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Activation of the transcription factor NF-KB in GH_3 pituitary cells

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Abstract

Since several genes expressed in the pituitary can bind the transcription factor NF-KB, its presence and regulation was examined in the GH_3 pituitary cell line. An electrophoretic mobility shift assay using nuclear extracts and an oligonucleotide probe corresponding to the Ig KB binding site was employed to identify activated NF-KB. One complex possessed properties characteristic of NF-KB: co-migration with an NF-KB complex and binding specificity restricted to NF-KB binding DNA sequences. Antibodies to the NF-KB subunits NFKB1p50 (p50) and RelA (p65) interacted with the extract-DNA complex. Activation of NF-KB in GH_3 cells was increased by PMA or the cytokine tumor necrosis factor alpha. A synergy between PMA and TNF or a calcium mobilizing agent was seen in NF-KB activation. Further TNF activation was enhanced by TRH. These observations indicate the presence of NF-KB in GH_3 cells and demonstrate its activation by hormones/second messengers that act on pituitary cells.

Keywords: Pituitary; NF-kappa-B; Phorbol myristate acetate; Tumor necrosis factor; Thyrotropin-releasing hormone; GH_3

1. Introduction

NF-KB is a transcription factor which has a wide tissue distribution and which has a role in the inducible expression of many genes (see Liou and Baltimore, 1993 for review). NF-KB is composed of a heterodimer of NF-KB1p50 and RelA. These two proteins belong to the rel family which in addition includes Rel, RelB, and NF-KB2. Family members can form homodimers and heterodimers. The specific dimer composition establishes its affinity for the particular NF-KB DNA binding sequence within the promoter/enhancer region of a gene (Nolan and Baltimore, 1992). Most cells contain NF-KB in a transcriptionally inactive form within the cytoplasm; the NF-KB is bound to an inhibitory subunit, IKB. Upon cell stimulation, NF-KB dissociates from IKB and then translocates to the nucleus where it can subsequently bind to DNA and alter gene expression (Baeuerle and Baltimore, 1988). The IKB subunit sequesters NF-KB in the cytosol by blocking its nuclear localization sequence and, in addition, prevents NF-KB from binding to DNA (Beg et al., 1992). The dissociation of IKB leads to an activation of the NF-KB.

The factors known to induce the activation of NF-KB include phorbol esters (Sen and Baltimore, 1986b), which activate protein kinase C, and several cytokines including tumor necrosis factor alpha (TNF) (Meichle et al., 1990). Both the activation of protein kinase C and the binding of cytokines have been shown to affect pituitary function as evidenced, in part, by increased hormone secretion. In addition, genes are expressed in the anterior pituitary which contain NF-KB binding sites in their promoter regions-regulatory genes such as interleukin 2 (Arzt et al., 1992), interleukin 2 receptor (Arzt et al., 1993), and interleukin 6 (Vankelecom et al., 1989; Spangelo et al., 1990) as well as ubiquitous genes such as c-myc (Duyao et al., 1990) and vimentin (Lilienbaum et al., 1990). Consequently, NF-KB may have a role in controlling gene expression in the anterior pituitary. This study sought to examine the presence and activation of NF-KB in the GH_3 pituitary cell line, a well characterized model for pituitary cell function. GH_3

The nomenclature used to describe the NF-KB/rel gene products follows the proposals recently made (Nabel and Verma (1993) Genes Dev. 7, 2063). NF-KB1 = p50, RelA = p65, NF-KB = the heterodimer p50p65, Rel = Rel and IKB is used here to refer to any member of the IKB family.

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cells were found to have NF-KB and, further, NF-KB activation was seen in response to well established inducers such as phorbol myristate acetate (PMA) and TNF. In addition an augmentation of the response induced by PMA and TNF was produced, respectively, by calcium and thyrotropin-releasing hormone (TRH), agents which had no effect on NF-KB activation by themselves.

