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Intracellular calcium changes induced by the endozepine triakontatetraneuropeptide in human polymorphonuclear leukocytes: role of protein kinase C and effect of calcium channel blockers

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Abstract

Background: The endozepine triakontatetraneuropeptide (TTN) induces intracellular calcium ($[Ca^{++}]_i$) changes followed by activation in human polymorphonuclear leukocytes (PMNs). The present study was undertaken to investigate the role of protein kinase (PK) C in the modulation of the response to TTN by human PMNs, and to examine the pharmacology of TTN-induced Ca^{++} entry through the plasma membrane of these cells.

Results: The PKC activator 12-*O*-tetradecanoylphorbol-13-acetate (PMA) concentration-dependently inhibited TTN-induced $[Ca^{++}]_i$ rise, and this effect was reverted by the PKC inhibitors rottlerin (partially) and Ro 32-0432 (completely). PMA also inhibited TTN-induced IL-8 mRNA expression. In the absence of PMA, however, rottlerin (but not Ro 32-0432) per se partially inhibited TTN-induced $[Ca^{++}]_i$ rise. The response of $[Ca^{++}]_i$ to TTN was also sensitive to mibefradil and flunarizine (T-type Ca^{++}-channel blockers), but not to nifedipine, verapamil (L-type) or ω-conotoxin GVIA (N-type). In agreement with this observation, PCR analysis showed the expression in human PMNs of the mRNA for all the α1 subunits of T-type Ca^{++} channels (namely, α1G, α1H, and α1I).

Conclusions: In human PMNs TTN activates PKC-modulated pathways leading to Ca^{++} entry possibly through T-type Ca^{++} channels.

Introduction

Triakontatetraneuropeptide [diazepam-binding inhibitor (DBI) 17–50, TTN] is one of the major endogenous peptides generated through the cleavege of DBI, a

neuropeptide also known as acyl-CoA-binding protein [1]. DBI and DBI-derived peptides are also called endozepines, after the ability of at least some of them to recognize the diazepam binding site on the $GABA_A$ receptor, and are widely distributed in the CNS and in peripheral organs [2,3]. In particular, immune tissues express DBI [4] and evidence exists that TTN and its related peptides octadecaneuropeptide (DBI 33–50, ODN) and eiksoneuropeptide (DBI 51–70, ENP) can affect the immune response. In particular, TTN and ODN stimulate the production of tumor necrosis factor (TNF) alpha, interleukin (IL)-1 beta, IL-8, granulocyte/macrophage colony-stimulating factor, IL-6 and IL-8 in human monocytes [5,6], and ODN enhances the LPS-induced secretion of IL-6 in human peripheral blood mononuclear cells [7].

In human polymorphonuclear leukocytes (PMNs) we previously showed that TTN rises intracellular calcium ($[Ca^{++}]_i$) and stimulates chemotaxis, O_2^- generation, phagocytosis and IL-8 production [8,9]. Pharmacological analysis of TTN-induced $[Ca^{++}]_i$ changes and IL-8 production suggested that these responses are brought about through a pertussis toxin (PTX) sensitive, G protein-coupled receptor linked to phospholipase C (PLC), which once activated induces both Ca^{++} release from thapsigargine-sensitive intracellular stores and Ca^{++} entry from the extracellular space [9]. In those experiments, preliminary evidence also suggested the possible involvement of protein kinase (PK) C, inasmuch as the PKC inhibitor calphostin C significantly affected (although did not completely block) TTN-induced effects.

The present study was therefore undertaken to investigate more in-depth, by use of a pharmacological approach, the role of PKC in the modulation of the response to TTN by human PMNs, and to better characterize TTN-induced Ca^{++} entry through the plasma membrane by testing its sensitivity to different Ca^{++}-channel blockers. The results show that the stimulatory effect of TTN is profoundly affected by PKC activation, and suggest that different PKC isoforms may play distinct roles. Evidence is also provided that TTN-induced Ca^{++} entry is specifically sensitive to T-type Ca^{++}-channel blockers. The possibility that T-type Ca^{++}-channels may represent the target for the effects of these drugs on human PMNs is further supported by the observation that these cells express the mRNAs for T-type Ca^{++}-channel α1 subunits.

