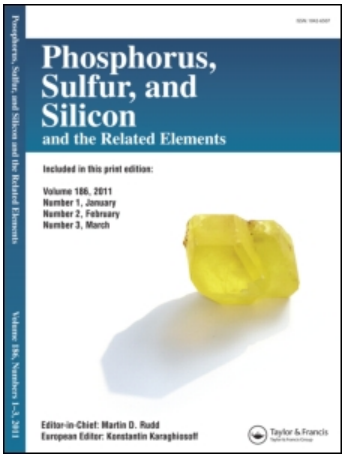

Phosphorus, Sulfur, and Silicon and the Related Elements
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

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To cite this Article Kim-Park, Wan K. , Moore, Michelle A. and Kowolik, Michael J.(1999) 'Regulating Phospholipase C Activity in Human Neutrophils', Phosphorus, Sulfur, and Silicon and the Related Elements, 144: 1, 675 — 679
To link to this Article: DOI: 10.1080/10426509908546335
URL: http://dx.doi.org/10.1080/10426509908546335

Phosphorus, Sulfur and Silicon, 1999, Vol. 144-146, pp. 675-679
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Regulating Phospholipase C Activity in Human Neutrophils

WAN K. KIM-PARK, MICHELLE A. MOORE and
MICHAEL J. KOWOLIK

Dept. of Oral Biology, Indiana University School of Dentistry, Indianapolis, IN 46202

Bacterial-derived formylated peptide, (FMLP) stimulates the respiratory burst activity of human neutrophils via phospholipase C (PLC) activation followed by increased production of second messengers, IP_3 and DG [1]. One synthetic bisphosphonate, clodronate was tested to see how it might affect Ca^{2+}-mediated activation of the neutrophil respiratory burst. Clodronate itself did not significantly change the respiratory burst, measured by Luminol-dependent chemiluminescence (CL). However, clodronate inhibited the FMLP-mediated stimulation of CL significantly ($p<0.001$). A selective inhibitor of PLC, quinacrine, alone inhibited CL significantly ($p<0.0001$) but with clodronate the inhibition was potentiated. The sensitivity to EGTA-treatment with clodronate indicated that clodronate is a Ca^{2+} mobilizing agent. Furthermore, clodronate-mediated CL was sensitive ($p<0.001$) to inhibitors of protein kinase C or tyrosine kinase and potentiated with vanadate treatment. Data suggests possible involvement of bisphosphonate in regulating phospholipase C activity in human neutrophils, probably via Ca^{2+}-mediated phosphorylation of the subunit of PLC.

Keywords: Neutrophil; Phospholipase C; Bisphosphonate

INTRODUCTION

Bisphosphonates have a back bone of P-C-P, similar to pyrophosphate (PPi) but are stable to enzymatic hydrolysis. They inhibit bone resorption [2], and so have been tested mainly in bone and calcified tissues. In addition, an anti-inflammatory action has been suggested [3] and established in an in vitro model with dichloromethylene bisphosphonate (clodronate) and ZK 90695, an experimental bisphosphonate, using human peripheral neutrophils [4]. Neither drug altered control cell respiratory burst activity when tested alone unless the cells were primed by hydroxyapatite (HA) particles. When the concentration of clodronate was high (10^{-4} M), it inhibited CL generation in HA-primed cells but the lower concentrations, 10^{-5} -10^{-13} M, stimulated CL generation. Since the study was performed with PMA- or zymosan-mediated

stimulation following the HA-priming, it was of interest to determine if there is a possible involvement of bisphosphonate in neutrophil respiratory burst activity via membrane-mediated receptor stimulation. A known stimulant, FMLP, stimulates CL generation via PLC activation [1]. The current study was focused on the Ca^{2+} flux which is a determinant of NADPH oxidase activity and the possible role of bisphosphonate in regulating neutrophil respiratory burst activity. In addition, various inhibitors of the kinase/phosphatase cycle were also tested in the presence of clodronate. The effect of bisphosphonate on GM-CSF primed cell respiratory burst activity was compared with other stimulants to deduce some detail of the underlying mechanisms, including any possible involvement of a G-protein cascade in regulating Ca^{2+} flux prior to activation of the NADPH oxidase.

