Vol. 144, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1987 Pages 152-159

INHIBITION BY 1-(5-ISOQUINOLINESULFONYL)-2-METHYLPIPERAZINE, AN INHIBITOR OF PROTEIN KINASE C, OF ENZYME INDUCTION BY GLUCOCORTICOID AND OF NUCLEAR TRANSLOCATION OF GLUCOCORTICOID-RECEPTOR COMPLEXES

Hiroshi Kido, Naomi Fukusen, and Nobuhiko Katunuma*

Department of Enzyme Chemistry, Institute for Enzyme Research, School of Medicine, The University of Tokushima, Tokushima 770, Japan

Received February 25, 1987

SUMMARY: Induction of tyrosine aminotransferase by glucocorticoid in rat hepatocytes was inhibited concentration-dependently by 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), an inhibitor of protein kinase C, but not by N-[2-(methylamino)-ethyl]-5-isoquinolinesulfonamide dihydrochloride, an inhibitor of cyclic nucleotide dependent protein kinases. H-7 also inhibited the accumulation of glucocorticoid-receptor complexes in the nuclear fraction with associated accumulation of these complexes in the cytoplasmic fraction, but did not affect incorporation of glucocorticoid into hepatocytes. These results indicate that protein kinase C may be essential in translocation of glucocorticoid-receptor complexes to the nuclei.

We recently reported that 1,2-racemic dioctanoyl glycerol and 12-o-tetradecanoyl-phorbol-13-acetate, potent activators of protein kinase C, markedly enhanced the induction of tyrosine aminotransferase (TAT) (EC 2.6.1.5) and ornithine decarboxylase (EC 4.1.1.17) by glucocorticoids *in vivo* and *in vitro*, whereas they had no effect on the activity of TAT in the absence of glucocorticoid (1,2). These findings provided indirect evidence that protein kinase C is involved in the mechanism of glucocorticoid action and thus that activators of protein kinase C amplify the actions of glucocorticoid.

Abbreviations: TAT, tyrosine aminotransferase; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; H-8, N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide.

* To whom correspondence should be addressed.

0006-291X/87 $1.50

Several recent investigations have shown that the properties of steroid hormone receptors and a 90-KDa nonsteroid-binding protein that associates with steroid receptors are modulated by phosphorylation and dephosphorylation (3-7). But the physiological role of phosphorylation in the functions of these proteins and the nature of the protein kinase(s) that is involved in phosphorylation of these proteins under physiological conditions are unknown. To elucidate the role of protein kinase(s) and of activators of protein kinase C in the mechanism of glucocorticoid action, in this work we studied the effects of an inhibitor of protein kinase C, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) (8-10), and of an inhibitor of cyclic nucleotide dependent protein kinases, N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-8)(8), on the induction of TAT by glucocorticoid. Results showed that H-7 inhibited the induction of TAT by glucocorticoid, but that H-8 did not. We also found that the accumulation of glucocorticoid-receptor complexes in the nuclei of primary cultures of adult rat hepatocytes after addition of [^{3}H]dexamethasone was inhibited dose-dependently by H-7.

MATERIALS AND METHODS

Materials : Male Wistar strain rats weighing 150-180 g were used. H-7, H-8 and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide were obtained from Seikagaku Kogyo Co. Bt_2cAMP, dexamethasone and glucagon were from Sigma Chemical Co. and [1,2,4(n)- ^{3}H]-dexamethasone (specific activity 60 Ci/mmole) and NCS tissue solubilizer were from Amersham.

Cell Culture, Labelling Conditions and Preparation of Cytoplasmic and Nuclear fractions of Hepatocytes : Hepatocytes were isolated from adult male Wistar strain rats by perfusion of the liver with collagenase (11), and were suspended at a density of 5×10^5 cells/ml in 6-cm dishes in Williams medium E containing 10% fetal calf serum, 10^{-6} M dexamethasone and 10^{-7} M insulin at 37°C under 5% CO_2 in air. After 48h, the medium was replaced by hormone-free medium containing 10% fetal calf serum. [^{3}H]Dexamethasone (10^{-8} M) and/or various concentrations of H-7 was added to 1-day cultures in serum-free medium and the cells were cultured. At the indicated times, the hepatocytes were washed three times with

