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Immunology

Protein kinases C-β and C-ε link the mast cell high-affinity receptor for IgE to the expression of c-*fos* and c-*jun*

(signal transduction/Fc_ε receptor type I/gene expression)

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ABSTRACT **In this report we identify the specific isozymes of protein kinase C (PKC) that are involved in c-*fos* and c-*jun* mRNA accumulation in the rat basophilic leukemia cell line RBL-2H3. These cells could be largely depleted of the endogenous PKC isozymes by chronic treatment with phorbol 12-myristate 13-acetate followed by permeabilization of the cells with streptolysin O. The reconstitution of these cells with defined concentrations of either PKC-β or PKC-ε up to 10 nM and 20 nM, respectively, induced c-*fos* and c-*jun* in a dose-dependent manner. At high concentrations of PKC-β and -ε the induction of c-*fos* and c-*jun* was independent of the aggregation of the high-affinity IgE receptors (Fc_ε type I receptors). In contrast, at limiting concentrations of these two PKC isozymes, 1 nM, the increase in c-*fos* and c-*jun* mRNAs was dependent on the aggregation of the Fc_ε type I receptors. Unlike PKC-β and -ε, PKC-α and PKC-δ failed to reconstitute c-*fos* and c-*jun* induction at any dose over the range examined. We conclude that PKC-β and PKC-ε serve as a link between the cell surface receptor and gene expression.**

Although initially regarded as a single enzyme, protein kinase C (PKC) is now known to represent a family of isozymes that differ in dependence on Ca^{2+} and diacylglycerol, in substrate specificity, and in topological localization (1).

The activation of this family of enzymes may in part lead to the modulation of gene expression due to the ability to effect changes in the activity of transcription factors such as AP-1 (2–6). Two major components of this complex are c-Jun (7, 8) and c-Fos (9–11). These proteins, the products of the c-*jun* and c-*fos* protooncogenes, form a stable heterodimer via a coil–coil interaction at the putative leucine zipper domain (9, 10) and can regulate transcription through the AP-1 binding site (9, 11).

The aggregation of the high-affinity IgE receptor (Fc_ε receptor type I, $Fc_\varepsilon RI$) results in direct interactions of the receptor with molecules that initiate diverse signal transduction pathways (12). These pathways lead to the release of mast cell granule contents and to late responses such as the increase in c-*fos* and c-*jun* expression and modulation of cytokine gene expression (13–17). In rat basophilic leukemia (RBL) cells a full secretory response to antigen was preferentially reconstituted by either PKC-β or PKC-δ (18) whereas inhibition of phosphatidylinositol hydrolysis was preferentially mediated by PKC-α and -ε (19). Evidence for a link between the signal transduction initiated by $Fc_\varepsilon RI$ aggregation and mast cell DNA synthesis comes from recent observations that the increase in PKC activity due to receptor aggregation is associated with a decrease in mast cell DNA synthesis (13, 20). However, the relationship between $Fc_\varepsilon RI$ aggregation, PKC, protooncogene expression, and DNA synthesis is still unclear. Thus, to further investigate the regulation of AP-1 component expression in mast cells, we initiated the present study on permeabilized RBL-2H3 cells.

MATERIALS AND METHODS

Cell Culture and Chronic Treatment with Phorbol 12-Myristate 13-Acetate (PMA). RBL-2H3 cells (5×10^5) were cultured for 48 hr in six-well plates (Costar) as described (18), with one modification. Cultures were incubated in the absence or presence of PMA (20 ng/ml). The adherent cells were then washed with serum-free culture medium and incubated overnight at 37°C in a 5% CO_2 atmosphere in the presence or absence of PMA.

Cell Permeabilization and Reconstitution. Cells were permeabilized with streptolysin O (1.5 units/ml; Murex Diagnostics, Dartford, U.K.) and washed as described (18). After 10 min of streptolysin O treatment, 98% of the cells were permeabilized as determined by loss of trypan blue exclusion. The permeabilized and washed cells were reconstituted for 30 min with various concentrations of either a mixture of purified rat brain PKC (α, β, and γ isozymes) or with individual recombinant isozymes derived from the baculovirus expression system (18).

