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ERYTHROID DIFFERENTIATION FACTOR STIMULATES HYDROLYSIS OF POLYPHOSPHOINOSITIDE IN FRIEND ERYTHROLEUKEMIA CELLS

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Summary We examined an early action of erythroid differentiation factor (EDF), a polypeptide which induces differentiation of Friend murine erythroleukemia (MEL) cells. (Eto et al., Biochem. Biophys. Res. Commun. 142: 1095-1103, 1987). In MEL cells, EDF caused a rapid and transient increase in cytoplasmic concentration of free calcium, $[Ca^{2+}]_c$. EDF increased $[Ca^{2+}]_c$ even in the absence of extracellular calcium. When [^{3}H]inositol-labeled MEL cells were incubated with EDF, EDF rapidly increased radioactivity in inositol trisphosphate, bisphosphate and monophosphate. EDF also increased [^{3}H] diacylglycerol in [^{3}H]arachidonate-labeled MEL cells. These results indicate that EDF increases $[Ca^{2+}]_c$ by stimulating hydrolysis of polyphosphoinositide.

Friend murine erythroleukemia (MEL) cells are virally transformed erythroid precursor cells which provide a model system to study the mechanism for erythroid differentiation. Various chemical agents induce MEL cells to terminally differentiate along the erythroid lineage: when cultured with dimethyl sulfoxide or hexamethylenebisacetamide, MEL cells

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become committed to differentiate and start accumulating hemoglobin (1, 2).

We have recently isolated a polypeptide factor, from conditioned medium of phorbol ester treated human monocyte-like cell line THP-1, which induces differentiation in MEL cells (3). This factor, termed as erythroid differentiation factor (EDF), is a homodimer of a molecular weight of 25 KDa, the subunit of which is identical to β_A-chain of porcine inhibin. The maximum effect of EDF is obtained at as low as nanomolar range (3). Hence, EDF appears to be a novel inducer of differentiation, which acts via a receptor-mediated mechanism. In an attempt to clarify the mechanism of action of EDF, we examined the effect of EDF on cell calcium metabolism since Cantley and colleagues postulated that calcium plays an important role in differentiation of MEL cells (4). The present results indicate that EDF increases cytoplasmic free calcium concentration by generating inositol trisphosphate, the putative mobilizer of calcium from the non-mitochondrial trigger pool.

Materials and Methods

Cell Culture Friend MEL cells F5-5, established from the DDD mouse by Ikawa et al. (5), were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum and maintained in a humidified incubator under an atmosphere of 95% air and 5% CO .

Measurement of Changes In Cytoplasmic Free Calcium Cytoplasmic free calcium concentration, $[Ca^{2+}]_c$, was monitored by measuring aequorin luminescence (6). Aequorin was loaded by the method of Morgan and Morgan (7) as described previously (8). Aequorin-loaded cells were incubated in modified Hanks solution containing 137 mM NaCl, 3.5 mM KCl, 4.2 mM $NaHCO_3$, 0.33 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.5 mM $CaCl_2$, 20 mM Hepes/NaOH (pH 7.4) equilibrated with O_2 gas. When cells were stimulated in calcium free medium, calcium free

modified Hanks solution containing 1 mM EGTA was employed. Aequorin luminescence was measured as described previously (8). Cell suspension containing 5 x 10^6 cells in 1 ml was applied into a cuvette and incubated at 37 C under constant stirring. Aequorin luminescence is not calibrated in terms of free calcium concentration since spatial distribution of calcium is not known (8). Traces presented are representative of at least three experiments.

Measurement of Inositol Phosphates MEL cells were labeled with [^{3}H]inositol by incubating in inositol-free Ham's F-12 medium containing 0.5 % fetal calf serum and 2 uCi/ml [^{3}H]inositol for 24 hrs. Cells were then washed with Ham's F-12 medium and incubated in the same medium containing 10 mM LiCl. Ten min later, cells were stimulated by EDF for indicated time. The reaction was terminated by adding perchlorate to a final concentration of 10 %. Cells were homogenized by aspirating through a 26 G needle and centrifuged at 200 x g for 10 min. The supernatant was neutralized by adding 5 M KOH and applied onto an anion exchange column. Inositol phosphates were separated by the method of Berridge et al. (9).

Measurement of Diacylglycerol Production MEL cells were labeled with [^{3}H]arachidonic acid. Cells were incubated in Ham's F-12 medium containing 0.5% fetal calf serum and 2 uCi [^{3}H]arachidonic acid for 24 hrs. Cells were then washed with Ham's F-12 medium and resuspended in the same medium. Cells were then stimulated by EDF for indicated time. The reaction was terminated by adding chloroform/methanol (1:2, v/v). Lipids were extracted and neutral lipids were separated by thin layer chromatography as described previously (10).

Materials EDF was prepared as described previously (3). Aequorin was purchased from Dr. J. R. Blinks of Mayo Foundation (Rochester, MN). [^{3}H]Inositol and [^{3}H]arachidonic acid were obtained from New England Nuclear (Boston, MA)

Results and Discussion

We have recently shown that EDF is a potent inducer of differentiation in MEL cells (3). To examine whether EDF affects cellular calcium metabolism in MEL cells, aequorin-loaded MEL cells were incubated with EDF and changes in aequorin luminescence were monitored. As demonstrated in Fig. 1A, 3 nM EDF, which elicits the maximum differentiation-inducing activity

