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Inhibition of 12-*O*-Tetradecanoylphorbol-13-acetate Induction of c-*fos* mRNA by the Protein Kinase A Inhibitor *N*-[2-(*p*-Bromocinnamylamino)ethyl]-5-isoquinoline Sulfonamide

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ABSTRACT. The tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) can induce expression of many immediate-early genes, such as c-*fos* and c-*jun*. In this study, TPA increased c-fos mRNA, cellular cyclic AMP, and protein kinase A (PKA) activity in the first 30 min with similar inductive time courses. Treatment of NIH 3T3 cells with *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (H-89), a PKA specific inhibitor, suppressed TPA induction of PKA activity and c-fos mRNA in a concentration-dependent manner, but did not inhibit serum-induced transcription. H-89 did not inhibit TPA and serum induction of c-jun mRNA. H-89 interfered with TPA-stimulated serum-responsive element-binding activity in a concentration-dependent manner, but did not inhibit TPA-induced mitogen-activated protein kinase 1/2 activity or Elk-1 phosphorylation. TPA stimulation of a c-*fos* promoter reporter construct was inhibited by overexpression of the dominant negative regulatory protein of PKA. In deletion studies, the H-89 inhibitory element was found to be localized between –563 and –379 in the c-*fos* promoter region. These results suggest that H-89 will be very useful for investigating the molecular mechanism of TPA induction of c-fos. BIOCHEM PHARMACOL **58**;10:1639–1647, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. TPA; c-fos; H-89; PKA; cAMP; MAPK 1/2; SRE

H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide, is an isoquinoline sulfonamide that was synthesized first by Hidaka and colleagues [1]. H-89 exhibits a potent and selective inhibitory action on PKA§. Many studies have used H-89 to assess the function of PKA in cells [2–5].

Treatment of cells with the tumor promoter TPA leads to the induction of immediate-early gene expression [6]. The c-*fos* proto-oncogene has been one of the most extensively studied immediate-early genes. The transient induction of c-fos is one of the signals that cells are being released from G_0/G_1 to S phase [7], a process that is ongoing in the cell cycle. When the cell cycle is in progression, cellular metabolic pathways have to be enhanced simultaneously. Some of these metabolic pathways are controlled directly or indirectly by PKA activation. Because cAMP is a PKA activator, the amount of cAMP should be elevated in the G_0/G_1 phase. Several studies have shown that cAMP is elevated in the G_0/G_1 phase [8, 9]. cAMP has also been reported to be increased by TPA treatment in mouse skin during the G_0/G_1 stage [10, 11]. This elevating effect may be the result of the activation of adenylate cyclase by PKC [12, 13]. Induction of both cAMP and c-fos occurs during the G_0/G_1 stage. This suggests that there is a relationship between the c-fos induction and the increase of PKA activity.

PKC, a serine/threonine kinase, can be activated by TPA [14, 15]. Studies of TPA-mediated signal transduction have shown that PKC can phosphorylate c-Raf, leading to c-Raf activation [16–18]. The phosphorylated c-Raf activates MAPK kinases, followed by phosphorylation of MAPK [17–25]. Two of the known MAPK isoforms are MAPK 1 (42 kDa) and MAPK 2 (44 kDa). Activated MAPK 1/2 can phosphorylate transcription factors [26–32]. After this phosphorylation, it can stimulate some transcriptional factors, such as TCF, at the SRE in the c-*fos* promoter. Alterations of the SRE binding property are essential for turning on TPA-induced c-*fos* transcription [33, 34].

In this study, we used H-89 to examine the relationship between PKA and c-fos mRNA induction. Our results demonstrated that H-89 inhibits c-fos mRNA induction

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§ *Abbreviations*: PKA, protein kinase A; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; cAMP, cyclic AMP; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; TCF, ternary complex factor; β-Gal, β-galactosidase; SSC, 0.15 M NaCl, 0.015 M sodium citrate; CAT, chloramphenicol acetyltransferase; SRE, serum-responsive element; EMSA, electrophoretic mobility shift assay; and GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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and that this inhibitory effect contributes to the suppression of PKA activity, rather than the inhibition of MAPK activities. Our results also show that the element responsive to H-89 is localized between –563 and –379 relative to the transcription initiation site.

MATERIALS AND METHODS

Materials

DMEM and FCS were purchased from Seromed. Antibiotics (100 U/mL of penicillin, 100 U/mL of streptomycin) were purchased from Biological Industries. The cAMP [^{3}H] assay kit (TRK 432), enhanced chemiluminescence reagent, and polyvinylidene difluoride membranes were obtained from Amersham. Bicinchoninic acid (BCA) protein assay reagent and a colorimetric PKA assay kit (Spinzyme format) were purchased from Pierce. Zeta-probe nylon filters were obtained from Bio-Rad, GeneFECTor^{TM} was purchased from VennNova, and CAT and β-Gal ELISA assay kits were obtained from Boehringer Mannheim. The oligonucleotides (SRE, CRE, SP-1) were purchased from Santa Cruz Biotechnology Inc. Anti-MAPK 1/2, phospho-specific MAPK 1/2, Elk-1, and phospho-specific Elk-1 antibodies were purchased from New England Biolabs. H-89 was obtained from BioMol. Unless otherwise indicated, all other chemicals were purchased from the Sigma Chemical Co.

