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RESEARCH ARTICLE

The Role of Protein Kinase C Isoforms in the Formation of Neutrophil Extracellular Traps

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Abstract– Neutrophils release decondensed nuclear chromatin or neutrophil extracellular traps (NET) in response to a great number of physiological and pharmacological stimuli. However, apart from the host defensive function, NETs play an essential role in the pathogenesis of various autoimmune, inflammatory, and malignant diseases. Therefore, understanding the molecular mechanisms of NET formation, usually leading to the neutrophil death (NETosis), is important to control the consequences of aberrant or excessive NET release. Protein kinase C (PKC) is a serine/threonine kinase that is involved in a variety of neutrophil functions, but its role in NETosis is not well understood. Since five PKC isoforms (α, βI, βII, δ, and ζ) have been described in human neutrophils, we studied their contribution to NETosis and oxidative burst using inhibitory analysis. Using specific PKC isoform inhibitors, we have shown that PKCβ, PKCδ, and PKCζ are involved in the oxidative burst and NETosis activated by calcium ionophore A23187, while PKCβ is involved in the oxidative burst and NETosis upon cell activation by diacylglycerol mimetic phorbol 12-myristate 13-acetate.

Keywords: *human neutrophils*, *neutrophil extracellular traps*, *NETosis*, *oxidative burst*, *protein kinase C isoforms*
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Neutrophils are the most abundant white blood cells in the circulation and the first line of defense against invading pathogens. As professional phagocytes, neutrophils contain antimicrobial enzymes in granules and are responsible for such effector functions as phagocytosis and degranulation in inflammation foci, and they also express oxidative burst consisting in reactive oxygen species (ROS) generation upon the activation of enzymatic complex NADPH oxidase. A new effector function of neutrophils first observed by Takei et al. [1] and comprehensively investigated in the laboratory of Zychlinsky [2] is the release of Neutrophil Extracellular Traps (NETs). NETs consist of decondensed chromatin coated with histones, antimicrobial enzymes and cationic peptides of granules, as well as cytosolic proteins [2, 3]. The process of NET formation leading to programmed cell death was called NETosis [4].

Subsequently, it became evident that apart from host defensive function, NETs play a pivotal role in the pathogenesis of numerous autoimmune, inflammatory, and malignant diseases [5–8]. Therefore, understanding the molecular mechanisms of NETosis is important for controlling the consequences of dysregulated or excessive NET release.

The classical NETosis proceeds as a multistage pathway, which involves activation, the generation of ROS by NADPH oxidase, hydrogen peroxide-mediated dissociation of "azurosomes" – protein complexes formed in the membranes of azurosome granules [9], the leakage of serine proteases (neutrophil elastase – NE, cathepsin G and azurocidin) and myeloperoxidase (MPO) from the azurosomes into the cytoplasm, the migration of NE and MPO to the nucleus where, along with peptidyl-arginine deiminase 4 (PAD4) citrullinating histones, they promote decondensation of nuclear chromatin [2].

The formation of NETs can be activated by various physiological stimuli such as bacteria, fungi, protozoa, viruses, and bacterial cell wall products (lipopolysaccharides). NETs can be also induced by antibodies [10], cytokines (IL-8, IL-1β, TNF-α) [3, 11], microcrystals [12], calcium and potassium ionophores [13], as well as pharmacological stimuli including phorbol esters.

Most of these stimuli trigger NETosis dependent on ROS generated by the multicomponent enzymatic complex NADPH oxidase. NADPH oxidase becomes active after the assembly of four cytosolic subunits (p47phox, p67phox, p40phox, and Rac2) with transmembrane subunits (gp91phox and p22phox), and it is tightly regulated by the phosphorylation with protein kinase C [14].

