Protein Kinase Inhibitor H7 Blocks the Induction of Immediate-Early Genes zif268 and c-fos by a Mechanism Unrelated to Inhibition of Protein Kinase C but Possibly Related to Inhibition of Phosphorylation of RNA Polymerase II*

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1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H7) has often been used in combination with protein kinase inhibitor (N-(2-guanidinoethyl)-5-isooquinolinesulfonamide) (HA1004) to assess the contribution of protein kinase C (PKC) to cellular processes, including the induction of gene expression. This use of H7 and HA1004 is based upon the fact that H7 inhibits PKC more potently than HA1004 in *in vitro* assays. Thus, although both compounds are broad spectrum protein kinase inhibitors, inhibition by H7, but not by HA1004, has often been interpreted as evidence for the involvement of PKC in the cellular process under study. Here we describe experiments that show that this interpretation is not correct with regard to the induction of two immediate-early genes, zif268 and c-fos, in PC12D cells. In these studies we confirmed that H7, but not HA1004, potently blocks the induction of zif268 and c-fos mRNA by nerve growth factor, carbachol, phorbol ester, Ca\(^{2+}\) ionophore, or forskolin. Surprisingly, however, H7 has no effect on the ability of these agents to activate mitogen-activated protein kinase (MAPK), an upstream activator of zif268 and c-fos gene expression. H7 also does not inhibit preactivated MAPK in *in vitro*. Taken together, these results suggest that H7 blocks gene expression by acting at a site downstream from MAPK. H7 has previously been shown to block transcription in *in vitro* by blocking the phosphorylation of the carboxyl-terminal domain of RNA polymerase II (Yankulov, K., Yamashita, K., Roy, R., Egly, J.-M., and Bentley, D. L. (1995) *J. Biol. Chem.* 270, 23922–23925). In this study, we show that pretreating PC12D cells with H7, but not with HA1004, significantly reduces levels of phosphorylated RNA polymerase II *in vivo*. These results suggest that H7 blocks gene expression by inhibiting the phosphorylation of RNA polymerase II, a step required for progression from transcription initiation to mRNA chain elongation.

The immediate-early genes zif268 (also termed NGFI-A, egr-1, krox24, TIS8; reviewed in Ref. 1) and c-fos (2) encode transcription factors that have been proposed to function as “third messengers” in intracellular signal transduction cascades that convert information conveyed by extracellular stimuli into genomic responses that underlie growth, differentiation, and long term changes in the behavior of cells (3, 4). We have previously shown that NGFI-A (1) and the carbachol (carbamylcholine) cause the rapid induction of zif268 mRNA in PC12D cells (5). Induction of zif268 mRNA by NGF is mediated by the high affinity NGF receptor, TrkA, which activates the Ras/MAPK cascade (6). Induction by carbachol is mediated by the m1 subtype of muscarinic acetylcholine receptor, which activates phospholipase C to produce the second messengers inositol 1,4,5-trisphosphate and diacylglycerol (5). Increased intracellular levels of inositol 1,4,5-trisphosphate trigger the release of \( \text{Ca}^{2+} \) from internal stores, which in turn open \text{Ca}^{2+} \text{ capacitative influx} channels in the cell membrane, resulting in a sustained influx of extracellular \text{Ca}^{2+}. Increased levels of diacylglycerol activate PKC. Both the sustained increase in intracellular \text{Ca}^{2+} \text{ and the activation of PKC contribute to the induction of zif268 mRNA (5), at least in part by activating the MAPK cascade (81). Activation of the MAPK cascade is therefore a common element in the intracellular signaling events leading to gene expression that are initiated by NGF and carbachol in PC12D cells.

In the course of investigating the involvement of PKC in the induction of zif268 mRNA by NGF and carbachol, we compared the effects of pretreating PC12D cells with the protein kinase inhibitor H7 (7) with pretreatment of the cells with the related compound HA1004 (8). Both H7 and HA1004 are broad spectrum protein kinase inhibitors, but H7 inhibits PKC more potently than HA1004 (K\(_i\) values = 6 and 40 \(\mu\)M for H7 and HA1004, respectively) *in vitro* assays (7). Based upon this difference, many investigators have used these inhibitors in combination to evaluate the role of PKC in various cellular processes, including the induction of gene expression. In many
of these studies, inhibition by H7 in the absence of inhibition by HA1004 was taken as evidence for a role for PKC in the process under investigation. The data presented in this paper, however, shows that inhibition of gene expression by H7 does not necessarily imply that PKC is involved. Rather, we found that although H7 potently inhibits the induction of zif268 and c-fos mRNAs following activation of PKC with phorbol ester, it fails to prevent activation of MAPK by phorbol ester. This shows that H7 can block the induction of gene expression without blocking PKC.

Examination of the literature indicates that H7 blocks the induction of a broad spectrum of rapidly inducible genes by a variety of stimuli, including stimuli not previously associated with the activation of PKC. These observations suggest that H7 may block a site, different from PKC, that is universally required for the induction of rapidly inducible genes. A previous report that H7 blocks transcription in vitro by inhibiting the phosphorylation of RNA polymerase II by a TFIII-associated kinase (9), led us to examine the effect of H7 on phosphorylation of RNA polymerase II in vivo. In the present study we show that pretreatment of PC12D cells with H7 significantly reduces levels of phosphorylated RNA polymerase II in vivo, suggesting that H7 blocks gene expression by directly inhibiting transcription.