Cell Culture

NIH 3T3 cells were grown in DMEM supplemented with 10% FCS and antibiotics. Before various treatments, 80% confluent cells were serum-starved in 0.5% FCS–DMEM for 24–36 hr.

Measurement of Cellular cAMP

The cellular contents of cAMP were measured using the cAMP [^{3}H] assay kit according to the manufacturer's instructions. The protein content was determined with a BCA protein assay reagent using BSA as a standard.

Measurement of PKA Activity

PKA activity was measured using the colorimetric PKA assay kit. The treated cells were washed with ice-cold PBS twice, resuspended in lysis buffer (50 mM Tris–HCl, 2.5 mM EDTA, 1 mM $MgCl_2$, 10 mM NaF, 10% glycerol, pH 7.2), sonicated, and centrifuged. Supernatants then were taken as lysates. Thirty micrograms of cell lysate was used to measure PKA activity. PKA activity assays were performed following the manufacturer's instructions included with the colorimetric PKA assay kit.

Total RNA Isolation and Northern Blot Analysis

NIH 3T3 cells were grown in 10-cm dishes to approximately 80% confluence and then were serum-starved for 24–36 hr. After pretreatment with or without H-89 for 1 hr, 100 ng/mL of TPA was added directly to the culture medium for 30 min. The treated cells were harvested, and RNA was isolated as described by Chomczynski and Sacchi [35] and slightly modified by us [36]. For northern blots, 30 μg of total RNA per lane was fractionated by electrophoresis on 1.2% agarose gel containing 6.7% formaldehyde. RNAs were transferred to a Zeta-probe nylon filter, baked, prehybridized, and hybridized for 12–16 hr at 42° in hybridization buffer (6X SSC, 50% deionized formamide, 10X Denhardt's solution, 10 mM EDTA, 0.1% SDS) with a probe concentration of $\sim 1 \times 10^6$ cpm/mL, as described previously [36]. The filters were washed successively in 2X SSC, 0.1% SDS; 0.5X SSC, 0.1% SDS; and 0.1X SSC, 0.1% SDS washing buffer at room temperature for 15 min. Finally, filters were exposed to x-ray film and scanned to estimate the density of the bands.

Constructs and Transient Transfection Assays

The FC-1 CAT construct contains the c-*fos* 5′ sequence from –2250 to +43 in front of the pSV2-CAT, which was deleted from the SV2 promoter sequence [37]. The deletion constructs, FC-2, FC-3, and FC-4, had sequences removed from positions –1400 to –563, –379, and –121, relative to the transcription initiation site, respectively. The dominant negative (NPKA) and wild-type (WT) PKA regulatory protein expression vectors were prepared by other researchers using a previously described technique [38]. pRSV-β-Gal was used as an internal control. Cells were transfected by GeneFECTor^{TM} according to the recommendations provided by the manufacturer. After transfection, cells were serum-starved for 24 hr before TPA treatment. Cellular extracts were prepared 6 hr after treatment. CAT and β-Gal expressions were measured using CAT and β-Gal ELISA assay kits (Boehringer Mannheim) as recommended by the manufacturer, and the amount of β-Gal expression was standardized in each of the extracts. Values (CAT/β-Gal) were corrected for the amount of protein in each extract. Each experiment was performed at least three times.

EMSAs

The nuclear extracts were prepared from serum-starved and treated cells as described by Andrews and Faller [39]. The oligonucleotides, SRE, CRE, and SP-1 were used in the binding assays. Oligonucleotides were labeled with polynucleotide kinase and [γ-^{32}P]ATP and used as probes. EMSAs were performed according to the manufacturer's instructions, using 4 μg of nuclear protein. Competition assays were performed to determine specific binding bands. In the competition assays, excess concentrations of unlabeled