In accordance with the conventional model, the activation of PKC is associated with translocation of enzyme from the cytosol to the membrane compartment. Following activation, PKC phosphorylates serine and threonine residues on the subunits of NADPH oxidase. The mammalian PKC family includes 12 isoforms which are grouped into three subfamilies according to their domain structure and regulation [15]. The conventional PKCs include PKCα, PKCβI, PKCβII, and PKCγ, and they require binding to phosphatidylserine, diacylglycerol (DAG) and calcium for their activation. They are also activated by the mimetic of DAG, PMA (phorbol 12-myristate 13-acetate). The novel PKCs (nPKC) isoforms include PKCδ, PKCε, PKCη, and PKCθ. These kinases are calcium-insensitive and are activated by DAG or PMA. The atypical (aPKCs) isoforms are PKCζ, PKCτ/λ, they are calcium insensitive and do not respond to PMA or DAG. However, they are activated by phosphoinositide-dependent protein kinase-1, phosphatidylinositol 3-kinases and its product phosphatidylinositol-3,4,5-trisphosphate. As was shown in numerous studies, human neutrophils contain five PKC isoforms (α, βI, βII, δ, and ζ) [16, 17].

It has been shown before that PKC isoforms are involved in various neutrophil functions, such as oxidative burst, adhesion, degranulation, phagocytosis and apoptosis [18]. However, little is known about the involvement of PKC isoforms in NETosis activated by various physiological and pharmacological stimuli. In our study, we comprehensively investigated the impact of conventional, novel, and atypical PKC isoforms in NETosis of human neutrophils activated by calcium ionophore A23187 and phorbol ester PMA.

MATERIALS AND METHODS

Reagents. PMA, A23187, chelerythrine (inhibitor of all PKC isoforms), rottlerin (PKCδ inhibitor), LY333531 (PKCβ inhibitor), PKCζ pseudosubstrate (PKCζ inhibitor), verapamil, luminol were purchased from Sigma-Aldrich (USA). SYBR Green and ProLong Gold antifade mountant were obtained from Thermo Fisher Scientific (Invitrogen, USA).

Isolation of primary human neutrophils. All experiments with blood were conducted according to the Helsinki Declaration on the Ethical Principles for Medical Research (2000) and the European Council Convention Protocol on Human Rights and Biomedicine (1999), and were approved by the local committee on ethics. Peripheral blood of healthy donors or patients with chronic granulomatous disease (CGD) was taken in the morning on an empty stomach in polypropylene tubes with heparin (20 ME/ml of blood). Neutrophils were isolated by centrifugation in a single-stage Ficoll-Hypaque density gradient (d = 1.077 g/cm^3) at 400 g for 25 min and room temperature, as described before [19]. Thereafter, the bulk of the erythrocytes were removed from the suspension by sedimentation with Dextran. The contaminating erythrocytes were lysed in a cold hypotonic sodium chloride solution (0.2%) for 30 s followed by reduction to physiological saline with hypertonic sodium chloride (1.6%). Isolated neutrophils were suspended in complete medium consisting of RPMI 1640 supplemented with 10 mM HEPES, 2 mM L-glutamine, and 1% heat inactivated fetal calf serum (FCS). The resulting cells were represented by 98% granulocytes, and their viability was more than 99%, as judged by 0.1% Trypan blue dye exclusion. Neutrophils were stored for 1 h at 4°C before the experiment.

Luminol-amplified chemiluminescence assay. The luminol-amplified chemiluminescence ~~(CL)~~ assay was used to detect total (intra- and extracellular) ROS, as previously described [20].

Induction and fluorescence detection of NETs. Fluorescent microscopy was used to detect NETs. For that, freshly isolated neutrophils (2×10^5 cells/mL) were allowed to adhere on round coverslips located in 24-well plate in a volume of 500 μL of complete medium (FCS, 1%) for 30 min at 37°C. Neutrophils were incubated in the wells with one of PKC inhibitors for 30 min at 37°C and 5% CO_2. NET formation was induced by 30 nM PMA or 2 μM A23187 for 2 h 40 min and 4 h, respectively. After the incubation, samples were fixed with 4% paraformaldehyde for 15 min. Slides were immersed in SYBR Green diluted 1:10,000 in PBS according to the manufacturer's recommendation and stained for 7 min at room temperature in the dark. The total number of cells and the number of netotic cells were counted in each field of view, and the percentage of NETosis was calculated in several fields of view.

Statistical analysis. Statistical analysis of the results was conducted using one-way ANOVA followed by a multiple comparison Bonferroni test used to evaluate the intergroup differences. The data in text and figures are expressed as mean ± standard error of the mean. Statistically significant P values are indicated in the figures as follows: *—$p < 0.05$; **—$p < 0.01$; ***—$p < 0.001$.