**EXPERIMENTAL PROCEDURES**

**Materials**—H7 was purchased from Seikagaku Kogyo and Calbiochem. This H7 is the authentic compound originally described by Hidaka et al. (7) and not the less potent iso-H7, which has sometimes been sold under the H7 label (10). HA1004, H8, and H89 were from Seikagaku Kogyo. NGF, carbachol, ATP, chloramphenicol, Ca[+]-ionophore A23187, thapsigargin, forskolin, dieodeoxyforskolin, and dimethyl sulfoxide were purchased from Wako Chemical Industries. K252a and staurosporine were from Kyowa Medex Co., Inc. GF109203X, PD98059, and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) were purchased from Calbiochem. PMA, 4-α-PMA, acetyl-CoA, and S-acetylcoenzyme A synthetase were from Sigma. γ-[32P]ATP and [α-32P]dCTP were obtained from Amersham Pharmacia Biotech and [3H]sodium acetate was from NEN Life Science Products. Anti-Erk-1 and anti-Erk-2 antibodies were obtained from Santa Cruz Biotechnology. Restriction enzymes and other reagents for plasmid construction were purchased from Toyobo Corp., Takara Shuzo Co., and New England Biolabs. Murine zif268 cDNA (ATCC number 63027), murine c-fos genomic DNA (ATCC number 41041), and the expression vector pBLCAT2 were obtained from the American Tissue Culture Collection. Human cyclophilin cDNA was a gift from Toshio Watanabe (Tohoku University), and pE6F-BOS (11) was a gift from Shigeki Nagata (Osaka Bioscience Institute).

**Cell Culture**—PC12D cells (12), a rapidly differentiating subline of rat pheochromocytoma-derived PC12 cells (13), were a gift from Mamoru Sano (Dept. of Biology, Faculty of Medicine, Kyoto Prefectural University of Medicine). PC12D cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui) supplemented with 5% fetal bovine serum, 5% horse serum, 0.1% sodium bicarbonate, 3.6 mM glucose, 10 units/ml penicillin, 45 μg/ml streptomycin at 37 °C under 5% CO2. Non-differentiated PC12D cells were used in all of the experiments. Dishes, and Northern analysis was carried out as described previously (5). The amount of RNA in each sample was determined by optical density of the RNA solution in suspended in the ethidium bromide-stained RNA in denaturing gel used for Northern blot analysis. Unless noted, 10 μg of total cellular RNA was electrophoresed in each lane. After blotting onto Pall Biodyne type B transfer membranes (0.45-μm pore size), hybridization was carried out simultaneously using DNA probes prepared from zif268, c-fos (coding regions), and cDNA fragments isolated from agamose gels and labeled using the Amersham Pharmacia Biotech Oligolabeling kit and [α-32P]dCTP. The intensities of bands in Northern blots were quantified using a Fuji Bioimaging analyzer BAS2000.

**Plasmid Construction**—An expression vector, pGLizI420, containing a firefly luciferase reporter gene linked to the rat zif268 promoter was constructed using pGL2 (Promega). Briefly, the zif268 promoter region (from -842 to 0 base pairs) containing 6 SRE sites and 2 CRE sites was amplified by polymerase chain reaction using synthetic oligonucleotide primers (forward primer corresponding to nucleic acid residues 121-149 of the rat zif268 promoter in the numbering system of Changelon et al. (Ref. 16; GenBank™ accession number J04154), 5'-AACACATATAAGGAGACAGAGATCC-3', backward primer containing a synthetic EcoRI site followed by nucleic acid residues 941-920 (14) of the rat zif268 promoter). This fragment was inserted into EcoRI excised pGL2. An expression vector containing the bacterial chloramphenicol acetyltransferase (CAT) gene and a polyadenylation signal derived from SV40. This fragment was inserted into EcoRI excised pGL2. An expression vector containing the bacterial chloramphenicol acetyltransferase (CAT) gene and a polyadenylation signal derived from SV40. This fragment was isolated, blunt-ended, and cloned into the XbaI site (after converting XbaI-cut ends to blunt ends) of pEF-BOS. The resulting vector, pEF-CAT, was used as an internal control in transfection experiments using zif268-luciferase expression vectors.

