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DIACYLGLYCEROL KINASE INHIBITORS R59022 AND DIOCTANOYLETHYLENE GLYCOL POTENTIATE THE RESPIRATORY BURST OF NEUTROPHILS BY RAISING CYTOSOLIC Ca^{2+}

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The diacylglycerol kinase inhibitors, R59022 and dioctanoylethylene glycol (diC_8-eg), potentiate stimulation of the respiratory burst by the chemotactic tripeptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) in human neutrophils. However, in contrast to the potentiation observed in intact cells, neither R59022 nor diC_8-eg enhanced the effect of fMLP on O_2 consumption in electropermeabilized neutrophils, under conditions where cytosolic $[Ca^{2+}]$ was held constant using EGTA. In unstimulated, intact cells treatment with the diacylglycerol kinase inhibitors elicited an increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$). The results suggest that enhancement of the respiratory burst by diC_8-eg and R59022 is mediated by a rise in $[Ca^{2+}]_i$, rather than by inhibition of diacylglycerol kinase.

Activation of neutrophils by the tripeptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) results in a burst of O_2 consumption to support the production of O_2^- by a membrane-bound NADPH oxidase. Stimulation of this respiratory burst is associated with phosphoinositide hydrolysis, an increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$), and the production of diacylglycerol, but the exact steps required to activate the oxidase remain unclear (1, 2). Studies to determine the role of the activation of protein kinase C by diacylglycerol for the stimulation of O_2^- production have produced contradictory results. Protein kinase C inhibitors do not inhibit fMLP stimulation of O_2^- production under conditions where the response to exogenously added diacylglycerol is blocked, indicating that the fMLP pathway is not mediated by protein kinase C (3, 4, 5). On the other hand, treatment of neutrophils with the diacylglycerol kinase inhibitor R59022, which prolongs the stimulation of

ABBREVIATIONS

$[Ca^{2+}]_i$, cytosolic Ca^{2+}; diC_8, 1,2-dioctanoylglycerol; diC_8-eg, dioctanoylethylene glycol; fMLP, N-formyl-methionyl-leucyl-phenylalanine; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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protein kinase C by diacylglycerol by preventing the phosphorylation of diacylglycerol to phosphatidic acid, potentiates fMLP-induced O_2^- production, suggesting that fMLP is able to act through protein kinase C (6, 7).

The role of the increase in $[Ca^{2+}]_i$ in the activation of the respiratory burst is also unclear, but Ca^{2+} changes do not appear to play a primary role in signalling. Elevation of $[Ca^{2+}]_i$ by means of ionophores is a poor stimulus of O_2^- production, though it potentiates the response of other stimuli including fMLP (8), suggesting that Ca^{2+} may play a role as a modulator rather than as an essential second messenger. In electropermeabilized neutrophils, fMLP stimulates O_2 consumption under conditions where $[Ca^{2+}]_i$ remains fixed at resting levels (5). Electropermeabilization renders the plasma membrane permeant to inorganic ions and small molecules (approximately ≤ 1000 daltons), allowing equilibration with Ca^{2+}-buffering agents such as EGTA and facilitating stabilization of $[Ca^{2+}]_i$ at the desired level. To examine the role of protein kinase C in fMLP activation independently of changes in $[Ca^{2+}]_i$, we have treated electropermeabilized cells with the diacylglycerol kinase inhibitors dioctanoylethylene glycol (diC_8-eg) (9) and R59022 (10). Both of these inhibitors were found to potentiate fMLP-induced O_2 consumption in intact, but not in electropermeabilized human neutrophils. Measurements of $[Ca^{2+}]_i$ in intact cells suggest that elevation of $[Ca^{2+}]_i$ by the inhibitors may be responsible for the potentiation of the O_2^- response.

