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The effect of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), an inhibitor of protein kinase C, on the nuclear binding of [^{3}H]dexamethasone and on the phosphorylation of glucocorticoid receptor was studied in rat liver slices to ascertain the role of protein kinase C in the expression of glucocorticoid action. H-7 reduces the nuclear binding of [^{3}H]dexamethasone in rat liver slices. It does not affect the extent of phosphorylation of glucocorticoid receptor both in the absence or in the presence of glucocorticoid. These findings indicate that protein kinase C may be involved in the nuclear binding of glucocorticoid receptor but does not directly influence the receptor phosphorylation.

Glucocorticoids modulate gene expression through a cascade of regulatory events initiated by high affinity binding to their intracellular receptors. The hormone-receptor complexes traverse the nuclear membrane and bind to their acceptor sites known as glucocorticoid regulatory elements, usually located 100–300 bp upstream from the RNA polymerase start site, ultimately causing change in the transcription of specific mRNAs (1,2). The glucocorticoid receptor is a phosphoprotein and the phosphorylation of the receptor has been studied in a number of cells, tissues, and animals under a variety of conditions (3–5). However, the protein kinase(s) involved in phosphorylation of the glucocorticoid receptor has not been clearly identified, nor has the role of receptor phosphorylation in DNA binding/transactivation, and recycling of the receptor (2,3).

We have previously observed that modulators of protein kinase C(PKC)[3] reg-

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[3] Abbreviations used: H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; PKC, protein kinase C; Bt_2cAMP, dibutyryl-adenosine 3′,5′-cyclic monophosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GR, glucocorticoid receptor.

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ulate the expression of glucocorticoid action in target cells and tissues (6). H-7 and sphingosine (both inhibitors of PKC) have been reported to inhibit the induction of tyrosine aminotransferase and tryptophan oxygenase by dexamethasone but not the induction of tyrosine aminotransferase by dibutyryl cyclic AMP (Bt_2cAMP) in primary culture of rat hepatocytes (7,8). H-7 markedly inhibited the accumulation of glucocorticoid-receptor complexes in the nuclear fraction of rat hepatocytes (7). To assign the functional site of PKC in mediation of glucocorticoid action, we studied and report in this paper the *in vivo* effect of H-7 on the nuclear binding of [^{3}H]dexamethasone in rat liver slices and on the phosphorylation of glucocorticoid receptor.

MATERIALS AND METHODS

Materials. Male Wistar rats of 120–150 g were adrenalectomized 7–8 days before experiments and kept on laboratory chow and saline *ad libitum.* H-7 was obtained from Seikagaku Kogyo Co. [6,7-^{3}H]Dexamethasone (sp act 1.7 TBq/mmol) and [6,7-^{3}H]dexamethasone mesylate (sp act 1.7 TBq/mmol) were from DuPont–New England Nuclear; ^{32}P orthophosphate (sp act 336.4 MBq/nmol; carrier-free) was from Amersham Corp. All other chemicals were of the purest analytical grade commercially available.

Buffers. Buffer A: 25 mM Hepes/128 mM NaCl/6.3 mM KCl/2.8 mM $CaCl_2$/1.3 mM $MgCl_2$/10 mM glucose/0.5 mM each of pyruvate, glutamate, and succinate (pH 7.4); buffer B: 20 mM Tris/250 mM sucrose/25 mM KCl/10 mM $MgCl_2$/2 mM DTT/10 mM sodium molybdate (pH 7.5); buffer C: 10 mM Tris/250 mM sucrose/1 mM EDTA/20 mM sodium molybdate/2 mM DTT/50 μM leupeptin/50 μM chymostatin/100 μM E-64 (pH 7.43); buffer D: 10 mM Tris/1 mM EDTA/10% glycerol/20 mM sodium molybdate/50 mM NaCl (pH 7.6); buffer E: buffer D having 0.5% Triton X-100 and 0.1% SDS (pH 7.6).

In vivo nuclear binding of [^{3}H]dexamethasone. Adrenalectomized rats were killed by cervical dislocation. Livers were removed, rinsed in ice-cold saline, and sliced by a sterilized stainless steel disposable scalpel to be equal sizes (1–2 mm^2). The slices (0.5 g) were washed twice with buffer A, finally suspended in 2.5 ml of the same buffer, and incubated at 37°C with shaking (9,10). After 5 min, H-7 (100 μM) was added to one group followed by the addition of [^{3}H]dexamethasone (100 nM). After another 25 min, the slices were washed twice with buffer B and homogenized in 2.0 ml of the same buffer. The homogenate was centrifuged at 800*g* for 10 min. The crude nuclear pellet was washed once with buffer B having 0.5% Triton X-100 and twice with buffer B without detergent. Last, the nuclear pellets were suspended in 2.0 ml of buffer B and 0.5 ml was counted using Amersham ACSII (Aguous Counting Scintillant). The above nuclear suspension (0.1 ml) was used for the measurement of DNA (11). The specific binding was calculated by subtracting the radioactivity bound in the presence of a 500-fold excess of the unlabeled dexamethasone from that bound in the presence of the labeled hormone alone.