RESULTS AND DISCUSSION

Protein kinase C is involved in oxidative burst and NETosis induced by various stimuli. In order to elucidate, whether NETosis of human neutrophils activated by A23187 and PMA is dependent on PKC func-

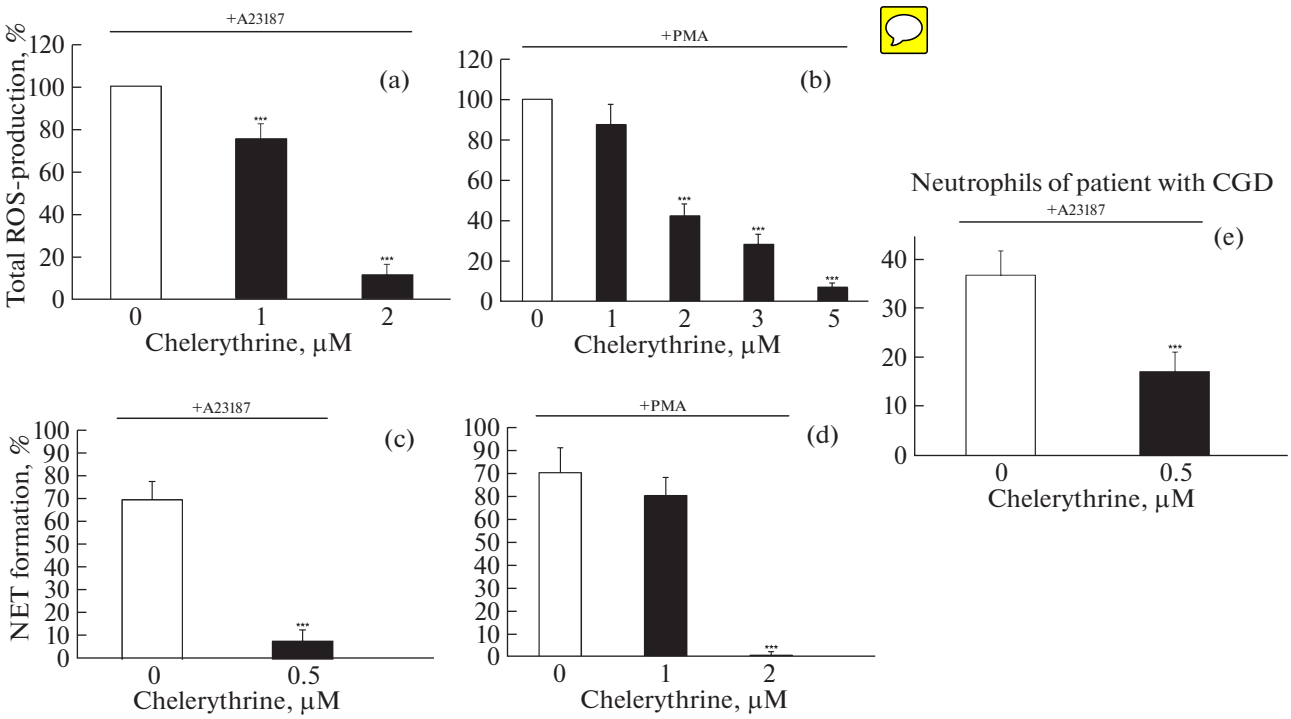

Fig. 1. Evaluation of of protein kinase C involvement in oxidative burst and NETosis of human neutrophils. Freshly isolated human neutrophils were treated with specific PKC inhibitor chelerythrine for 30 min. The oxidative burst was induced by 2 μM A23187 (A) or 30 nM PMA (B). ROS formation was calculated as the area under the curves luminol-amplified chemiluminescence and expressed as a percentage of the control (100%, stimulated neutrophils). To investigate NETosis, neutrophils of healthy donors were treated at coverslips with chelerythrine for 30 min. NET formation was induced by 2 μM A23187 (C) or 30 nM PMA (D) for 4 h and 2 h 40 min, respectively. Cells were fixed with 4% paraformaldehyde and stained by SYBR Green to visualize chromatin. ($n = 5$). ***—$p < 0.001$. (E) Neutrophils isolated from the patients with CGD were treated with 0.5 μM chelerythrine for 30 min. NET formation was induced by 2 μM A23187 for 4 h. ($n = 3$). ***—$p < 0.001$.

tion, a specific inhibitor of all PKC isoforms, chelerythrine, was applied. The effect of chelerythrine was also estimated in the model of oxidative burst induced by A23187 and PMA using luminol-amplified chemiluminescence assay.