2. Materials and methods

2.1. GH_3 cells and preparation of cell extracts

GH_3 cells obtained from the American Tissue Culture Collection (Rockville, MD) were grown in Ham's F10 medium supplemented with 2.5% fetal calf serum and 15% horse serum. For preparation of crude nuclear cell extracts, GH_3 cells grown in 100-mm plastic petri dishes were removed from the dish by scraping into a lysis buffer (100 mM Hepes, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride). After a 15-min incubation at 4°C, 25 μl of 10% NP40 was added and the nuclei were pelleted by centrifugation (15 000 × g, 30 s). The nuclei were then resuspended and incubated for 20 min in 50 μl of extraction buffer (50 mM Hepes, pH 7.8, 50 mM KCl, 300 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride, 10% glycerol). After centrifugation (15 000 × g, 5 min) the crude nuclear extract was removed and frozen at −70°C until use (modified from Schreiber et al., 1989). When whole cell extracts were used, cells were suspended in 500 μl of extraction buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 100 mM KCl, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, 0.1 mM p-aminobenzamidine, 10 μg/ml aprotinin) homogenized and centrifuged at 15 000 × g for 5 min (Korner et al., 1989). The supernatant (whole cell extract) was removed and frozen at −70°C until use.

2.2. Electrophoretic mobility shift assay (EMSA)

Nuclear or whole cell extract (10–25 μg protein) was incubated in a reaction buffer of 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2% NP40, 5% glycerol containing 800 ng dIdC and approximately 50 fmol of ^{32}P-labeled, double-stranded KB oligonucleotide (10 000–25 000 cpm) in a volume of 15 μl for 20 min at ambient temperature. Ten microliters of reaction mixture was then added to a pre-equilibrated 4% polyacrylamide gel and run in a buffer of 6.7 mM Tris–HCl (pH 7.5), 3.3 mM Na-acetate, 1 mM EDTA at 8 mV/cm for 2.5 h. X-Ray film was exposed to the dried gel with an intensifying screen at −70°C. After development, band density was quantified by a Molecular Dynamics densitometer using ImageQuant software.

Each experiment was repeated at least three times and yielded similar results. One representative experiment is presented. The statistical significance of treatment effects was analyzed using the non-parametric Kruskal–Wallis test.

Table 1
Consensus NF-KB DNA binding sequence 5′ GGGRN NYYCC 3′

Sequence of KB probe	AGCTTCAGA	GGGAC	TTTCC	TCTGA
B1 probe	TGGTGG	GGGAG	CCTCC	GGCGCGCCGCCC
B2 probe	GCTGTG	GGGAC	GTCCC	CTCCCGCCGCCC
Int probe	TTCTC	GGGGT	TCCTC	ATTGTGCCGCCC

R = purine, Y = pyrimidine, and N = any nucleotide.

2.3. Oligonucleotide probes

The probe used in the EMSA was the double-stranded oligonucleotide corresponding to the NF-KB binding site from the immunoglobulin kappa locus KB (Sen and Baltimore, 1986a). This oligonucleotide was labeled using polynucleotide T4 kinase and [γ-^{32}P]ATP.

For displacement studies, unlabeled KB oliognucleotide was used as well as several double-stranded oligonucleotide sequences with varying affinity for NF-KB-like DNA binding proteins (Korner et al., 1989) (Table 1).

2.4. Materials

Antibodies directed against NF-KB1p50 and RelA were obtained from M. Scott, Rockefeller University. Antibody for recombinant human Rel was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Recombinant human TNF was purchased from Bachem California, Torrance, CA; okadaic acid from LC Laboratories, Woburn, MA; [γ-^{32}P]ATP from NEN, Boston, MA; all other reagents were obtained from Sigma, St. Louis, MO.

3. Results

3.1. Presence of NF-KB-like DNA binding protein in nuclear extract of GH_3 cells

Fig. 1 represents a typical electrophoretic mobility shift assay using GH_3 nuclear extract and a ^{32}P-labeled KB oligonucleotide. Lane 1 contains nuclear extract of spleen tissue which is used as an approximate marker for NF-KB-DNA complex. Lanes 2–11 contain nuclear extract of separate cultures of GH_3 cells. Typically three bands of protein-DNA complex were observed. The slowest protein-DNA complex (A) co-migrated with that of NF-KB-DNA (compare spleen extract, lane 1 with lanes 2–11). Two additional, faster migrating bands were also noted (B and C). The free probe migrated to the bottom of the gel. Band density of the NF-KB-like DNA binding complex was low in untreated cultures (lanes 2–5) but exposure to PMA for 1 h markedly increased it (lanes 9–11 versus 2–5). Densitometric analysis of this gel indicated that band density of the A complex was significantly increased by PMA treatment. TRH produced a minimal increase (lanes 2–5 versus lanes 6–8) which was not statistically different from con-