Materials and Methods

Drugs and chemicals

TTN was obtained from Neosystem SA (Strasbourg, France); Bovine serum albumine (BSA), HEPES, EDTA, EGTA, Trizma Base, 12-*O*-tetradecanoylphorbol-13-acetate (PMA), 4α-phorbol-12,13-didecanoate (α-PMA), rottlerin, Ro 32-0432 (hydrochloride), L-verapamil, ω-conotoxin GVIA, flunarizine, and mibefradil (hydrochloride) were obtained from Sigma Aldrich (St. Louis, MO). Fura-2/AM was obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Dextran and Ficoll-Paque Plus were obtained from Pharmacia Biotech (Uppsala, Sweden). All other reagents and solvents were from Merck (Darmstadt, Germany). All solutions were daily freshly prepared from stock solutions stored at -20°C until use.

Cell preparation

PMNs were isolated from venous blood obtained from healthy volunteers using heparinized tubes. Whole blood was allowed to sediment on dextran at 37°C for 30 min. Supernatant was recovered and PMNs were isolated by Ficoll-Paque Plus density-gradient centrifugation. Contaminating erythrocytes were eliminated by 10 min hypotonic lysis in distilled water with added (g/l): NH_4Cl 8.248, $KHCO_3$ 1.0, EDTA 0.037. Cells were then washed three times in NaCl 0.15 M and resuspended in 1 ml Ca^{++}/Mg^{++}-free PBS (composition as follows [g/l]: NaCl 8.000, KCl 0.200, $Na_2HPO_4xH_2O$ 1.288, KH_2PO_4 0.200) with added 0.25% BSA. Purity and viability of PMNs preparations were >95% and no platelets or erythrocytes could be detected either by light microscopic examination or by flow cytometric analysis.

Measurement of $[Ca^{++}]_i$

PMNs were resuspended at the concentration of 2×10^6/ml and incubated at 37°C with 5 μM Fura-2/AM (stock solution, 1 mM in dimethyl sulfoxide). After 30 min, cells were washed thrice by gentle centrifugation (5 min, 300 g), resuspended in PBS supplemented with 10 mM HEPES, 10 mM glucose, 0.25% BSA and 1 mM $CaCl_2$ and placed in a thermostatically controlled (37°C) cuvette equipped with a cuvette stirrer for $[Ca^{++}]_i$ measurement. Fluorescence measurements were performed using a spectrofluorimeter (Perkin-Elmer LS-50B, Perkin Elmer Instruments, Bridgeport, CT). Excitation of Fura-2 was performed at 340 and 380 nm, with excitation band widths set at 5 nm. The ratio of emitted fluorescence signals (510 nm) was used to calculate the cytosolic free Ca^{++} concentration according to Grynkiewicz et al. [10] and calibration was performed by the addition of 0.5% Triton X 100 and 1 mM $CaCl_2$ (max) or 45 mM TRIS and 50 mM EGTA/TRIS (min). In each experiment, $[Ca^{++}]_i$ changes were calculated as the difference (Δ) between the highest values (peak levels) reached after addition of the agent and the mean 1-min pretreatment values (resting levels).

PCR analysis

Total RNA was extracted from 1×10^6 PMNs by Perfect RNA Eukaryotic Mini kit (Eppendorf, Hamburg, Germany). The kit utilizes a chaotropic guanidinium isothiocyanate solution for cell lysis and rapid inactivation of cellular RNAses. RNA is subsequently bound to the matrix

Table 1: PCR primers used for the detection of IL-8 and of T-type Ca^{++}-channel α1 subunits.

	Primer sequence	Annealing temperature	PCR product	Ref.
IL-8	5'-CCACCCATGGCAAATCCATGGC-3' 5'-TCTCAGCCCTCTTCAAAAACTTCTC-3'	65°C	289	[31]
α1G	5'-CCTGGACTTCTTCACGATGT-3' 5'-CCAGGTCTGCTGGGTCAGAG-3'	63°C	395	[32]
α1H	5'-TGTTCGTGACGGACTCGAATT-3' 5'-AGTGCACAGAGGCAACGGA-3'	63°C	436	[32]
α1I	5'-TTCCCCTACACCGGAACGG-3' 5'-TAGTAACGGTTCCAGTTGA-3'	50°C	227	[32]
HPRT	5'-CCTGCTGGATTACATTAAAGCACTG-3' 5'-CTTCGTGGGGTCCTTTTCACCAGC-3'	65°C	370	Genbank n. M26434
β-actin	5'-GGAAATAGGGGTTAGCAC-3' 5'-CTCATGTGCGCCTACTTA-3'	56°C	929	Genbank n. AJ005353

