THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 27, Issue of July 5, pp. 16111–16118, 1996

Printed in U.S.A.

Inhibition of HSP70 Expression by Calcium Ionophore A23187 in Human Cells

AN EFFECT INDEPENDENT OF THE ACQUISITION OF DNA-BINDING ACTIVITY BY THE HEAT SHOCK TRANSCRIPTION FACTOR*

(Received for publication, November 27, 1995, and in revised form, April 10, 1996)

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Heat shock proteins (HSPs) are induced in mammalian cells in a variety of pathophysiological states and have an important role in cytoprotection *in vitro* and *in vivo*. In this study, we report that the calcium ionophore A23187, a glucose-regulated protein (GRP) inducer, dramatically inhibits HSP70 synthesis and *HSP70* mRNA transcription after induction by heat shock, sodium arsenite, or prostaglandin A_1 treatment in human K562 cells. A23187 does not suppress, and it actually prolongs, the DNA-binding activity of the human heat shock transcription factor (HSF), while it alters HSF1 phosphorylation in heat shock-treated cells. To inhibit HSP70 expression, A23187 needs to be present during heat shock, while treatment before or after heat shock does not affect *HSP70* mRNA transcription. The GRP inducer thapsigargin, which specifically inhibits the endoplasmic reticulum Ca^{2+}-ATPase, has no effect on heat-induced HSP70 synthesis, indicating that A23187 inhibitory activity is not due to depletion of intracellular calcium stores and is independent of the concomitant induction of GRP genes. Inhibition of HSP70 expression is correlated with alterations in HSF1 phosphorylation in heat-shocked cells, but not in sodium arsenite-treated cells, indicating that different mechanisms may be involved in mediating A23187 inhibitory activity.

Eukaryotic and prokaryotic cells exposed to temperatures a few degrees above the physiological level exhibit a finely regulated and highly conserved cellular response by inducing the synthesis of a specific set of proteins referred to as heat shock proteins (HSPs)[1] (1). In mammalian cells, several HSPs are expressed during normal growth conditions and can be induced by biologically active molecules like hemin (2) and prostaglandins (3), whereas others are expressed upon stress-activated regulation of transcriptional and translational switches during exposure to adverse environmental conditions, which include, besides heat shock, hypoxia (4), glucose deprivation (5), virus infection (6), and the presence of transition metal ions, sulfhydryl reagents, or amino acid analogs (1, 7, 8). Induction requires the activation and translocation to the nucleus of a transregulatory protein, the heat shock transcription factor (HSF), which binds to heat shock elements (HSEs) in the promoters of heat shock genes (9). In yeast cells, HSF is constitutively bound to DNA and undergoes stress-dependent phosphorylation associated with transcriptional competence. On the other hand, in vertebrate cells, HSF exists as an inactive non-DNA-binding form, which is rapidly converted to the DNA-binding form upon heat shock or other environmental stresses (9). Several HSFs have been identified in mammalian cells (9). Activation of heat shock transcription factor 1 (HSF1), which is the primary component of the HSF DNA-binding activity present in cells exposed to heat shock, requires oligomerization, acquisition of DNA-binding activity, and localization to the nucleus (9). HSF1 has also been shown to be phosphorylated upon heat stress in different types of cells (10).

The molecular mechanisms responsible for signal transduction that leads to HSF activation and phosphorylation in heat-shocked cells have not been completely elucidated. It has been suggested that a calcium-dependent metabolic process is involved in the generation of the heat shock signal, and calcium has been shown to activate HSF DNA-binding activity *in vitro* (11) and to be essential for multistep activation of the heat shock factor in permeabilized murine cells (12) and for induction of heat shock protein synthesis in rat hepatoma cells (13). Increased Ca^{2+} mobilization has also been recently shown to be an early event in the signal transduction pathway mediating the activation of *HSP70* gene expression by prostaglandin A_2 (14).

The calcium ionophore A23187, which is known to permeabilize the plasma membrane and to induce the leakage of Ca^{2+}, has been shown to increase the expression level of the αB -crystallin in chondrocytes (15) and is known to induce the expression of a set of glucose-regulated protein (GRP) genes, but not *HSP70*, through the depletion of Ca^{2+} from the intracellular Ca^{2+} stores (16, 17).

In this report, we have investigated the effect of A23187 treatment on *HSP70* gene expression and HSF activation by heat shock and other inducers in K562 human erythroleukemia cells. We demonstrate that the calcium ionophore dramatically inhibits *HSP70* gene expression in heat-shocked cells as well as after treatment with sodium arsenite or prostaglandin A_1. The calcium ionophore does not suppress HSF DNA-binding activity induced by either heat shock or the other inducers, while it inhibits the formation of the phosphorylated high molecular mass form of HSF1 in heat shock-treated cells. In the presence of A23187, HSF remains in an activated DNA-binding state for a longer period than in the heat shock-treated control.

* This work was supported by grants from the Italian Ministry of Public Health (IX AIDS Research Project) and from the Italian National Research Council (Progetto Finalizzato "ACRO"). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: HSPs, heat shock proteins; HSF, heat shock transcription factor; HSE, heat shock element; GRP, glucose-regulated protein; PAGE, polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; PGA_1, prostaglandin A_1.

EXPERIMENTAL PROCEDURES

Cell Culture—K562 human erythroleukemia cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics at 37 °C in a 5% CO_2 humidified atmosphere. For heat shock treatment, flasks were submersed in a temperature-controlled water bath (Grant Instruments Ltd., Cambridge, United Kingdom) at 45 ± 0.01 °C for 20 min (immersion depth, 4 cm; $t_{½}$ = 1.5 min). The A23187 calcium ionophore (Sigma) was dissolved in ethanol as a 1 mM stock solution and stored at −20 °C. Control medium contained the same concentration of ethanol diluent. Prostaglandin A_1 (Cayman Chemical Co., Inc., Ann Arbor, MI) was dissolved in absolute ethanol (10 mg/ml) and diluted to the appropriate concentration in culture medium immediately before use. Sodium *meta*-arsenite and thapsigargin were obtained from Sigma.

Protein Synthesis and Western Blot Analysis—K562 cells were labeled with L-[^{35}S]methionine (5 μCi/10^6 cells) as described previously (3). After cell lysis, the radioactivity incorporated into trichloroacetic acid-insoluble material was determined, and samples containing the same amount of radioactivity were separated by SDS-PAGE in a vertical slab gel apparatus (3% stacking gel, 10% resolving gel) and processed for autoradiography and densitometric analysis as described (3).