**Transfection of PC12D Cells and Assay of Reporter Genes**—Transfections were performed using LipofectAMINE™ reagent (Life Technologies, Inc.) essentially as recommended by the manufacturer. Cells were seeded in 6-cm plastic culture dishes (Corning or Iwaki Glass) at a density of 4 × 10^5 cells/dish and cultured for 1 day prior to transfection. 0.5 μg of pEF-CAT DNA, 2.5 μg of luciferase expression vector DNA, 13.8 μl of LipofectAMINE™ reagent were added to each dish of cells, and incubated for 4 h, prior to adding the medium containing twice the normal concentration of serum. After incubation overnight, the cells in each 6-cm dish were resuspended and distributed into 12 × 1.1-cm wells. The following day, the medium was replaced with normal DMEM, and the cells were cultured for 1 more day. Drugs were added after 24 h, and cell culture medium changes were made the day after 4 h. Luciferase expression was carried out using the Promega Luciferase or Packard LucLite™, and luciferase activities were quantified using a Packard Tri-Carb or Top count scintillation counter as described in the manuals supplied by Promega and Packard. Background luciferase

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3 E. Kumahara and D. Saffen, unpublished observations.
expression was determined using cells transfected with pGL2, which lacks a promoter for luciferase gene expression. Transfection efficiency was determined by cotransfection with pEF-CAT. CAT activities were measured as described by Nordeen et al. (17), and these values were used to calculate normalized luciferase activities for each sample.

Assay of RNA Polymerase II Phosphorylation in Vivo—PC12D cells were labeled with 32P in vivo as described (18). Briefly, PC12D cells grown to 80–90% confluency in 10-cm culture plates were washed twice with 10 mM Tris (pH 7.0)-buffered saline (TBS) and overlaid with 5 ml DMEM, 100 mM Tris, 192 mM glycine, 10% methanol, and 0.02% SDS. Following the current was increased to 45 mA per gel. Proteins were then electrophoresed in 20 cm) in buffer containing 0.5% bisacrylamide stock solution) containing 0.5% acrylamide stacking gel containing 125 mM Tris-Cl (pH 8.0), 2.5 mM magnesium acetate, 2 mM CaCl2, 0.05 mM EDTA, 0.1 mM DTT, 0.25 M sucrose, 0.2 mM PMSF, and 12.5% glycerol and treated with 0.5% SDS. Following electrophoresis, gels were stained with Coomassie blue R-250 for 3 min to remove debris. Nuclei extracts (15 μg/lane) were resolved in 10% SDS, boiled for 3 min, and centrifuged at 15,600 g for 5 min. Cells were resuspended in 1 ml of buffer containing 10 mM Tris-Cl (pH 7.9), 1 mM CaCl2, 1.5 mM MgCl2, 0.25 M sucrose, 0.2 mM PMSF, 0.5% Triton X-100, homogenized with Dounce homogenizer, and centrifuged at 800 X-100 g for 5 min. Nuclei pellets were washed once with 1 ml of the above buffer lacking Triton X-100. Pellets were resuspended in 100 μl of buffer containing 25 mM Tris-Cl (pH 8.0), 2.5 mM magnesium acetate, 2 mM CaCl2, 0.05 mM EDTA, 0.1 mM DTT, 0.25 M sucrose, 0.2 mM PMSF, and 12.5% glycerol and treated with 10 μg of DNase and RNase on ice for 30 min. Nuclei were lysed by adding 100 μl of 2% SDS, boiled for 3 min, and centrifuged at 15,600 g for 3 min to remove debris. Nuclei extracts (15 μl/lane) were resolved by SDS-PAGE (5% polyacrylamide stacking gel containing 125 mM Tris-Cl (pH 6.8), 0.1% SDS; 5% polyacrylamide resolving gel (prepared from 28.5% acrylamide + 0.5% bisacrylamide stock solution) containing 375 mM Tris-Cl (pH 8.8), 0.1% SDS; running buffer containing 25 mM Tris, 250 mM glycine, 0.1% SDS). Electrophoresis was carried out at 25 mA per gel until the tracking dye entered the resolving gel, after which the current was increased to 45 mA per gel. Proteins were then electrothermally blotted (0.5, 45 min) onto polyvinylidene difluoride membranes (immobilon™ transfer membrane, Millipore) using a Ni-hon Eido Western blotting apparatus (20 × 20 cm) in buffer containing 100 mM Tris, 192 mM glycine, 10% methanol, and 0.02% SDS. Following transfer, the membranes were blocked by incubation in phosphate-buffered saline containing 5% skim milk and 0.5% Tween 20 overnight at room temperature. Phosphorylated proteins were detected by autoradiography using Fuji RX-U x-ray film. The membranes were then exposed to 0.02 μg/ml antibodies that recognize the carboxyl-terminal domain (CTD) of RNA polymerase II (C-21, catalog number sc-900, Santa Cruz Biotechnology) in phosphate-buffered saline containing 0.5% skim milk and 0.05% Tween 20 for 2 h at room temperature, washed 3 times (10 min each) with buffer, and incubated in buffer containing anti-rabbit IgG antibodies cross-linked with horseradish peroxidase (Jackson ImmunoResearch, catalog number 111-035-003; 5000-fold final dilution) for 2 h at room temperature. After washing 3 times for 20 min/wash and 3 times for 10 min/wash with buffer, immune complexes were visualized by enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech).

