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Barium modulates c-*fos* expression and post-translational modification

(PC12 cells/transcriptional regulation/calmodulin/protooncogene/calcium channels)

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ABSTRACT **The addition of exogenous barium ions to PC12 rat pheochromocytoma cells elicits a transient induction of the c-*fos* gene. Induction is antagonized by extracellular calcium and the dihydropyridine calcium channel blockers, and it is attenuated in the presence of calmodulin inhibitors. Thus, barium appears to enter the cell through a voltage-dependent calcium channel and, either directly or indirectly, interacts with calmodulin to stimulate c-*fos* expression. This property of barium is not shared by a range of di- and trivalent cations examined. Agents that induce the c-*fos* gene in PC12 cells may be divided into two broad categories. The first comprises polypeptide growth factors and phorbol esters, which give rise to a c-fos protein that undergoes extensive post-translational modification. The second, which comprises depolarizing agents and barium ions, acts via calcium channels and yields a c-fos protein that undergoes less extensive post-translational modification. These different forms of c-fos protein can be distinguished on the basis of their apparent molecular weights on sodium dodecyl sulfate/polyacrylamide gels.**

Modulation of transmembrane ion transport has frequently been shown to be a primary event in the coupling of extracellular signals to intracellular events. Thus, specific ion fluxes play essential roles in such diverse phenomena as stimulus–secretion (1), stimulus–mitosis (2), and stimulus–contraction (3) coupling. Recently, we demonstrated that gating of calcium ions mediated the coupling of membrane depolarizing stimuli to transcriptional activation of the *fos* protooncogene (c-*fos*) in PC12 rat pheochromocytoma cells (4). In this system, elevation of intracellular calcium is believed to activate a calmodulin/calmodulin kinase system to induce c-*fos* expression. However, an alternative mode of coupling intracellular calcium to transcription would be via a calcium-sensitive potassium channel. To investigate this possibility, PC12 cells were exposed to a number of agents that modulate various potassium channels. Among these was barium, a blocker of the calcium-dependent potassium channel (5). We report here that extracellular barium is one of the most potent inducers of c-*fos* in PC12 cells yet identified. However, the c-fos protein synthesized in barium-treated cells undergoes less post-translational modification than in nerve growth factor (NGF)-stimulated PC12 cells. Pharmacological evidence is also presented that addresses the nature of the coupling mechanism of barium to c-*fos* transcription.

MATERIALS AND METHODS

Materials. NGF (7S) was obtained from Collaborative Research (Waltham, MA). Trifluoperazine and chlorpromazine were purchased from Sigma. Nisoldipine and benzodiazepine were synthesized by the chemical division of Hoffmann–La Roche. All other chemicals were of the highest quality available from commercial sources.

Cell Culture. PC12 cells (6) were cultured in RPMI medium (GIBCO) containing 10% horse serum, 5% calf serum, and 4 mM L-glutamine. For experimentation, cells were seeded onto 35-mm Petri dishes (Falcon) and allowed to attach and proliferate for 48 hr. Immediately prior to experimentation this medium was removed and replaced with 1 ml of incubation buffer (basal medium) or 1 ml of Dulbecco–Vogt-modified Eagle's medium (DMEM) lacking methionine. Basal medium consisted of 20 mM Hepes, 146 mM NaCl, 4.2 mM KCl, 0.5 mM $MgCl_2$, and 0.2% glucose.

Detection of c-fos Protein. Cells were incubated in 35-mm Petri dishes at 37°C for 15 min with [^{35}S]methionine (≈800 μCi·mmol^{-1}, Amersham; 1 Ci = 37 GBq) at 300 μCi·ml^{-1}. Preparation of cell lysates, immunoprecipitation, NaDodSO$_4$/polyacrylamide gel electrophoresis, and fluorography were as previously described (7, 8). The fos-specific antibodies used were affinity purified by using a peptide whose sequence was specified by the c-*fos* nucleotide sequence (8).

RNA Isolation and Analysis. Cells were cultured in 150-cm^2 flasks. For the duration of the incubation periods growth medium was replaced with 15 ml of basal medium containing the described additions. RNA isolation, electrophoresis on agarose/formaldehyde gels, transfer to nitrocellulose, and hybridization were as described (9, 10). The concentration of RNA in the gels was monitored by ethidium bromide staining prior to transfer (11). Probes specific for Ha-c-*ras* (12) and the entire c-*fos* coding region (13) were prepared by nick-translation.