For immunoblot analysis, equal amounts of protein for each sample were separated by SDS-PAGE (8% resolving gel) and blotted onto nitrocellulose. Alternatively, whole cell extracts (50 μg/sample), prepared as described (20), were resuspended in 25 μl of reaction mixture containing 2.5 μl of 10 × phosphatase buffer (500 mM Tris-Cl (pH 9.0), 10 mM $MgCl_2$), and 12 units of calf intestinal phosphatase in the presence or absence of the phosphatase inhibitor NaH_2PO_4 (10 mM) and incubated at 37 °C for 1 h. The reactions were stopped by adding 25 μl of 2-fold concentrated SDS-PAGE sample buffer, and samples were heated at 100 °C for 5 min. Samples were then separated by SDS-PAGE (8% resolving gel) and blotted onto nitrocellulose as described (3). The filters were incubated with a rabbit polyclonal antibody against HSF1 (kindly provided by Dr. R. Morimoto, Northwestern University, Evanston, IL), and the bound antibody was detected by horseradish peroxidase-linked secondary antibodies as described previously (3).

Northern Blot Analysis—Total RNA was isolated following the procedure of Chomczynski and Sacchi (18), fractionated on 1% agarose-formaldehyde gels, and transferred to nylon membranes as described previously (19). Membranes were hybridized with ^{32}P-labeled probes, prepared by random-primed DNA synthesis of the human *HSP70* gene (plasmid pH2.3) (19). After stripping, filters were rehybridized with a ^{32}P-labeled probe derived from a cDNA fragment (1400 base pairs; *Pst*I) of the rat glyceraldehyde-phosphate dehydrogenase (GAPDH) gene as a loading control.

Electrophoretic Mobility Shift Assay (EMSA)—Whole cell extracts (10^7 K562 cells) were prepared, and binding reactions were performed using a specific heat shock element oligonucleotide as described (20). Briefly, extracts (10 μg/sample) were mixed with 0.1 ng of a ^{32}P-labeled HSE oligonucleotide and 0.5 μg of poly(dI-dC) (Pharmacia Biotech Inc.) in 25 μl of binding buffer (10 mM Tris-Cl (pH 7.8), 50 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, and 5% glycerol). After a 30-min incubation at room temperature, HSF·HSE complexes were analyzed by nondenaturing 4% polyacrylamide gel electrophoresis and autoradiography as described (20), and the amount of shifted HSE probe was quantified by Molecular Dynamics PhosphorImager analysis. For *in vitro* HSF DNA binding inhibition experiments, extracts from 10^7 K562 cells, either kept at 37 °C or heat-shocked at 45 °C for 20 min and then allowed to recover for 1 h at 37 °C, were added to the binding reaction mixture pretreated for 15 min at room temperature with 5, 10, or 20 μM A23187 or the corresponding amount of ethanol diluent. After an additional 30-min incubation at room temperature, HSF·HSE complexes were detected as described above.

Run-on Transcription Assay—In vitro run-on transcription reactions were performed in isolated K562 nuclei as described (21). ^{32}P-Labeled RNA was used for hybridization with nitrocellulose filters containing plasmids for the *HSP90* (pUCHS801; StressGen Biotechnologies Co., Victoria, British Columbia, Canada), *HSP70* (pH2.3) (22), *HSC70* (pHA7.6) (20), *GRP78/BiP* (pHG23.1) (20), or the *GAPDH* gene as a control. Following hybridization, filters were visualized by autoradiography, and the radioactivity was quantitated by a PhosphorImager.

RESULTS

A23187 Inhibits Heat Shock-induced HSP70 Synthesis in K562 Cells—To investigate the effect of the A23187 calcium ionophore on HSP70 synthesis and mRNA accumulation, K562 cells were treated with A23187 (5 μM) or ethanol diluent and either kept at 37 °C or heat-shocked at 45 °C for 20 min. A23187 was kept during the recovery period at 37 °C. At different times (0.5, 1, 2, 4, and 6 h) after heat shock, aliquots of cells (10^7 cells) were collected from each culture, and total RNA was extracted as described under "Experimental Procedures." The same amount of RNA was fractionated on agarose gels, blotted onto nylon filters, and hybridized to ^{32}P-labeled probes for human *HSP70* mRNA and rat *GAPDH* mRNA as a control. Fig. 1*A* shows that in heat-shocked K562 cells, *HSP70* mRNA starts accumulating 30 min after heat treatment, reaching a maximum at 4 h of the recovery period at 37 °C. *HSP70* mRNA levels were found to be dramatically decreased in heat-shocked K562 cells treated with A23187, while no difference in GAPDH transcript accumulation was found.

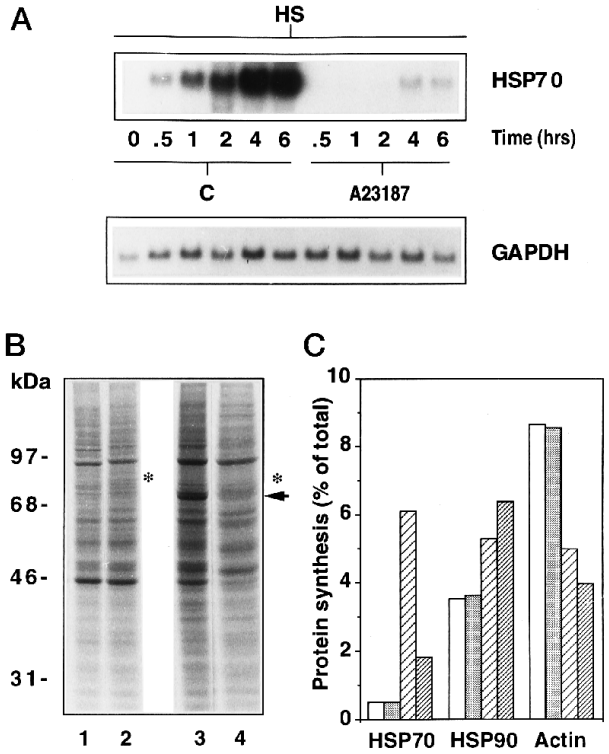

FIG. 1. **A23187 inhibits *HSP70* mRNA accumulation and HSP70 protein synthesis after heat shock.** *A*, K562 cells were treated with A23187 (5 μM) or ethanol diluent (control (*C*)) immediately before heat shock treatment (*HS*; 45 °C, 20 min). Total RNA was extracted soon after heat shock (time 0) or at different times during the recovery period at 37 °C and processed for Northern blot analysis using ^{32}P-labeled pH2.3 (HSP70) or GAPDH (1400 base pairs; *Pst*I) probes. *B*, K562 cells were treated with A23187 (5 μM; *lanes 2* and *4*) or ethanol diluent (*lanes 1* and *3*) and subjected to heat shock treatment as described above. Soon after the end of the heat shock period, cells were labeled with L-[^{35}S]methionine (5 μCi/10^6 cells) for 6 h at 37 °C. Samples containing the same amount of radioactivity were processed for SDS-PAGE and autoradiography. HSP70 is indicated by the *arrow*. *Asterisks* indicate GRP78. *C*, synthesis of HSP70, HSP90, and actin was determined by densitometric analysis and is expressed as percent of total protein synthesis. □, control; ▨, A23187; , heat shock; ▨, heat shock in the presence of A23187.