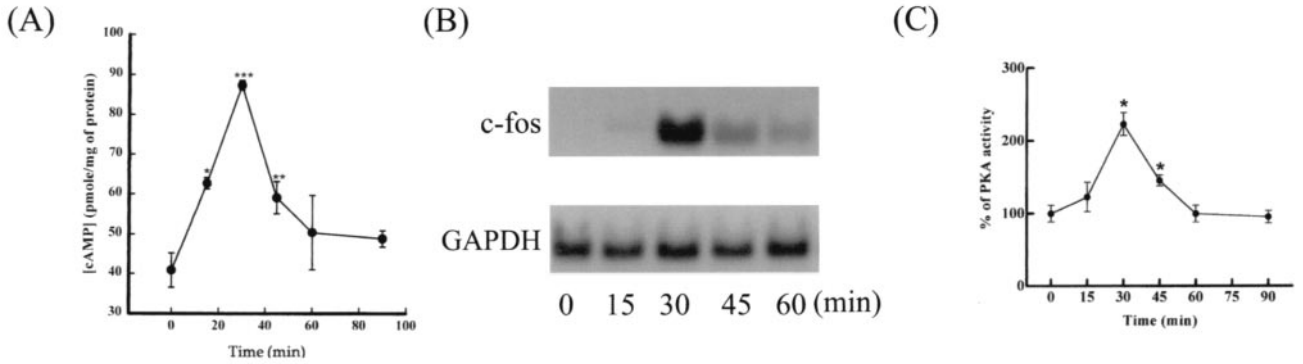

FIG. 1. Time course of the induction of cAMP (A), c-fos mRNA (B), and PKA activity (C) by TPA. NIH 3T3 cells were grown on 0.5% FCS–DMEM for 24 hr, and 100 ng/mL of TPA was added directly to the culture medium. The treated cells were harvested at the indicated times. (A) The cAMP content assays were performed and the content levels were determined; values are means ± SEM of three independent experiments. Key: (*) $P < 0.05$; (**) $P < 0.005$; and (***) $P < 0.0005$ compared with time zero. (B) Twenty-five micrograms of total RNA per well was electrophoresed on 1.2% agarose gel containing 6.67% formaldehyde. Northern blotting was performed as described in Materials and Methods. GAPDH was used as the internal control. Results were obtained from three independent experiments; only one representative experiment is shown. (C) PKA activities were determined using a colorimetric PKA assay kit, and are expressed as a percentage of time-zero cells. Values are means ± SEM of three independent experiments. The time-zero value was 0.055 ± 0.00065 OD_{570}/30 μg protein. Key: (*) $P < 0.05$ compared with time zero.

wild-type or mutant oligonucleotides were added to the reaction mixtures. The intensities of SRE-specific binding bands were scanned using a densitometer.

Preparation of Cellular Extracts for Immunoblotting and In-gel Kinase Assay

The cellular extracts prepared from both serum-starved and treated cells were modified according to the method described by McMahon and colleagues [40]. Briefly, serum-starved and treated cells were washed twice with ice-cold PBS (0.125 M NaCl, 10 mM Na_2HPO_4, 3 mM KH_2PO_4, pH 7.2), scraped, and then lysed in Gold lysis buffer [10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL of aprotinin, 10 μg/mL of leupeptin, 1 mM sodium orthovanadate, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 100 μM β-glycerophosphate, 20 mM Tris, pH 7.9, 137 mM NaCl, 5 mM EDTA]. The lysates were vortexed for 30 sec and then were centrifuged. Supernatants were used for assay. Concentrations were measured using the BCA protein assay reagent with BSA as a standard.

Immunoblotting

Fifty micrograms of cellular lysate was separated by 10% SDS–PAGE. After the separation, the proteins were transferred onto polyvinylidene difluoride membranes. MAPK 1/2, phospho-specific MAPK 1/2, Elk-1, and phospho-specific Elk-1 were detected with anti-MAPK 1/2, phospho-specific MAPK 1/2, Elk-1, and phospho-specific Elk-1 antibodies, followed by a secondary horseradish peroxidase-linked antibody. Signals from an enhanced chemiluminescence reagent followed by exposure to x-ray films were used to measure protein levels.

In-gel Kinase Assay

The samples were prepared and separated as described above, except for the use of 10% SDS–PAGE containing 200 μg/mL of myelin basic protein. After electrophoresis, the gels were washed successively in buffer P [20% (v/v) 2-propanol, 50 mM Tris, pH 8.0], buffer A (5 mM 2-β-mercaptoethanol, 50 mM Tris, pH 8.0), and buffer B (6 M guanidine HCl in buffer A) for 1 hr in each buffer at room temperature, and then were incubated with buffer C [0.04% (v/v) Tween-40 in buffer A] for 16 hr at 4°. After 16 hr, the gels were incubated in reaction buffer (40 mM HEPES, pH 7.4, 2 mM dithiothreitol, 5 mM $MgCl_2$, 300 μM sodium orthovanadate, 100 μM EGTA, 100 μCi [γ-^{32}P]ATP) for 1 hr at room temperature, washed in washing buffer (5% trichloroacetic acid, 1% sodium pyrophosphate), dried, and exposed to x-ray films at -70° [41, 42]. The intensities of ^{32}P-labeled proteins were scanned using a densitometer.