RESULTS

Induction of c-*fos* by Barium Ions. A previous study (4) led us to believe that calcium ions played a central role in the induction of c-*fos*. However, an alternative explanation would be that depolarizing agents, and their presumed induced calcium flux, activate further biophysical processes that ultimately lead to c-*fos* activation. One such mechanism would involve effects on calcium-dependent potassium channels (5). Since these are an important class of ion channels in nerve cells, we examined the influence of potassium channel blockers on c-*fos* induction. Two classical inhibitors of potassium channels, tetraethylammonium ions and 4-aminopyridine (5), had no effect on potassium-induced c-*fos* expression. Remarkably, a third potassium channel blocker, barium chloride (14, 15), elicted a large induction of c-*fos* even in the absence of any other inducing agent (Fig. 1*A*). This effect was highly specific for barium ions and was not seen with cadmium, cesium, cobalt, magnesium, manganese, lanthanum, strontium, tin, or zinc ions in concentrations up to 10 mM. Since cesium ions should block the same potas-

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Abbreviation: NGF, nerve growth factor.

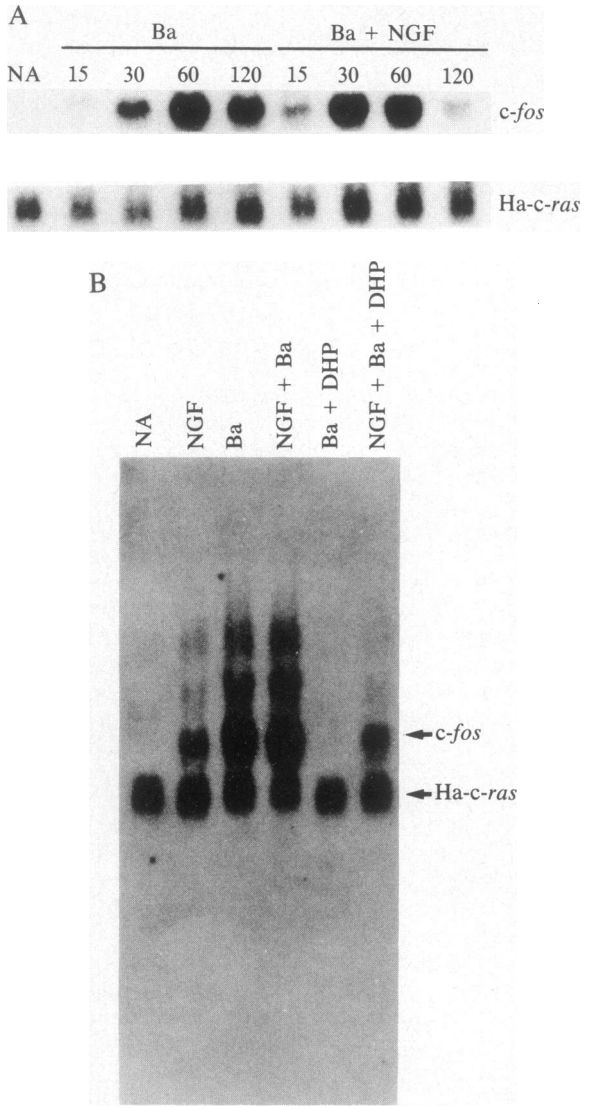

FIG. 1. Induction of c-fos mRNA by using barium. (*A*) PC12 cells were incubated in basal medium with no additions (NA), with 1 mM barium chloride for 15, 30, 60, and 120 min, or with 1 mM barium chloride plus NGF at 200 ng·ml^{-1} for 15, 30, 60, and 120 min. RNA was isolated and 10-μg fractions were analyzed by gel electrophoresis and transferred to nitrocellulose. The filter was hybridized with c-*fos* and Ha-c-*ras* probes. (*B*) PC12 cells were incubated for 30 min in basal medium with no additions (NA), NGF at 200 ng·ml^{-1}, 1 mM barium chloride, 1 mM barium chloride plus 30 μM nisoldipine, a dihydropyridine (DHP), or NGF at 200 ng·ml^{-1} plus 1 mM barium chloride plus 30 μM nisoldipine. RNA was isolated and 10-μg fractions were analyzed by gel electrophoresis and transferred to nitrocellulose. The filter was hybridized with c-*fos* and Ha-c-*ras* probes.

sium channels as barion ions (5), we tentatively conclude that barium does not induce c-*fos* by inactivating a potassium current.

After 30-min exposure to barium, the level of c-*fos* mRNA exceeds that in cells treated with NGF alone (Fig. 1*B*). Under these conditions no significant alterations occur in the level of Ha-c-*ras* mRNA (Fig. 1), and only modest alterations in the levels of c-*myc* mRNA were observed. It should also be noted that barium does not induce c-*fos* expression in fibroblasts (unpublished data).