RESULTS

H7 Blocks the Induction of zif268 and c-fos mRNA by NGF and Carbachol—As shown in Fig. 1, levels of both zif268 and c-fos mRNAs are low or undetectable in unstimulated cells but increase rapidly following exposure to NGF or carbachol, reaching high levels after 45 min. The fact that NGF-stimulated increases in zif268 and c-fos mRNA are blocked by the inhibitor K252a (19, 20) indicates that these inductions require the activation of the tyrosine kinase of the high affinity NGF receptor, TrkA. Likewise, the ability of atropine to block the inductions of zif268 and c-fos mRNA are blocked by the inhibitor H7 (19) indicates that these inductions require the involvement of muscarinic acetylcholine receptors. Pretreatment of the cells for 30 min with 100 μM H7 completely blocks the induction of zif268 and c-fos mRNAs by NGF and carbachol. By contrast, pretreatment with 100 μM HA1004 has essentially no effect on these inductions. Neither H7 nor HA1004 affect background levels of zif268 and c-fos mRNAs.3 As shown in Fig. 2, H7 blocks the induction of zif268 and c-fos mRNAs in a dose-dependent manner, with complete inhibition observed at concentrations of 50 μM and greater.

The complete inhibition of zif268 and c-fos mRNA inductions by H7 is surprising for two reasons. First, although NGF has previously been reported to activate PKC in PC12 cells (21, 22), the inductions of zif268 and c-fos mRNAs were not expected to require the activation of PKC, since down-regulation of PKC by prolonged exposure to phorbol ester has only a small effect on...
the induction of these mRNAs by NGF. We have also previously observed (5) that induction of zip268 mRNA by NGF is not affected by the specific PKC inhibitor GF109203x (23, 24). Second, although we have previously shown that PKC contributes to m₁ muscarinic acetylcholine receptor-mediated induction of zip268 mRNA in PC12D cells, this induction is only partially blocked by pretreatment with GF109203x (5). Thus, the total block of zip268 and c-fos gene induction by H7 seems to be too large an effect. To understand better how H7 blocks gene expression, we examined its effect on the induction of zip268 and c-fos mRNAs by additional agents.

H7 Also Blocks the Induction of zip268 and c-fos mRNAs by Phorbol Ester, Ca²⁺ Ionophore, and Forskolin—Phorbol ester is expected to stimulate increases in immediate-early gene mRNA by activating PKC, and therefore this induction would be expected to be inhibited by H7. By contrast, activation of immediate-early gene expression by elevated levels of intracellular Ca²⁺ in PC12 cells has not previously been suggested to require PKC. Rather, activation of Ca²⁺/calmodulin kinases (25, 26) and/or activation of MAPK cascade (27, 28) is thought to be sufficient. Similarly, forskolin, which increases intracellular levels of cAMP by stimulating adenylate cyclase, is thought to activate gene expression via activation of PKA (29) and/or the MAPK cascade (30–33). The experiment depicted in Fig. 3, however, shows that H7 inhibits the induction of zip268 and c-fos mRNAs by each of these agents. These results strongly suggest that H7 blocks gene expression by acting at a site distinct from PKC.

MAPK Contributes to the Induction of zip268 and c-fos RNAs by NGF, Carbachol, Phorbol Ester, and Increases in Intracellular Ca²⁺ or cAMP—MAPK is likely to play a central role in the induction of zip268 and c-fos by NGF and carbachol in PC12 cells. MAPK functions as part of the Ras/MAPK cascade (Ras-Raf-MEK-MAPK), which transmits signals from tyrosine kinase- and G protein-linked membrane receptors to the nucleus (6, 34, 35). Activated MAPK enters the nucleus where it phosphorylates and activates “ternary complex” transcription factors, e.g. Elk-1 or SAP-1, which stimulate gene expression by forming a complex with serum response factor bound to the serum response element (SRE) (36–38). The c-fos promoter contains 1 SRE (2) and the zip268 promoter contains 6 SREs (16, 39), which have been shown to play a role in the induction of zip268 mRNA by serum and NGF (16, 40, 41). In PC12 cells, MAPK has previously been shown to be strongly activated by NGF (42–45), phorbol ester (44), and by elevated levels of intracellular Ca²⁺ (27, 28, 46) or cAMP (30–33). Together, these observations suggest that activation of MAPK plays a central role in the induction of c-fos and zip268 mRNAs by each of these agents in PC12 cells. Evidence supporting this inference is shown in the Northern blot depicted in Fig. 4, where induction of c-fos and zip268 RNAs by each of the agents tested was inhibited by pretreating PC12D cells with PD098059 (48, 49), a specific inhibitor of MEK, the immediate upstream activator of MAPK.

Concentrations of H7 Sufficient to Block the Induction of zip268 and c-fos mRNAs Do Not Block the Activation of MAPK—The data in Fig. 5 show that concentrations of H7 that block the induction of zip268 and c-fos mRNAs have no effect on the activation of MAPK. Pretreatment with HA10054 also does not block activation of MAPK. The inability of H7 to block the activation of MAPK by phorbol ester is particularly surprising, since H7 is expected to inhibit PKC. To determine if PKC can be effectively blocked in PC12D cells, we examined the effects of pretreatment with the specific PKC inhibitor GF109203x. The data in Fig. 6 show that pretreatment with GF109203x is effective in blocking both phorbol ester-mediated induction of zip268 and c-fos mRNAs and phorbol ester-mediated activation of MAPK. That phorbol ester activation of immediate-early gene expression and MAPK activation is mediated by PKC is supported by the observation that exposure to the same concentration of a less active phorbol ester, 4a-PMA, failed to increase zip268 and c-fos mRNA levels or activate MAPK. These results are also consistent with the conclusion that H7 blocks zip268 and c-fos gene expression by acting at a site distinct from PKC.