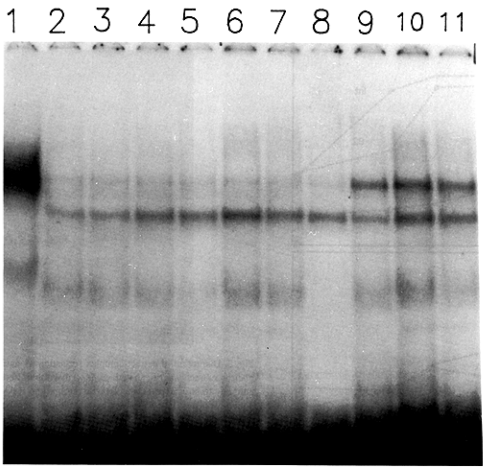

Fig. 1. Electromobility shift assay of nuclear extracts from GH_3 cells. 100 mm dishes of GH_3 cells were incubated for 1 h in DME/0.1% BSA plus treatments. Nuclear extracts were incubated with ^{32}P-labelled KB probe and the reaction mixture was then separated through a 4% acrylamide gel. Lane 1, nuclear extract of spleen; lanes 2–11 nuclear extracts of GH_3 cells: 2–5 control, 6–8 TRH 100 nM, 9–11 PMA 100 nM. A–C complexes of extract and oligonucleotide.

trol. Band densities of B and C complexes showed no significant pattern of change as a consequence of treatment.

3.2. Characterization of NF-KB-like DNA binding complex in GH_3 cell extract

The inducible DNA binding activity in nuclear extracts of GH_3 cells is consistent with the presence of NF-KB in its co-migration with recombinant NF-KB-DNA complex and its induction by PMA. Further characterization was carried out to identify its properties. The specificity of the interaction between GH_3 nuclear extract and the probe was examined by displacement of the extract-DNA complex in the EMSA with excess, unlabeled DNA (Fig. 2) Increasing mass of unlabeled KB, the oligonucleotide used as probe, caused a decrease in band density of the A complex (NF-KB-like DNA complex) while it did not alter band densities of complexes B or C (data not shown). A related oligonucleotide B1, which is known to bind NF-KB (Korner et al., 1989), also displaced the NF-KB-like DNA complex (Fig. 2, upper). A similar oligonucleotide B2, which binds NF-KB poorly (Korner et al., 1989; Grandison and Pfaff, unpublished), did not displace the NF-KB-like DNA complex at a 7-fold excess and displaced it only to a limited extent at a 20-fold excess. The oligonucleotide Int which does not bind NF-KB at all (Grandison and Pfaff, unpublished) was unable to affect binding except at a 200-fold excess where non-specific displacement might be expected. Complex B was readily inhibited by all three oligonucleotides suggestive of low capacity, non-specific binding (Fig. 2, lower). Thus complex A shows a specificity for DNA sequence: only those oligonucleotides which bind NF-KB can displace it while similar sequences of DNA do not.

In order to analyze the NF-KB-like DNA complex, antibodies to NF-KB/rel proteins were used to identify components of the complex. Inclusion of antiserum against NF-KB1p50 in the protein DNA binding reaction resulted in a loss of complex A (Fig. 3) consistent with the presence of NF-KB1p50 in the complex. Similarly antiserum against RelA also diminished the NF-KB-like DNA complex whereas antiserum against Rel had no effect. These observations are consistent with the presence of NF-KB1p50-RelA-DNA in band A. Additionally no band corresponding to A was observed in extracts from the cytosol of untreated or treated GH_3 cells (data not shown). However, treatment of cytosolic extract with formamide was able to induce the appearance of a protein-DNA complex which co-migrated

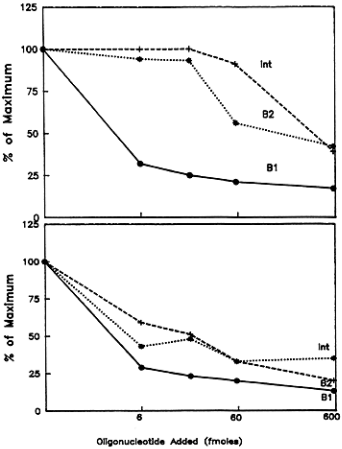

Fig. 2. Displacement of protein-DNA complex by unlabelled oligonucleotides. Nuclear extracts from PMA-treated GH_3 cells were incubated with radiolabelled KB probe and increasing amounts of unlabeled oligonucleotides. Upper panel: displacement of the A complex (NF-KB-like-DNA) by the double stranded oligonucleotides B1, B2 and Int. Lower panel: displacement of the B complex.