of the column, washed to remove contaminants and then eluted with molecular biology grade water. In the present study, the amount of extracted RNA was estimated by spectrophotometry at 260 nm. Total RNA was reverse transcribed and cDNA was amplified using a one-step RT-PCR reaction kit (Finnzymes, Espoo, Finland). Briefly, 1 μg total RNA was added to a reaction mixture consisting of 5 μl RT-10x reaction buffer, 1 μl $MgCl_2$ 50 mM, 1 μl deoxynucleotide triphosphate mixture (10 nM each), 1 μl specific primer (Invitrogen, San Giuliano Milanese, MI, Italy), 1 μl avian myeloblastosis virus RT 5 U/ml, 1 μl thermostable DNA polymerase (DYNazyme™ II DNA polymerase) 1 U/ml. Diethylpyrocarbonate-treated water was added up to a final volume of 50 μl. PCR was then brought about by using a thermocycler (GeneAmp PCR System 2400, Perkin Elmer Instruments). For the analysis of IL-8 mRNA, 30 cycles of PCR were performed according to the following steps: 48°C, 30 min (once); 94°C, 30 s 65°C, 45 s and 72°C, 45 s. For the analysis of T-type Ca^{++}-channel α1 subunit mRNAs, 35 cycles of PCR were performed according to the following steps: 45 min at 48°C, then 30 s at 94°C, 30 s at 63°C, 1 min at 72°C, followed by a 10 min-extension period at 72°C. At the end, the reaction mixture was kept for 15 min at 72°C and finally chilled at -4°C until analysis, which was performed on a 10 μl aliquot of the PCR product by electrophoretical separation on a 2% agarose gel and subsequent visualization by ethidium bromide staining (BIORAD, Hercules, CA). Quantification of the amount of RT-PCR products was carried out by densitometric analysis of photographic negatives of the agarose gel by use of a software for image analysis (Multi-Analyst, BIORAD). For selection of the primers, we referred to the National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/ database and to published literature. Selected primers are shown in Table 1. Data were finally presented as optical density ratio (in arbitrary units), with respect to expression of the mRNA for the housekeeping gene (hypoxanthine-guanine phosphorybosyl transferase [HPRT] for IL-8 and β-actin for T-type Ca^{++}-channel α1 subunits).

Statistical analysis

Data are shown as means ± standard deviation (SD) of the mean. Statistical significance of the differences among groups was assessed by two-tailed Student's *t* test or by ANOVA followed by Bonferroni post test for paired or unpaired data, as appropriate. The concentration-response relationship of PMA was analyzed by nonlinear regression using a commercial software (Prism 2.0, GraphPad Software Inc., San Diego, CA, USA) and a sigmoidal concentration-response curve was fitted to find the mean value of the EC_{50} (i.e., the concentration which elicited 50% of the maximal response) together with its 95% confidence interval (C.I.).

Results

Effect of PKC ligands

In agreement with previous studies [8,9], in human PMNs TTN 100 μM induced a rapid and transient rise of $[Ca^{++}]_i$ and increased the expression of IL-8 mRNA. The PKC activator PMA (but not its inactive analogue α-PMA) reduced the effect of TTN 100 μM on $[Ca^{++}]_i$ rise in a concentration-dependent fashion (Fig. 1). PMA also reduced TTN-induced IL-8 mRNA expression (control: 0.44 ± 0.15; TTN 100 μM: 1.04 ± 0.23, $P < 0.01$ vs control; TTN + PMA 100 ng/ml: 0.28 ± 0.07, not significant vs control, $P < 0.001$ vs TTN alone), while also in this regard α-PMA was completely ineffective (TTN + α-PMA 100 ng/ml: 0.82 ± 0.28, $P < 0.05$ vs control, not significant vs TTN alone). The mean EC_{50} value (with 95% C.I.) for PMA-induced reduction of TTN-induced $[Ca^{++}]_i$ rise was 0.06 (0.05–0.07) ng/ml. PMA however failed to completely abolish the effect of TTN, which even in the presence of PMA 1–100 ng/ml was able to evoke a slow and progressive rise of $[Ca^{++}]_i$ about 20% of that in the presence of TTN alone (Fig. 1). The effect of PMA was concentration-dependently