Cell Activation and RNA Extraction. IgE sensitization was carried out (18) in the absence or presence of PMA. The cells were then either permeabilized or not and washed (18). Incubation was at 37°C with defined concentrations of 2,4-dinitrophenyl bovine serum albumin (DNP-BSA) for various times. For all experiments reactions were stopped by the addition of RNA extraction buffer (5 M guanidine thiocyanate/0.5% *N*-lauroylsarcosine/25 mM sodium citrate) and the purified RNA was recovered by centrifugation through a CsCl gradient. In another set of experiments, IgE sensitized, PMA-treated and permeabilized cells were activated (DNP-BSA) in the absence or presence of the following PKC inhibitors: calphostin C (Kamiya Biochemical, Thousand Oaks, CA), 100 nM; Ro31-7549 (kindly given by M. Beaven; provided by Eisai, Ibaraki, Japan), 10 μM; and peptide inhibitor of PKC (Peninsula Laboratories), 50 μM. These concentrations have been shown to specifically inhibit PKC activity in RBL cells (21).

Immunoblotting of PKC Isozymes. For immunoblotting experiments, boiling tricine/SDS sample buffer (NOVEX, San Diego) was added to the plates after experimental manipulations were completed. Proteins were resolved in tricine/SDS/10% polyacrylamide gels and transferred to nitrocellulose membranes. Immunoreactive proteins were

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Abbreviations: RBL, rat basophilic leukemia; DNP, 2,4-dinitrophenyl; BSA, bovine serum albumin; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; $Fc_\varepsilon RI$, Fc_ε receptor type I (high-affinity receptor for IgE).

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detected as described with antibodies specific for the individual PKC isozymes (22).

Reverse Transcription–PCR Amplification and Detection of cDNA. RNA from each sample was reverse transcribed (23). The cDNA library from each treatment was divided into three samples of 10–100 ng each in a 50-μl reaction volume. The mixtures were amplified essentially as described (23), with an annealing temperature of 60°C. Samples were amplified for up to 28 cycles. The oligonucleotide primers for the control rat cyclophilin were 5′-GCT-GAT-GGC-GAG-CCC-TTG-GGT-C-3′ (sense) and 5′-ACC-AGT-GCC-ATT-ATG-GCG-TGT-G-3′ (antisense), corresponding to bases 76–264 (24). The rat c-*fos* oligonucleotide primers were 5′-AGC-CGA-CTC-CTT-CTC-CAG-CAT-3′ (sense) and 5′-CAG-ATA-GCT-GCT-CTA-CTT-TGC-3′ (antisense), corresponding to bases 235–533 (25). The rat c-*jun* oligonucleotide primers were 5′-GCG-CCG-CCG-GAG-AAC-CTC-TGT-C-3′ (sense) and 5′-CAG-CTC-CGG-CGA-CGC-CAG-CTT-G-3′ (antisense), corresponding to bases 577–1227 (26).

A portion (10 μl) of the PCR mixture was resolved in an agarose gel. Ethidium bromide-stained bands were identified on a UV table and photographed to obtain a negative image (Polaroid type 55 film). The veracity of the amplified cDNA was confirmed by transfer of the DNA to Hybond-N membrane (Amersham) and probing with 700 ng of oligonucleotide probe conjugated to fluorescein-11-dUTP (ECL Random Prime; Amersham). The rat cyclophilin oligonucleotide probe was 5′-CTA-TAA-GGG-TTC-CTC-CTT-TCA-C-3′ (bases 182–203) (24). The rat c-*fos* oligonucleotide probe was 5′-GTC-TCC-TCC-GTG-GCC-CCA-TCG-C-3′ (bases 378–399) (25). The rat c-*jun* oligonucleotide probe was 5′-GAT-GCC-CTC-AAC-GCC-TCG-TTC-C-3′ (bases 950–971) (26).

RESULTS

Depletion of PKC in RBL Cells and Effect on Protooncogene Expression. PKC activity is involved in the expression of the protooncogenes c-*fos* and c-*jun* in mouse mast cells stimulated by antigen (13, 20). Similarly, in RBL cells depletion of PKC by prolonged exposure to PMA drastically reduced the accumulation of mRNA for these two protooncogenes. Quantitation of c-*fos* and c-*jun* mRNAs was by PCR using cyclophilin mRNA as an internal control. The three amplified cDNA products were subjected to Southern blot hybridization using specific probes to determine the veracity of amplification. Bands of the size predicted for the amplified cDNA were obtained: 189 bp for cyclophilin, 299 bp for c-*fos*, and 651 bp for c-*jun* (data not shown). The linear relation between template concentrations (10–80 ng of cDNA) and the amplification of these three products, using 22 or 28 cycles, was determined for each experiment (data not shown). With 22 cycles of amplification, a decrease of 85% and 93%, respectively, for c-*jun* and c-*fos* mRNAs was observed in antigen-stimulated cells pretreated with PMA for 72 hr (Fig. 1). No significant change in the level of cyclophilin mRNA was detected after the PMA treatment. These results suggest that the expression of c-*fos* and c-*jun* is dependent on the presence of PKC activity in these cells.