In a parallel experiment, immediately after heat shock in the presence of 5 μM A23187 or ethanol diluent, the culture medium was replaced with methionine-free RPMI 1640 medium containing 2% dialyzed fetal calf serum and A23187 or control diluent. After 15 min, L-[^{35}S]methionine (5 μCi/10^6 cells) was added, and the cultures were incubated at 37 °C for the following 6 h. While it moderately affected protein synthesis in unstressed K562 cells, A23187 was found to further decrease heat shock-mediated inhibition of protein synthesis in these cells. Samples containing an equal amount of radioactivity were

processed for SDS-PAGE and autoradiography, and synthesis of HSP70 was determined by densitometric analysis as described (19). As expected, A23187 treatment induced GRP78 synthesis in unstressed K562 cells (Fig. 1*B*). In heat-shocked K562 cells, accordingly to the decrease in the *HSP70* mRNA level shown above, A23187 was found to dramatically suppress heat shock-induced HSP70 synthesis (Fig. 1, *B* and *C*).

Effect of A23187 on HSP70 Transcription and HSF Activation by Heat Shock—To investigate whether treatment with the calcium ionophore affected HSF activation and *HSP70* mRNA transcription, K562 cells, treated with A23187 or control diluent, were either kept at 37 °C or subjected to heat shock (45 °C, 20 min), and at different times during the recovery period at 37 °C, aliquots of cells were collected for determination of HSF DNA-binding activity by EMSA. In the same experiment, the transcription rate of the *HSP70* gene as well as of the *HSC70*, *HSP90*, *GRP78*, and *GAPDH* genes was analyzed by run-on assay. Under the conditions examined, HSF·HSE complex formation was detected as early as 20 min after heat shock, continued for at least 1 h, and declined 3 h after the return to the physiological temperature (Fig. 2*A*). A23187 did not induce HSF DNA-binding activity in the absence of heat shock (data not shown). In heat shock-treated cells, treatment with A23187 did not alter either the kinetics or the extent of HSF·HSE complex formation up to 60 min after heat treatment, while it appeared to maintain HSF in an activated DNA-binding state up to 3 h after heat shock (Fig. 2*A*). The transcription rates of the *HSP90*, *HSP70*, *HSC70*, and *GRP78* genes in isolated nuclei from heat-shocked untreated or A23187-treated cells are shown in Fig. 2*B*. Heat shock rapidly induced the transcription of all heat shock genes tested and, to a lesser extent, also induced *GRP78* gene transcription. A23187 treatment was found to suppress *HSP90*, *HSC70*, and *HSP70* gene transcription, while it enhanced the *GRP78* gene transcription rate as expected (Fig. 2, *B* and *C*). In A23187-treated cells, *HSP70* gene transcription was inhibited by ~50% at 20 min after heat shock and by 90% after 30 min at 37 °C (Fig. 2*C*). In the same experiment, the level of *HSP70* mRNA accumulation was dramatically decreased in A23187-treated cells as determined by Northern blot analysis (Fig. 2*D*).

To determine whether A23187 treatment could be affecting HSF1 phosphorylation, K562 cells, treated with A23187 or control diluent, were either kept at 37 °C or subjected to heat shock (45 °C, 20 min), and at different times during the recovery period at 37 °C, aliquots of cells were collected for determination of HSF DNA-binding activity by EMSA or for Western blot analysis using polyclonal anti-HSF1 antibodies. As described above, HSF·HSE complex formation was detected 20 min after heat shock and declined 3 h after the return to 37 °C (Fig. 3*A*). As previously shown in different cell lines (10), HSF DNA-binding activity in heat shock-treated K562 cells was associated with a significant increase (~12 kDa) in HSF1 molecular mass as determined by immunoblot analysis (Fig. 3, *A* and *B*). A unique band of HSF1 of ~78 kDa was detected in unstressed K562 cells. Immediately after the 20-min heat shock, HSF1 consisted of two closely sized proteins (82 and 86 kDa); a third form of HSF1 with a slightly higher molecular mass (~90 kDa) was found from 30 to 60 min after heat shock. Three hours after the return to the physiological temperature, no difference in the molecular mass of HSF1 in unstressed and heat shock-treated K562 cells was found, concomitant with the loss of HSF DNA-binding activity. The increase in HSF1 molecular mass has been previously shown to be the result of HSF1 phosphorylation during activation (10). To investigate whether the increase in HSF1 molecular mass in heat-shocked K562 cells was due to HSF1 phosphorylation, K562 cells were either kept at 37 °C or subjected to heat shock (45 °C, 20 min) and allowed to recover at 37 °C for 1 h. Whole cell extracts were then treated with calf intestinal phosphatase for 1 h at 37 °C in the presence or absence of the phosphatase inhibitor NaH_2PO_4. In the absence of calf intestinal phosphatase, the high molecular mass form (90 kDa) of HSF1 was detected in heat-shocked cells. Fig. 3*E* shows that when cell lysates were treated with phosphatase, the 90-kDa HSF1 band almost completely disappeared, while the 78-kDa band could be detected. The addition