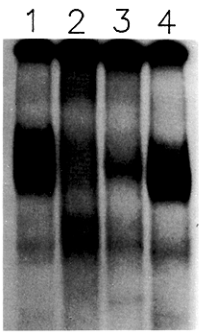

Fig. 3. Disruption of extract-complex formation by antibodies. GH_3 cell nuclear extract was incubated with normal rabbit serum (lane 1),with antibody to NFKB1p50 (lane 2), with antibody to RelA (lane 3) or with antibody to Rel (lane 4) for 1 h before addition of labeled KB oligonucleotide.

with NF-KB-DNA (data not shown). Nuclear localization of activated NF-KB and induction of NF-KB binding activity in cytosolic extract by denaturing agents is characteristic of NF-KB (Sen and Baltimore, 1986a). These observations further indicate that GH_3 cells contain NF-KB.

3.3. Activation of NF-KB in GH_3 cells

As indicated, treatment of GH_3 cells with the phorbol ester PMA resulted in the activation of NF-KB (Figs. 1 and 4). Another well characterized activator of NF-KB, TNF, was also effective in GH_3 cells (Fig. 4). The combination of PMA and TNF was more effective in activating NF-KB than either agent alone (Fig. 4).

The best characterized, physiological stimulus for GH_3 cells is TRH which, after binding to its receptor, activates phosphatidase C to generate the second messengers inositol 1,4,5-trisphosphate and diglycerol (Gershengorn, 1986). In turn, digylcerol activates protein kinase C, the same enzyme activated by PMA. However, unlike PMA, TRH is a poor stimulus for NF-KB activation in GH_3 cells (Fig. 1). Variation in time of TRH exposure (0.5–3 h) did not alter TRH response (data not shown). While TRH and PMA both activate protein kinase C, TRH simultaneously raises intracellular calcium as a results of increased inositol 1,4,5-trisphosphate. To determine whether increased calcium would alter protein kinase C activation of NF-KB, cells were exposed to PMA plus A23187, a calcium ionophore. The combination of PMA plus A23187 did not reproduce the smaller response seen with TRH. PMA itself activated NF-KB (Fig. 5) and even in the presence of A23187, PMA

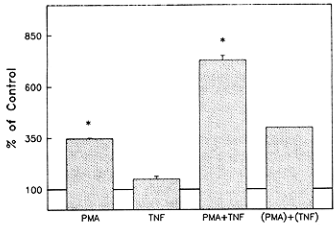

Fig. 4. Activation of NF-KB by PMA, TNF and the combination of PMA plus TNF. Cells were exposed to PMA (100 nM, bar 1), TNF-α (10 ng/ml, bar 2) or PMA plus TNF (bar 3). Bar 4 represents the calculated sum of the response produced by PMA alone plus TNF alone [(PMA – control) + (TNF – control) + control]. Mean plus or minus the range of 2–3 replicates is presented. This is one representative experiment of three. *Indicates significant treatment effect.

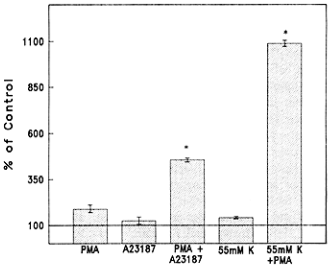

Fig. 5. Activation of NF-KB by PMA plus A23187 or by PMA in depolarized cells. Cells were incubated in Earle's balanced salt solution/0.1% BSA with PMA (100 nM, bar 1), with A23187 (1 μM, bar 2) or the combination of PMA plus A23187 (Bar 3); or in Earle's balanced salt solution/55 mM potassium/0.1% BSA (bar 4) or the combination of 55 mM potassium plus PMA (bar 5). Mean plus or minus the range of 2–3 replicates is presented. This is one representative experiment of three. *Indicates significant treatment effects.

still activated NF-KB. In fact, the response to PMA plus A23187 was greater than the response to either agent alone. A second method used to increase cytosolic calcium was depolarization which opens voltage regulated calcium gates. While depolarization itself had little effect on activation of NF-KB, the combination of depolarization plus PMA produced a greater response than PMA alone (Fig. 5).