H7 Does Not Inhibit PKA in PC12D Cells—Although H7 has been reported to potently block PKA activity in vitro (Kᵣ = 3 μM) (7), the failure of H7 to block PKC in PC12D cells suggests that it may also not be effective in blocking PKA. To demonstrate that PKA in PC12D cells can be pharmacologically blocked, we pretreated the cells with the specific PKA inhibitor H89 (47). The results depicted in Fig. 7 show that pretreatment with 30 μM H89 blocks both forskolin-stimulated induction of zip268 and c-fos mRNAs and forskolin-stimulated activation of MAPK. The specificity of the effects of forskolin is demonstrated by the lack of mRNA induction and MAPK activation by the related compound dideoxyforskolin, which does not activate adenylate cyclase. These results suggest that the ability of H7 to block the induction of c-fos and zip268 mRNA is unrelated to PKA.

H7 Does Not Inhibit Preactivated MAPK—The results presented so far show that although H7 potently inhibits the induction of zip268 and c-fos mRNAs, this is not caused by inhibition of PKC or PKA or by blocking the activation of MAPK. To determine if the block of mRNA induction is caused by inhibition of MAPK itself, we examined the ability of H7 to inhibit preactivated MAPK in vitro. As shown in Fig. 8, preactivated MAPK is essentially unaffected by H7, even at high

4 T. Ebihara and D. Saffen, unpublished observations.
concentrations of the inhibitor. Thus, it is unlikely that H7 blocks the induction of immediate-early gene RNAs by blocking MAPK. Rather, these results suggest that H7 acts at a site downstream from MAPK.

**H7 Blocks NGF-mediated Induction of a Luciferase Reporter Gene Linked to zif268 Promoter**—The data in Fig. 9 show that preincubation with H7, but not HA1004, blocks the induction of luciferase reporter gene linked to the zif268 promoter. By contrast, GF109203x and H89 have only a small effect on the induction of the luciferase reporter by NGF. The ability of H7 to block the induction of gene expression mediated by the zif268 promoter in a heterologous system suggests that the mechanism of inhibition involves the functioning of the promoter rather than post-transcriptional controls such as transcriptional pausing, which is mediated by sequences in the first intron of the c-fos gene (50, 51). The small effects of GF109203x and H89 have only a small effect on the induction of the luciferase reporter by NGF.
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**FIG. 8.** H7 does not inhibit preactivated MAPK in vitro. Activated forms of p42 and p44 MAPK were isolated by immunoprecipitation from PC12D cells that had been stimulated with water (W) or 5 ng/ml NGF for 5 min. The indicated concentrations of H7 or HA1004 were added to the standard in vitro MAPK assay reaction mix containing buffer, immunoprecipitated MAPK, [γ-32P]ATP, and myelin basic protein (MBP). Reaction mixtures were incubated at 30 °C for 30 min, and the incorporation of 32P into myelin basic protein was determined by scintillation spectroscopy. H7, dark gray box; HA1004, dotted box.

**FIG. 9.** H7 blocks NGF-mediated induction of a luciferase reporter gene linked to the zif268 promoter. PC12D cells transfected with pGL2zif268, an expression vector containing a luciferase reporter gene under the control of the zif268 promoter, were pretreated with 100 μM HA1004 (HA), 1 μM GF109203x (GF), 30 μM H89, or 100 μM H7 for 30 min prior to stimulation with water (W) or 5 ng/ml NGF. Cells were harvested 4 h after stimulation and luciferase assays performed as described under “Experimental Procedures.”

and H89 on the expression of the reporter gene suggest that neither PKC nor PKA is essential for activation of the zif268 promoter by NGF.

**H7 Inhibits the Phosphorylation of RNA Polymerase II in Vivo**—The data presented up to this point show that H7 does not inhibit zif268 and c-fos gene expression by blocking the activation of MAPK or by directly inhibiting MAPK. Rather, the site at which H7 acts seems to be downstream from MAPK. One possibility we considered was that H7 blocks gene expression, not by inhibiting a step in the intracellular signaling cascade, but rather by inhibiting some step essential for transcription. A search of the literature for kinases known to be essential for transcription elongation (9, 54), and blocking CTD phosphorylation with H7 inhibited transcription of human immunodeficiency virus and c-myc mRNAs by RNA polymerase II in vitro (9). Based upon these results we decided to determine if H7 blocks the phosphorylation of RNA polymerase II in vivo. The experiment depicted in Fig. 10A shows that pretreatment of PC12D cells with H7 specifically reduces levels of phosphorylated RNA polymerase II, as evidenced by (i) a reduction in the intensity of 32P-labeled RNA polymerase II detected by autoradiography (left panel, upper band within the bracket) and (ii) a reduction in levels of the “shifted-up” form of RNA polymerase II detected by immunochemical staining (right panel, upper band within the bracket). The slowly migrating, “shifted-up” form of RNA polymerase II has been previously shown to correspond to the phosphorylated form of the enzyme (18). By contrast, pretreatment of PC12D cells with HA1004 does not reduce levels of phosphorylated RNA polymerase II or change its electrophoretic mobility (Fig. 10, B and C). Inhibition of RNA polymerase II phosphorylation similar to that obtained with H7 is also observed following pretreatment of PC12D cells with two additional compounds known to block phosphorylation of the CTD of RNA polymerase II in vitro, the protein kinase inhibitor H8 (7, 55) and the classic RNA polymerase II inhibitor DRB (9, 56) (Fig. 10B). By contrast, the MEK inhibitor PD098059 and broad spectrum protein kinase inhibitor staurosporine have no effect on phosphorylation of RNA polymerase II (Fig. 10B). Taken together, these data suggest that H7 blocks the induction of zif268 and c-fos mRNAs in vivo by inhibiting transcription elongation.