ice-cold Tyrode buffer but without divalent cations, harvested with a rubber policeman in 1.5 ml of Buffer A [0.25 M sucrose in 20 mM Tris-HCl buffer (pH 7.5), containing 25 mM KCl, 10 mM $MgCl_2$ and 2 mM dithiothreitol], and disrupted by 60 strokes of a Dounce homogenizer. The nuclear fraction was obtained by centrifugation of the homogenate at 800 xg for 10 min. This fraction was washed four times with 3 ml of buffer A to remove unbound glucocorticoid with thorough mixing of the nuclear pellet each time in a Vortex mixer followed by centrifugation at 800 x g for 10 min. The washed nuclear pellet was dissolved in 0.5 ml of NCS tissue solubilizer and kept at room temperature overnight, and 400 µl of the solution was used for measurement of radioactivity. The cytoplasmic faction was obtained by centrifugation of the supernatant of the first centrifugation at 105,000 x g for 30 min. The amount of protein-bound [^{3}H]dexamethasone in the cytoplasmic faction was determined by the adsorption technique of Beato and Feigelson (12). Specific binding to receptor proteins was determined by comparing the amounts of bound radioactivity in the cytoplasmic and nuclear fractions derived from cells treated with labeled dexamethasone with or without 500-fold excess of unlabeled dexamethasone (13).

Enzyme and Protein Assays : TAT activity in cytoplasmic fractions was measured by the method of Granner and Tomkins (14) and protein concentration was determined by the method of Lowry et al. (15).

RESULTS AND DISCUSSION

Effects of Inhibitors of Protein Kinases on Induction of TAT by Dexamethasone : Isoquinolinesulfonamide derivatives have been reported to bind directly to protein kinase C, cyclic AMP-dependent and cyclic GMP-dependent protein kinases, with different affinities (8). Of these derivatives, H-7 is the most potent inhibitor for protein kinase C and also inhibits cyclic nucleotide-dependent protein kinases (8), while H-8 is the most potent inhibitor of cyclic nucleotide-dependent protein kinases and has markedly higher affinities to cyclic nucleotide-dependent protein kinases than to other kinases (8). To clarify the nature of the protein kinases involved in the mechanism of action of glucocorticoid, we examined the effects of H-7 and H-8 on the induction of TAT by glucocorticoid in primary cultures of adult rat hepatocytes. we also examined the effects of these inhibitors on enzyme induction by Bt_2cAMP or glucagon to confirm the specificities of their effects.

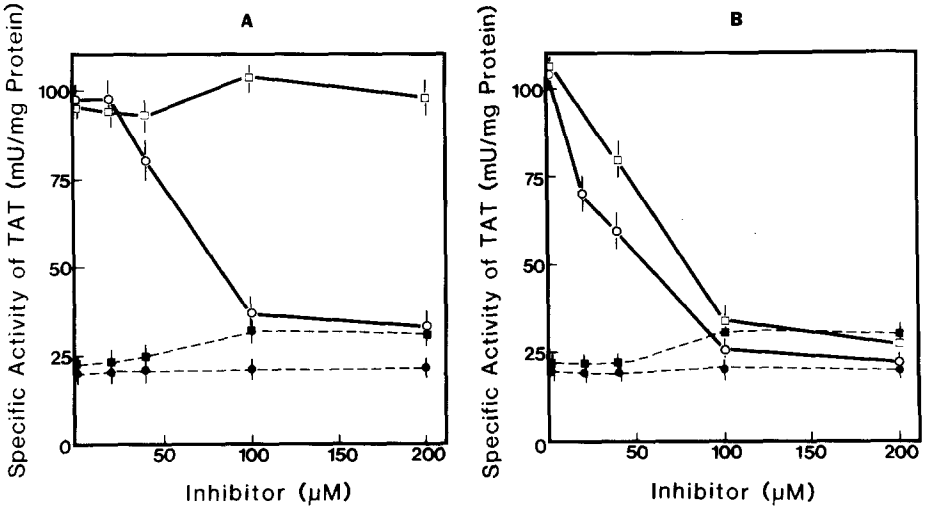

Fig. 1. Effects of H-7 and H-8 on TAT inductions by dexamethasone (A) and by Bt_2cAMP (B) in rat hepatocytes. The preparation of primary hepatocytes and incubation conditions are described in "Materials and Methods". A, TAT activities at the indicated concentrations of H-7 with (○) or without (●) dexamethasone (10^{-7} M) and those at the indicated concentrations of H-8 with (□) or without (■) dexamethasone (10^{-7}M). B, TAT activities at the indicated concentrations of H-7 with (○) or without (●) Bt_2cAMP (10^{-4}M) and at the indicated concentrations of H-8 with (□) or without (■) Bt_2cAMP (10^{-4}M). Values are means from three dishes. Bars, S.D.