METHODS

Reagents and Solutions: R59022 was from Calbiochem. diC_8-eg was from Molecular Probes. Both were prepared as 10^{-2} M stocks in DMSO. fMLP (Sigma) was used as a 10^{-3} M stock in DMSO. At the concentrations used, DMSO alone had no effect on O_2 consumption or $[Ca^{2+}]_i$. Na^+ solution (pH 7.3) contained (in mM) 140 NaCl, 10 HEPES, 10 glucose, 5 KCl, and either 2 $CaCl_2$ or 1 EGTA, as indicated. Permeabilization medium (pH 7.0) contained (in mM) 140 KCl, 10 HEPES, 10 glucose, 1 ATP, 1 $MgCl_2$, 1 EGTA, and 0.193 $CaCl_2$ to give a final free $[Ca^{2+}]$ of 100 nM. All solutions were adjusted to 290 ± 5 mosM.

Cell Isolation and Permeabilization: Neutrophils were isolated from fresh human blood by dextran sedimentation followed by Ficoll-Hypaque gradient centrifugation (11). Contaminating red cells were removed by ammonium chloride lysis. The purified neutrophils were suspended in solution RPMI 1640 (HCO_3^--free, with 25 mM HEPES) at room temperature until use. To permeabilize, 8 x 10^6 cells were suspended in permeabilization medium and subjected to 2 discharges of 5 kV/cm from a Bio-Rad Gene Pulser as outlined in (5).

O_2 consumption: O_2 consumption was measured as in (5) with a Model 53 biological oxygen monitor (Yellow Springs Instruments). Intact cells (6 x 10^6) were suspended in 2 ml of Na^+ solution and permeabilized cells (4 x 10^6) were suspended in 2 ml of permeabilization medium containing 2 mM NADPH and 100 μM GTP. Where indicated, R59022 or diC_8-eg was added 1.5 to 2 min prior to the addition of fMLP. O_2 consumption was calculated using a solubility coefficient of 0.024 ml O_2 ml^{-1} medium at 37°C.

Cytoplasmic Ca^{2+} determinations: $[Ca^{2+}]_i$ was measured in cells loaded with 1 μM indo-1 acetoxymethylester as outlined in (12). Indo-1 loaded cells (3 x 10^6 ml^{-1}) were suspended in 1 ml of Na^+ medium at 37°C, and fluorescence was monitored with excitation at 331 nm and emssion at 410 nm, using 3 and 15 nm slits, respectively. Where indicated, cells were loaded with 1 μM fura-2 acetoxymethylester and fluorescence monitored with excitation at 335 nm and emission at 510 nm. Calibration was performed using ionomycin and Mn^{2+} as decribed in (13). All compounds were tested for autofluorescence.

All measurements were performed at 37°C and were repeated with blood from at least three different donors. Data are presented as means ± one SE of the number of determinations indicated in parenthesis.

RESULTS AND DISCUSSION

In intact neutrophils, 10^{-6} M fMLP stimulated a transient burst of O_2 consumption after a short lag (approximately 0.5 min), as illustrated in Fig. 1. The maximum rate of O_2 consumption was 2.1

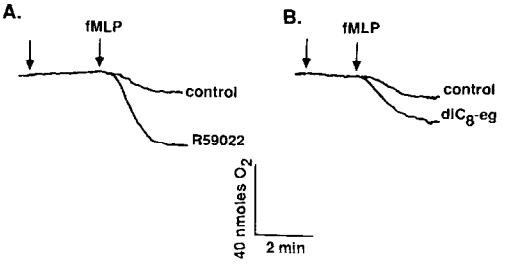

Figure 1: Effect of diacylglycerol kinase inhibitors on fMLP-induced O_2 consumption in intact neutrophils. Intact cells (6 x 10^6) were suspended in 2 ml Na^+ medium containing 2 mM Ca^{2+} at 37°C with stirring 1-2 min before the start of the trace. 20 μM R59022 (A) or 20 μM diC_8-eg (B) was added to the indicated trace at the first arrow. No addition was made to control traces at the first arrow. 10^{-6} M fMLP was added to both traces at the second arrow. O_2 consumption was measured as outlined in Methods.