**DISCUSSION**

In this study we show that H7 blocks the induction of zif268 and c-fos mRNA in PC12D cells by NGF, carbachol, phorbol ester, and agents that increase intracellular Ca2+ or cAMP (Figs 1–3 and 5) but fails to block the activation of MAPK, an enzyme that contributes to the induction of these mRNAs (Fig. 4). The inability of H7 to block the activation of MAPK by phorbol ester is surprising, since H7 is a potent inhibitor of PKC- and PKA-dependent kinase (cdk) MO15/Cdk-7) that phosphorylates the CTD of RNA polymerase II (9, 52–54). Phosphorylation of the CTD of RNA polymerase II is required for efficient RNA chain elongation (9, 54), and blocking CTD phosphorylation with H7 has been shown to inhibit transcription of human immunodeficiency virus and c-myc mRNAs by RNA polymerase II in vitro (9). Based upon these results we decided to determine if H7 blocks the phosphorylation of RNA polymerase II in vivo. The experiment depicted in Fig. 10A shows that pretreatment of PC12D cells with H7 specifically reduces levels of phosphorylated RNA polymerase II, as evidenced by (i) a reduction in the intensity of 32P-labeled RNA polymerase II detected by autoradiography (left panel, upper band within the bracket) and (ii) a reduction in levels of the “shifted-up” form of RNA polymerase II detected by immunochemical staining (right panel, upper band within the bracket). The slowly migrating, “shifted-up” form of RNA polymerase II has been previously shown to correspond to the phosphorylated form of the enzyme (18). By contrast, pretreatment of PC12D cells with HA1004 does not reduce levels of phosphorylated RNA polymerase II or change its electrophoretic mobility (Fig. 10, B and C). Inhibition of RNA polymerase II phosphorylation similar to that obtained with H7 is also observed following pretreatment of PC12D cells with two additional compounds known to block phosphorylation of the CTD of RNA polymerase II in vitro, the protein kinase inhibitor H8 (7, 55) and the classic RNA polymerase II inhibitor DRB (9, 56) (Fig. 10B). By contrast, the MEK inhibitor PD098059 and broad spectrum protein kinase inhibitor staurosporine have no effect on phosphorylation of RNA polymerase II (Fig. 10B). Taken together, these data suggest that H7 blocks the induction of zif268 and c-fos mRNAs in vivo by inhibiting transcription elongation.

The inability of H7 to block PKC and PKA in vivo could be caused by an inability to efficiently penetrate the cells. Thus, H7 may not reach sufficient concentrations to block PKC or PKA, even though concentrations sufficient to block gene expression are attained. By contrast, the more potent PKC inhibitor GF109203x (in vitro Ki = 14 nM; Ref. 23) and PKA inhibitor H89 (in vitro Ki = 48 nM; Ref. 47) evidently do reach sufficient intracellular concentrations to block PKC and PKA, respectively (Figs. 6 and 7). The observation that HA1004, a potent inhibitor of PKA in vitro (Ki = 2.3 μM; Ref. 7), fails to block the activation of MAPK or induction of zif268 and c-fos mRNAs by forskolin in vivo suggests that HA1004 may also not efficiently permeate the cells. If it is true that H7 fails to block intracellular PKC and PKA because only a small amount enters the cells, then its ability to block mRNA induction may depend upon high affinity binding of H7. Inhibition by H7 apparently takes place very rapidly, since H7 was found to be equally effective at inhibiting the induction of c-fos and zif268 mRNAs when added 30 min before, at the same time, or up to 5 min after stimulation with NGF.3