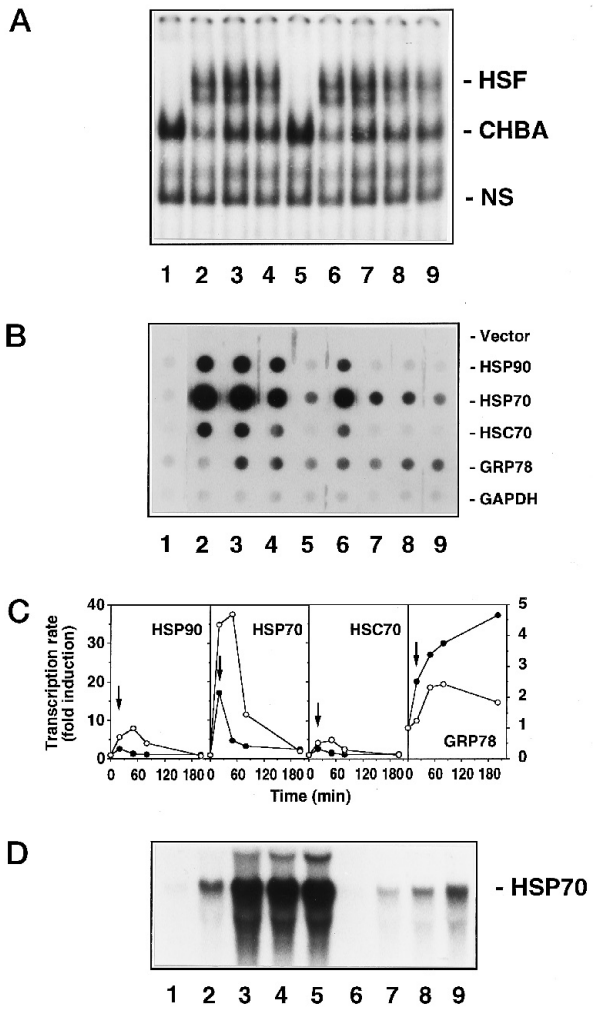

FIG. 2. **Effect of A23187 on HSF activation and HSP and GRP gene transcription rate in heat-shocked K562 cells.** *A*, K562 cells were either kept at 37 °C (*lane 1*) or subjected to heat shock at 45 °C for 20 min in the absence (*lanes 2–5*) or presence (*lanes 6–9*) of A23187 (5 μM). A23187 was kept during the recovery period at 37 °C. Soon after the end of the heat shock (*lanes 2* and *6*) or at 30 (*lanes 3* and *7*), 60 (*lanes 4* and *8*), and 180 (*lanes 5* and *9*) min during the recovery period at 37 °C, whole cell extracts were analyzed for HSF DNA-binding activity by EMSA using the HSE sequence from the human *HSP70* promoter. *HSF*, induced form of DNA-binding activity; *CHBA*, constitutive HSE-binding activity; *NS*, nonspecific protein-DNA interactions. Free probe is not shown. *B*, nuclei isolated from aliquots of the samples described in *A* were subjected to *in vitro* run-on transcription analysis as described under "Experimental Procedures." The genes analyzed are listed on the right. The plasmid vector pAT153 was used as a nonspecific hybridization control. *C*, shown is the quantitative evaluation by PhosphorImager analysis of the transcription rates of the samples in *B*. Transcription rate values were normalized against respective *GAPDH* gene transcription levels and are expressed as -fold induction with respect to control cells. ○, heat shock; ●, heat shock in the presence of A23187. *Arrows* indicate the end of the heat shock period. *D*, total RNA was extracted from aliquots of the samples described in *A* and processed for Northern blot analysis using ^{32}P-labeled pH2.3 (HSP70) or GAPDH (1400 base pairs; *Pst*I) probes. *GAPDH* mRNA levels were identical in all samples (data not shown).

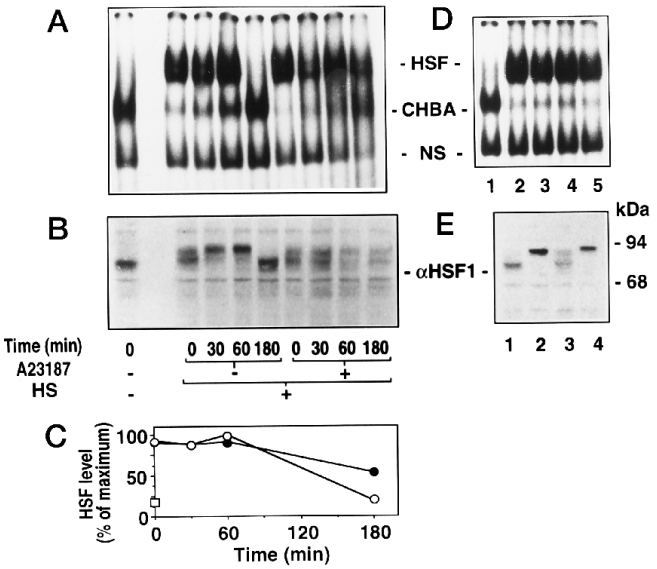

FIG. 3. **Effect of A23187 on HSF DNA-binding activity and phosphorylation.** *A*, K562 cells were either kept at 37 °C (*HS*, −) or subjected to heat shock at 45 °C for 20 min (*HS*, +) in the absence (*A23187*, −) or presence (*A23187*, +) of the calcium ionophore A23187 (5 μM). A23187 was kept during the recovery period at 37 °C. Soon after the end of the heat shock (time 0) or at 30, 60, and 180 min during the recovery period at 37 °C, whole cell extracts were analyzed for HSF DNA-binding activity by EMSA using the HSE sequence from the human *HSP70* promoter. *HSF*, induced form of DNA-binding activity; *CHBA*, constitutive HSE-binding activity; *NS*, nonspecific protein-DNA interactions. *B*, HSF1 phosphorylation. An equal amount of protein (50 μg) from the samples described in *A* was separated by SDS-PAGE and processed for Western blot analysis using a rabbit polyclonal antibody against HSF1 (*αHSF1*). *C*, quantitative evaluation of HSF·HSE complex formation by PhosphorImager analysis of the samples in *A*. Values are expressed as a percentage of the maximum level. □, control, 37 °C; ○, heat shock; ●, heat shock in the presence of A23187. *D*, effect of A23187 on HSF DNA-binding activity *in vitro*. Extracts from 10^7 K562 cells, either kept at 37 °C (*lane 1*) or heat-shocked at 45 °C for 20 min and then allowed to recover for 1 h at 37 °C (*lanes 2–5*), were added to the EMSA binding reaction mixture pretreated for 15 min at room temperature with 5 (*lane 3*), 10 (*lane 4*), or 20 μM (*lane 5*) A23187 or the corresponding amount of ethanol diluent (*lane 2*). After an additional 30-min incubation at room temperature, HSF·HSE complexes were detected as described under "Experimental Procedures." *E*, phosphatase treatment. Whole cell extracts (50 μg) from heat-shocked (*lanes 2–4*) or control (*lane 1*) K562 cells were processed for Western blot analysis using the HSF1 antiserum either directly (*lanes 1* and *2*) or after calf intestinal phosphatase digestion (*lanes 3* and *4*) in the presence (*lane 4*) or absence (*lane 3*) of the phosphatase inhibitor NaH_2PO_4. Sizes are indicated in kilodaltons.

of the calf intestinal phosphatase inhibitor NaH_2PO_4 to the reaction mixture prevented the decrease in HSF1 molecular mass, indicating that the high molecular mass band represents a phosphorylated form of HSF1 in K562 cells.

As shown above, A23187 did not alter the extent of HSF·HSE complex formation, while it maintained HSF in an activated DNA-binding state up to 3 h after heat shock (Fig. 3, *A* and *C*). A23187 treatment had no effect on the heat-induced modification of HSF1 at 20 min after heat shock (time 0), while the third form of HSF1 with a higher molecular mass was not detected in the presence of A23187 between 30 and 60 min after heat exposure, suggesting that HSF1 phosphorylation is a multistep process and that A23187 could be selectively affecting some of these events. A23187 treatment also appears to moderately decrease the amount of HSF1, starting at 60 min after the return to the physiological temperature (Fig. 3*B*). Similar results were recently shown by Nagai *et al.* (23) in HeLa cells after treatment with the flavonoid quercetin, which was also found to inhibit HSF1 phosphorylation and to decrease the amount of HSF1. The decrease in the HSF1 level is unlikely to be related to the A23187-induced inhibition of protein synthesis since cycloheximide treatment was found not to significantly reduce the amount of HSF1 for up to 12 h (23). It was suggested that inhibition of HSF1 phosphorylation could lead to a more rapid HSF1 degradation in heat-shocked cells (23). It should be pointed out that, different from the control, the molecular mass of the two forms of HSF1 appeared to be constant in the presence of A23187 up to 3 h after heat shock (Fig. 3*B*). This was associated with the maintenance of the HSF DNA-binding state. The addition of A23187 (5–20 μM) *in vitro* during the EMSA binding assay had no effect on heat shock-induced HSF activation (Fig. 3*D*).