The concentrations of the various PKC isozymes in RBL cells cultured in medium containing fetal bovine serum were recently reported (19). Since the RBL cells in the present experiments were deprived of serum to reduce the basal levels of c-*fos* and c-*jun* expression, we determined the concentration of PKC-α, -β, -δ, and -ε in these cells. Western blots (ECL detection; Amersham) were subjected to quantitative densitometry under linear conditions and the levels were compared with recombinant PKC standards derived from the baculovirus expression system. The predominant PKC isozymes present in the RBL cells under the conditions of serum deprivation were α and β (Table 1). In contrast to the values reported for nondeprived RBL cells, the concentration of the δ isozyme was almost 6-fold less, and the concentration of PKC-α was 3-fold greater. No significant difference was observed for PKC-β and -ε. Additionally, immunoblots revealed an average decrease of 90% in the levels of all of the isozymes in RBL cells treated for 72 hr with PMA and a further decrease (of 95%) was observed when the cells were also permeabilized (Fig. 2).

Protooncogene Response in Permeabilized PMA-Treated Cells. To explore which of the PKC isozymes were involved

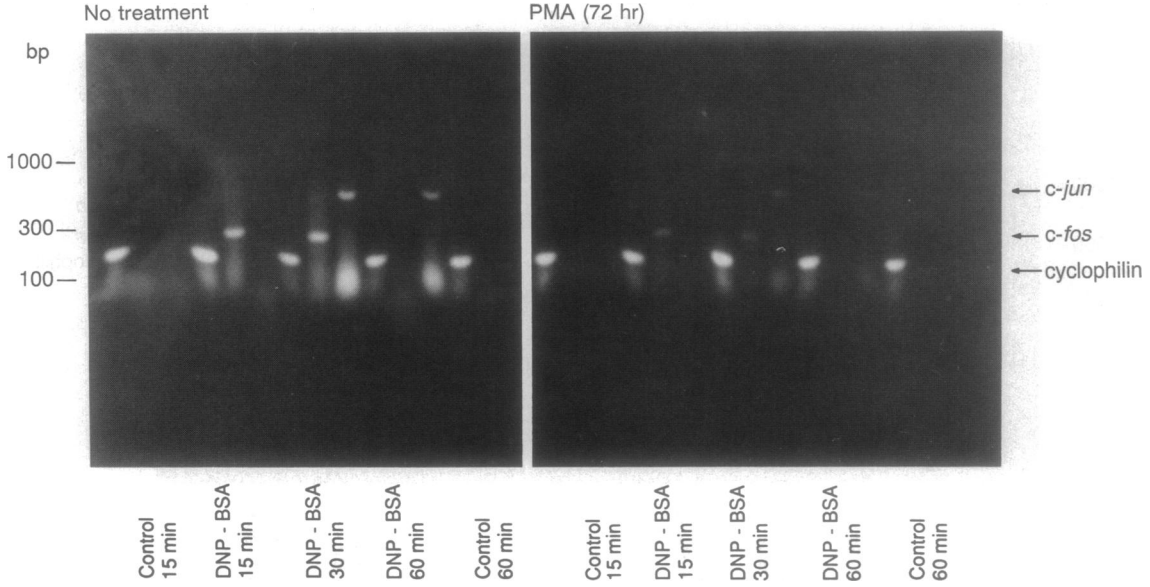

FIG. 1. Effect of chronic PMA treatment on the accumulation of c-*fos* and c-*jun* mRNAs in serum-deprived RBL cells activated by antigen, as detected by ethidium bromide staining of PCR-amplified cDNA. IgE-sensitized RBL cells were deprived of serum overnight, incubated in the presence (*Right*) or absence (*Left*) of PMA for 72 hr, and then activated by antigen (DNP-BSA, 100 ng/ml) for various periods of time. Cytoplasmic RNA was extracted and reverse transcribed, and the cDNAs were subjected to 22 cycles of PCR as described in *Materials and Methods*. Triplicate lanes represent one treated sample amplified with the respective oligonucleotides whose products are indicated at right. Results of one representative experiment out of three performed are shown.

