PKCε Is Involved in Granulocyte–Macrophage Colony-Stimulating Factor Signal Transduction: Evidence from Microphysiometry and Antisense Oligonucleotide Experiments†

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ABSTRACT: We have used microphysiometry and antisense methodology to show that the ε isoenzyme of protein kinase C (PKC) is involved in the signal transduction pathway of granulocyte–macrophage colony-stimulating factor (GM-CSF) in a human bone marrow cell line, TF-1. These cells require GM-CSF or a related cytokine for proliferation. When the cells are appropriately exposed to GM-CSF, they exhibit a burst of metabolic activity that can be detected on the time scale of minutes in the microphysiometer, a biosensor-based instrument that measures the rate at which cells excrete protons. These cells express PKCα and -ε, as determined by Western blot analysis. Treatment with isoenzyme-specific antisense oligonucleotides inhibits expression appropriately, but only inhibition of PKCε appreciably diminishes the burst of metabolic activity induced by GM-CSF. Consistent with the involvement of PKCε, GM-CSF appears to activate phospholipase D and does not cause a detectable increase in cytosolic [Ca^{2+}].

Since being established from the bone marrow of a human erythroleukemia patient by Kitamura et al. (1979), the TF-1 cell line has proven to be useful model system with which to study signal transduction by hemopoietic growth factors (Kitamura et al., 1991).

We[1] (Wada et al., 1992) have used a novel biosensor-based instrument, the microphysiometer (Parce et al., 1989; McConnell et al., 1992), to detect integrative physiological responses of these cells to cytokines. For example, GM-CSF[2] induces a burst of metabolic activity that occurs at physiologically relevant concentrations and is inhibitable by antibodies to GM-CSF. The abilities of GM-CSF and five other cytokines to elicit such responses correlate very well with their abilities to cause the proliferation of these cells.

The microphysiometer has been shown to detect the activation of a wide variety of cellular receptors in a pharmacologically relevant manner, for example, the T-cell (Nag et al., 1992), kainate glutamate (Rayley-Susman et al., 1992), epidermal growth factor (Owicki et al., 1989), m_1-muscarinic receptor (Owicki et al., 1989), and β_2-adrenergic receptor (Owicki et al., 1989). It does so by detecting changes, sometimes weak and transient, in the rates at which cells excrete protons. Extracellular acidification reflects the rate of production of the acidic products of energy metabolism (principally lactic acid and CO_2) and the regulation of intracellular pH (Owicki & Parce, 1992). Changes in extracellular acidification caused by receptor activation therefore reflect changes in these processes.

Our earlier work (Wada et al., 1992) suggested that PKC was involved in the GM-CSF signal transduction in these cells: pretreatment with a phorbol ester inhibited the GM-CSF acidification response. The response was also suppressed by calphostin C, an inhibitor of PKC.

PKC is a family of multiple isoforms having closely related but different structures (Ono et al., 1988; Nishizuka, 1988; Parker et al., 1989). Some cells express only one of the isoforms identified to date (Pelosin et al., 1987; Rose-John et al., 1988; Heidenreich et al., 1990) while the majority coexpress multiple isoforms (Strulovici et al., 1989, 1991; Kiley et al., 1990; Pfeffer et al., 1990). It is anticipated that each of these isoforms mediates distinct cellular responses. Biochemically, PKCα, -$\beta_{I/II}$, and -γ exhibit subtle differences and are known as Ca^{2+}- and phospholipid-dependent kinases (Jaken & Kiley, 1987) whereas PKCδ, -ε, and -η are phospholipid-dependent yet Ca^{2+}-independent kinases (Kiley et al., 1990; Leibersperger et al., 1990; Bacher et al., 1991).

The present paper strengthens the conclusion that PKC is involved in the response of TF-1 cells to GM-CSF by demonstrating that staurosporine, another PKC inhibitor, also inhibits the acidification response. More importantly, this work identifies an isoenzyme of PKC that is involved in GM-CSF signal transduction by showing that the response can be inhibited by treatment with antisense oligonucleotide. Evidence is also provided that the activation of phospholipase D is involved in the process.