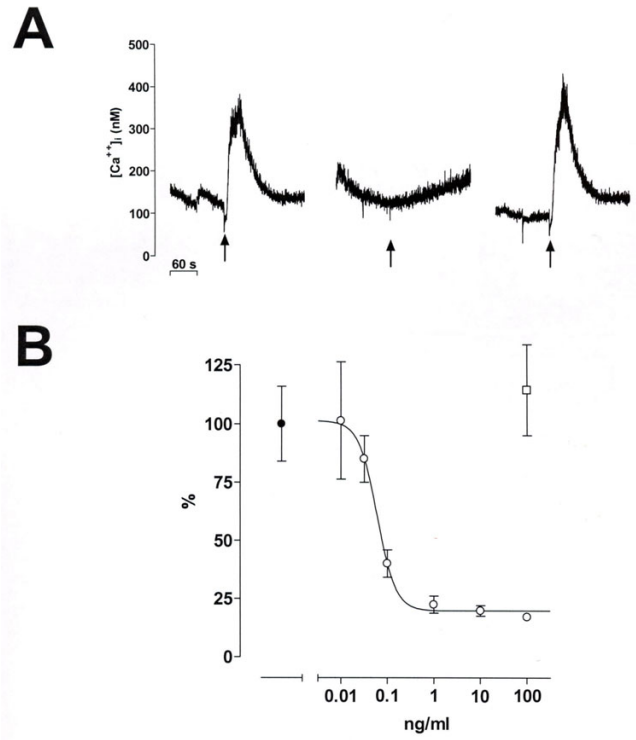

Figure 1
Effect of TTN on $[Ca^{++}]_i$ in human PMNs. ***Panel A***: Representative tracings showing the effect of TTN 100 μM (added at arrow) on $[Ca^{++}]_i$ in FURA-2-loaded cells under standard conditions (left) and after 60-s incubation with PMA 100 ng/ml (center) or α-PMA 100 ng/ml (right). ***Panel B***: Concentration-response relationship for the effect of PMA (empty circles) and lack of effect of α-PMA (empty square) on TTN 100 μM-induced $[Ca^{++}]_i$ rise. Data are expressed as percentage of the effect of TTN alone (filled circle). Each point is the mean ± SD of 3 separate experiments.

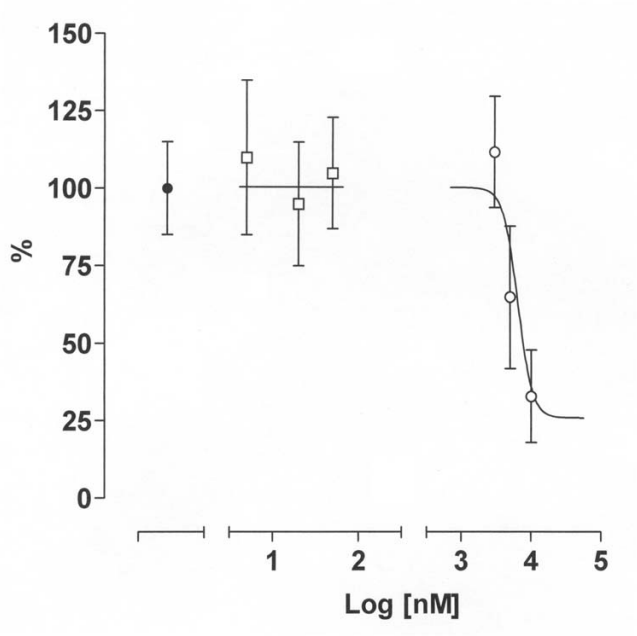

Figure 2
Concentration-response relationship for the effect of rottlerin (right panel, empty circles) and lack of effect of Ro 32-0432 (central panel, empty squares) on TTN 100 μM-induced $[Ca^{++}]_i$ rise. Data are expressed as percentage of the effect of TTN alone (left panel, filled circle). Each point is the mean ± SD of 3 separate experiments.

Table 2: Effect of rottlerin and of Ro 32-0432 on PMA-dependent inhibition of the $[Ca^{++}]_i$ rise induced by TTN. Data are expressed as percentage of the effect of TTN alone, and are shown as the mean ± SD of at least 3 separate experiments. * = *P* < 0.05, ** = *P* < 0.01 vs TTN alone; # = *P* < 0.01 vs TTN + PMA.