Table 1. PKC isozymes in serum-deprived RBL-2H3 cells

Isozyme	Concentration, nM	
	Present study	Ref. 19*
α	240	85
β	30	35
δ	10	60
ε	20	12.5

Values represent concentrations in whole cell extracts after overnight deprivation of serum (average of duplicate samples in each of two separate experiments with a standard deviation from the mean in the range of 5–10%). Quantitation was done as described (22). The software NIH IMAGE 1.52 (written by Wayne Raspband) was used for quantitation.
*For cells grown in the presence of fetal bovine serum.

in c-*fos* and c-*jun* mRNA accumulation, we used permeabilized PMA-treated RBL cells. The concentration of PKC isozymes remaining after this treatment was 2–8% of that in untreated cells (data not shown).

Unlike the intact cells, the permeabilized cells required 28 cycles of amplification to detect a comparable cyclophilin signal (Fig. 3 vs. Fig. 1, control). Nonetheless, after 28 cycles the amplified cDNA subfragments of c-*fos* and c-*jun* were readily detectable though greatly reduced (91%) as compared with the intact nontreated cells (Fig. 3 vs. Fig. 1, DNP-BSA). The same level of response to antigen for c-*fos* and c-*jun* mRNA accumulation, as determined by densiometric quantitation, was observed in permeabilized control cells as well as in permeabilized PMA-treated cells (data not shown). However, since a more complete depletion of the PKC isozymes was achieved in permeabilized PMA-treated cells (Fig. 2), we used this system for the subsequent reconstitution experiments.

The retention of c-*fos* and c-*jun* induction in response to antigen in the permeabilized PMA-treated cells was unexpected (Fig. 3, DNP-BSA). It was possible, however, that this might reflect the 2–8% of PKC remaining in the cells. To assess this possibility, we examined the ability of three PKC inhibitors to block c-*fos* and c-*jun* induction: Ro31-7549, which acts on the ATP binding site (27), calphostin C, which binds to the regulatory domain (28), and a pseudosubstrate

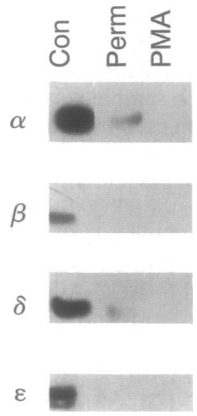

FIG. 2. Western blot for PKC content in serum-deprived RBL cells after permeabilization or chronic treatment with PMA and permeabilization. Cells were incubated in the absence (Perm) or presence (PMA) of PMA (72 hr). The cells were then permeabilized with streptolysin O (1.5 units/ml) and washed. Control (Con) cells were incubated under the same conditions but were not treated with PMA or permeabilized. Total cell extracts were prepared and analyzed. Results of one representative experiment out of four are shown.

peptide inhibitor (29). Conditions that inhibit PKC activity in the RBL cells in a specific manner were utilized (21). The individual inhibitors were added to permeabilized IgE-sensitized, PMA-treated cells prior to the addition of the antigen. Each of the PKC inhibitors suppressed the induction of c-*fos* and c-*jun* mRNA accumulation in response to antigen stimulation (Fig. 3), suggesting that the remaining PKC was sufficient to initiate the accumulation of c-*fos* and c-*jun* mRNAs in response to antigen stimulation.

Reconstitution. The significant reduction in protooncogene expression in the permeabilized PMA-treated cells compared with intact cells led us to attempt to reconstitute a full response. The addition of either 10 nM or 20 nM PKC (mixture of α, β, and γ isozymes, purified from rat brain) stimulated the induction of c-*fos* and c-*jun* mRNA accumulation in the absence of antigen stimulation (Fig. 4*A*). As with