MATERIALS AND METHODS

Chemicals. Unless otherwise noted, chemicals were purchased from Sigma (St. Louis, MO). Antisense 15-mer oligodeoxynucleotides to the PKC isoenzymes were synthesized by Operon Technologies (Alameda, CA) on the basis of sequences obtained from the GenBank database. No human PKCε sequence was available, but a partial human PKCα sequence permitted construction of an antisense oligonucleotide beginning at nucleotide 41. Oligonucleotides based on rat PKCα and -ε sequences began at the start codon. Following are the antisense sequences used (5′ → 3′): human PKCα,

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[1] DeVries et al., Abstract, International Cytokine Workshop, Stresa, Italy, Nov 1991; Indelicato et al., submitted for publication.

[2] Abbreviations: AM, acetoxymethyl ester; $[Ca^{2+}]_i$, intracellular (cytosolic) free calcium ion concentration; FBS, fetal bovine serum; GM-CSF, granulocyte–macrophage colony-stimulating factor; HSA, human serum albumin; PAF, platelet-activating factor; pH_i, intracellular (cytosolic) pH; PKC, protein kinase C; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.

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CGGTTGGCCACGTCC; rat PKCα, GTAAACGTCAGC-CAT; rat PKCϵ, ATTGAACACTACCAT.

Cells. TF-1 cells were a gift from S. Indelicato (Schering-Plough, Bloomfield, NJ) and were cultured in RPMI 1640 medium (Irvine Scientific, Irvine, CA) supplemented with 10% FBS, 2 mM sodium pyruvate, and 1 ng/mL recombinant human GM-CSF (Sandoz/Schering-Plough Corp., Bloomfield, NJ).

Treatment of Cells with Antisense Oligonucleotides. Cells were seeded at 1×10^6/mL in 2 mL of medium and grown in the presence or absence of 15 μM oligonucleotide, replaced with fresh medium and oligonucleotide every 24 h. Twenty-four hours prior to an experiment in the microphysiometer the cells were transferred to medium that was identified except that it lacked GM-CSF; such cytokine deprivation has been shown to potentiate GM-CSF responses in the microphysiometer. Control cells were not exposed to oligonucleotide but otherwise were treated similarly (including daily change of medium).

Western Blot Analysis. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis and immunoblot analysis were performed exactly as described before (Strulovici et al., 1989). Antisera to PKCα and -ϵ were a generous gift from Dr. Berta Strulovici (Syntex Research, Palo Alto, CA).

Microphysiometry. The microphysiometer is based on a pH-sensitive silicon sensor, the light-addressable potentiometric sensor (Hafeman et al., 1988), which forms one surface of a flow chamber (volume of several microliters) in which cultured cells are immobilized. Culture medium is supplied by a pump to maintain cell viability. Periodically the pump is stopped, and the pH in the chamber decreases, typically by 0.05–0.1 pH unit in a minute. This rate of acidification is recorded by a computer that controls the instrument, and flow is resumed, restoring the original pH. For more instrumental details, see Parce et al. (1989) or McConnell et al. (1991).

The microphysiometer was loaded with low-buffered RPMI 1640 medium (Molecular Devices Corp., Menlo Park, CA) containing 1 mg/mL endotoxin-free HSA (Miles, Elkhart, IN), and each flow chamber of the instrument was assembled with 10^6 TF-1 cells embedded in a fibrin clot (Parce et al., 1989; McConnell et al., 1991). The acidification rate was measured every 135 s (flow on at 100 μL/min for 90 s, flow off for 15 s, flow off with acidification rate measured for 30 s). The chamber temperature was 37 °C.

Acidification rate was monitored until steady (typically 30 min), at which time a sham treatment (culture medium without GM-CSF) was performed for 10 min. Then, GM-CSF was added at 5 ng/mL for 10 min.

Absolute acidification rates can vary from chamber to chamber, for example, by about ±20% due to fluctuations in the number of cells in the ~300-nL part of the cell chamber near the active site of the sensor. To correct for this, data are normalized within a chamber by expression as percent of basal (unstimulated) acidification rate. For identically prepared chambers from the same flask of cells on the same day, we have found the standard deviation of the peak stimulation of acidification by GM-CSF to be 3.5% of the basal acidification rate (Wada et al., 1992). To compare cells with differing culture history prior to use in the microphysiometer, as in Figure 3B, we have previously found (Wada et al., 1992) that statistical consistency is improved if the peak response to GM-CSF is instead normalized to the peak of an unrelated response, the acidification increase that occurs when cells are exposed to 1 μg/mL calcium ionophore ionomycin administered 90 min after removal of GM-CSF.