Although TRH cannot activate NF-KB to the same extent as PMA, TRH like PMA can interact with TNF to augment TNF activation of NF-KB. The augmentation of

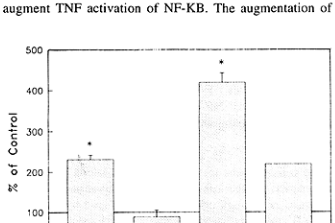

Fig. 6. Activation of NF-KB by TNF and TRH. Cells were incubated with DME/0.1% BSA with TNF (10 ng/ml, bar 1) with TRH (100 nM, bar 2) or with TNF plus TRH (bar 3). Bar 4 represents the sum of responses produced by TNF alone and TRH alone. Mean plus or minus the range of three replicates is presented. One of three replicate experiments. *Indicates significant treatment effects.

TNF activation by TRH was less than the augmentation by PMA (Fig. 6). Other stimuli known to activate GH_3 cells or to activate NF-KB in other cell types were examined and found to induce little or no change in NF-KB activation in GH_3 cells. These included okadaic acid 1–100 nM, 1 h (Rieckman et al., 1992), a phosphatase inhibitor; forskolin 1 μM, 0.5–2 h (Shirkaqa et al., 1989), an activator of adenylyl cyclase; and hydrogen peroxide 150 and 600 μM, 1 h (Staal et al., 1990; Schreck et al., 1991; Toleano and Leonard, 1991), an oxidant.

4. Discussion

GH_3 pituitary tumor cells contain NF-KB DNA binding proteins. Electrophoretic mobility shift assays demonstrate that extract from GH_3 cells interacts with a double-stranded oligonucleotide corresponding to the KB locus of immunoglobulin, a NF-KB binding sequence. One of the complexes formed from the extract and the labeled oligonucleotide, complex A (Fig. 1), displayed properties characteristic of NF-KB. (a) Its mobility was similar to the mobility of the NF-KB1p50-RelB-DNA complex formed with spleen extract and slightly slower than p50 homodimer-DNA complex. (b) The labeled oligonucleotide formed a complex only with crude nuclear extract and not with cytosolic extract. This is consistent with the observation that cytosolic NF-KB is associated with IKB which prevents its ability to bind to DNA (Baeuerle and Baltimore, 1988). (c) Treatment of whole cell extract with the denaturing agent formamide increased formation of the extract-DNA complex. This is consistent with the ability of formamide to dissociate IKB from NF-KB thereby allowing NF-KB to bind to DNA (Sen and Baltimore, 1986b). (d) There was little NF-KB in untreated GH_3 cells but treatment with phorbol ester or the cytokine TNF substantially increased extract-DNA complex formation. Low basal concentration and induction by PMA and TNF are well characterized features of NF-KB regulation (Meichle et al., 1990). (e) The extract-DNA complex displayed DNA sequence specificity. Oligonucleotide sequences known to bind NF-KB decreased the formation of the complex between extract and labeled oligonucleotide as would be expected from competitive displacement with unlabeled oligonucleotide. In contrast, oligonucleotide sequences which did not bind NF-KB were poor displacers of extract-DNA complex formation. Thus the interaction between GH_3 cell extract and oligonucleotide displayed specificity for the oligonucleotide sequence. (f) Finally, antibodies directed against NF-KB1p50 and RelA reduced the formation of the extract-DNA complex. An antibody to Rel did not alter complex formation. Thus, the extract-oligonucleotide complex contains immunoreactive NF-KB1p50 and RelA but not Rel.

It is clear that GH_3 cells not only contain NF-KB but that stimulation of GH_3 cells results in the activation of NF-KB: its release from IKB which is associated with its tran-

slocation into the nucleus and its ability to bind to cognate DNA sequences. Treatment of GH_3 cells with the phorbol ester PMA activated NF-KB (Figs. 1, 4, 5). Activation of protein kinase C by PMA is a well characterized stimulus for hormone release from GH_3 cells. The cytokine TNF also activated NF-KB (Figs. 4, 5) and has been found to affect hormone secretion from these cells (Koike et al., 1991; Nash et al., 1992). Further, PMA and TNF acted synergistically to induce greater activation than that produced by either agent alone (Fig. 4). Synergy for activation of NF-KB by PMA and TNF has been observed in other cell types (Osborn et al., 1989) and has been attributed to the different pathways employed by these stimuli. PMA activates protein kinase C while TNF binds to its receptor with the activation of sphingomyelinase and the generation of ceramide (Schultz et al., 1992).