± 0.3 nmoles $O_2 \cdot 10^6$ $cells^{-1} \cdot min^{-1}$ (n=5) and the burst was complete within 2 min, after which O_2 consumption returned to approximately the basal rate. The addition of either R59022 or diC_8-eg alone did not stimulate O_2 consumption, but both diacylglycerol kinase inhibitors potentiated the response of fMLP. When 20 μM R59022 was added to the cell suspension 1.5- 2 min prior to the addition of fMLP, the maximum rate of O_2 consumption elicited by fMLP increased to 4.6 ± 0.5 nmoles $O_2 \cdot 10^6$ $cells^{-1} \cdot min^{-1}$ (n=5), without affecting the lag time or the duration of the response (Fig. 1A). This represents a 120% increase in the rate of O_2 consumption, which is comparable with the enhancement reported previously (6, 7). Another diacylglycerol kinase inhibitor, diC_8-eg, showed a smaller but still significant enhancement of fMLP-induced O_2 consumption (Fig.1B). When 20 μM diC_8-eg was added to the cells before fMLP, the maximum rate of fMLP-induced O_2 consumption increased to 3.2 ± 0.1 nmoles $O_2 \cdot 10^6$ $cells^{-1} \cdot min^{-1}$ (n=5). Although this represents only a 50% enhancement, smaller than that produced by R59022, the increase was still significant ($p < 0.005$).

As previously described, the respiratory burst elicited in electropermeabilized neutrophils is larger than in intact cells, despite the absence of an increase in $[Ca^{2+}]_i$, (ref. 5 and Fig. 2). When fMLP is used as a stimulus, this is reflected in both an increased maximum rate of O_2 consumption and a prolonged duration of the response, which contrasts with the rapid termination of the burst in intact cells. As discussed in (5), this may reflect the diffusion of a soluble inhibitor out of the permeabilized cells. In contrast to the results obtained in intact cells, neither R59022 nor diC_8-eg potentiated the respiratory burst in permeabilized cells. The addition of R59022 prior to the addition of fMLP did not significantly ($p > 0.1$) enhance the rate or duration of fMLP-induced O_2 consumption (Fig. 2A). The maximum rate of O_2 consumption was 11.9 ± 1.4 nmoles $O_2 \cdot 10^6$ $cells^{-1} \cdot min^{-1}$ (n=6) in the absence of R59022 compared with 14.4 ± 2.4 nmoles $O_2 \cdot 10^6$ $cells^{-1} \cdot min^{-1}$ (n=5) in cells treated with R59022. Electropermeabilized cells treated with diC_8-eg were in fact slightly inhibited compared to untreated cells (8.8 ± 1.7 nmoles $O_2 \cdot 10^6$ $cells^{-1} \cdot min^{-1}$, n=5; Fig. 2B). Failure of the diacylglycerol kinase inhibitors to potentiate the fMLP response in the permeabilized cells was not due to the cells having reached a maximum rate of O_2 consumption in the absence of the inhibitors. This was demonstrated by using the synthetic diacylglycerol 1,2-dioctanoylglycerol (diC_8), which stimulated O_2 consumption to a rate of 21.4 ± 1.6 nmoles

$O_2 \cdot 10^6$ cells^{-1}·min^{-1} (n= 6) in electropermeabilized cells (Fig. 2C). A similar rate, 22.5 ± 1.0 nmoles $O_2 \cdot 10^6$ cells^{-1}·min^{-1} (n= 4), was induced by another protein kinase C activator, 12-O-tetradecanoylphorbol-13-acetate (TPA). These rates are almost twice as large as those obtained with fMLP, indicating that neither protein kinase C activation by diacylglycerol nor saturation of the NADPH oxidase are limiting the rate of O_2 consumption induced by fMLP.