To determine whether the effect of A23187 on HSF activation and *HSP70* mRNA accumulation was reversed upon removal of the drug or was due to irreversible cell damage, K562 cells were treated with A23187 (5 μM) or ethanol diluent for 30 min. After repeated washings to remove the calcium ionophore, cells were exposed to a temperature of 45 °C for 20 min. In the same experiment, cells that had not been pretreated with A23187 were exposed to the calcium ionophore either during the 20-min heat shock or during both the heat shock and the recovery period at 37 °C. At 1 h after the return to the physiological temperature, aliquots of cells were collected for determination of HSF DNA-binding activity and Western blot analysis using the HSF1 antiserum. *HSP70* mRNA levels were also determined in the same samples, collected 3 h after heat shock. As shown in Fig. 4, pretreatment of K562 cells with 5 μM A23187 before heat shock had no effect on *HSP70* mRNA accumulation, HSF DNA-binding activity, or the heat-induced modification of HSF1 protein, indicating that the effects described above are not due to generalized irreversible cell damage caused by the calcium ionophore. As described above, treatment with A23187 during and after heat shock did not alter HSF DNA-binding activity, while it decreased the level of *HSP70* mRNA accumulation at 3 h after heat shock treatment. Similar dramatic effects were obtained when K562 cells were treated with A23187 only during the 20-min heat shock (Fig. 4, compare *lanes 2* and *4*), indicating that A23187 affects an early event in the signaling pathway for HSP70 induction and that the presence of the calcium ionophore during the recovery period at 37 °C is not necessary in order to achieve maximal inhibition of HSP70 expression. Treatment with A23187 only during or during and after heat shock resulted in an alteration in the electrophoretic mobility of the HSF1 stress-induced form. In fact, in the absence of A23187 treatment or in cells that had been treated with A23187 before heat shock, a highly phosphorylated form of HSF1 with a molecular mass of ~90 kDa was detected (Fig. 4*B*, *lanes 2* and *3*). On the other hand, in cells that had been treated with A23187 during or during and after heat shock, HSF1 consisted of two closely sized proteins (82 and 86 kDa), again indicating that HSF1 is not fully phosphorylated in the presence of the calcium ionophore (Fig. 4*B*, *lanes 4* and *5*). Finally, treatment with A23187 only after heat shock had no effect on *HSP70* mRNA accumulation up to 3 h after heat treatment (data not shown).

Inhibition of HSP70 Gene Expression by A23187 Is Not Specific for Heat Shock Induction—Synthesis of HSP70 can be induced by a variety of molecules, including sodium arsenite (7), amino acid analogs (8), hemin (2), and cyclopentenone prostaglandins (3, 20). To determine whether inhibition of *HSP70* gene expression by A23187 is limited to induction during heat shock or could be obtained also at 37 °C, the ability of A23187 to interfere with *HSP70* gene expression was tested in K562 cells treated with prostaglandin A_1 (PGA_1) or sodium *meta*-arsenite ($NaAsO_2$). Soon after the addition of 5 μM A23187 or ethanol diluent, K562 cells were treated with PGA_1

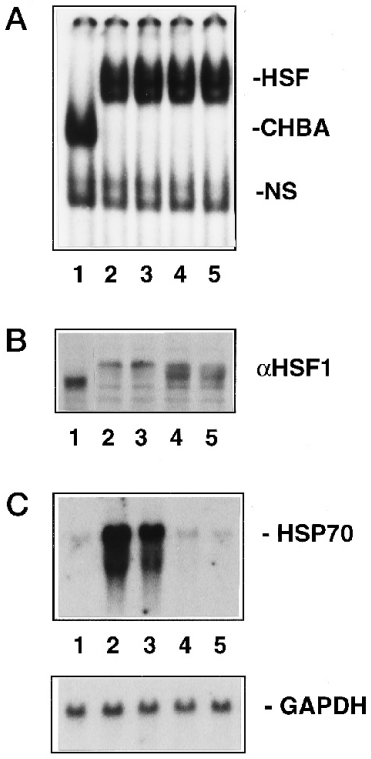

FIG. 4. **Presence of A23187 during heat shock is necessary for inhibition of HSP70 expression.** K562 cells were either kept at 37 °C (*lane 1*) or heat-shocked at 45 °C for 20 min (*lanes 2–5*) in the presence or absence of 5 μM A23187. *Lane 2*, untreated heat-shocked cells; *lane 3*, cells pretreated with A23187 for 30 min before heat shock; *lane 4*, cells treated with A23187 only during heat shock; *lane 5*, cells treated with A23187 during heat shock and the following 1-h recovery phase. *A*, after 1 h of recovery at 37 °C, whole cell extracts were subjected to EMSA. *HSF*, induced form of DNA-binding activity; *CHBA*, constitutive HSE-binding activity; *NS*, nonspecific protein-DNA interactions. *B*, an equal amount of protein (50 μg) from the samples described above was separated by SDS-PAGE and processed for immunoblot analysis for HSF1 detection. *C*, after a 3-h incubation at 37 °C, total cellular RNA was extracted and subjected to Northern blot analysis using ^{32}P-labeled pH2.3 (HSP70) or GAPDH (1400 base pairs; *Pst*I) probes.

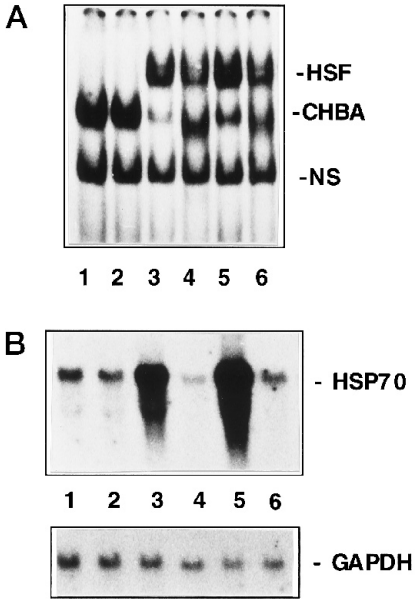

FIG. 5. **A23187 inhibits *HSP70* mRNA accumulation after treatment with other inducers.** K562 cultures were treated with prostaglandin A_1 (6 μg/ml; *lanes 3* and *4*), sodium *meta*-arsenite (2×10^{-5} M; *lanes 5* and *6*), or ethanol diluent (*lanes 1* and *2*) in the absence (*lanes 1*, *3*, and *5*) or presence (*lanes 2*, *4*, and *6*) of 5 μM A23187. *A*, after a 3-h incubation at 37 °C, whole cell extracts were subjected to EMSA. *HSF*, induced form of DNA-binding activity; *CHBA*, constitutive HSE-binding activity; *NS*, nonspecific protein-DNA interactions. *B*, total cellular RNA was extracted from samples collected after a 3-h incubation at 37 °C and subjected to Northern blot analysis using ^{32}P-labeled pH2.3 (HSP70) or GAPDH (1400 base pairs; *Pst*I) probes.