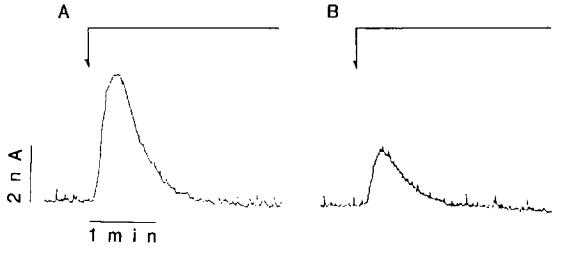


Figure 1. Effect of EDF on Cytoplasmic Free Calcium Concentration

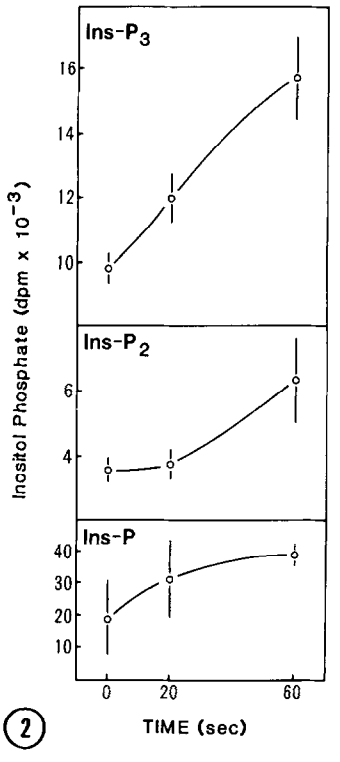
Aequorin-loaded MEL cells were stimulated by 3 nM EDF as indicated by the arrow in the presence (A) and absence (B) of extracellular calcium. EDF was added as indicated by the arrow.

in MEL cells (3), caused a rapid increase in aequorin luminescence. EDF-induced increase in luminescence was observed after an apporoximately 10 sec lag time. A peak in aequorin luminescence was observed at 30 sec and the luminescence returned to the basal value within 2 min. Thus, EDF caused a rapid but transient rise in aequorin luminescence in MEL cells. To determine whether EDF mobilizes calcium from an intracellular pool, the effect of EDF on $[Ca^{2+}]_c$ was measured in calcium free medium. As shown in Fig. 1B, EDF elicits a rapid and transient increase in $[Ca^{2+}]_c$ in calcium-free medium even though the increment was slightly smaller. The ED_{50} of EDF to which induce differentiation was 125 pM (3). At 100 pM, EDF induced a small increase in $[Ca^{2+}]_c$ (data not shown). These results indicate that an early action of EDF in MEL cells is to increase $[Ca^{2+}]_c$ by mobilizing calcium from an intracellular pool.

Recent studies have shown that calcium mobilizing agonists cause hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P_2) and generate inositol trisphosphate (Ins-P_3), the putative mobilizer of

calcium from an intracellular non-mitochondrial pool (11). In the next set of experiments, we examined whether EDF increased Ins-P_3. In unstimulated MEL cells labeled with [^{3}H]inositol, radioactivity in Ins-P_3 fraction was relatively high. Since we did not separate isomers of Ins-P_3, the composition of isomers in the Ins-P_3 fraction was not known. When MEL cells were incubated with 3 nM EDF, radioactivity in Ins-P_3 increased within 20 sec (Fig 2). EDF also elevated [^{3}H]-Ins-P_2. Radioactivity in Ins-P_1 fraction increased slowly in response to EDF. These results suggest that EDF increases inositol polyphosphates by stimulating hydrolysis of polyphosphoinositide. To confirm this point, we measured production of diacylglycerol, another product of hydrolysis of PtdIns4,5-P_2, in [^{3}H]arachidonic acid-labeled MEL cells. As shown in Fig 3, [^{3}H]diacylglycerol increased in response to EDF. Although the increase was not detected at 20 sec, a peak in [^{3}H]diacylglycerol was observed at 60 sec. At 5 min, radioactivity was still greater than unstimulated value. Thus, EDF caused a rapid accumulation of both Ins-P_3 and diacylglyerol by stimulating hydrolysis of PtdIns4,5-P_2.

The present results demonstrate that EDF increases $[Ca^{2+}]_c$ in MEL cells by causing release of calcium from non-mitochondrial pool. To our knowledge, this is the earliest alteration in MEL cell metabolism caused by an inducer of differentiation. A potent action of EDF on $[Ca^{2+}]_c$ described in this study favors the proposal by Cantley and colleagues that calcium plays a key role in causing differentiation of MEL cells. Furthermore, the



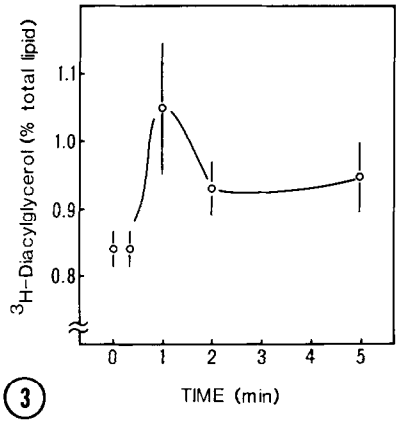


Figure 2. Effect of EDF on the Production of Inositol Phosphates

[^{3}H]Inositol-labeled MEL cells were incubated with 3 nM EDF for indicated time in the presence of 10 mM lithium chloride. Inositol phosphates were analysed as described in Methods. Values are the mean ± S.E. for three determinations and representative of three experiments with similar results.

Figure 3. Effect of EDF on Diacylglycerol Production

[^{3}H] Arachidonic acid-labeled MEL cells were incubated with 3 nM EDF for indicated time. Diacyglycerol was measured as described in Methods. Values are the mean ± S.E. for four determintations and representative of two experiments with similar results.

fact that EDF causes hydrolysis of polyphosphoinositide in MEL cells suggests that EDF acts by receptor-mediated calcium messenger system. EDF thus provides a useful system to elucidate a role of calcium in differentiation of MEL cells.

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