Role of Voltage-Dependent Calcium Channels. Induction of c-*fos* by barium is completely blocked by the calcium channel blocker nisoldipine, whereas NGF induction is essentially unaffected (Fig. 1*B*). These results have also been confirmed by monitoring c-fos protein synthesis. Furthermore, the induction of c-fos protein by barium is markedly enhanced by depletion of extracellular calcium (Fig. 2). Taking this result together with the dihydropyridine sensitivity (Fig. 1*B*), it appears that barium enters the cell via a voltage-dependent calcium channel and there stimulates c-*fos* expression. In fact, it is known that barium is translocated very efficiently through the voltage-dependent calcium channel in PC12 cells (16), and calcium is known to antagonize barium-induced conductance changes in myocytes (17).

Routinely, we used barium at a concentration of 1 mM; however, as little as 50 μM still evoked an appreciable induction of c-*fos*. When barium is used in DMEM or in phosphate buffer, an insoluble barium phosphate precipitate is formed. This does not inhibit c-*fos* induction, as a similar induction was obtained using a Hepes-buffered (phosphate-free) medium (Fig. 2), in which no precipitation was observed.

Calmodulin Mediates Barium Induction of c-*fos*. The next question to be addressed was that if barium does not act via a potassium channel, what is the mechanism by which it induces c-*fos* transcription? Thus, barium-stimulated PC12 cells were treated with a number of pharmacological agents that interfere with various intracellular messenger systems. Of the substances tested, calmodulin antagonists such as trifluoperazine (18) consistently abolished barium-induced c-*fos* expression (Fig. 3). Calmodulin inhibitors do not block NGF-induced c-*fos* expression; however, they do block induction by depolarizing agents, such as 50 mM potassium (Fig. 3), which are calcium- and calmodulin-dependent (4). At the concentrations of calmodulin antagonists employed in these studies we observed no alteration of c-*fos* induction by phorbol esters, which act through protein kinase C. Thus, an alternative site of action of the calmodulin inhibitor, namely protein kinase C (19), is precluded. In addition, a more specific calmodulin inhibitor, W7 (20), also blocks barium-induced c-*fos* expression (unpublished data). The data, therefore, suggest that barium, once having entered the cell, activates c-*fos* by a calmodulin-dependent mechanism. Studies of the ion dependence of brain calmodulin have shown that barium cannot substitute for calcium (21). Thus, it is

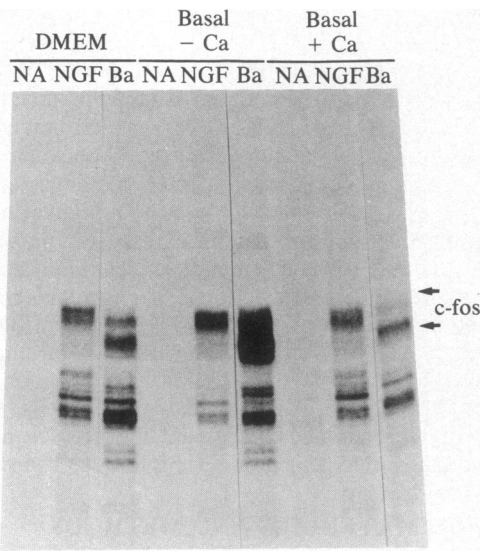

FIG. 2. Induction of c-fos protein by barium is inhibited by extracellular calcium. PC12 cells were incubated in methionine-free DMEM, basal medium in the absence of calcium (Basal − Ca), and basal medium in the presence of 1.1 mM calcium chloride (Basal + Ca) with no additions (NA), NGF at 200 ng·ml^{-1}, or 1 mM barium chloride for 30 min before labeling with [^{35}S]methionine. Arrows indicate the positions of the differentially modified c-fos proteins.