As shown in Fig. 1a,b, the treatment of neutrophils with increasing doses of chelerythrine resulted in significant and concentration-dependent inhibition of oxidative burst induced by both stimuli. NETosis stimulated by A23187 and PMA was also inhibited by chelerythrine in a concentration-dependent manner (Fig. 1c,d; Appendix 1A) indicating its dependence on PKC activation in both methods of stimulation.

We hypothesized that PKC, in addition to phosphorylation of NADPH oxidase subunits (oxidative burst), can phosphorylate some other targets playing a pivotal role in the activation of NETosis. To test this hypothesis, we used neutrophils isolated from the blood of patients suffering from CGD due to full-mutations of NADPH oxidase. These CGD neutrophils are unable to generate oxidase-dependent ROS and they do not release NETs in response to many stimuli including PMA (Appendix 1B), however, they form NETs in response to A23187 (2 μM). Incubation of neutrophils from patients with CGD in the presence of chelerythrine for 30 min before stimulation of NETosis by A23187 resulted in remarkable suppression of NETosis (Fig. 1e and Appendix 1B) indicating the participation of additional PKC substrates in this process.

In conclusion, our results confirmed that PKC is involved in NADPH oxidase-dependent oxidative burst induced by A23187 and PMA. In NET-forming signaling cascade, PKC, in addition to NADPH oxidase subunits, phosphorylates some other unknown targets.

The role of various PKC isoforms in oxidative burst and NETosis. We were interested to elucidate, which PKC isoforms are involved in NETosis stimulated by A23187 and PMA, given that the contradictory reports have been obtained before [21, 22]. Considering that only five PKC isoforms (α, βI, βII, δ, and ζ) have been described in human neutrophils and PKCα is found in minor amounts in these cells [23], we used selective inhibitors of PKCβ (LY333531), PKCδ (rottlerin) and PKCζ (PKCζ pseudosubstrate) isoforms.

The effects of inhibitors were assessed in the model of oxidative burst induced by A23187 and PMA. As shown in Fig. 2a,b, inhibitors of PKCβ and PKCδ suppressed A23187-induced oxidative burst significantly