While PMA can activate NF-KB in the GH_3 cell, the physiological significance of this relates to receptor ligands which can activate protein kinase C like PMA. Perhaps most relevant to anterior pituitary function would be the effect of releasing factors. To this end, the action of TRH on NF-KB activation in GH_3 cells was examined. By itself TRH did not produce a consistent, significant activation of NF-KB. In this respect it differed from the action of PMA even though both agents activate protein kinase C. PMA does so directly by binding to protein kinase C whereas TRH activates phosphatidase C to generate diglyceride which like PMA activates protein kinase C. Although both PMA and TRH lead to activation of protein kinase C, TRH in addition leads to the generation of inositol 1,4,5-tris phosphate, a mobilizer of cytosolic calcium. In order to determine whether the concurrent mobilization of calcium along with activation of protein kinase C would reproduce the action of TRH, cells were exposed to PMA along with the calcium ionophore A23187 or exposed to PMA during potassium depolarization. Increased cytosolic calcium induced by either the ionophore or opening of voltage regulated calcium gates during depolarization had a small effect on NF-KB activation. However, in combination with PMA both methods for increasing cytosolic calcium actually enhanced PMA-induced activation of NF-KB activation. Therefore failure of TRH to induce NF-KB activation can not be attributed to calcium mobilization cancelling the stimulation produced by activation of protein kinase C. Rather, the failure of TRH to activate NF-KB most likely derives from inadequate stimulation by TRH of those pathways which lead to activation of NF-KB. While PMA produces a continuous and prolonged activation, TRH has been reported to produce limited activation of protein kinase C (Kiley et al., 1991). When measured by protein kinase C translocation from the cytosol to the membrane or by protein kinase C autophosphorylation, TRH produced three transient phases of protein kinase C activation, at 15 s at 10 min and then at approximately 6 h (Kiley et al., 1991). Consistent with this interpretation, it was found that short-term exposure of cells to PMA was a poor stimulus for activation of NF-KB (data not shown). While TRH alone does not induce NF-KB activation, it was effective in augmenting the activation produced by TNF. The synergy between TRH and TNF is consistent with the synergy observed between PMA and TNF. Although TRH produces only slight protein kinase C activation, apparently it, along with the concurrent calcium mobilization, is sufficient to enhance TNF-induced activation of NF-KB. Thus, TRH, the major physiological stimulus for hormone release from lactotrophs, might also be considered as a co-regulator of cytokine-induced NF-KB activation in these cells.

Other agents including cyclic AMP (Shirakaga et al., 1989), inhibitors of protein phosphatase (Rieckman et al., 1992), and hydrogen peroxide (Staal et al., 1990; Schreck et al., 1991; Toleano and Leonard, 1991), calcium ionophores (Novak et al., 1990) which have been reported to induce activation of NF-KB in various cells were examined for their ability to affect NF-KB in GH_3 cells. No induction of NF-KB activation was produced by increased cyclic AMP resulting from exposure to forskolin, by inhibition of phosphatases during treatment with okadaic acid or by increased cytosolic calcium. The activation of NF-KB by cyclic AMP, phosphatase or calcium while seen in some cells is not as universally noted as is that for PMA or tumor necrosis factor. Calcium, however, did significantly enhance the action of PMA. The degree of enhancement by calcium is of a much greater magnitude (approximately sixfold-high K + PMA/PMA, Fig. 5) than has been previously noted in other cell types (Novak et al., 1990; Nelson et al., 1993). However, the infrequency of examination of the interaction between PMA and calcium on NF-KB activation and the lack of precisely defined conditions such as extent of calcium mobilization in the different cell types precludes conclusions about the significance of this large response in the GH_3 cell. No response to an enhanced oxidative state resulting from treatment with 150 or 600 μM hydrogen peroxide was found. Other cells have also been reported to fail to respond to H_2O_2 (Stylianou et al., 1992). These demonstrations of specificity for the second messengers which activate NF-KB in different cells emphasize the tight regulation of NF-KB activation which exists within cells and the complex interactions that must exist to achieve it.

In conclusion, GH_3 cells have NF-KB which in unactivated cells remains in the cytosol in a latent form. Upon stimulation of the cells by strong activation of protein kinase C or through other pathways utilized by cytokines, NF-KB can be activated so that it translocates to the nucleus where it can now bind to cognate DNA sequences and influence gene expression. The full role of NF-KB in GH_3 cells or in the anterior pituitary is at present unappreciated. Physiological situations during which NF-KB might be involved based on the genes which possess NF-KB binding sites within their promoter regions would include cell growth or immunological challenge. The release of cytokines during immunological challenge could act alone

or in combination with releasing factors to activate NF-KB and thereby bring about altered gene expression.

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