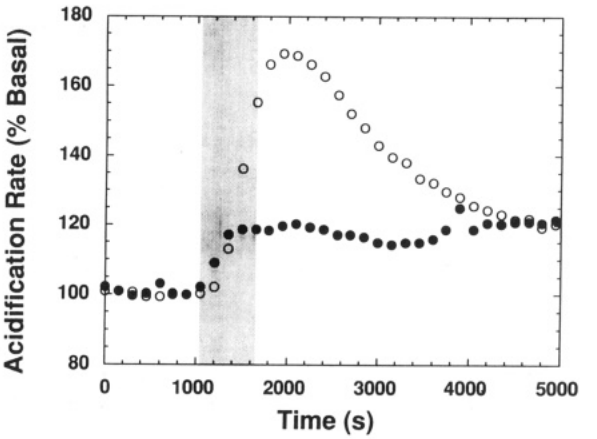

FIGURE 1: Metabolic response of TF-1 cells to GM-CSF and inhibition by staurosporine. TF-1 cells were treated with 5 ng/mL GM-CSF for 10 min (shaded period in figure) in the absence (open symbols) and presence (closed symbols) of 100 nM staurosporine (5-min preincubation and present with GM-CSF), and the acidification rate was measured as described under Materials and Methods. The experiment was repeated four times, each time yielding an inhibition at least as great as that shown.

Measurement of Intracellular [Ca^{2+}]. TF-1 cells that had been GM-CSF-starved overnight were centrifuged and resuspended in low-buffered RPMI 1640 medium supplemented with 10 mM HEPES and then incubated at room temperature for 45 min with 5 μM fura-2, AM (1 mM in DMSO). The cells were then centrifuged and resuspended in a balanced salt solution (NaCl, 138 mM; KCl, 5 mM; KH_2PO_4, 0.11 mM; Na_2HPO_4, 0.81 mM; $MgCl_2$, 0.05 mM; $CaCl_2$, 1.3 mM; glucose, 10 mM; HEPES, 10 mM; pH 7.4) and transferred to a stirred cuvette kept at 37 °C in the sample compartment of a PTI Model 4000 ratiometric fluorometer (South Brunswick, NJ). $[Ca^{2+}]_i$ was monitored ratiometrically using 340 and 380 nm as the excitation wavelengths and 510 nm as the emission wavelength. $[Ca^{2+}]_i$ was calibrated with the equation of Grynkiewicz et al. (1985) with R_{min}, R_{max}, S_{f2}, and S_{b2} determined empirically for each experiment using ionomycin and EGTA in the salt solution.

Choline Assay. Briefly, cells were incubated in suspension with 10 ng/mL GM-CSF. After various reaction times the cells were added to excess phosphate-buffered saline at 0 °C and rapidly collected by centrifugation. Cells were lysed in hypotonic lysis buffer; cytosolic fractions were collected, and cytosolic choline levels were determined by using choline kinase to synthesize radiolabeled choline phosphate from choline and [γ-^{32}P]ATP (2–10 Ci/mmol, from Du Pont/NEN Research Products, Boston, MA) according to the method of Wang and Haubrich (1975) as modified by Thompson et al. (1990).

RESULTS AND DISCUSSION

Response of TF-1 Cells to GM-CSF and Inhibition by Staurosporine. Exposure to 5 ng/mL GM-CSF for 10 min causes the extracellular acidification rate of TF-1 cells to approximately double within 5–10 min (Figure 1). Thereafter, the acidification rate gradually falls, remaining significantly above basal levels 45–60 min after the initial exposure to GM-CSF.

These results are consistent with our earlier observations (Wada et al., 1992). There we kinetically resolved the response into low-amplitude short-lived and higher-amplitude long-lived components. The former is associated with the activation of the Na^+/H^+ antiporter. The latter requires the presence