The fact that H7 does not effectively block PKC- and PKA-mediated activation of MAPK in PC12D cells suggests that it inhibits the induction of zif268 and c-fos mRNAs by acting at a site unrelated to PKC or PKA. The observation that H7 also does not block preactivated MAPK (Fig. 8) suggests that H7 blocks the induction of zif268 and c-fos mRNAs by acting at a site downstream from MAPK or at a site unrelated to the...
MAPK intracellular signaling cascade. Recent work has shown that MAPK activates two additional kinases that may be important for the induction of c-fos mRNA in PC12 cells. The serine/threonine kinases RSK-1 (p90RSK) and RSK-2 (CREB kinase) are activated upon phosphorylation by MAPK and then enter the nucleus and activate the transcription factors serum response factor (57) and CREB (58, 59), respectively. Studies by Greenberg and co-workers (58) have shown that, although SRE and CRE function independently when linked to heterologous minimal promoters, both are required for activation of c-fos gene expression by the native c-fos promoter. Thus, it may be that activation of c-fos and zif268 gene expression requires the activation of RSK-1 and/or RSK-2 in addition to activation of MAPK. Significantly, Yin and Yang (60) have shown that H7 inhibits preactivated RSK-1 and/or RSK-2 in addition to activation of MAPK. Recent work has shown that H7 is blocking mRNA induction by inhibiting mRNA splicing. Nevertheless, our data do not rule out the possibility that H7 blocks gene expression by interfering with the processing of mRNA. The observation that H7 blocks the activation of the zif268 promoter linked to a luciferase reporter gene (Fig. 9), however, suggests that its effects are not restricted to the native zif268 gene.

A survey of published papers that use H7 to study gene expression reveals that H7 blocks the induction of a broad spectrum of rapidly inducible mRNAs by diverse stimuli. For example, H7 blocks the induction of mRNA by cytokines and lymphokines, including the induction of tumor necrosis factor mRNA by interleukin-1β (62) and the induction of immediately-early genes by interleukin-6 (60, 63–65), interleukin-1β (60, 66), α- and γ-interferons (67), leukemia inhibitory factor (60, 68), and tumor necrosis factor (67). H7 also blocks mRNA induction by trophic/growth factors, including the induction of c-fos (69) and neuropeptide Y (70) mRNA by NGF, junB mRNA (71), and platelet-derived growth factor A and B chain mRNAs (72) by transforming growth factor (TGF)-β, and c-fos mRNA by growth hormone (73). Additional examples, include the inhibition by H7 of the induction of c-fos and c-jun mRNAs by H2O2 (74) and the induction of metallothionein mRNA induction by Cd2+ or Zn2+ (75).

In many studies using H7, inhibition of gene expression has been taken as evidence for the involvement of PKC. Whereas PKC certainly functions in the induction of some genes, there...
are many cases where the authors have noted that the inhibition of mRNA induction by H7 is not consistent with a role for PKC. For example, Nakajima and Wall (63), Kahle (64), and Cook and McCormick (65) reported that down-regulation of PKC by long-term exposure to phorbol ester failed to block the gene expression under study, even though H7 was effective. Authors of other studies have also concluded that H7 blocks inducible gene expression by blocking a kinase distinct from PKC (60, 66).

In a few studies, H7 had no effect on gene expression or, instead, stimulated gene expression. In most of these cases, the lack of inhibition can in retrospect be accounted for by the use of iso-H7, rather than authentic H7 (76, 77), or very low concentrations of authentic H7 (78, 79). In almost every study to date, authentic H7 used at concentrations between 50 and 100 μM has been found to block gene induction. Together, these results suggest that H7 may be inhibiting some step that is generally required for the induction genes.

An attractive hypothesis to explain the breadth of the inhibitory activity of H7 is that it affects some aspect of the general transcriptional machinery, rather than the kinase cascades that activate specific transcription factors. Consistent with this idea is the observation that H7 blocks the kinase associated with the general transcription factor TFIIB, which phosphorylates the carboxyl-terminal domain of RNA polymerase II (9, 52). TFIIB participates in the late stages of transcription initiation, and the associated kinase is required for efficient elongation mRNA by RNA polymerase II (9, 52). Inhibition of TFIIB kinase with H7, in fact, blocks transcriptional elongation of human immunodeficiency virus and c-myc RNAs by RNA polymerase II in vitro (9).

Based upon these observations, we decided to determine whether pretreatment of PC12D cells with H7 affects the phosphorylation of RNA polymerase II. The results depicted in Fig. 10 show that a 30-min exposure to H7 is sufficient to reduce significantly levels of phosphorylated RNA polymerase II in both unstimulated cells and cells stimulated with NGF. By contrast, pretreatment of the cells with HA1004 has no effect on phosphorylation of RNA polymerase II. To our knowledge, this is the first demonstration that H7 inhibits the phosphorylation of RNA polymerase in vitro, although it has previously been shown to block c-myc transcriptional elongation in vitro (80). Together, these results suggest that H7 blocks the induction of gene expression by inhibiting elongation of RNA transcripts.

A scheme indicating the site at which H7 inhibits gene expression is depicted in Fig. 11. Further work will be required to prove this model, but the data in this study provide a plausible explanation for the general inhibition of gene expression by H7 and suggest that caution must be taken when interpreting the effects of H7 on inducible gene expression.