RESULTS

We attempted to demonstrate whether cellular cAMP could be induced by treating NIH 3T3 cells with TPA. As shown in Fig. 1A, we found that cellular cAMP in quiescent NIH 3T3 cells was induced by 100 ng/mL of TPA and reached the highest content, 87.25 ± 1.17 pmol/mg protein, at 30 min. It then declined to a basal level similar to the control level of 40.87 ± 7.3 pmol/mg protein during the following 30 min. We also analyzed PKA activity during

TPA stimulation. As shown in Fig. 1C, the highest PKA activity was about 2-fold that of control. The time courses of cAMP induction and PKA activation were similar to that of TPA induction of c-fos mRNA (Fig. 1B). These results suggest that PKA activation might be correlated with c-fos induction. After quiescent NIH 3T3 cells were treated with various concentrations of H-89, a PKA-specific inhibitor, 1 hr prior to the addition of TPA, it was observed that H-89 suppressed TPA induction of c-fos mRNA in a concentration-dependent manner, whereas H-89 did not inhibit TPA induction of c-jun mRNA (Fig. 2A, lanes 3–7). A 15 μM concentration of H-89 completely inhibited c-fos mRNA induction, but did not inhibit TPA induction of c-jun mRNA (Fig. 2A, lane 6). A 15 μM concentration of H-89 also inhibited induction of c-fos mRNA by forskolin, which activates PKA through elevation of cAMP levels by stimulation of adenylate cyclase (Fig. 2A, lanes 11 and 12). A 15 μM concentration of H-89 did not suppress serum induction of c-fos and c-jun mRNAs (Fig. 2A, lanes 9 and 10). We also investigated whether H-89 inhibits TPA-induced PKA activity. As shown in Fig. 2B, we found that H-89 inhibited TPA-induced PKA activity in a concentration-dependent manner. A 20 μM concentration of H-89 inhibited TPA-induced PKA activity completely. We also observed that H-89 did not inhibit TPA-induced PKC activity and cAMP elevation (data not shown).

The activation of MAPK 1/2 is crucial to the ability of TPA to induce c-fos mRNA [43, 44]. To determine whether H-89 contributed to inhibition of the activation of MAPK 1/2, we performed immunoblotting to determine the level of activated MAPK 1/2. As shown in Fig. 3A (lane 2), the level of activated MAPK1/2 was influenced by TPA (Fig. 3A, lane 2). The activation of MAPK 1/2 pp(42/44) induced by TPA was not inhibited by H-89 (Fig. 3A, lanes 4–6), and 20 μM H-89 did not induce MAPK 1/2 activation (Fig. 3A, lane 3). To determine whether H-89 could inhibit MAPK activity as well, an in-gel kinase assay was performed. As shown in Fig. 3B, we found that H-89 did not inhibit TPA-induced MAPK activity, and treatment with 20 μM H-89 alone did not alter MAPK activity as compared with control cells. We also examined whether the phosphorylation of Elk-1, which is a substrate of MAPK 1/2, was suppressed by H-89. As shown in Fig. 3C, H-89 did not inhibit TPA-induced Elk-1 phosphorylation.

To directly test whether PKA was essential for TPA to induce c-fos mRNA, cells were cotransfected with FC-1 CAT containing the c-*fos* promoter region and expression vectors coding for dominant-negative (NPKA) or wild-type (WT) PKA regulatory proteins. As shown in Table 1, we found that TPA induced an approximately 1.8-fold increase in FC-1 CAT activity in the presence of the WT vector. Expression of NPKA resulted in a dramatic decrease in TPA-stimulated FC-1 CAT activity. The c-*fos* promoter contains SRE, which is known to mediate induction of this promoter by TPA. This led us to investigate whether the