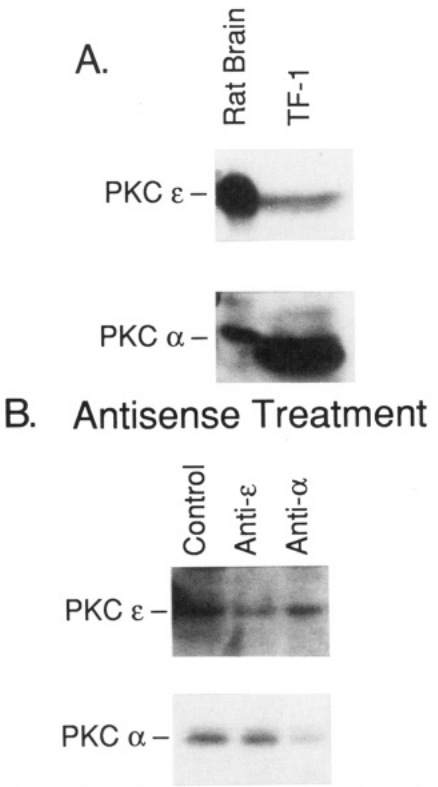

FIGURE 2: Expression of PKC isoenzymes in TF-1 cells. (A) Total cell homogenates (200 μg of protein per lane for TF-1 cells and 20 μg per lane for rat brain) were subjected to SDS/PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies specific for PKCα and PKCε as described under Material and Methods. Immunoblots were labeled with ^{125}I protein A and exposed to Kodak XAR film for 7 days at −70 °C. Rat brain was loaded as a control. PKC isoforms are indicated. (B) TF-1 cells were treated with or without PKC–antisense oligonucleotides for 5 days as described under Materials and Methods, and immunoblot analysis was performed as described above. Cell treatments are indicated in the figure, and PKC isoforms are identified. The experiment was repeated twice with similar results, and representative Western blots are shown.

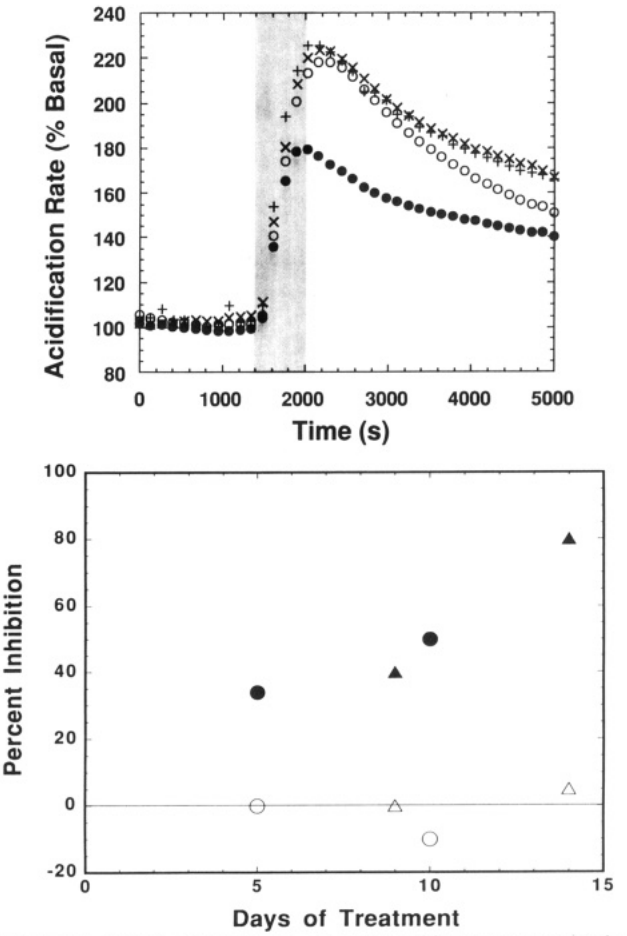

FIGURE 3: Effect of antisense oligonucleotide treatment on the acidification response of TF-1 cells to GM-CSF. (A, top) TF-1 cells were treated in the presence or absence of antisense oligonucleotide to PKCα or PKCε for varying periods (5 days shown here) as described under Materials and Methods. Cells were then challenged with GM-CSF (5 ng/mL for 10 min; shaded period in figure), and the acidification rate was measured as described under Materials and Methods. Antisense treatment: control, open circles; human α, crosses; rat α, ×'s; rat ε, filled circles. (B, bottom) Time course of the inhibitory effects of antisense treatment on the GM-CSF response of TF-1 cells, represented in percent inhibition compared to control cells. TF-1 cells were treated in the presence or absence of antisense oligonucleotides to PKCα or -ε for up to 14 days. For each time point, cells were treated with 5 ng/mL GM-CSF for 10 min, and the acidification rate was measured as described above. Percent inhibition was determined by first normalizing GM-CSF responses to ionomycin responses within each chamber as described under Materials and Methods and then comparing the maximum normalized GM-CSF-mediated acidification response of antisense-treated cells to that of control cells for each time point. Simply normalizing responses to basal rates rather than using this procedure would have made a significant difference at only one data point, substantially decreasing the apparent inhibition for the 14-day incubation with anti-PKCε. Four experiments were performed for each antisense treatment, one for each of four durations of exposure. There were two independent series of experiments (two durations each), indicated by the circular and triangular symbols. The open symbols show the (lack of) inhibition of PKCα and the filled symbols the progressive inhibition for PKCε.