METHODS AND MATERIALS

Neutrophils were harvested from the buffy coats, separated from healthy human donor blood from the Central Indiana Regional blood Center, by a standard method [5]. The average cell yield was 2.2×10^7 cells/ml in 10 ml of RPMI medium. The viability of the cells was determined by Trypan Blue staining. The harvested neutrophils were primed with 10^{-11} M granulocyte macrophage colony-stimulating factor (GM-CSF) or Formyl-Met-Leu-Phe (FMLP) during preincubation for 15 minutes at 37° C. The respiratory burst activity was measured as Luminol-dependent chemiluminescence (CL) following stimulation with various stimulants in millivolts (mV) over 60 minutes at 37° C, and results compared as integrals (mV. min).

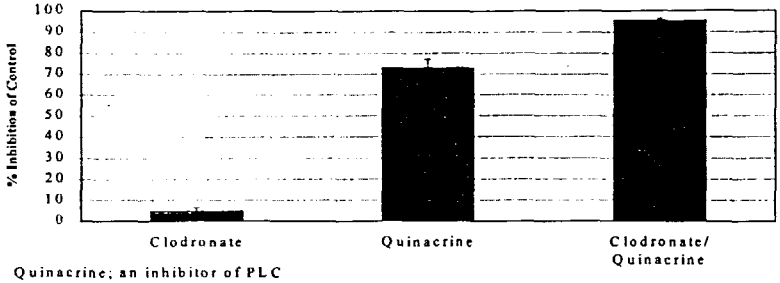
Pretreatment of neutrophils with Ca^{2+} chelators or inhibitors of enzymes was performed for 15 minutes at 37^0 C during the preincubation phase. In control experiments, preincubation was done with only primers. Each protocol was repeated at least 6 times with three replicates of each variable in every run. Statistical analysis was made by student's t-test in paired two-tail tests.

RPMI 1640, PMA. Luminol (5-amino-2,3-dihydro-1,4-phthlazinedione crystalline), D-2-amino-7-phosphnoheptanoate (AP7), N-methyl-D-aspartate (NMDA), sodium vanadate, Ro31-8220, genistein, Pertussis toxin and HISTOPAQ-1119 were purchased from Sigma Chemical Co. (St. Louis, MO). BAPTA and quinacrine were purchased from Cal Biochem. Clodronate was a gift from Dr. Hyvönen, Kuopio, Finland.

RESULTS

Clodronate alone did not change CL significantly but it potentiated quinacrine-mediated inhibition of CL. (Figure 1).

Figure 1. Effect of clodronate on CL



Quinacrine; an inhibitor of PLC
Data expressed as mean of 10 experiments (in triplicate) +/- S.E.M.

As shown in Table 1, the clodronate-mediated CL generation was sensitive to kinase inhibitors and when the endogenous phosphatase was inhibited by sodium vanadate, there was a significant ($p<0.0001$) stimulation of CL generation.

Table 1. Clodronate with inhibitors of kinases and phosphatase

	% increase	% decrease
Control	0	0
Clodronate		4.5 ± 5.130
Clodronate/Va	324.3 ± 29.83	
Clodronate/BIMH		44.6 ± 8.64
Clodronate/Gen		56.4 ± 0.729
Clodronate/BIMH/ Gen.		50.8 ± 1.11

Data expressed as mean of 6 experiments (in triplicates) ± SEM. Va; Sodium Vanadate, inhibitor of Tyrosine Phosphatase. BIMH; inhibitor of PKC. Genistein; inhibitor of Tyrosine Kinase

As shown in Table 2, clodronate-mediated CL generation was inhibited significantly ($p<0.0001$) by the pretreatment with EGTA. Furthermore, the EGTA-treatment potentiated the quinacrine-mediated inhibition of PLC on CL generation.

