cells were functional. This is in agreement with the work of Prossnitz et al. [36], who showed that FPR transfection of undifferentiated HL-60 cells by FPR cDNA led to a FMLP-induced increase in $[Ca^{2+}]_i$. The fact that FMLP could not induce superoxide-ion generation after 24 h to 3 days of CTx treatment clearly revealed that this elevation in cyclic AMP was not sufficient to allow complete differentiation of these U937 cells. This finding was corroborated by the absence of differentiation markers (CD14, CD68, CD11b) in U937 cells treated with CTx for up to 3 days, whereas these markers were present in RA/VD-differentiated cells (results not shown).

In summary, our results indicate that an elevation of cyclic AMP levels increases the oxidative burst induced by FMLP and C5a in differentiated U937 cells. In the case of FMLP responses, we demonstrate that their potentiations coincide with an enhancement in the number of FMLP-binding sites, also corresponding to an increase in FPR mRNA. The increased FPR and FPRII expression induced by elevated cyclic AMP levels, which occurs independently of the U937-cell differentiation process, suggests simultaneous over-expression of these adjacent genes on chromosome 19. The fact that CTx treatment also enhanced the C5a-induced oxidative burst supports the hypothesis that cyclic AMP generally regulates the clustered chemotactic receptors, including FPR, FPRII and the C5a receptor [11]. Regulation of the FPR gene is still poorly understood, but no cyclic AMP response element has been found in the first 100 bp upstream from its start codon. It should now be determined whether cyclic AMP up-regulates transcription of the chemotactic receptor cluster through a presently unknown cyclic AMP response element, or whether it acts by gene transactivation, as previously suggested for FPR only [11]. Our results also indicate that the FMLP response of differentiated U937 cells is directly linked to the number of available FPRs. Moreover, our data established that, after CTx treatment of undifferentiated

cells, FMLP stimulates an increase in $[Ca^{2+}]_i$. This might provide an interesting model for studying the final steps involved in triggering of the oxidative response induced by FMLP.

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