When hepatocytes were incubated with dexamethasone (10^{-7}M) or Bt_2cAMP (10^{-4} M), the specific activity of TAT increased rapidly to a peak after incubation for 4 hr. The enzyme activity induced by dexamethasone remained constant unless dexamethasone was removed, but that induced by Bt_2cAMP gradually decreased after incubation for 6 hr (data not shown). Thus the effects of these inhibitors on enzyme induction by dexamethasone and Bt_2cAMP were studied after incubation for 4 hr. The induction of TAT by dexamethasone (10^{-7} M) was inhibited concentration-dependently by H-7, inhibition being almost complete at concentrations of above 100 μM, as shown in Fig. 1-A. However, H-8 had little inhibitory effect on induction of the enyzme. In contrast, both H-7 and H-8 caused concentration-dependent inhibition of the induction of TAT by Bt_2cAMP, as shown in Fig. 1-B. These inhibitors had similar effects on induction of the enzyme by glucagon (10^{-7} M) (data not

shown). These results support the conclusion that the effects of glucagon and Bt_2cAMP are adequately explained by the action of cyclic AMP-dependent protein kinase (16). The results also show that sufficient H-8 is incorporated into hepatocytes to inhibit the protein kinase but not to inhibit the induction of TAT by glucocorticoid. Thus the finding that H-7 inhibited enzyme induction by glucocorticoid suggests that the induction is mediated by protein kinase C. Moreover, the induction of TAT by dexamethasone was not inhibited by N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride (17,18), which inhibits calmodulin, at concentrations of 20 to 200 μM (data not shown).

Effect of H-7 on the Process of Glucocorticoid Action : To determine which process in the induction of TAT by glucocorticoid is inhibited by H-7, we examined the subcellular distribution of glucocorticoid-receptor complexes in primary hepatocytes after their treatment with [^{3}H]dexamethasone with or without H-7.

When primary hepatocytes were incubated with [^{3}H]dexamethasone, the radioactivity of receptor-bound [^{3}H]dexamethasone in the cytosol increased rapidly, reaching a maximum after incubation for 30 min, and then gradually decreased, as shown in Fig. 2-B. On the other hand, the radioactivity of [^{3}H]dexamethasone in the nuclei increased linearly with decrease in the radioactivity of receptor-bound [^{3}H]dexamethasone in the cytosol. When primary hepatocytes were incubated with [^{3}H]dexamethasone plus 100 μM H-7, the radioactivity of receptor-bound [^{3}H]dexamethasone in the cytosol also increased rapidly to a peak after incubation for 30 min, but then decreased very slowly with little increase in the radioactivity in the nuclei. After incubation for 4 hr, about 20% and 80% of the receptor-bound [^{3}H]dexamethasone in cells without H-7 treatment became located in the cytosol and the nuclei, respectively. In