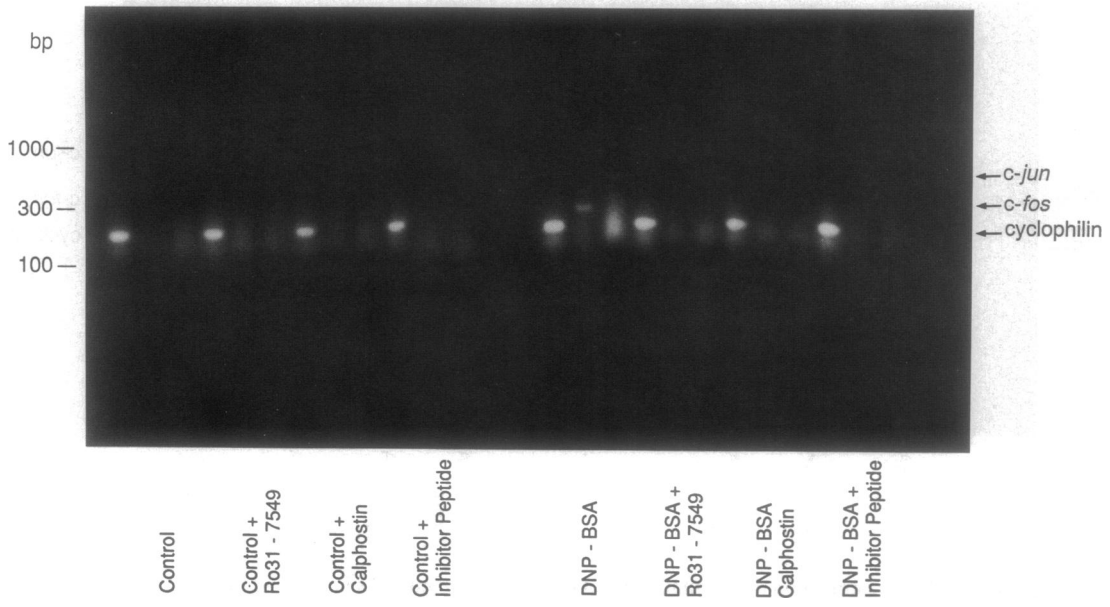

FIG. 3. Effect of PKC inhibitors on the accumulation of c-*fos* and c-*jun* mRNAs, as detected by ethidium bromide staining. RBL cells were deprived of serum overnight, treated with PMA for 72 hr, IgE-sensitized, and permeabilized. The cells were then activated by antigen for 30 min in the absence or presence of the indicated PKC inhibitors at concentrations described in *Materials and Methods*. The PCR-generated cDNA was subjected to 28 cycles of PCR. Triplicate lanes represent one treated sample. Results of one representative experiment of three are shown.