TTN 100 μM	100.0 ± 15.5
TTN 100 μM + PMA 1 ng/ml	29.3 ± 15.5**
TTN 100 μM + PMA 1 ng/ml	
+ rottlerin 3 μM	25.0 ± 18.3**
+ rottlerin 5 μM	47.4 ± 15.1*
+ rottlerin 10 μM	61.5 ± 14.2*
TTN 100 μM + PMA 1 ng/ml	
+ Ro 32-0432 5 nM	60.0 ± 13.2**
+ Ro 32-0432 20 nM	89.5 ± 14.7#
+ Ro 32-0432 50 nM	102.3 ± 15.1#

reverted by 5-min preincubation with rottlerin 3–10 μM and with Ro 32-0432 5–50 nM. However, the reversion induced by Ro 32-0432 was complete, while that induced by rottlerin was only partial (Table 2). In the absence of PMA, rottlerin (but not Ro 32-0432) per se was also able to inhibit TTN 100 μM-induced $[Ca^{++}]_i$ rise in a concentration-dependent fashion (Fig. 2). The mean EC_{50} value (with 95% C.I.) of rottlerin was 5.77 (3.91–8.51) μM. PMA, α-PMA, Ro 32-0432, and rottlerin at the concentrations used had per se no significant effect on the parameters under study (data not shown).

Effect of Ca^{++} channel blockers and evidence for the presence of T-type Ca^{++} channels in human PMNs

Preincubation for 5 min with the chemically unrelated T-type Ca^{++} channel blockers flunarizine or mibefradil significantly reduced TTN 100 μM-induced $[Ca^{++}]_i$ rise down to about 50% of that induced by TTN alone. On the contrary TTN 100 μM-induced $[Ca^{++}]_i$ rise was affected neither by the L-type Ca^{++} channel blockers nifedipine and verapamil nor by the N-type Ca^{++} channel blocker ω-

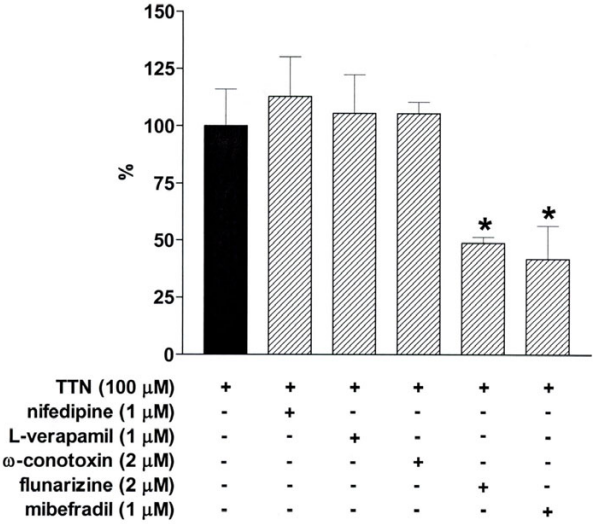

Figure 3
Effect of different Ca^{++} channel blockers on TTN-evoked $[Ca^{++}]_i$ rise in human PMNs. Data are expressed as percentage of the effect of TTN alone. Each bar is the mean ± SD of 4 experiments. * = $P < 0.01$ vs TTN alone.

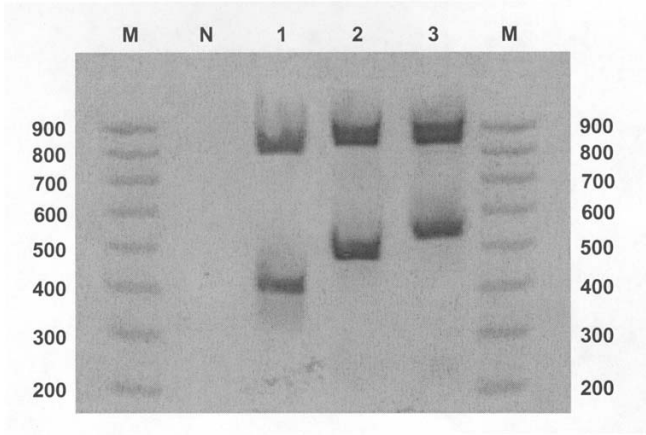

Figure 4
Expression of mRNAs for T-type Ca^{++} channel α1G (lane 1), α1H (lane 2), and α1I (lane 3) subunits in human PMNs. Data are from one representative of 3 separate experiments. N, negative control (no RNA); M, molecular weight markers.

conotoxin GVIA (Fig. 3). None of the Ca^{++} channel blockers had any effect per se on $[Ca^{++}]_i$ at the concentrations used in this study (data not shown).