Preparation of antibody. The glucocorticoid receptor antibody was raised in New Zealand white rabbits using a hemocyanin conjugate (12,13) of a synthetic peptide (SVFSNGYSSPGMRPDVS) corresponding to amino acids 407 to 423 of

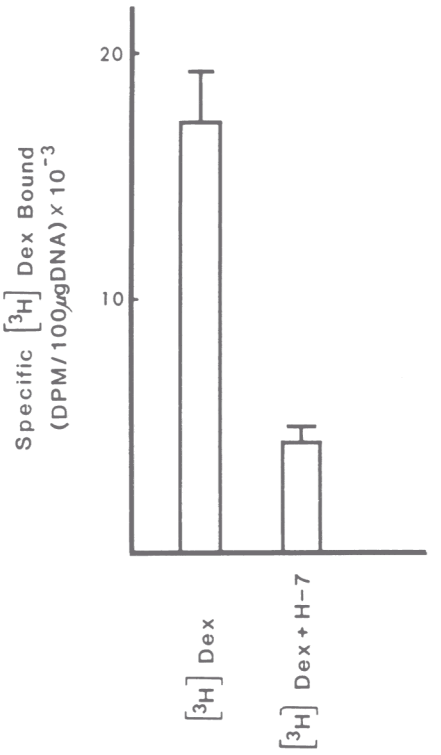

FIG. 1. Effect of H-7 on the nuclear binding of [^{3}H]dexamethasone in adrenalectomized rat liver slices. Specific binding of [^{3}H]dexamethasone (Dex) to the nuclei of control and H-7-treated slices was determined as mentioned under Materials and Methods. Values are means of three separate experiments with three to four samples each set. Bars = SD.

the rat glucocorticoid receptor (14). The peptide was synthesized in an automated peptide synthesizer 431A (Applied Biosystems) according to the manufacturer's instructions. The antibody was checked for the immunoprecipitation of radiolabeled glucocorticoid receptor and used in the present experiment.

Phosphorylation and immunoprecipitation of glucocorticoid receptor. The rat liver slices (0.7 g) were washed twice and suspended in 5.0 ml of buffer A (9,10). They were then incubated at 37°C with shaking in presence of 1.2 mCi ^{32}P orthophosphate. H-7 (100 μM) was added to one group after 10 min and the incubation was continued for 60 min. At the end, the slices were washed twice and homogenized in 4 vol of buffer C. The homogenate was centrifuged at 800*g* for 10 min. The resulting supernatant was further centrifuged at 105,000*g* for 1 h at 4°C. Fat-free clear cytosol was used to immunoprecipitate the receptor. Typically, 0.8 ml of cytosol was mixed with 0.4 ml of buffer D and 1:200 dilution of anti-GR serum and/or nonimmunized serum and incubated overnight at 0–4°C. The antibody–receptor complexes were adsorbed by addition of 50 μl of 50% protein A Sepharose 4B (Pharmacia) in buffer D for 2 h at 4°C with continuous rotation. Protein A Sepharose pellets were washed once with buffer D/E/E + 350 mM

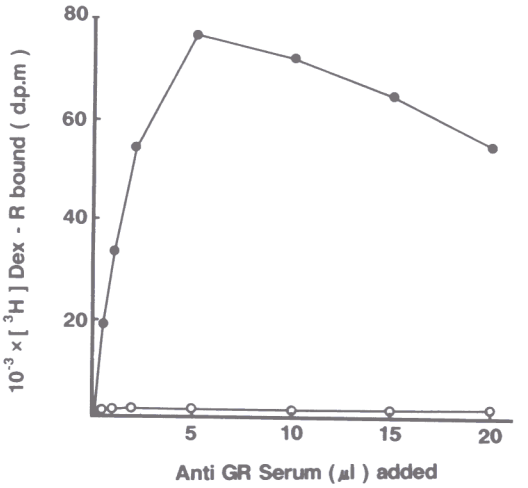

FIG. 2. Immunoprecipitation of rat liver glucocorticoid receptor using anti-GR serum. Adrenalectomized rat liver cytosol was incubated with [^{3}H]dexamethasone (100 nM) for 2 h at 0°C. The cytosol was then treated with dextran-charcoal to remove unbound hormone and used for the immunoprecipitation. Typically, 1.0 ml of the labeled cytosol was incubated overnight at 0–4°C with the indicated amounts of anti-GR serum (●) or nonimmunized serum (○). Immunoprecipitate was adsorbed using protein A Sepharose 4B, washed thrice with buffer D, and counted for bound radioactivity. Dex-R, dexamethasone receptor.