of a carbon source in the medium (glucose, but not glutamine, is sufficient) and presumably reflects an increased rate of glycolysis.

Several PKC inhibitors have been described (Tamaoki et al., 1986; Hannun & Bell, 1987; Davis et al., 1989); of these, staurosporine has been shown to be the most potent (Davis, et al., 1989). To determine whether PKC is involved in mediating the GM-CSF acidification response, we treated TF-1 cells in the microphysiometer with 5 ng/mL GM-CSF, with or without 100 nM staurosporine, and monitored extracellular acidification as described under Materials and Methods. As shown in Figure 1, staurosporine strongly inhibited the acidification increase mediated by GM-CSF. This concentration of staurosporine was determined not to be cytotoxic to these cells, as indicated by the constant acidification rate (Figure 1).

Expression of PKC Isoenzymes and Inhibition with Antisense Oligonucleotides. Whole-cell extracts from TF-1 cells were analyzed by immunoblot analysis using affinity-purified antibodies directed against synthetic peptides unique to the PKCα and -ε isoforms (Strulovici et al., 1991). As shown in Figure 2A, TF-1 cells express both PKCα and -ε. To determine which PKC isoforms are involved in GM-CSF signaling, we attempted specifically to reduce or inhibit the expression of PKCα and -ε by treating the TF-1 cells with antisense oligonucleotides specific for each of these isoforms. TF-1 cells were grown with or without antisense oligonucleotides for 5 days. Western blot analysis was performed on these cells to determine the amount of immunoreactive PKC remaining after treatment. As seen in Figure 2B, the amount of immunoreactive PKCα is significantly reduced in the human PKCα–antisense-treated cells when compared with either control cells or the PKCε–antisense-treated cells. Similarly, the amount of PKCε is significantly reduced in the PKCε–antisense-treated cells when compared with either control cells or human PKCα–antisense-treated cells. The rat

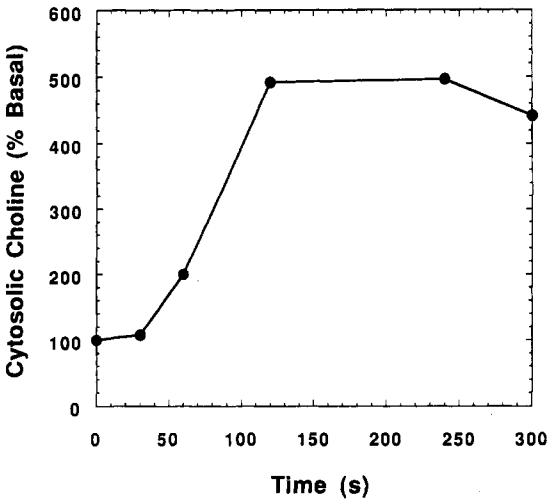

FIGURE 4: Effect of GM-CSF on cytosolic choline concentrations in TF-1 cells. TF-1 cells were treated with 10 ng/mL GM-CSF for the indicated time periods, and the cytosolic fractions were measured for free choline concentration as described under Materials and Methods. The experiment was repeated three times, each displaying identical kinetics (peak choline levels were reached at 2 min) and similar basal choline levels; the maximum level of cytosolic choline varied between 500 and 800 pmol/10^7 cells. A representative experiment is shown.

PKCα–antisense-treated cells showed comparable levels of inhibition (data not shown). Therefore, this treatment was successful in reducing the levels of the targeted PKC isoforms.