(6 μg/ml), $NaAsO_2$ (2×10^{-5} M), or control diluent. Three hours after treatment with PGA_1 or $NaAsO_2$, aliquots of cells were collected for determination of HSF DNA-binding activity and *HSP70* mRNA accumulation. Fig. 5 shows that both PGA_1 and sodium arsenite treatment induced HSF DNA-binding activity in K562 cells. Levels of *HSP70* mRNA were increased several-fold in PGA_1- and $NaAsO_2$-treated cells as compared with control cells 3 h after treatment, while no difference in *GAPDH* levels was found. A23187 treatment dramatically reduced the levels of *HSP70* mRNA in both PGA_1- and $NaAsO_2$-treated cells (Fig. 5*B*), while, as previously shown for heat-shocked cells, it did not affect HSF DNA-binding activity (Fig. 5*A*).

The kinetics of HSF activation was then studied in K562 cells treated with $NaAsO_2$ in the presence or absence of the calcium ionophore. HSF DNA-binding activity was detected by 60 min and was unmodified up to 3 h after the beginning of $NaAsO_2$ treatment (Fig. 6*A*). Western blot analysis of the same samples showed the presence of a unique band of ~78 kDa in unstressed K562 cells. The presence of higher molecular mass forms of HSF1 (~82, 88, and 90 kDa) was detected at 1, 2, and 3 h after the beginning of $NaAsO_2$ treatment, respectively. A23187 did not affect HSF DNA-binding activity and did not appear to affect HSF1 phosphorylation in this case. Only a modest change (~88 kDa, as compared with 90 kDa in the control) in the molecular mass of the stress-induced form of HSF1 was detected in the presence of the calcium ionophore at 3 h after treatment (Fig. 6*B*).

To determine whether the reduced *HSP70* mRNA accumulation in A23187-treated cells could be a consequence of an increased turnover of the *HSP70* mRNA, K562 cells were treated with $NaAsO_2$ or control diluent in the presence or absence of A23187. The heat shock genes were allowed to transcribe for 3 h after $NaAsO_2$ addition. At this time, the transcription inhibitor actinomycin D (10 μg/ml) was added to the cultures, and the total cytoplasmic RNA was extracted at different times and processed for Northern blot analysis. As expected, levels of *HSP70* mRNA at 3 h after $NaAsO_2$ addition were much lower in A23187-treated cells compared with untreated cells (Fig. 6*C*); however, A23187 treatment did not alter the rate of *HSP70* mRNA degradation (Fig. 6*D*).

Effect of A23187 Is Not Mimicked by the GRP78 Inducer Thapsigargin—The calcium ionophore A23187 induces the expression of GRP genes through the depletion of Ca^{2+} from the intracellular Ca^{2+} stores (17). The sesquiterpene lactone thapsigargin, which is known to specifically inhibit the endoplasmic reticulum Ca^{2+}-ATPase (24) and causes a discharge of the intracellular Ca^{2+} stores, has also been shown to induce the transcription of two GRP genes (*GRP78/BiP* and *GRP94*) as effectively as A23187 and with kinetics similar to that of the calcium ionophore (17).

To evaluate whether the induction of GRPs by A23187 could interfere with heat-induced HSP70 synthesis, K562 cells were treated with thapsigargin (0.1–1 μM) or control diluent and either kept at 37 °C or heat-shocked at 45 °C for 20 min. Immediately after heat shock, the culture medium was replaced with methionine-free RPMI 1640 medium containing 2% dialyzed fetal calf serum and thapsigargin or control diluent. After 15 min at 37 °C, L-[^{35}S]methionine (5 μCi/10^6 cells) was added, and cells were incubated at 37 °C for the following 6 h, after which time, samples were processed for SDS-PAGE and autoradiography. Thapsigargin was found to induce GRP78 synthe-

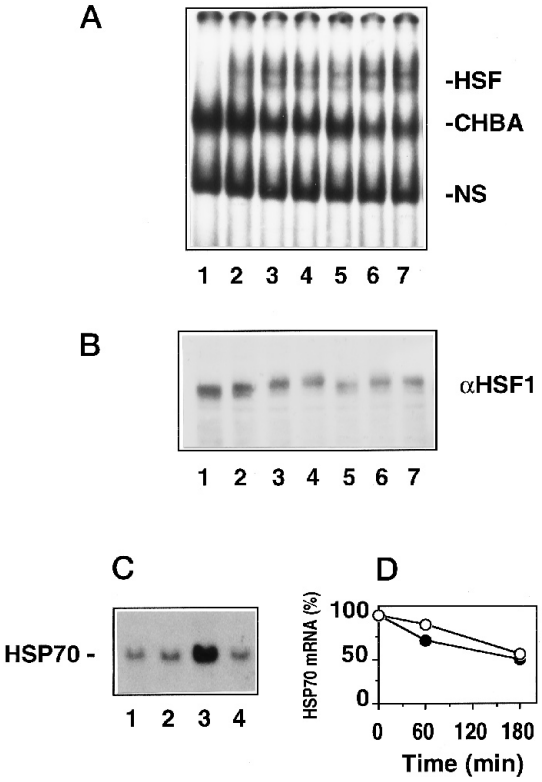

FIG. 6. **Effect of A23187 on HSF activation and on *HSP70* mRNA accumulation and stability in K562 cells treated with sodium arsenite.** *A*, K562 cells were treated with control diluent (*lane 1*) or with 2×10^{-5} M $NaAsO_2$ (*lanes 2–7*) in the absence (*lanes 2–4*) or presence (*lanes 5–7*) of the calcium ionophore A23187 (5 μM). At 60 (*lanes 2* and *5*), 120 (*lanes 3* and *6*), and 180 (*lanes 4* and *7*) min after the addition of $NaAsO_2$, whole cell extracts were analyzed for HSF DNA-binding activity by EMSA using the HSE sequence from the human *HSP70* promoter. *HSF*, induced form of DNA-binding activity; *CHBA*, constitutive HSE-binding activity; *NS*, nonspecific protein-DNA interactions. *B*, shown are the results of HSF1 phosphorylation. An equal amount of protein (50 μg) from the samples described in *A* was separated by SDS-PAGE and processed for Western blot analysis using a rabbit polyclonal antibody against HSF1 (*αHSF1*). *C*, total cellular RNA was extracted from K562 cells treated for 3 h with $NaAsO_2$ (*lanes 3* and *4*) or control diluent (*lanes 1* and *2*) in the presence (*lanes 2* and *4*) or absence (*lanes 1* and *3*) of A23187 (5 μM). Samples were subjected to Northern blot analysis using the HSP70 probe. *GAPDH* gene mRNA accumulation was equal in all samples (not shown). *D*, to evaluate the effect of A23187 on *HSP70* mRNA stability, K562 cells were treated with $NaAsO_2$ for 3 h in the presence (●) or absence (○) of 5 μM A23187. After this time, cells were treated with actinomycin D (10 μg/ml) and incubated at 37 °C. Cytoplasmic mRNA was extracted soon after (time 0) or at 60 and 180 min after actinomycin D addition and processed for Northern blot analysis using the ^{32}P-labeled HSP70 probe. The radioactivity was quantitated using a PhosphorImager. Values are expressed as percent of the respective control levels at time 0.