The finding that the diacylglycerol kinase inhibitors can potentiate the fMLP-induced respiratory burst in intact, but not in permeabilized neutrophils, suggests that either fMLP can act through protein kinase C in the intact, but not the permeabilized cells or, alternatively, that the effects of R59022 and diC_8-eg are not mediated by their inhibition of diacylglycerol kinase. The former hypothesis seems unlikely, since in both intact and permeabilized cells fMLP stimulation is sensitive to pertussis toxin, indicating that at least the initial steps of activation are identical in the two systems (unpublished observations). Moreover, in both intact and permeabilized cells the fMLP response is unaffected by the protein kinase C inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7), consistent with the reponse being mediated by a similar pathway in the two systems (5, 14). In addition, the observation that both TPA and diC_8 can elicit large responses in the permeabilized cells indicates that protein kinase C is functional in this system. Thus, it is more likely that R59022 and diC_8-eg are acting by a mechanism other than inhibition of diacylglycerol kinase.

Neutrophils can be primed to enhance production of O_2^- by the addition of low concentrations of the Ca^{2+} ionophore ionomycin. Priming concentrations of ionomycin are not by themselves stimulatory, but potentiate the respiratory burst produced by a subsequent stimulus such as fMLP. Because some fatty acid derivatives such as sphinganine and diC_8 have been reported to increase $[Ca^{2+}]_i$ by a non-specific or undefined mechansim (15, 16), it was possible that the diacylglycerol kinase inhibitors were also increasing $[Ca^{2+}]_i$ in the intact cells. Such an increase would not occur in the permeabilized cells where $[Ca^{2+}]$ is fixed, which could explain the differential effects of the inhibitors in the two systems. This possibility was tested by loading intact cells with the fluorescent

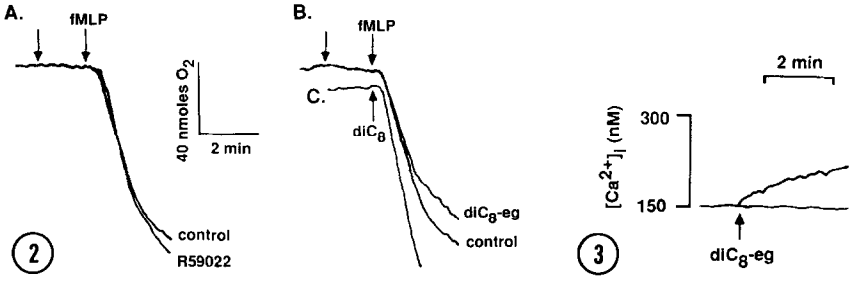

Figure 2: Effect of diacylglycerol kinase inhibitors on fMLP-induced O_2 consumption in electropermeabilized neutrophils. Electropermeabilized cells (4 X 10^6) were suspended in 2 ml of permeabilization medium containing 2 mM NADPH and 100 μM GTP at 37°C with stirring 1-2 min before the start of the trace. (A) 20 μM R59022 was added to the indicated trace at the first arrow. 10^{-6} M fMLP was added to both traces at the second arrow. (B) 20 μM diC_8-eg was added to the indicated trace at the first arrow. 10^{-6} M fMLP was added to both traces at the second arrow. (C) 1.5 μM diC_8 was added at the arrow. O_2 consumption was measured as outlined in Methods.

Figure 3: Effect of diC_8-eg on $[Ca^{2+}]_i$ in neutrophils. $[Ca^{2+}]_i$ was measured using the fluorescent indicator indo-1 as outlined in Methods. 3 X 10^6 cells loaded with 1 μM indo-1 were suspended in 1 ml of Na^+ medium containing 2 mM Ca^{2+} at 37°C with stirring 1-2 min before the start of the trace. 20 μM diC_8-eg was added to the top trace at the arrow.