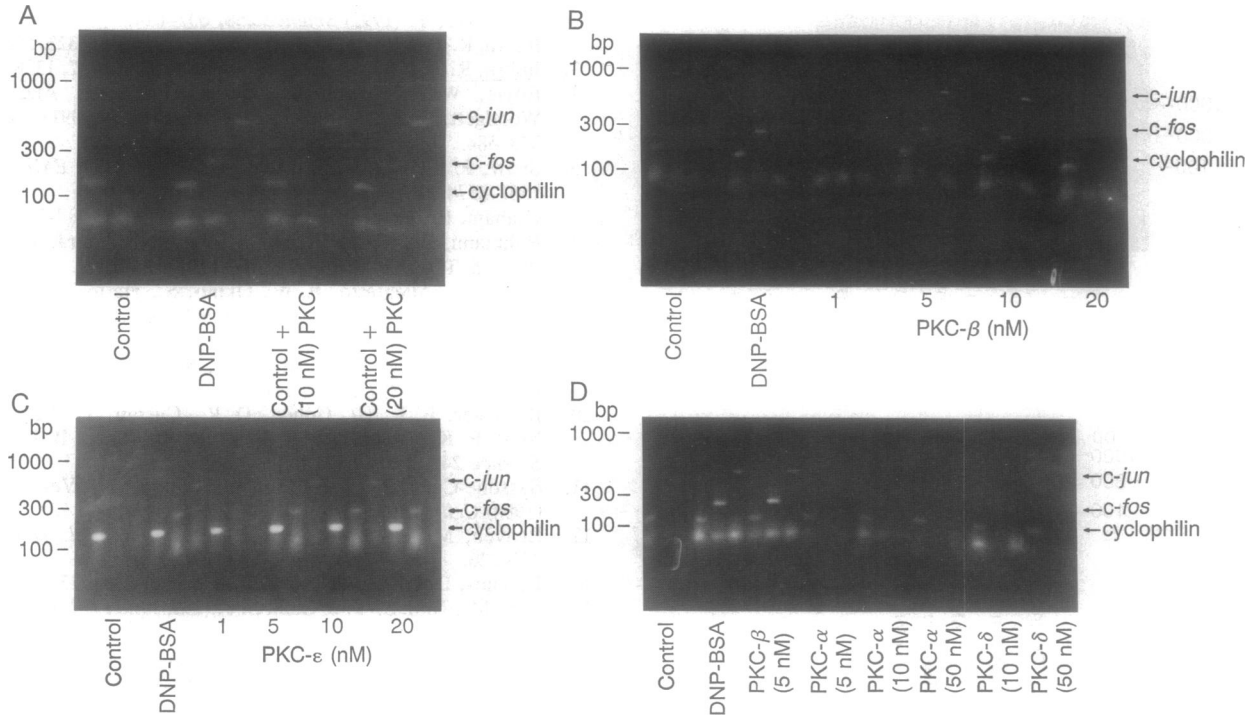

FIG. 4. Reconstitution of c-*fos* and c-*jun* mRNA accumulation by addition of PKC isozymes, as detected by ethidium bromide staining of the PCR-amplified product. RBL cells were deprived of serum overnight, treated with PMA for 72 hr, permeabilized, and reconstituted for 30 min with purified PKC isozymes. (*A*) Reconstitution with a mixture of purified rat brain-derived PKC-α, -β, and -γ. (*B*) Reconstitution with recombinant PKC-β derived from baculovirus expression. (*C*) Reconstitution with recombinant PKC-ε derived from baculovirus expression. (*D*) Reconstitution with recombinant PKC-β, -α, and -δ derived from baculovirus expression. The PCR-generated cDNA was subjected to 28 cycles of amplification. Triplicate lanes represent one treated sample. Results of one representative experiment out of three are shown.

secretion, high levels of PKC were capable of reconstituting an antigen-independent response suggesting the presence of endogenous activators of PKC (18). Activation of the PKC-reconstituted permeabilized cells with antigen did not result in further increases in protooncogene expression. To investigate which of the PKC isozymes were responsible for the increase in protooncogene expression, the permeabilized PMA-treated RBL cells were reconstituted with the individual recombinant isozymes of PKC. Addition of defined concentrations of either PKC-β or PKC-ε for 30 min resulted in a dose-dependent increase in c-*fos* and c-*jun* mRNAs (Fig. 4 *B* and *C*). Maximal reconstitution was achieved at 5–10 nM PKC-β or -ε, and at these concentrations the induction of protooncogene expression was independent of $Fc_\varepsilon RI$ aggregation. For PKC-ε, a 20 nM concentration maintained the maximal c-*fos* and c-*jun* induction. In contrast to PKC-ε, a concentration of 20 nM PKC-β suppressed the induction. PKC-α and PKC-δ did not reconstitute the induction at any dose within the range examined (Fig. 4*D*). We cannot exclude the possibility that the latter isozymes might reconstitute a response at higher concentrations; however, under conditions where the relative ratio of the *in vivo* concentrations was maintained, both PKC-α and -δ failed to reconstitute a response.

Role of the Receptor. The dependence on the $Fc_\varepsilon RI$ for the induction of c-*fos* and c-*jun* mRNA accumulation was further investigated. Maximal c-*fos* and c-*jun* mRNA accumulation was obtained with DNP-BSA at 10 ng/ml in permeabilized IgE-sensitized, PMA-treated cells (Fig. 5). With the antigen at 1 ng/ml, no signal was detected. In addition, with either PKC-β or PKC-ε at 1 nM, reconstitution of protooncogene expression was not achieved in the absence of antigen (Fig. 5). Therefore, we used these conditions to determine whether receptor activation could be linked to protooncogene expression. A reconstitution of the response was observed when 1 nM PKC-β or PKC-ε together with antigen at 1 ng/ml was added to the permeabilized PMA-treated cells (Fig. 5). In contrast, the addition of PKC-δ or PKC-α (1 nM and 10 nM, respectively) along with antigen at 1 ng/ml did not result in induction of c-*fos* and c-*jun* mRNA accumulation (data not shown). These results show that PKC-β and PKC-ε can be linked to the accumulation of c-*fos* and c-*jun* mRNAs through the activation of $Fc_\varepsilon RI$.

DISCUSSION

Activation of $Fc_\varepsilon RI$ initiates a well-documented cascade of biochemical events (reviewed in ref. 12). Many of these events are common to the activation of a variety of receptors, but the sequence of events and their physiological significance remain to be deciphered.