Table 2. EGTA-sensitivity of clodronate (% inhibition of control)

Control	0
EGTA	78.00 ± 4.933
Quinacrine	77.33 ± 4.372
EGTA/quinacrine	93.33 ± 3.180

Data expressed as mean of 3 experiments (in triplicates) ± S.E.M.

Since it was suggestive that clodronate can mobilize Ca^{2+} from the extracellular media (Table 2), it was of interest to know if clodronate affects a putative Ca^{2+} channel in PMNs, a NMDA channel [6]. Clodronate was tested with a selective inhibitor of the NMDA site, AP7. The inhibition was similar to NMDA, in inhibiting CL generation with AP7 treatment (Table 3).

Table 3. Clodronate and NMDA Ca^{2+} channel

	% increase	% decrease
Control	0	0
Clodronate	0.5 ± 4.33	0
Clodronate/AP7	0	43.0 ± 1.247
NMDA	1.75 ± 9.369	0
NMDA/AP7	0	41.67 ± 5.193
NMDA/Clodronate	8.25 ±8.436	

AP7; inhibitor of NMDA-site, data expressed as mean of 6 experiments (in triplicates) ± SEM

DISCUSSION AND CONCLUSIONS

In the present study, clodronate (10^{-7} M) stimulated CL production in primed neutrophils when the endogenous phosphatase was inhibited by vanadate-pretreatment in a EGTA-sensitive manner. The data suggest that endogenous phosphatase activity in neutrophils is high and it may be a pace maker between kinase-mediated activation of the NADPH oxidase by dephosphorylation-mediated control of possible over-activation of the respiratory burst activity. In addition, quinacrine-treatment inhibited the clodronate-mediated CL generation almost completely, up to 95%, while quinaqcrine alone inhibited CL generation of the control cells by 74%. It would be logical to hypothesize that clodronate may activate membrane-bound PLC via the NMDA Ca^{2+} channel-mediated phosphorylation of the enzyme. If the NMDA receptor-mediated Ca^{2+} influx is so high (excessive) as to activate phosphatase, the quinacrine effect in

inhibiting the PLC becomes bigger, resulting in a significant inhibitory effect on NADPH oxidase activation. The sensitivity to quinacrine-treatment, AP7 and to Purtussis toxin supported the idea that clodronate can stimulate membrane-bound PLC via an NMDA receptor coupled to a G-protein cascade.

In this in vitro experimental system, a bisphosphonate, clodronate affected the activity of PLC via a Ca^{2+}-mediated phosphorylation/dephosphorylation mechanism, depending on the Ca^{2+} concentration increased by clodronate. It appears that clodronate acts on a NMDA channel to phosphorylate the subunits of PLC , prior to activation of the enzyme. Since the degree of inhibition with quinacrine or AP7 followed by clodronate was similar, this was further evidence. Importantly, the EGTA-sensitivity to clodronate-mediated CL generation demonstrated that clodronate is a Ca^{2+} mobilizing agent and the Ca^{2-} may come through a NMDA channel to phosphorylate the PLC subunit. Clodronate may have an affinity to an NMDA site or one in the vicinity. The important finding here is that the concentration of transiently increased Ca^{2+} (by clodronate) is crucial. If it is excessive, it will activate phosphatase dominantly, masking (or reducing) the effect of kinase. Clodronate may use a G-protein cascade as seen in the FMLP-mediated respiratory burst activity in neutrophils. It seems legitimate to conclude that clodronate may stimulate membrane-bound PLC via an NMDA receptor mediated phosphorylation and affect the NADPH oxidase complex through the kinase/phosphatase cycle, probably via a G-protein cascade. Bisphosphonate could be clinically useful as an anti-inflammatory agent when the conditions for the activation of neutrophil NADPH oxidase are met.

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