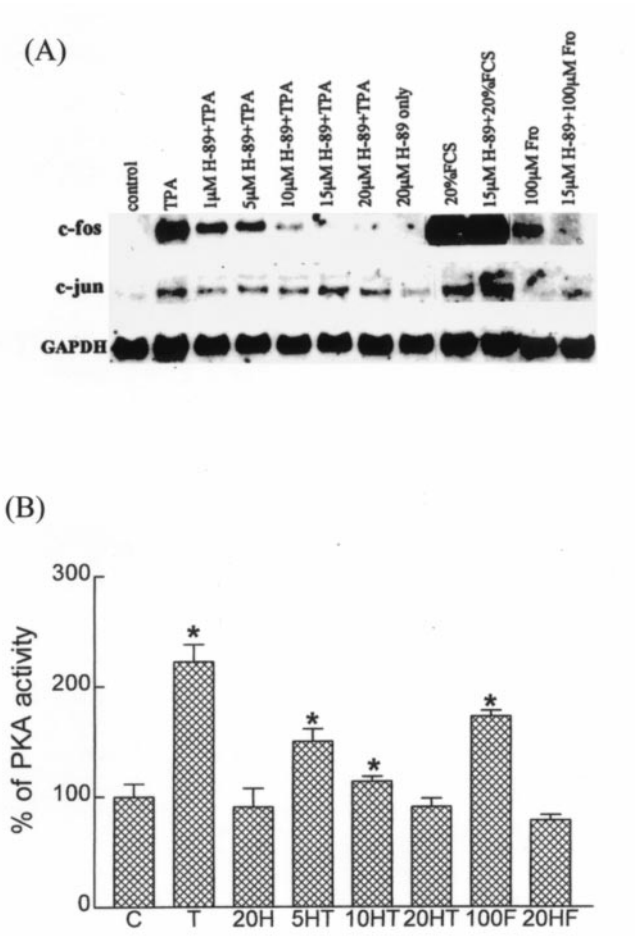

FIG. 2. Effects of H-89 on TPA induction of (A) c-fos and c-jun mRNAs and (B) PKA activity. (A) Total cellular RNAs were extracted from serum-starved 3T3 cells either without further treatment (lane 1) or treated as follows: 100 ng/mL of TPA only (lane 2); 20% FCS (lane 9) for 30 min; 20 μM H-89 only for 90 min (lane 8); pretreatment with H-89 (1 μM, lane 3; 5 μM, lane 4; 10 μM, lane 5; 15 μM, lanes 6 and 10; 20 μM, lane 7) for 1 hr, then 100 ng/mL of TPA (lanes 3–7) or 20% FCS (lane 10) for 30 min; 100 μM forskolin for 1 hr (lane 11); pretreatment with 15 μM H-89 for 1 hr, followed by the addition of 100 μM forskolin into the medium for 1 hr (lane 12). c-fos, c-jun, and GAPDH mRNAs were detected by northern blot analysis. GAPDH was used as an internal control. Results were obtained from three independent experiments; only one representative experiment is shown. (B) Total cellular protein was extracted from serum-starved cells. PKA activity was assayed in 30 μg cellular protein without treatment (C) or treated as follows: 100 ng/mL of TPA only for 30 min (T); 20 μM H-89 only for 90 min (20H); pretreatment with H-89 (5 μM, 5HT; 10 μM, 10HT; 20 μM, 20HT) for 1 hr, followed by 100 ng/mL of TPA; 100 μM forskolin for 1 hr (100F); pretreatment with 20 μM H-89 for 1 hr, followed by the addition of 100 μM forskolin into the medium for 1 hr (20HF). PKA activities were expressed as percentages of the untreated cells. Values are means ± SEM of three independent experiments. The control (100%) value was 0.055 ± 0.00065 OD_{570}/30 μg protein. Key: (*) $P < 0.05$ compared with the control.