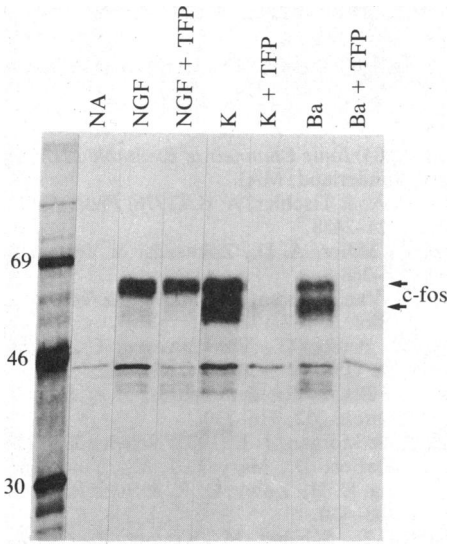

FIG. 3. Barium induction of c-*fos* is calmodulin dependent. PC12 cells were incubated in DMEM with no additions (NA), NGF at 200 ng·ml^{-1}, NGF at 200 ng·ml^{-1} plus 15 μM trifluoperazine (TFP), 50 mM potassium chloride, 50 mM potassium chloride plus 15 μM trifluoperazine, 1 mM barium chloride, or 1 mM barium chloride plus 15 μM trifluoperazine for 30 min before labeling with [^{35}S]methionine. The numbers on the left indicate the molecular weights of [^{14}C]methyl-labeled marker proteins (Amersham). Arrows indicate the positions of the c-fos proteins.

unlikely that barium activates calmodulin directly in PC12 cells, although we cannot exclude the possibility that the cells contain a unique barium-sensitive calmodulin species. However, given that calmodulin does seem to be involved, and since extracellular calcium is not required (Fig. 2), it would seem likely that barium elevates intracellular calcium by some other means. The most likely method of doing this would be via the release of calcium from intracellular stores, perhaps by utilizing the inositolphospholipid pathway (22). Lithium ions are known to retard the degradation of inositol phosphates by inhibiting their phosphatases (23). However, lithium does not induce c-*fos* in PC12 cells, indicating that barium probably does not elevate inositol 1,4,5-trisphosphate by such a mechanism. The turnover of the inositol phosphates in barium-treated PC12 cells remains to be investigated.

Effects on Post-Translational Modification. It appears either that barium-induced c-fos mRNA is more stable than that induced by NGF or that transcriptional activation occurs over a longer period of time, since c-fos mRNA is clearly detected 2 hr after the initial stimulation (Fig. 1*A*). This is also true of c-fos protein synthesis, which continues, although at lower levels, up to 4 hr after treatment with barium (Fig. 4*A*). While this difference is small, it is reproducible.

Surprisingly, c-fos protein induced by barium appears to have a lower apparent molecular weight than that induced by NGF or NGF plus benzodiazepine (Figs. 2, 3, and 4). Fig. 4*B* demonstrates that the barium-induced (smaller) c-*fos* protein is deficient in post-translational modification. The block in modification is not absolute; rather, it appears that the process is slowed in barium-treated cells. However, simultaneous addition of barium and NGF, which gives enhanced levels of induction (Fig. 1*A*), permits rapid modification of c-fos protein (unpublished data). Thus, while barium is an extremely potent inducer of c-*fos*, it appears to be deficient in stimulating processes related to the post-translational modification of the gene product.

The absolute degree of post-translational modification of the c-*fos* protein varied from experiment to experiment. However, in general, agents that induced c-*fos* could be grouped into two categories: (*i*) those that induced a highly modified c-fos protein such as NGF, fibroblast growth factor, NGF plus benzodiazepines, and phorbol esters; and (*ii*) those that induced a less-modified c-fos protein such as potassium, barium, calcium ionophore, veratridine, and BAY K 8644 (4, 11).

The profile of proteins precipitated from stimulated PC12 cells by using fos-peptide antibodies is quite complicated. Some of these proteins represent cross-reacting cellular antigens that are also induced by the various treatments, while others are proteins complexed with c-fos such as p39 (8). The time course of induction and rate of post-translational modification of these proteins may differ from those of c-fos [e.g., see the 46-kDa antigen in cells treated with NGF plus benzodiazepine (Fig. 4*A*)].