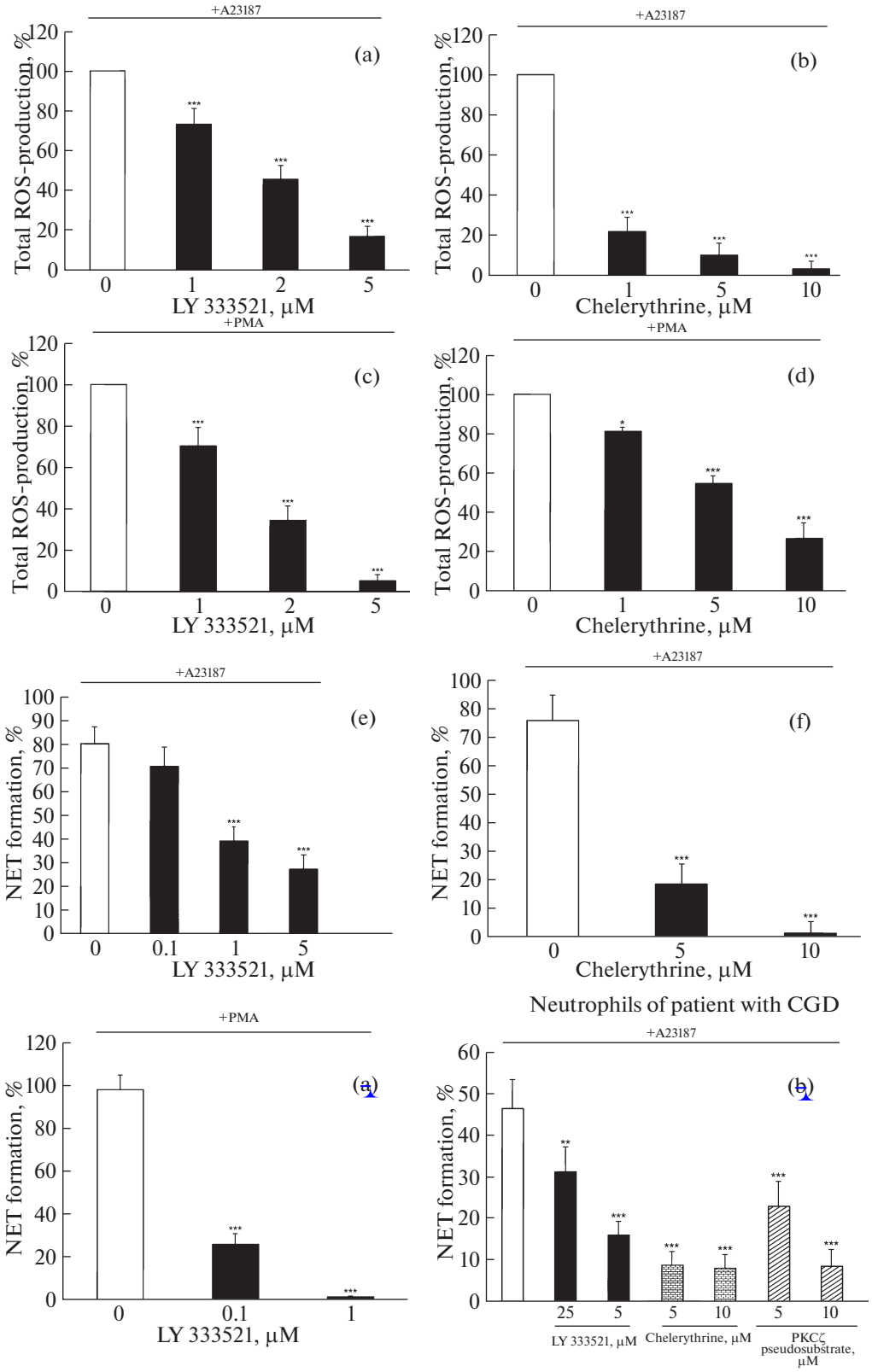

Fig. 2. Evaluation of PKC isoforms involvement in oxidative burst and NETosis induced by various stimuli. Neutrophils were treated with selective inhibitors of PKC isoforms LY333531 (PKCβ inhibitor) (a,c,e,g,h), rottlerin (PKCδ inhibitor) (b,d,h), or PKCζ pseudosubstrate (PKC ζ inhibitor) (F, H) for 30 min. The oxidative burst and NETosis were induced and analyzed as shown in Fig. 1. ($n = 3$). *—$p < 0.05$; ***—$p < 0.001$.