The activation of PKC has been described in response to a large variety of physiological stimuli presumably acting through specific cellular receptors (1). For $Fc_\varepsilon RI$, PKC was shown to translocate to the membrane in response to antigen activation (30, 31). Translocation peaked at 15 sec after activation and persisted at low levels 10 min after the activation signal (31). This evidence presents an argument for a dual role of PKC, suggesting that PKC participates in early and late events that ultimately result in mast cell secretion.

Recent studies by Ozawa and colleagues present further evidence for the dual nature of the PKC response to antigen activation, showing that secretion is dependent on the presence of either PKC-β or PKC-δ (18). Furthermore, PKC-α or PKC-ε are negative regulators of the activation of phospholipase C-γ1 (19). In this study we present evidence for the induction of the transcription factors c-Fos and c-Jun in response to PKC-β and PKC-ε. A common feature shared in these studies is (*i*) the ability of two different PKC isozymes to mediate each function and (*ii*) one Ca^{2+}-dependent as well as one Ca^{2+}-independent isoform is capable of inducing the response. The implications for both a Ca^{2+}-dependent and a

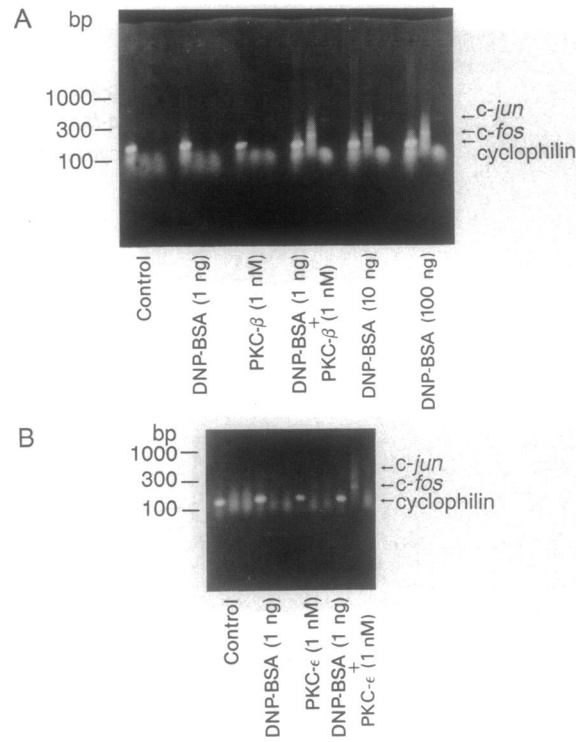

FIG. 5. Ethidium bromide detection of $Fc_\epsilon RI$-linked induction of c-*fos* and c-*jun* mRNA accumulation in response to PKC-β and -ϵ reconstitution. RBL cells were deprived of serum overnight, treated with PMA for 72 hr, permeabilized, and activated by antigen (DNP-BSA) and/or reconstituted for 30 min with recombinant PKC-β (*A*) or PKC-ϵ (*B*). The PCR-generated cDNA was subjected to 28 cycles of PCR. Triplicate lanes represent one treated sample. Results of one representative experiment out of two are shown.

Ca^{2+}-independent isoform being capable of carrying out the same function remain unclear. However, this duality would provide the cell with the ability to mediate intracellular signaling in the presence or absence of Ca^{2+} mobilization. Moreover, while mast cell exocytosis is inhibited in the absence of extracellular Ca^{2+}, many early signaling events, such as receptor and other substrate phosphorylation and activation, still occur (32, 33).

We propose that the induction of c-*fos* and c-*jun* expression observed in RBL cells activated through $Fc_\epsilon RI$ is mediated by the activation of PKC-β and/or PKC-ϵ. Whether PKC exerts its effect by enhancing the transcription of the protooncogenes or the stability of the mRNA remains to be determined. We cannot exclude the possibility that the induction of c-*fos* and c-*jun* may require other signaling molecules or an alternative pathway that can be inhibited by mechanistically different inhibitors of PKC. Nevertheless, it is evident that the depletion of PKC leads to a loss of protooncogene expression. Therefore, at a minimum PKC is required for the activation of other signaling molecules that lead to c-*fos* and c-*jun* induction.

This study was done while E.R. was on sabbatical in the Section on Chemical Immunology.

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