Northern blot analysis provided evidence for the expression of the mRNA for all the α1 subunits of T-type Ca^{++} channels (namely, α1G, α1H, and α1I) in human PMNs (Fig. 4).

Discussion

The endozepine TTN behaves as a chemoattractant factor for human PMNs, resulting in a typical pattern of cell activation, which includes a rise of $[Ca^{++}]_i$, with subsequent IL-8 mRNA expression and release of this proinflammatory chemokine, chemotaxis, induction of oxidative metabolism and phagocytosis [8,9]. In the present study we have further characterized the pharmacological profile of the response to TTN by human PMNs, showing that PKC exerts a complex modulation of TTN-induced $[Ca^{++}]_i$ rise and that TTN-induced Ca^{++} entry in these cells is sensitive to T-type Ca^{++} channel blockers. A tentative synopsis of the experimental evidences obtained so far is given in Fig. 5, which should also be taken as a reference frame for the subsequent discussion of the findings of this study.

In our experiments, the PKC activator PMA prevented both TTN-induced $[Ca^{++}]_i$ rise and IL-8 mRNA expression. Involvement of PKC was confirmed by the low EC_{50} value of PMA, by the inactivity of its negative control α-PMA, as well by the ability of two chemically unrelated PKC inhibitors such as Ro 32-0432 and rottlerin to revert this response. These observations stand for the existence of a PKC-operated inhibition of TTN-dependent pathways, a finding in line with the notion that in PMNs activation of PKC inhibits the signals responsible for mobilization of $[Ca^{++}]_i$ [11]. Interestingly however PMA did not completely prevent TTN-induced $[Ca^{++}]_i$ rise, and a residual effect of the peptide (around 20% of the maximal effect) was still evident even in the presence of high concentrations of PMA (see Fig. 1). This is in line with our previous results showing that the PKC inhibitor calphostin C per se significantly reduced the effects of TTN in human PMNs, suggesting that activation of PKC was also part of the signaling process triggered by this peptide in PMNs [9].

PKC consists of a family of at least 12 serine/threonine kinases, which are currently divided into three main groups based on their structure and substrate requirements, namely: conventional (Ca^{++}-dependent and activated by both phosphatidylserine [PS] and diacylglycerol [DAG], represented by α, βI, βII, and γ), novel (Ca^{++}-independent and regulated by PS and DAG, represented by δ, ε, η, and θ), and atypical (Ca^{++}-independent and regulated by PS but not by DAG, represented by ζ and ι/λ) [12]. Recent studies have investigated the expression and role of PKC isoforms in human PMNs, showing in these cells the presence of conventional (βI, βII, and α, although in lower amounts), as well as novel (δ) PKC [13], and that different isoforms subserve distinct functions: conventional isoforms regulate PMA-stimu-