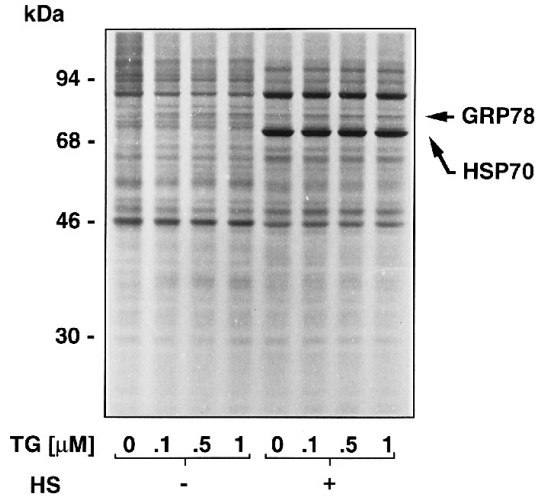

FIG. 7. **Effect of thapsigargin on HSP70 protein synthesis.** K562 cells, treated with 0.1, 0.5, or 1 μM thapsigargin (*TG*) or control diluent, were either incubated at 37 °C (*HS*, −) or subjected to heat shock at 45 °C for 20 min (*HS*, +). Soon after the end of the heat shock period, cells were labeled with L-[^{35}S]methionine (5 $\mu Ci/10^6$ cells) for 6 h at 37 °C. Samples containing the same amount of radioactivity were processed for SDS-PAGE and autoradiography. The GRP78 and HSP70 proteins are indicated.

sis in both unstressed and heat-shocked cells, even at the lower concentration tested (0.1 μM) (Fig. 7). Induction of GRP78 synthesis appeared to have no effect on HSP70 synthesis in heat-shocked K562 cells, even at the highest concentration of thapsigargin (1 μM). The fact that the inhibition of HSP70 synthesis by A23187 cannot be mimicked by thapsigargin also indicates that the inhibitory effect of A23187 is not due to depletion of intracellular calcium stores.

DISCUSSION

Induction of heat shock genes can be activated by exposure to a variety of adverse environmental conditions, which include, besides hyperthermia, hypoxia, virus infection, and glucose deprivation as well as the presence of several molecules, including sulfhydryl reagents, amino acid analogs, or transition metal ions (1, 9). Heat shock gene expression can also be induced in unstressed cells by biologically active molecules, including hemin (2), arachidonic acid (25), and specific cyclooxygenase and lipoxygenase products (3, 20, 26). Recently, the ability of several nonsteroidal anti-inflammatory drugs, including sodium salicylate, aspirin, and indomethacin, to activate the heat shock transcription factor has been described in human cells (27–29). Interestingly, induction of HSF DNA-binding activity at physiological temperatures was not sufficient for transcriptional activation of HSP genes, indicating a multistep activation control mechanism (27). Nonsteroidal anti-inflammatory drugs have also been shown to lower the threshold for HSP induction at supraphysiological temperatures (29) and to modulate HSP70 expression after brief heat shock (28).

While several molecules have been shown to induce the heat shock response in mammalian cells, only a few compounds, belonging to the flavonoid group, have been shown to inhibit HSP70 expression (30–33). In particular, the flavonoid quercetin has been shown to inhibit HSP synthesis in a human colon carcinoma cell line after heat shock (30) and in human monocyte-macrophages during erythrophagocytosis (32). We have also recently shown that quercetin is able to transiently inhibit HSP70 expression in K562 cells after heat shock or prostaglandin A_1 treatment (19, 33). In this case, the block was exerted at the transcriptional level and was not associated with loss of HSF DNA-binding activity (33).

In this report, we describe that the calcium ionophore A23187 is a potent inhibitor of *HSP70* gene expression after induction by heat shock, sodium arsenite, or prostaglandin A_1 treatment. The calcium ionophore does not suppress HSF DNA-binding activity induced by either heat shock or the other inducers, confirming previous observations that suggest that HSF1 oligomerization and DNA binding are uncoupled from transcriptional activation (27). Actually, in the presence of A23187, HSF remained in an activated DNA-binding state for a longer period than in the heat shock-treated control. The calcium ionophore inhibited instead the formation of the phosphorylated high molecular mass form of HSF1 in heat shock-treated cells, indicating an effect on HSF1 phosphorylation.

The mechanism by which A23187 inhibits HSF1 phosphorylation in heat-shocked cells is unknown. The calcium ionophore

could directly affect a kinase involved in HSF1 phosphorylation. A23187 has been shown to modulate the activity of several kinases, including microtubule-associated protein-2 kinase in B cells (34), tyrosine kinase in rat liver (35), and Ca^{2+}/calmodulin-dependent protein kinases and protein kinase C in several cell types (36, 37). The kinase responsible for HSF1 phosphorylation has not yet been identified, and it has been recently suggested that several kinases could in fact be involved in this process (38). HSF1 phosphorylation could then be a multistep process, and A23187 could selectively affect some of these events. It should be pointed out that the protein kinase C inhibitor staurosporine was recently shown to inhibit HSP expression in human colon carcinoma cells, and a role for this kinase in HSF1 phosphorylation has been hypothesized (39).