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REFERENCES

1. Gaszler, A., and Sukhatme, V. P. (1995) Proc. Nucleic Acid Res. Mol. Biol. 50, 191–224
2. Angel, P., and Herrlich, P. (eds) (1994) The Fos and Jun Families of Transcription Factors, CRC Press, Inc., Boca Raton, FL
3. Beckmann, A. M., and Wilce, P. A. (1997) Neurochem. Int. 31, 477–510
4. Herrera, D. G., and Robertson, H. A. (1996) Prog. Neurobiol. (New York) 50, 83–107
5. Eibihara, T., and Saffern, D. (1997) Neurochem. 68, 1001–1010
6. Segal, R. A., and Greenberg, M. E. (1996) Annu. Rev. Neurosci. 19, 463–489
7. Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. (1984) Biochemistry 23, 5036–5041
8. Asano, T., and Hidaka, H. (1984) J. Pharmacol. Exp. Ther. 221, 141–145
9. Yankulov, K., Yamashita, K., Roy, E., Egly, J.-M., and Bentley, D. L. (1995) J. Biol. Chem. 270, 23922–23925
10. Quick, J., Ware, J. A., and Driedger, P. E. (1992) Biochem. Biophys. Res. Commun. 187, 657–663
11. Mizushima, S., and Nagata, S. (1999) Nucleic Acids Res. 18, 5322
12. Katoh-Semba, R., Kitajima, S., Yamazaki, Y., and Sano, M. (1987) J. Neurosci. Res. 17, 36–44
13. Greene, L. A., and Tischler, A. S. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2434–2438
14. Cook, S. J., and McCormick, P. (1993) Science 262, 1069–1072
15. Howe, L. R., Leevers, S. J., Gomez, N., Nakielny, S., Cohen, P., and Marshall, C. J. (1992) Cell 71, 335–342
16. Changelis, P. S., Peng, P., King, T. C., and Milbrandt, J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 377–381
17. Nordeen, S. K., Green, P. P., and Fowlkes, D. (1987) RNA (N. Y.) 6, 173–178
18. Cadena, D. L., and Dahmus, M. E. (1987) J. Biol. Chem. 262, 12468–12474
19. Knusel, B., and Hefti, F. (1992) J. Neurochem. 59, 1987–1996
20. Berg, M. M., Sternberg, D. W., Parada, L. F., and Chao, M. V. (1992) J. Biol. Chem. 267, 13–16
21. Hama, T., Hwang, K. P., and Guroff, G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 2353–2357
22. Heasley, L. E., and Johnson, G. L. (1989) J. Biol. Chem. 264, 8666–8662
23. Triepel, C., Puntiet, P., Coste, H., Beliveau, P., Grand-Perret, T., Ajakanje, M., Baudet, V., Boissin, P., Boursier, E., Lorillolr, F., Duhamel, L., Charon, D., and Kirilovsky, J. (1991) J. Biol. Chem. 266, 15771–15781
24. Martiny-Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Koch, G., Hug, H., Marne, D., and Schachtete, C. (1993) J. Biol. Chem. 268, 9194–9197
25. Enaden, H., and Soderling, T. R. (1994) J. Biol. Chem. 269, 20872–20877
26. Johnson, C. M., Hill, C. S., Chawla, S., Treisman, R., and Bading, H. (1997) J. Neurosci. 17, 6189–6202
27. Rusanescu, G., Qi, H., Thomas, S. M., Brugge, J. S., and Hagleoua, S. (1995) Neuron 15, 1415–1425
28. Xia, Z., Dudek, H., Miranti, C. K., and Greenberg, M. E. (1996) J. Neurosci. 16, 5425–5436
29. Meinkoth, J. L., Alberts, A. S., Wente, W., Fantozzi, D., Taylor, S. S., Hagiwara, M., Montminy, M., and Feramisco, J. R. (1993) Mol. Cell. Biochem. 127/128, 179–186
30. Frodin, M., Peradali, P., and Van Obberghen, E. (1994) J. Biol. Chem. 269, 6207–6214
31. Erhardt, P., Troppmair, J., Rapp, U., and Cooper, G. (1995) Mol. Cell. Biol. 15, 5524–5530
32. Peradali, P., Frodin, M., Bari, J. V., Calleja, Y., Scimone, J. C., Filoux, C., Calythy, G., and Van Obberghen, E. (1995) FEBS Lett. 357, 290–296
33. Vossler, M. R., Yao, H., York, R. D., Pan, M. G., Rim, C. S., and Stork, P. J. (1995) Cell 80, 73–82
34. Seger, R., and Krebs, E. G. (1995) FASEB J. 9, 726–735
35. Sugden, P. H., and Clerk, A. (1997) Cell Signalling 9, 337–351
36. Gille, H., Sharroros, A. D., and Shaw, P. E. (1992) Nature 358, 414–417
37. Hipskind, R. A., Bacarrini, M., and Nordheim, A. (1994) Mol. Cell. Biol. 14, 6219–6231
38. Treisman, R. (1994) Curr. Opin. Genet. Dev. 4, 96–101
39. Tsai-Morris, C. H., Can, X. M., and Sukhatme, V. P. (1988) Nucleic Acids Res. 1201–1206

FIG. 11. Working model, H7 blocks transcription of c-fos and zif268 mRNAs by inhibiting phosphorylation of RNA polymerase II. PKC and PKA function upstream of MAPK, which activates gene expression by phosphorylating ternary complex factor. Ternary complex factor stimulates transcription by forming a ternary complex with the transcription factor serum response factor, which is prebound to the SRE. There is 1 SRE in the c-fos promoter and 6 SRE in the zif268 promoter (shown in diagram). H7 blocks elongation of nascent mRNAs by inhibiting phosphorylation of RNA polymerase II.