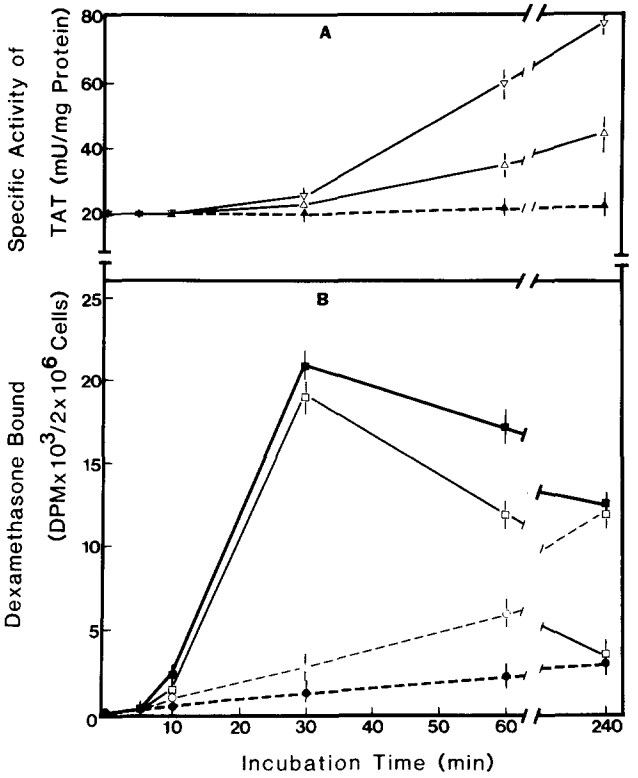

Fig. 2. Time courses of the effects of H-7 on TAT induction (A) and on the distribution of glucocorticoid receptor (B) in primary hepatocytes incubated for 0 to 4 hr with [^{3}H] dexamethasone. Preparations of cytosolic and nuclear fractions and measurements of specific binding of [^{3}H]dexamethasone to the cytosolic and washed nuclear fraction are described in "Materials and Methods". A, TAT activities in cytosolic fractions of hepatocytes with 10^{-8} M [^{3}H]dexamethasone (△), 10^{-8} M [^{3}H]dexamethasone plus 100 μM H-7 (▲) and 10^{-7} M [^{3}H]dexamethasone (▽) at the indicated times. B, receptor-bound [^{3}H]dexamethasone in cytosolic fractions of hepatocytes with 10^{-8} M [^{3}H]dexamethasone with (■) or without (□) 100 μM H-7 and in nuclear fractions of hepatocytes with 10^{-8} M [^{3}H]dexamethasone with (●) or without (○) 100 μM H-7. Values are means from three dishes. Bars, S.D.

contrast, in cells treated with H-7 about 80% and 20 % of the receptor-bound [^{3}H]dexamethasone were found in the cytosol and nuclei, respectively. The activity of TAT was increased 1.6-fold and 2.5-fold by incubations for 4h with 10^{-8} M and 10^{-7} M [^{3}H]dexamethasone, respectively, compared with the activities on incubations without dexamethasone and the induction by 10^{-8} M [^{3}H]dexamethsone was almost completely inhibited by 100 μM H-7 (Fig. 2-A).

The correlations between the extent of induction of TAT and the radioactivities of receptor-bound [^{3}H]dexamethasone in the