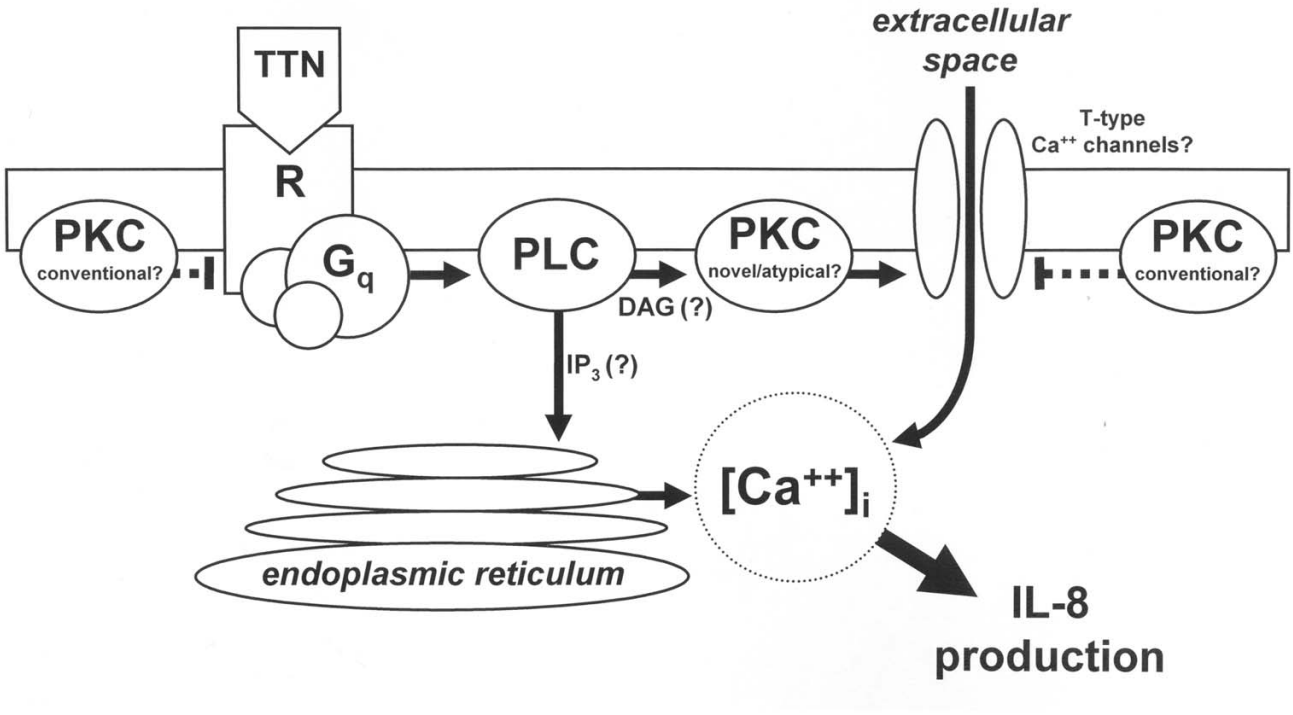

Figure 5
Schematic representation summarizing the putative signaling pathways acted upon by TTN in human PMNs. TTN likely activates G protein-coupled membrane receptors, which in turn signal to PLC- and PKC-dependent pathways. PKC may exert both positive and negative modulation of TTN signaling, eventually depending on the specific isoform involved. Increased $[Ca^{++}]_i$ then occurs through both Ca^{++} release from intracellular stores and Ca^{++} entry, possibly through T-type Ca^{++} channels. The picture is based upon the results of the present as well as of previous studies [8,9].

lated cytoskeletal association and activation of NADPH oxidase, while novel isoforms modulate other responses that involve cytoskeletal components [14].

In our experiments, we have used the phorbol ester compound PMA, which penetrates the cytoplasmic membrane to directly bind and activate PKC [15]. PMA however does not show a high degree of selectivity for PKC isoforms, thus resulting in activation of all PKC in the cell. In the present study, we have therefore investigated the ability of the PKC inhibitors Ro 32-0432 and rottlerin to revert the effect of PMA on TTN-induced $[Ca^{++}]_i$ rise, as well as to affect per se this response. Rottlerin has been shown to inhibit PKCδ with some selectivity over other PKC isoforms, while Ro 32-0432 is selective for conventional isoforms (reviewed in [12]). According to our results, both drugs resulted in the reversion of PMA-induced inhibition of TTN-induced $[Ca^{++}]_i$ rise (Table 2), however only rottlerin also inhibited per se the effect of TTN (Fig. 2). The EC_{50} of rottlerin in this regard was 5.77 μM, which is in good agreement with the reported IC_{50} for PKCδ (3–6 μM) [12]. On the contrary, Ro 32-0432 in the 5–50 nM concentration range (which has been reported to be selective for conventional PKC isoforms [12]) had no effect per se on the response of human PMNs to TTN (Fig. 2). These findings, together with the observation that at variance with Ro 32-0432, rottlerin was only partially effective in reverting the effect of PMA (Table 2), may suggest that different isoforms of the enzyme play distinct roles in modulating the responses to TTN. While PKCδ seems to contribute to the rise of $[Ca^{++}]_i$ induced by TTN, activation of conventional PKC isoforms (possibly, βI, βII, and/or α) may inhibit this response. When all the PKC isoforms undergo activation, as in the case of treatment with PMA, inhibition prevails over facilitation. In future studies, this hypothesis will be tested by investigating the pattern of activation of different PKC isoforms occurring as a result of TTN stimulation in human PMNs.