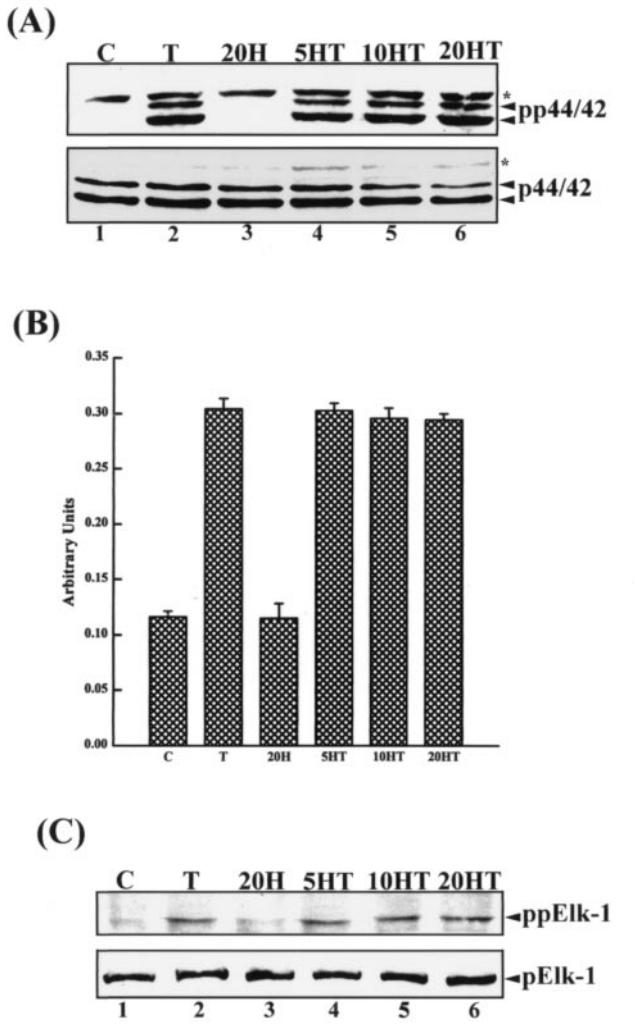

FIG. 3. Effects of H-89 on MAPK 1/2 (p42/44) (A and B) and Elk-1 (C). (A) Detection of activation of MAPK 1/2 by immunoblotting. Total cell lysates (50 μg) were prepared from serum-starved 3T3 cells and then stimulated as follows: no treatment (lane 1, C); 100 ng/mL of TPA only (lane 2, T); 20 μM H-89 only (lane 3, 20H); pretreatment with various concentrations of H-89: 5 μM (lane 4, 5HT), 10 μM (lane 5, 10HT), and 20 μM (lane 6, 20HT) for 1 hr, followed by the addition of 100 ng/mL of TPA directly into the culture medium for 30 min. p42/44 or pp42/44 were analyzed by immunoblotting. pp42/44 were active forms of p42/44. The asterisk (*) indicates non-specific binding. Results were obtained from three independent experiments; only one representative experiment is shown. (B) In-gel kinase assays of MAPK 1/2 activity. Treatments and notations are as described for panel A. Arbitrary units: densities of x-ray films/densities of p42/44 immunoblots. Values are means ± SEM of three independent experiments. (C) Immunoblotting detection of Elk-1. Treatments and notations are as described for panel A.

H-89 inhibitory effect influenced SRE binding activity. In nuclear extracts prepared from serum-starved cells, the SRE binding complex appeared as shown in Fig. 4 (lane 2). Treatment with TPA alone caused a slight increase of SRE binding activity (Fig. 4, lane 3). On the other hand, treatment with 20 μM H-89 decreased SRE binding activity (Fig. 4, lane 4). Treatment with TPA and H-89 resulted in the gradual disappearance of SRE binding activity in a concentration-dependent manner (Fig. 4, lanes 5–7, and Table 2). In competitive assays, our data showed that the shifted bands (SB) were specific binding bands for SRE (Fig. 4, lanes 8–11). We tried to determine which region in the c-*fos* promoter was responsible for the H-89 inhibitory effect. When NIH 3T3 cells were transfected with constructs of the c-*fos* promoter driving the CAT reporter gene, FC-1, FC-2, FC-3, and FC-4 (Fig. 5A), we observed that the addition of 10 μM H-89 had an inhibitory effect on TPA induction of c-*fos* promoter activity. Inhibitory effects were observed with FC-1 and FC-2 CAT reporter plasmids, which retain the capacity for TPA induction, but not with the FC-3 plasmid, which is also TPA-inducible (Fig. 5B). A 100 μM concentration of forskolin induced FC-2 and FC-3 CAT activities, and a 10 μM concentration of H-89 repressed forskolin-induced FC-2 CAT activity, but not FC-3 activity (Fig. 5C).

TABLE 1. Effects of TPA-induced FC-1 CAT expression by PKA

Expression vectors	Treatments	Induction fold* (%)
Wild-type (WT, 2 μg)	Control	100
	+ TPA	180
Dominant negative	Control (2 μg)	100
	(NPKA (1 μg) + TPA	120
	NPKA(2 μg) + TPA	105

* Induction fold is expressed as a percentage of control in each expression vector experiment. Values are from three separate experiments. The control value (100%) was 1.67 ± 0.1586 $OD_{405-490}$/mg protein.

DISCUSSION

In this study, the cellular cAMP level in NIH 3T3 cells was found to be induced by TPA during the first 30 min. This result is similar to the results of other studies [10, 11] and suggests that PKC may directly or indirectly activate adenylate cyclase [12, 13]. Our data also showed that the inductive time courses of c-fos mRNA and PKA activity were very similar, and that H-89 suppressed TPA induction of c-fos mRNA and PKA activity in a concentration-dependent manner. However, treatment with 20 μM H-89 combined with TPA did not inhibit TPA-induced PKC activity or cAMP level (data not shown). In our NPKA transfection experiments, we found that transfection of NPKA inhibited TPA-induced but not serum-induced CAT activities (data not shown). These results were consistent with our results on H-89 inhibitory effects and suggest that the activation of PKA may be involved in mediating TPA induction of c-fos mRNA, and that the inhibition of TPA induction of c-fos mRNA by H-89 may account for the suppression of PKA activity. However, we cannot rule out the possibility that H-89 inhibits another