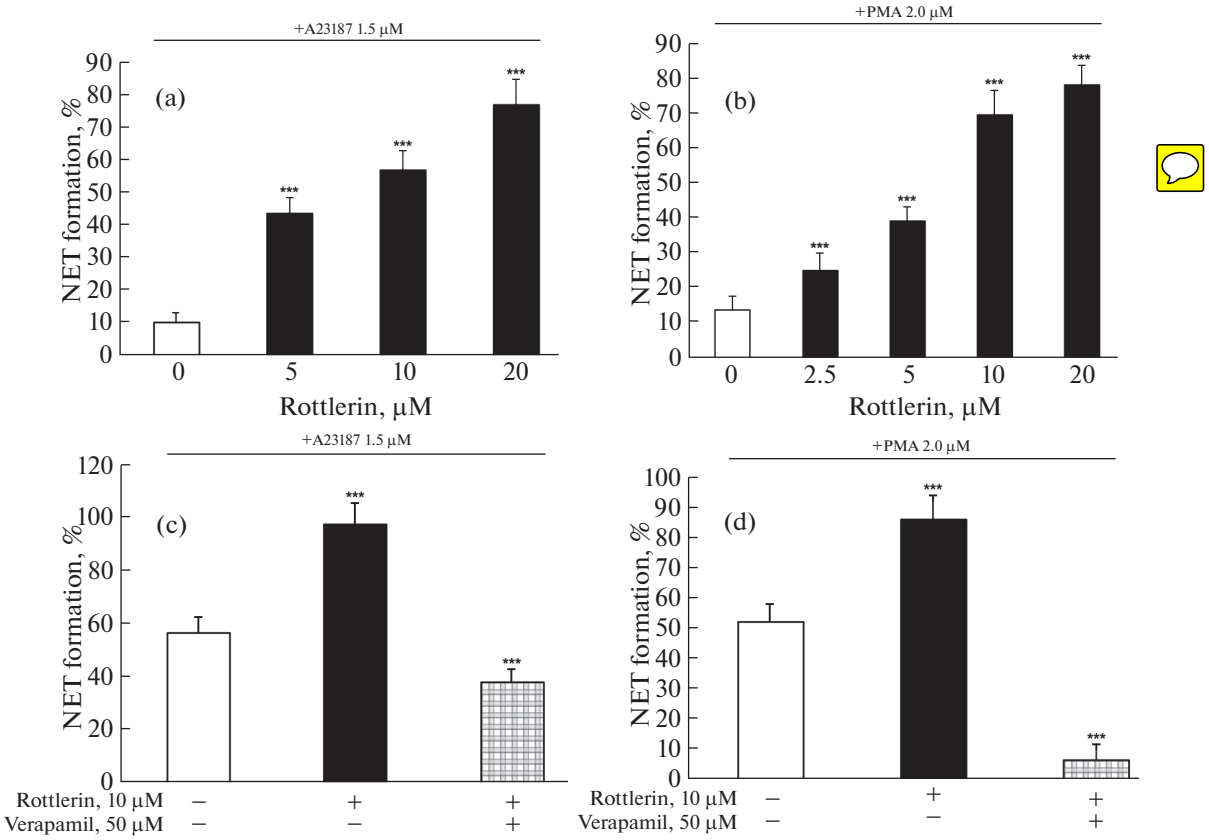

Fig. 3. Effect of rottlerin on NETosis induced by low doses of stimuli. (a, b) Healthy donor neutrophils were treated with increasing concentrations of rottlerin, then NETosis was stimulated by low doses of A23187 (1.5 μM) or PMA (20 nM) for 4 h and 2 h 40 min, respectively. (c, d) Neutrophils were treated with 50 μM verapamil for 10 min followed by 10 μM rottlerin treatment for another 30 min. NETosis was induced with 1.5 μM A23187 or 20 nM PMA for 4 h and 2 h 40 min, respectively. Cells were stained and analyzed as in Fig. 1. ($n = 3$). *** – $p < 0.001$.

and in a concentration-dependent manner (suppression by PKCζ inhibitor was not shown) indicating that all of them are involved in the phosphorylation of NADPH oxidase subunits. However, in the case of PMA-stimulated neutrophils, only inhibitors of PKCβ and PKCδ isoforms suppressed the oxidative burst (Fig. 2c,d), while the inhibitor of PKCζ isoform was not efficient (not shown). This result is consistent with the fact that the PKCζ isoform is insensitive to DAG.

The effects of the PKC inhibitors have been studied in the model of A23187- and PMA-stimulated NETosis. As shown in fig. 2e,f, NET formation induced by A23187 was suppressed after the treatment of donor neutrophils with the inhibitors of PKCβ and PKCζ isoforms effectively and in a concentration-dependent manner, in accordance with their suppressive effects on the oxidative burst. However, the inhibitor of PKCδ rottlerin, to our great surprise, was absolutely ineffective when added in a wide range of concentrations (not shown). In the case of PMA-stimulated NET formation, only inhibitor of PKCβ isoform LY333531 suppressed NETosis (Fig. 2g), while the PKCζ inhibitor and rottlerin were inefficient (not shown).