Few evidence exists about the possible contribution of membrane Ca^{++} channels to the effects of TTN on $[Ca^{++}]_i$. In rat astrocytes, TTN increases $[Ca^{++}]_i$ through a

peripheral-type benzodiazepine receptor-mediated opening of Ca^{++} channels which are sensitive to the L-type channel blocker nifedipine but not to the T-type channel blocker mibefradil nor to the N-type channel blocker ω-conotoxin GVIA [16], while in the frog adrenal gland, TTN-induced $[Ca^{++}]_i$ rise involves the activation of membrane receptors positively coupled to adenylyl cyclase through a cholera toxin-sensitive G protein, which in turn results in Ca^{++} influx which is inhibited by mibefradil but not by nifedipine or ω-conotoxin GVIA [17]. In our experiments, TTN-evoked $[Ca^{++}]_i$ rise was significantly reduced by mibefradil and also by flunarizine, another T-type Ca^{++} channel blocker structurally different from mibefradil, whereas nifedipine, verapamil (another L-type channel blocker) and ω-conotoxin GVIA had no effect. Interestingly, the effect of TTN on $[Ca^{++}]_i$ was not completely blocked, an evidence consistent with the idea that TTN increases $[Ca^{++}]_i$ through both release from intracellular stores and entry through the plasma membrane [8].

The sensitivity of TTN-induced $[Ca^{++}]_i$ rise to T-type channel blockers led us to investigate the expression of T-type Ca^{++} channels in human PMNs. Molecular studies have identified at least 3 voltage-gated Ca^{++} channel α1 subunits which share the biophysical and pharmacological properties of T-type channels, namely α1G, α1H, and α1I [18]. In the present study, northern blot analysis provided evidence for the expression of the mRNA for all these 3 α1 subunits in human PMNs (Fig. 4). We are not aware of other studies showing the expression of T-type Ca^{++} channels in human PMNs, either at the level of mRNA or of the protein subunits, and the present findings may therefore represent the first evidence that such channels are expressed by these cells. Nonetheless, whether true voltage-dependent T-type Ca^{++} channels occur and play a relevant role in human PMNs remains still largely to be established. Electrophysiological studies of Ca^{++} influx in non-excitable cells such as leukocytes, including PMNs, have provided evidence for the existence of receptor-mediated, non voltage-operated Ca^{++} entry in these cells [19]. Interesting observations however regard the possible occurrence, at least in lymphocytes, of non-voltage-operated Ca^{++} channels, with an amino acid sequence which is closely related to classical voltage-operated Ca^{++} channels (reviewed in [20]). In human PMNs flunarizine has been shown to inhibit Ca^{++} entry triggered by fMLP or by the Ca^{++} ionophore A23187 [21]. Available data, including those reported in the present study, seem thus to support the existence in human PMNs of Ca^{++} entry mechanisms which are closely related to T-type Ca^{++} channels, from both the structural and the pharmacological point of view. Such mechanisms are likely to be involved in the response of human PMNs to TTN, and may represent the target of T-type channel blockers such as flunarizine and mibefradil.

In conclusion, the present results together with previous studies [8,9] suggest that in human PMNs the endozepine TTN activates G protein-coupled membrane receptors, resulting in a signaling cascade which comprises PLC and PKC. This latter enzyme in particular may exert both positive and negative effects on TTN signaling, possibly depending on the specific isoform(s) involved. TTN-activated pathways finally result in increased $[Ca^{++}]_i$, due to both Ca^{++} release from intracellular stores and Ca^{++} entry possibly through T-type Ca^{++} channels. $[Ca^{++}]_i$ rise then signals for activation of cell function, including mRNA expression and release of the chemokine IL-8 (Fig. 5). DBI is released from nerve terminals and its fragments can be detected in liquor and peripheral blood [22]. Endozepines such as TTN may therefore contribute to the central nervous system-immune system cross-talk. In addition DBI, also called acyl-CoA binding protein, is widely distributed in many peripheral organs such as gut and endocrine cells of the pancreatic islets [23,24], liver, kidney [25], adrenals [26], adipose tissue, heart, muscles and mammary gland [27] of different species, and in circulating mononuclear cells [28], in red blood cells [29] and even in neoplastic cell lines [30]. High concentrations of DBI and/or of its processing products may therefore occur locally, e.g. as the result of leakage from damaged cells during tissue injury. DBI-derived peptides may thus also add to the multiple agents constituting the local microenvironment in inflamed tissues. Studies are therefore warranted to develop TTN-receptor ligands to assess the relevance of TTN-operated pathways as novel targets for the pharmacological modulation of PMNs during the inflammatory process.

Authors' contribution

FM and MC designed the study, performed spectrofluorimetric experiments and drafted the manuscript; MF performed RT-PCR experiments; SC and GF collaborated to spectrofluorimetric studies; AMF collaborated to the design of the study and to the analysis and interpretation of the data; SL and GMF collaborated to data interpretation and to the critical discussion of the manuscript. All Authors read and approved the final manuscript.

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