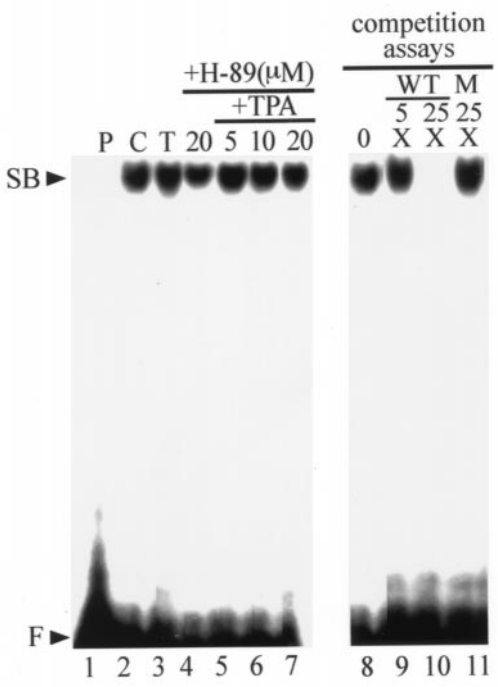

FIG. 4. Effects of H-89 on SRE-binding activity in 3T3 cells. EMSA of nucleoprotein complexes formed on SRE was conducted using 4 μg of nuclear protein. Nuclear protein was extracted from serum-starved cells and then stimulated as follows: no treatment (lanes 2 and 8, C); 100 ng/mL of TPA only (lane 3, T); 20 μM H-89 only (lane 4); pretreatment with various concentrations of H-89 containing 5 μM (lane 5), 10 μM (lane 6), and 20 μM (lane 7) for 1 hr, followed by the addition of 100 ng/mL of TPA directly to the culture medium for 30 min. Determination of a SRE-specific binding band was performed by the addition of excess concentrations of unlabeled wild-type or mutant oligonucleotides to the reaction mixtures. Lanes 9 and 10: the mixtures were incubated with 5-fold (lane 9) or 25-fold (lane 10) excess of unlabeled wild-type (WT) probe. Lane 11: the mixture was incubated with 25-fold excess of unlabeled mutant (M) probe. Lane 1: probe only. The positions of non-binding probe (F) and SRE-binding band (SB) are indicated by arrows. Results were obtained from three independent experiments; only one representative experiment is shown.

kinase(s) rather than PKA. Further experiments are needed to determine the inhibitory specificity of H-89.

In the TPA-mediated signal transduction pathway, phosphorylated PKC can activate Raf, and then activate MAPK kinase and MAPK 1/2 [14–25]. The activation of MAPK 1/2 is essential for TPA to induce c-fos mRNA [43–45] because it can phosphorylate a number of transcription factors, such as Elk-1 [28–32]. In our immunoblotting and in-gel kinase assays, treatment with H-89 did not inhibit TPA-induced MAPK 1/2 activities (Fig. 3B) or Elk-1 phosphorylation (Fig. 3C). This indicates that the inhibition of TPA-induced c-fos expression by H-89 did not occur through the inhibition of activation of MAPK 1/2 and its activity.

In this study, PKA did not disturb MAPK 1/2 activities. Several studies have reported that the preactivation of PKA

TABLE 2. Effects of H-90 on SRE-binding activity in 3T3 cells

Treatment	Relative intensities of SRE-binding activity* (%)
Control	100
100 ng/mL TPA	107
20 μM H-89	62
5 μM H-89 + TPA	92
10 μM H-89 + TPA	80
20 μM H-89 + TPA	71

* Relative intensities (%) were obtained from SRE/SP-1 specific band intensities. Values are from three separate experiments.

inhibits the stimulation of MAPK activity by growth factors and serum [46–50]. In addition, Linder and colleagues [11] reported that preinhibition of PKA results in the activation of MAPK. These conflicting results imply that PKA may regulate MAPK activity through different stimulators at different times of activation, and/or with different specificities among cell types. Further studies are needed to determine the nature of this regulatory mechanism.

Alterations of the SRE binding property are essential for turning on TPA-induced c-fos transcription [32, 34]. In our EMSAs, we found that some transcription factors already were bound to SRE in serum-starved nuclear extract (Fig. 4), and this observation is consistent with other studies [32, 34]. However, the one shifted band observed in this study was different from the findings of other studies, which showed shifting of multiple bands [32]. Because we used shorter oligonucleotides, 25-mers, than those studies [32], some transcription factors, such as TCF, may not have been stable enough to bind to the oligonucleotides. Therefore, the multiple bands may not have appeared in our gel shift data. After treatment with TPA, the SRE-binding intensity did not exhibit a dramatic change, which suggests that certain transcriptional factors may be displaced from SRE in the TPA-treated state, and that this change was not detectable in our EMSA experiments. As shown in Fig. 3, H-89 did not inhibit MAPK activity or Elk-1 phosphorylation. We assumed that Elk-1 normally may bind to SRE, and our preliminary data supported this assumption (data not shown). Results obtained following treatment with various concentrations of H-89 demonstrated that H-89 inhibited TPA-induced SRE binding activity in a concentration-responsive manner (Fig. 4). On the other hand, treatment with H-89 alone also decreased SRE binding. These results are not accounted for sufficiently by the inhibitory effects of H-89. Therefore, we analyzed the c-*fos* promoter sequence to determine which region was responsible for the inhibitory effect of H-89. In our c-*fos* promoter deletion constructs, the inhibitory effectiveness of H-89 depended on the sequence localized between –563 and –379 relative to the transcription initiation site. In the future, we will localize the H-89 responsive element in this region and find the transcription factor(s) involved in the inhibitory effect.