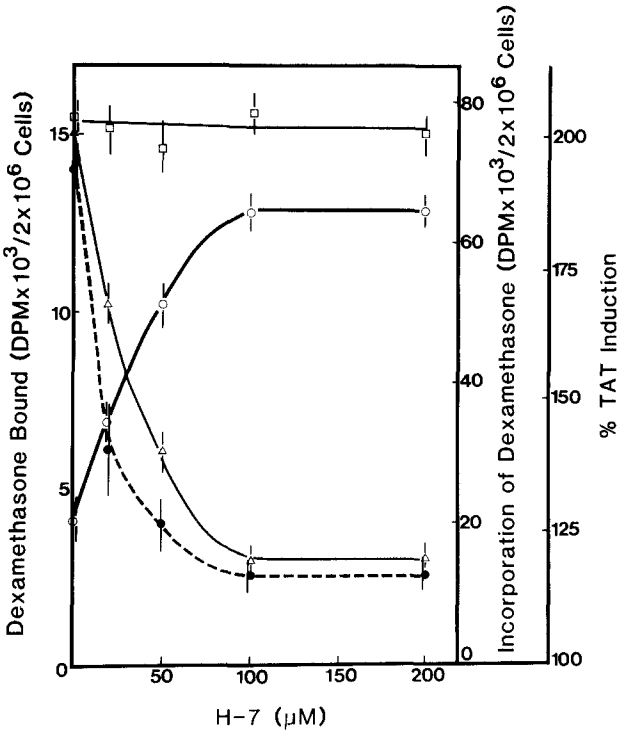

Fig. 3. Dose-response curves for inhibition of TAT induction and accumulation of receptor-bound [^{3}H]dexamethasone in nuclei of hepatocytes by H-7. Hepatocytes were incubated with 10^{-8} M [^{3}H]dexamethasone plus various concentrations of H-7 at 37°C for 4 hr. Hepatocytes were washed three times with Tyrode buffer but without divalent cations, solubilized in 0.5 ml of NCS tissue solubilizer and stood at room temperature overnight. Then 100 μl of the solution was used for measurement of the radioactivity incorporated into the cells (□). The rate of induction of TAT in the cytosolic fraction (△) is shown as a percentage of the activity of control cells not treated with dexamethasone. The specific bindings of [^{3}H]dexamethasone to the cytosolic (○) and nuclear (●) fractions. Values are means from three dishes. Bars, S.D.

nuclei and those of receptor-bound [^{3}H]dexamethasone in the cytosol were studied as a function of the concentration of H-7, as shown in Fig. 3. The induction of TAT by dexamethasone and the nuclear accumulation of receptor-bound [^{3}H]dexamethasone were both inhibited concentration-dependently by H-7 and the radioactivity in the cytosol conversely increased with decrease in the radioactivity in the nuclei, the combined radioactivities in the cytosol and nuclei being almost constant, regardless of the concentration of H-7. In addition, the total radioactivity incorporated into the cells was not affected by H-7. These results

indicate that H-7 inhibited the translocation of glucocorticoid-receptor complexes into the nuclei and consequently inhibited the induction of TAT by glucocorticoid, but that it did not inhibit the incorporation of glucocorticoid into the cells or the binding of glucocorticoid to its receptor. These findings indicate that protein kinase C may play an essential role in the translocation of glucocorticoid-receptor complexes to the nuclei.

ACKNOWLEDGEMENTS

We wish to thank Ms. E. Inai for expert secretarial assistance. This work was supported in part by a Grant-in-Aid (56880028) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Kido, H., Fukusen, N., Ishidoh, K., and Katunuma, N. (1986) Biochem. Biophys. Res. Commun. 138: 275-282.
2. Kido, H., Fukusen, N., Katunuma, N. (1987) Biochemistry, in press.
3. Leach, K.L., Dahmer, M.K., Hammond, N.D., Sando, J.J., and Pratt, W.B. (1979) J. Biol. Chem. 254: 11884-11890.
4. Barnett, C.A., Schmidt, T.J., and Litwack, G. (1980) Biochemistry 19: 5446-5455.
5. Singh, V.B., and Mondgil, V.K. (1985) J. Biol. Chem. 260: 3684-3690.
6. Sanchez, E.R., Toft, D.O., Schleinger, M.J., and Pratt, W.B. (1985) J. Biol. Chem. 260: 12398-12401.
7. Mendel, D.B., Bodwell, J.E., Gametchu, B., Harrison, R.W., and Munck, A. (1986) J. Biol. Chem. 261: 3758-3763.
8. Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. (1984) Biochemistry 23: 5036-5041.
9. Inagaki, M., Kawamoto, S., and Hidaka, H. (1984) J. Biol. Chem. 259: 14321-14323.
10. Kawamoto, S., and Hidaka, H. (1984) Biochem. Biophys. Res. Commun. 125: 258-264.
11. Tanaka, K., Sato, M., Tomita, Y., and Ichihara, A. (1978) J. Biochem. (Tokyo) 84: 937-946.
12. Beato, M., and Feigelson, P. (1972) J. Biol. Chem. 247: 7890-7896.
13. Raaka, B.M., Finnerty, M., Sun, E., and Samuels, H.H (1985) J. Biol. Chem. 260: 14009-14015.
14. Granner, D.K., and Tomkins, G.M. (1970) Methods in Enzymol. 17A : 633-637.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193: 265-275.
16. Hers, H.G., and Hue, L. (1983) Ann. Rev. Biochem. 52: 617-653.
17. Hidaka, H., Sasaki, Y., Tanaka, T., Endo, T., Ohno, S., Fuji, Y., and Nagata, T. (1981) Proc. Natl. Acad. Sci. USA, 78: 4354-4357.
18. Chafouleas, J.G., Bolton, W.E., Hidaka, H., Boyd, A.E., and Means, A.R. (1982) Cell 28: 41-50.