To be effective in suppressing HSP70 expression, A23187 needs to be present during heat shock (45 °C, 20 min) or during both heat shock and the recovery period at 37 °C. In fact, pretreatment with the calcium ionophore prior to the heat challenge had no effect on *HSP70* mRNA accumulation, HSF DNA-binding activity, or HSF1 phosphorylation, indicating that the inhibitory effect is not due to generalized irreversible cell damage caused by the calcium ionophore. Moreover, the fact that treatment with A23187 immediately after the end of the heat shock period also had no effect on *HSP70* mRNA accumulation indicated an effect on an early event of HSF activation. It should be emphasized that inhibition of HSP70 expression by A23187 is not specific for K562 cells. In fact, A23187 treatment caused a selective and dramatic inhibition of HSP70 synthesis, associated with induction of GRP78 synthesis, in a monkey epithelial cell line (MA104) after treatment with prostaglandin A_1.[2]

How does A23187 affect HSP70 induction? It is possible that in heat-shocked cells, the inhibitory effect could be mediated by the described alterations in HSF1 phosphorylation. The role of HSF phosphorylation in HSP gene transcriptional activation, however, is not clear. Hyperphosphorylation upon heat shock was reported to be the modification involved in the activation of HSF in yeast as well as in animal cells (40, 41), and phosphorylation of HSF1 was shown to be tightly correlated with its DNA-binding activity in human cells (10). Quercetin was recently shown to inhibit HSF1 phosphorylation without affecting HSF DNA-binding activity during the transient block of HSP70 expression in K562 cells (33), also suggesting a positive role of HSF1 phosphorylation in *HSP70* gene activation in these cells. Moreover, it has been recently shown that the lack of transcriptional activation of heat shock genes correlates with the lack of inducible serine phosphorylation of HSF1 in HeLa cells treated with nonsteroidal anti-inflammatory drugs, which induce HSF DNA-binding activity (42). The results described in the present report also indicate that in heat-shocked cells, a reduction in HSF1-inducible phosphorylation correlates with a decrease in the transcription rate of heat shock genes in A23187-treated cells. However, increases in phosphorylation do not appear to be absolutely required for acquisition of HSF1 transcriptional activity. In fact, amino acid analogs like azetidine can induce *HSP70* transcription without affecting HSF1 phosphorylation (10, 38), and it has been recently suggested that in human and yeast cells, phosphorylation of HSF serves as a regulatory mechanism to deactivate HSF, rather than being involved in its activation (43). The fact that, in $NaAsO_2$-stressed cells, A23187 inhibits *HSP70* mRNA transcription, even though the presence of a highly phosphorylated form of HSF1 is detected, suggests that other mechanisms, unrelated to HSF1 phosphorylation, may be involved in mediating A23187 inhibitory activity.

A23187 is known to act by permeabilizing the plasma membrane and by inducing the leakage of Ca^{2+}, which can have a wide range of pleiotropic effects. Evidence has accumulated that for a variety of cellular genes, A23187 elicits a transcriptional response through a rapid and transient increase of intracellular Ca^{2+}, with the implication of calmodulin kinase in the phosphorylation of transcription factors (44, 45). A23187 activation of c-*fos* transcription was shown to be dependent on a cAMP response element adjacent to the TATA element in PC12 cells (46). The mechanism of this induction involves the phosphorylation of the transcription factor cAMP response element-binding protein by a calmodulin kinase (46). On the other hand, a different mechanism for induction of GRP genes by A23187 has been shown, which involves endoplasmic reticulum Ca^{2+} discharge and a novel pathway in which a Ca^{2+} signal is transduced through redundant elements containing CCAAT box-like motifs flanked by GC-rich regions, without the involvement of cAMP response element-like elements (17). To investigate whether inhibition of HSP70 expression could be the result of a specific discharge of intracellular endoplasmic reticulum Ca^{2+} stores and could be associated with induction of GRP genes, we have studied the effect of the GRP inducer sesquiterpene lactone thapsigargin, which has been shown to specifically inhibit the endoplasmic reticulum Ca^{2+}-ATPase (24) and to rapidly increase cytosolic free Ca^{2+} without hydrolysis of inositol phospholipids (24, 47). Thapsigargin was found to induce GRP78 synthesis in K562 cells at concentrations as low as 0.1 μM in the absence of heat treatment as well as after heat shock. The presence of thapsigargin during heat shock, however, was found to have no effect on HSP70 synthesis even at much higher (1 μM) concentrations, indicating that the inhibitory effect of A23187 is not due to depletion of intracellular calcium stores and is independent of the concomitant induction of GRP genes. Similar results were also obtained when the inhibitory effect of A23187 was compared with the effect of thapsigargin in monkey epithelial cells.[2]

While depletion of intracellular Ca^{2+} stores appears not to be the event responsible for the A23187-mediated block of HSP70 induction, it should be pointed out that depletion of extracellular Ca^{2+} by treatment with the calcium chelator EGTA (10 mM), while having no effect on GRP78 induction, prevented A23187-mediated inhibition of HSP70 synthesis.[3] As anticipated in the Introduction, it has been previously suggested that a calcium-dependent metabolic process is involved in the regulation of the heat shock signal (11–14) as well as in the control of heat resistance (48). It has in fact been shown that hyperthermic sensitivity varies as a function of extracellular Ca^{2+} concentration in a biphasic manner, and the calcium ionophore A23187 was found to be a potent hyperthermic sensitizer in the presence of extracellular Ca^{2+}, while it had a much less dramatic effect in the absence of extracellular Ca^{2+} (48).

A different well known effect of A23187 is its ability to cause the release of eicosanoids, which are converted to cyclooxygenase or lipoxygenase products and may consequently stimulate the adenylate cyclase, giving rise to a mixed Ca^{2+}/cAMP signal (49, 50). The fact that arachidonic acid (25) and cyclopentenone prostaglandins (3, 20) are known to induce HSF activation, while cyclooxygenase and lipoxygenase inhibitors can modulate the heat shock response in human cells (27–33), suggests the interesting possibility that an eicosanoid-mediated signal could be involved in A23187 inhibitory activity.

The regulation of heat shock gene expression has attracted a great deal of interest because of its importance to the survival

[2] A. De Marco and M. G. Santoro, unpublished observation.

[3] G. Elia and M. G. Santoro, unpublished observation.

of both prokaryotic and eukaryotic organisms under stressful conditions. In mammalian cells, HSP expression is induced *in vivo* in mammalian brain and other organs during hyperthermia (51), ischemia (52), and exposure to neurotoxin (53). Abnormal expression of HSPs has been described in a variety of pathophysiological states, which include fever, inflammation, oxidant injury, viral infection, and cancer (54), and a cytoprotective role of HSPs and HSP70 in particular has been shown in several pathological conditions, including ischemia (55) and virus infection (6, 56). The cytoprotective effect of heat shock proteins has been related to their role in the folding, assembly, and intracellular translocation of newly synthesized proteins; refolding of partially denatured proteins; and ATP-dependent catalysis of protein assembly/disassembly reactions (1). The understanding of the mechanism by which mammalian cells detect physiological stress at the molecular level and transduce the stress signal to the transcriptional apparatus is an important goal in view of the possibility of pharmacological manipulation of the stress response. The finding that the calcium ionophore A23187 is a potent inhibitor of HSP induction could represent a new tool in the comprehension of heat shock gene regulation.

Acknowledgment—We thank Rick Morimoto for the kind gift of plasmids pH2.3, pHA7.6, and pHG23.1 and for polyclonal anti-HSF1 antibodies.

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Inhibition of HSP70 Expression by Calcium Ionophore A23187 in Human Cells: AN EFFECT INDEPENDENT OF THE ACQUISITION OF DNA-BINDING ACTIVITY BY THE HEAT SHOCK TRANSCRIPTION FACTOR

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J. Biol. Chem. 1996, 271:16111-16118.
doi: 10.1074/jbc.271.27.16111