Inhibition of Acidification Responses to GM-CSF by Antisense Oligonucleotides to PKCϵ but Not PKCα. TF-1 cells were treated with the PKC–antisense oligonucleotides in two series of experiments, each lasting up to 14 days. As seen in Figure 3A, the burst of acidification caused by 5 ng/mL GM-CSF is significantly diminished by treatment with antisense oligonucleotide to PKCϵ but not to PKCα–antisense. The PKCϵ–antisense treatments showed a consistent and progressive inhibition of the GM-CSF response corresponding to length of antisense treatment, up to 80% after 14 days (Figure 3B). These data suggest that PKCϵ may be involved in the signal transduction pathway associated with GM-CSF-mediated increase in the metabolic response observed with the microphysiometer.

The stimulation of acidification by GM-CSF has been shown to comprise at least two processes: a brief (<10 min) activation of the Na^+/H^+ antiporter and a larger and longer-lasting stimulation that involves the activity of glycolysis (Wada et al., 1992). On the basis of the amplitudes and shapes of the inhibited responses, the PKCϵ–antisense treatment inhibits the glycolytic component. The absence of initial broadening in the inhibited response suggests that the smaller antiporter component is also inhibited, but additional experiments need to be done to show this conclusively.

Increased Intracellular Choline after Exposure to GM-CSF but No Increase in Cytosolic [Ca^{2+}]. It has been suggested that the activation of PKCϵ may be associated with the hydrolysis of phosphatidylcholine, possibly by a phospholipase D, which contrasts with the well-described hydrolysis of phosphatidylinositol by a phospholipase C (Koide et al., 1992). If this is true for the present case, then it should be possible to detect increased levels of choline in the GM-CSF-activated cells. We detected an approximately 5-fold increase in free cytosolic choline within 2 min after exposure to 10 ng/mL GM-CSF (Figure 4). The amplitude and kinetics are similar to those observed for agonist-induced phosphatidylcholine hydrolysis in other human cells (Duronio et al., 1989; Thompson et al., 1990). GM-CSF fails to alter [Ca^{2+}] significantly under conditions where it elicits strong acidification and proliferative responses (Figure 5). The positive response to PAF (Figure 5) shows that the cells are capable of an agonist-induced increase in cytosolic [Ca^{2+}]. That GM-CSF increases cytosolic choline without increasing $[Ca^{2+}]_i$ strongly implicates phospholipase D in the transduction of the GM-CSF signal in TF-1 cells.

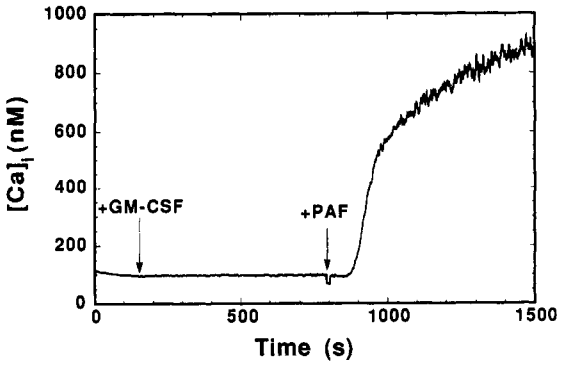

FIGURE 5: Effect of GM-CSF on cytoplasmic [Ca^{2+}] in TF-1 cells. GM-CSF (10 ng/mL) had no effect on cytoplasmic [Ca^{2+}] of cells suspended in a cuvette. Subsequent exposure to 5 μM PAF did elicit an increase in cytoplasmic [Ca^{2+}]. This experiment was repeated five times with similar results; a representative experiment is shown.

Conclusion. We have combined antisense methodology with microphysiometry, a novel biosensor-based means of detecting alterations of cellular physiology, to provide evidence that the ϵ isoenzyme of protein kinase C is involved in GM-CSF signal transduction in TF-1 cells. We have found that the signal transduction does not cause an increase in cytosolic [Ca^{2+}] but does involve an increase in intracellular choline, both of which are consistent with the involvement of phospholipase D, as might be expected for the activation of PKCϵ. The failure of antisense oligonucleotides against PKCα to inhibit cellular response to GM-CSF in the microphysiometer, together with the failure of GM-CSF to increase cytosolic [Ca^{2+}], suggests that this isoenzyme is not involved in GM-CSF signal transduction in these cells. Further studies on the effects of antisense of oligonucleotides on GM-CSF-driven proliferation, acidification responses, and the relationship between the two in TF-1 cells are in progress.

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Registry No. GM-CSF, 83869-56-1; PKC, 141436-78-4; phospholipase D, 9001-87-0.