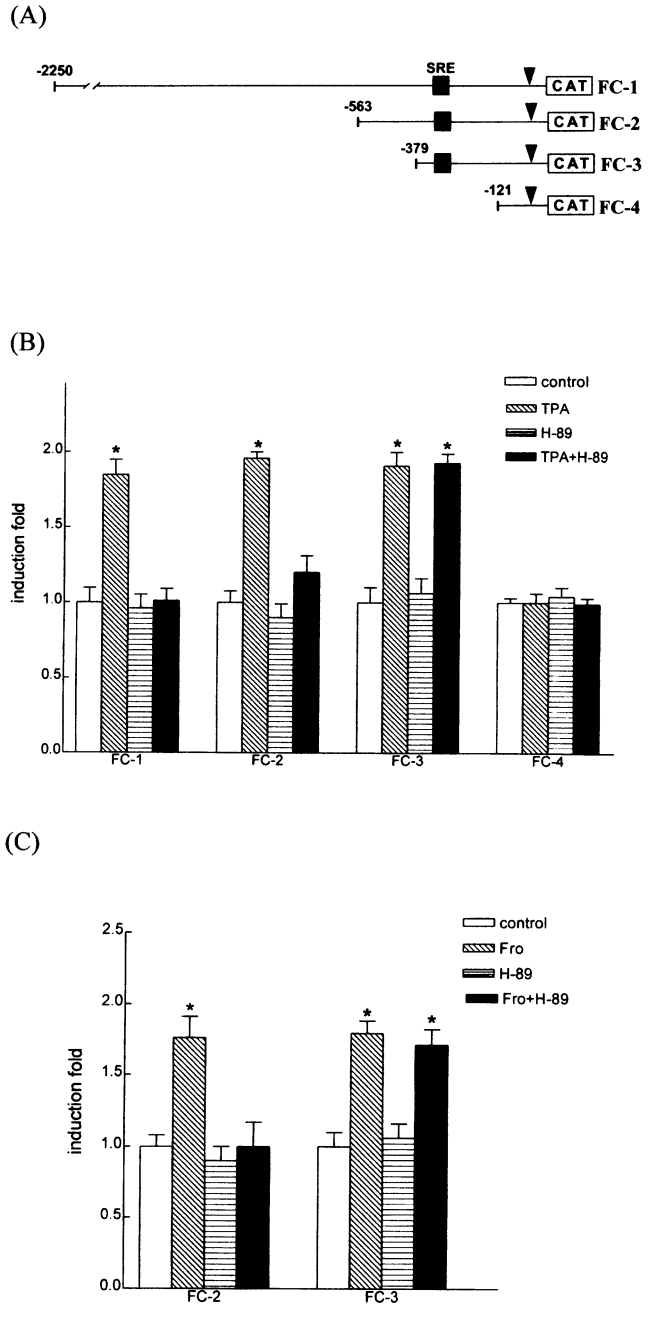

FIG. 5. Effects of H-89 on the c-*fos* promoter region. 3T3 cells were cotransfected with 2 μg of various deletion CAT constructs and 0.1 μg of pRSV-β-Gal, which was used as an internal control. The deletion of c-*fos* promoter CAT reporter constructs (A). Transfected cells were serum-starved for 24 hr and incubated with or without 10 μM H-89 for 1 hr followed by 6-hr induction of 100 ng/mL of TPA (B) or 100 μM forskolin (C). The fold induction was expressed as a multiple of the value for untreated cells in each c-*fos*-CAT construct. Values are means ± SEM of three independent experiments. Control values: FC-1, 1.67 ± 0.1586 $OD_{405-490}$/mg protein; FC-2, 1.52 ± 0.114 $OD_{405-490}$/mg protein; FC-3, 1.48 ± 0.148 $OD_{405-490}$/mg protein; and FC-4, 1.5 ± 0.045 $OD_{405-490}$/mg protein. Key: (*) $P < 0.05$ compared with control.

Although our data indicated that the inhibitory effect of H-89 may have resulted from transcription inhibition, we cannot rule out the contributory effect of an increase of mRNA instability. Further studies need to be performed to determine the relative contribution of each of these mechanisms. Interestingly, we found that H-89 did not inhibit TPA induction of c-jun. Recently, several studies have shown that free radicals induce c-jun [51–53]. Moreover, our preliminary data showed that TPA-induced free radicals and c-jun were not suppressed by staurosporine (data not shown). These findings imply that TPA induction of c-jun may be mediated by free radicals. In conclusion, the findings of this study indicate that the mechanisms of TPA induction of c-fos and c-jun are different in the first 30 min, and that H-89 is a very useful tool for investigating the molecular mechanisms by which TPA induces c-fos.

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