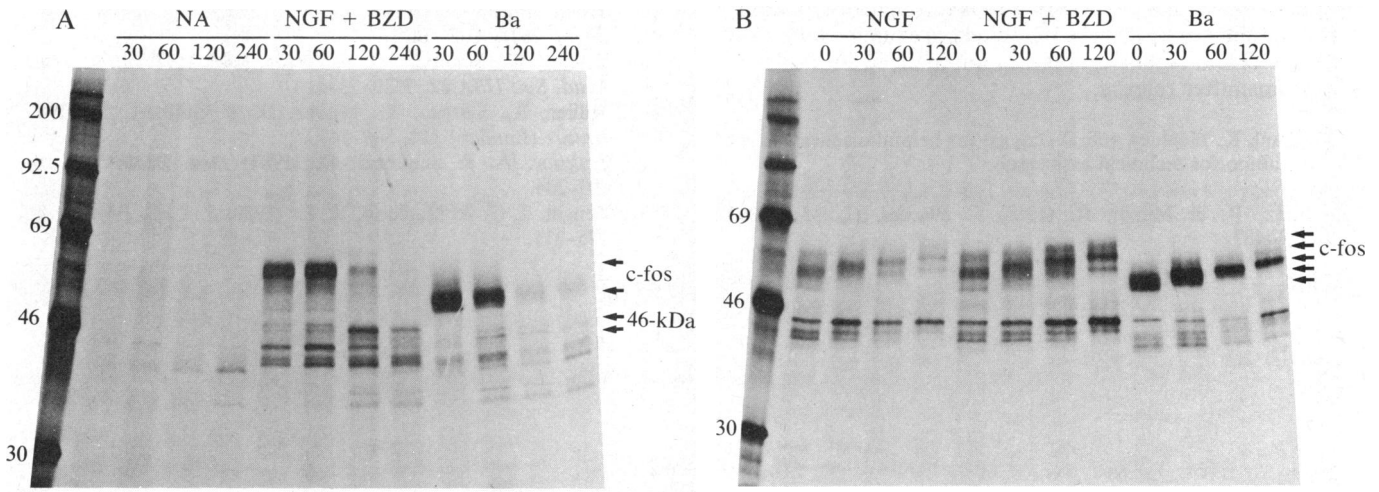

FIG. 4. (*A*) Time course of c-fos protein synthesis after induction. PC12 cells were incubated in basal medium with no additions (NA), NGF at 200 ng·ml^{-1} plus 100 μM benzodiazepine (7-3351) (NGF + benzodiazepine), or 1 mM barium chloride for 30, 60, 120, or 240 min before labeling with [^{35}S]methionine for a further 15 min. (*B*) Pulse–chase analysis of c-fos protein. PC12 cells were incubated in basal medium containing NGF at 200 ng·ml^{-1}, NGF at 200 ng·ml^{-1} plus 100 μM benzodiazepine (7-3351) (NGF + benzodiazepine), or 1 mM barium chloride for 30 min. Cultures were then labeled for 15 min with [^{35}S]methionine. After the pulse-labeling period the medium containing [^{35}S]methionine was removed and was replaced with basal medium supplemented with 1× nonessential amino acids (GIBCO) for 0, 30, 60, and 120 min before preparation of cell lysates. In *A* and *B*, arrows indicate the positions of the various forms of the c-fos protein and a 46-kDa fos-related protein. The numbers on the left indicate the molecular weights of marker proteins.

DISCUSSION

Transcriptional activation of the c-*fos* gene has been observed in many different biological situations. It has been linked to mitogenesis (10, 24–26), cellular differentiation (11, 27–29), and depolarization of neuronal cells (4, 27, 28). These data suggest a rather general role for the c-*fos* gene in coupling short-term events elicited by extracellular stimuli to long-term alterations in gene expression. We have used c-*fos* induction as an early nuclear marker to investigate stimulus–transcription coupling in PC12 cells. Proliferating PC12 cells may be induced to undergo a process resembling differentiation by addition of either NGF or depolarizing concentrations of potassium ions to their growth medium (6, 30). In our hands, the morphologies of the cells observed after the two treatments are dissimilar: potassium-treated cells were flatter and bore stumpy neurites. Furthermore, potassium treatment does not lead to an elevation of choline acetyltransferase activity as seen with NGF treatment (30). The rapid induction of the c-*fos* gene is the earliest known transcriptional consequence of treating PC12 cells with either agent (4, 11, 27, 28). Superficially this would indicate that if c-*fos* plays a role in the process of PC12 differentiation, it is at some early, common, stage of the induction process. However, the demonstration here that the protein product of c-*fos* is different in the two pathways suggests that different forms of the molecule may be responsible for eliciting alternative activation processes. Since c-fos is known to be a nuclear protein (7), it might be postulated that the two forms of the protein bind to different targets. Alternatively, as the c-fos protein is known to exist as a complex with other cellular proteins (8), it is possible that structural alterations affect the composition or stability of c-fos binding. Finally, c-fos may have an as-yet-unknown enzymatic activity that is regulated by the post-translational modifications documented here.

The mechanism by which barium activates c-*fos* transcription is unclear. It has been shown that barium can potentiate the evoked release of neurotransmitters from neurons (31), although the basis of this action is not understood. To produce this effect with barium, calcium must be present in the extracellular medium (31), which is in contrast to the results shown here (Fig. 2). Thus, it is unclear whether the two mechanisms of action are related. Since we show that barium exerts its effects on PC12 cells through a calmodulin-dependent process, it will be of interest to examine the action of calmodulin antagonists upon barium-induced alterations in neurotransmitter release.

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