Ca^{2+} indicator indo-1 and suspending the cells in Na^+ medium containing 2 mM Ca^{2+}. Addition of 20 μM diC_8-eg caused a slow increase in $[Ca^{2+}]_i$ that began immediately after addition of the inhibitor (Fig. 3). Two min following the addition of diC_8-eg, $[Ca^{2+}]_i$ had increased by 41 ± 8 nM (n=6). $[Ca^{2+}]_i$ continued to increase for up to 4 min, the longest time point measured. When intact neutrophils were suspended in Ca^{2+}-free Na^+ medium containing 1 mM EGTA, the increase in $[Ca^{2+}]_i$ elicited by diC_8-eg was reduced but not abolished, indicating that diC_8-eg causes both a release of Ca^{2+} from intracellular stores and an influx of extracellular Ca^{2+}. In Ca^{2+}-free medium, a transient increase in $[Ca^{2+}]_i$ of 17 ± 3 nM (n=3) was seen that peaked 1 min after the addition of diC_8-eg, followed by a slow decline (not illustrated). The intrinsic fluorescence of R59022 interfered with the measurement of $[Ca^{2+}]_i$ at the wavelengths used to detect indo-1. When fura-2 was used to measure $[Ca^{2+}]_i$, R59022 caused an increase in $[Ca^{2+}]_i$ that was qualitatively similar to that observed with diC_8-eg. In our hands the autofluorescence of R59022 was still sufficiently large to preclude accurate calibration of fura-2. However, Mege *et al* have reported that R59022 causes an increase $[Ca^{2+}]_i$ of approximately 60 nM in otherwise untreated rabbit neutrophils (6), which is similar to the increase we observed with diC_8-eg.

If the potentiation of the fMLP-induced respiratory burst in intact neutrophils by diC_8-eg and R59022 is due to an increase in $[Ca^{2+}]_i$ rather than to their ability to inhibit diacylglycerol kinase, it would be expected that reduction of the $[Ca^{2+}]_i$ change would also reduce the potentiation seen with the inhibitors. In Ca^{2+}-free medium, fMLP-stimulation of O_2 consumption in the intact cells reached a maximum rate of 3.7 ± 0.5 nmoles $O_2 \cdot 10^6$ $cells^{-1} \cdot min^{-1}$ (n=6). Following addition of 20 μM R59022 or 20 μM diC_8-eg, stimulation with fMLP induced O_2 consumption reaching maximal rates of 5.5 ± 0.8 nmoles $O_2 \cdot 10^6$ $cells^{-1} \cdot min^{-1}$ (n=6) and 4.3 ± 0.9 nmoles $O_2 \cdot 10^6$ $cells^{-1} \cdot min^{-1}$ (n=5), respectively. Thus, R59022 enhanced fMLP-induced O_2 consumption by 50% in the absence of extracellular Ca^{2+} compared to 120% in the presence of extracellular Ca^{2+}, while the enhancement by diC_8-eg in Ca^{2+}-free medium was not statistically significant ($p > 0.1$). These results contrast those of Mege *et al* (6), who found that R59022 actually produced a greater relative enhancement of O_2^- generation by rabbit neutrophils in Ca^{2+}-free compared to Ca^{2+}-containing media. However, the conditions used in the assay differed significantly from those used in this paper. The cells used by Mege *et al* (6) were incubated in the absence of extracellular Ca^{2+} for 30 min prior to the assay, and R59022 was added 30 min prior to the assay. These differences may account for the contrasting findings.

In summary, the results presented here suggest that the increases in $[Ca^{2+}]_i$ caused by diC_8-eg and R59022, rather than their inhibition of diacyglycerol kinase, mediate their potentiation of the respiratory burst in neutrophils. Priming by an increase in $[Ca^{2+}]_i$ can explain why R59022 and diC_8-eg are able to augment fMLP stimulation in intact cells but not in in permeabilized cells, where $[Ca^{2+}]$ is fixed. The reduced potentiation observed in the absence of extracellular Ca^{2+} also supports a role for increased $[Ca^{2+}]_i$ in the enhancement of the respiratory burst caused by diC_8-eg and R59022. In view of the results reported here, R59022 and diC_8-eg cannot be assumed to act specifically on the diacylglycerol kinase and caution should be exercised when these agents are used to examine the role of diacylglycerol in neutrophil activation.

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