Thus, the results show that although rottlerin inhibited the oxidative burst induced by A23187 and PMA, the NETosis was not suppressed in these conditions. We can explain this phenomenon by the residual function of NADPH oxidase in the case of PMA-induced NETosis, and the mitochondrial ROS formation in the case of A23187-induced NET release, as it occurs in neutrophils of patients with CGD [24].

We were also interested to find out whether PKC isoforms (β, δ, and ζ) are involved in the phosphorylation of targets, other than NADPH oxidase subunits, in NETosis pathway. For that, we investigated A23187-induced NETosis in neutrophils of patients with CGD. As shown in Fig. 2h and Appendix 2, the inhibitors of PKCβ, PKCδ, and PKCζ suppressed A23187-induced NETosis in these neutrophils. Thus, all three PKC isoforms are involved in the phosphorylation of targets, other than NADPH oxidase subunits, required for NET formation.

Pleiotropic effects of rottlerin in human neutrophils. In the previous section of our study we obtained con-

flicting data concerning the effects of rottlerin on various effector functions of neutrophils. On one hand, rottlerin inhibited A23187- and PMA-induced oxidative burst, and on the other hand, it was not effective in NETosis. In addition, rottlerin suppressed A23187-induced NETosis in neutrophils of patients with CGD. The explanation for these effects may be due to the fact that rottlerin, in addition to PKCδ inhibition, has pleiotropic effects on various cells [25].

Almost all immune reactions of neutrophils including oxidative burst and NETosis are known to require an elevation of cytoplasmatic Ca^{2+} at some stage. To generate calcium signals, neutrophils, as electrically non-excitable cells, mainly rely on store-operated Ca^{2+}-channels located in the plasma membrane [26]. However, it has become clear in recent years that neutrophils also possess voltage-gated Ca^{2+}-channels (VGCC) L-type [27], which are in general the signature of electrically excitable cells. In addition, as shown in a mouse model of the eye lens, rottlerin is an L-type calcium channel agonist [28].

We hypothesized that rottlerin contributed to the opening of these channels and an increase in the concentration of cytoplasmic Ca^{2+}, compensating for its effect as PKCδ inhibitor that reduces the activity of NADPH oxidase. To prove our hypothesis, we used A23187 and PMA at the concentrations that induced mild NETosis (Fig. 3). Adding of rottlerin in increasing concentrations under these conditions caused a dose-dependent elevation of NETosis (Fig. 3a,b). In order to confirm that rottlerin increases NETosis targeting L-type VGCCs, we applied a typical L-type VGCC antagonist, verapamil. As shown in Fig. 3C, D and Appendix 3, the enhancing effect of rottlerin was inhibited by verapamil in A23187- and PMA-induced NETosis. Interestingly, verapamil was even more effective than compensating for the Rottlerin-induced increase of NET formation. These data indicate that both stimuli can activate L-type VGCCs, which contributes significantly to the induction of NETosis.

In the preliminary experiments, we have shown that rottlerin does not induce NETosis in non-activated neutrophils (data not shown). This suggested that rottlerin acts as L-type VGCC agonist only upon the membrane depolarization (which accompanies neutrophil activation), as it occurs in excitable cells. Depolarization of the neutrophil membrane occurs upon NADPH oxidase activation due to its electrogenic function [26]. In neutrophils of CGD patients, membrane depolarization does not occur, which prevents L-type VGCC opening under the rottlerin action. Therefore, in the absence of a compensatory effect, rottlerin suppresses NETosis as an inhibitor of PKCδ.

Thus, our results show that PKCδ is critical for NETosis, at least in neutrophils of patients with CGD. At the same time, the obtained results do not allow assessing the role of PKCδ in NETosis of neutrophils isolated from healthy donors, since the PKCδ inhibitor rottlerin is also an L-type VGCC agonist and stimulates NETosis, compensating for its possible inhibitory effect.

In conclusion, our results show for the first time that calcium ionophore A23187 induces oxidative burst and NETosis with the participation of conventional (PKCβ), novel (PKCδ), and atypical (PKCζ) PKC isoforms (Figs. 2, 3). Our results show that the mimetic of DAG, PMA, stimulated oxidative burst with the participation of PKCβ and PKCδ (Fig. 2), while only PKCβ was proven to induce NETosis.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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SPELL: 1. citrullinating, 2. decondensation