NaCl/E/D. The pellets were then mixed with 20 μl of 1.5 × SDS-sample buffer, heated at 100°C for 6 min, and 15 μl of the extracts were subjected to electrophoresis.

Gel electrophoresis. SDS–PAGE was carried out by the method of Laemmli (15) in 4–20% gradient gels having 0.1% SDS (Daiichi Pure Chemicals Co., Japan) at 4°C. Gels were stained for 2 h with 0.1% Coomassie brilliant blue R250 in 50% methanol/10% acetic acid and destained overnight in 25% methanol/7.5% acetic acid. The gels were then dried using an Atto Gel Drying Processor (AE-3700) and exposed to X-ray film (Fuji Rx) with an intensifying screen for 10 days at −80°C. [^{14}C]Methylated protein molecular weight markers (Amersham) were used for autoradiography. The autoradiograms were also scanned using Shimadzu scanning densitometer (CS-9000).

RESULTS AND DISCUSSION

Glucocorticoids exert their biological effects in various target tissues by interacting with their cognate receptor protein. These biological effects are dependent on the presence of a functional receptor (16). In addition, glucocorticoid action on target cells may also be influenced by various modulators (6). Of these, the possible involvement of PKC in mediation of glucocorticoid action has been considered by this laboratory (17–19). The molecular mechanism for the regulatory action of PKC in glucocorticoid action is not yet known. Although the glucocorticoid receptor is a phosphoprotein (3–5), the functional roles of phosphorylation/dephosphorylation, kinase(s), and phosphatase(s) acting on receptors are

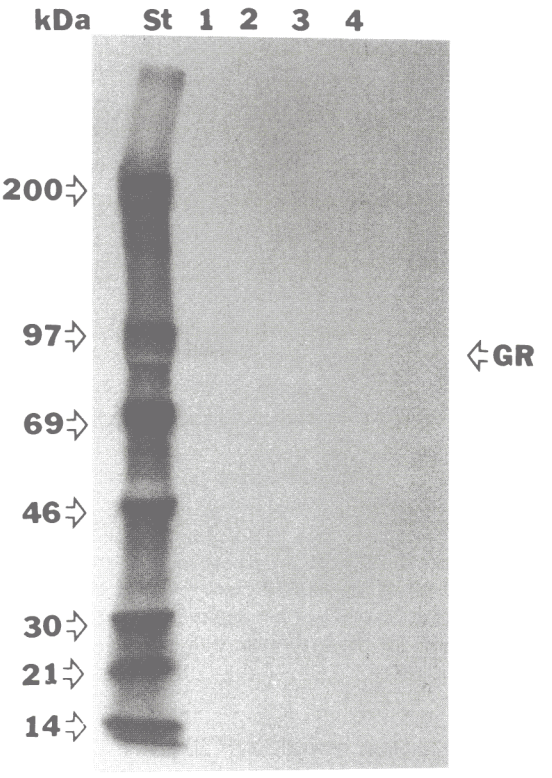

FIG. 3. Immunoprecipitation of affinity-labeled [^{3}H]dexamethasone mesylate-receptor complexes with anti-GR antibody. Adrenalectomized rat liver cytosol was incubated with [^{3}H]dexamethasone mesylate in the presence or absence of a 500-fold excess of nonradioactive dexamethasone for 2 h at 0°C. Unbound hormone was removed by dextran-charcoal treatment and the clear supernatant was used for immunoprecipitation. Labeled cytosol (1.0 ml) was incubated overnight at 0–4°C with 200-fold dilution of anti-GR serum and/or nonimmunized serum. The immunoprecipitate was adsorbed by protein A Sepharose, washed, and electrophoresed as described under Materials and Methods. After staining and destaining, the gel was impregnated for 20 min in Amersham fluorescence amplifier, dried, and subjected to autoradiography. The sample was incubated in the absence (lane 1) and in the presence (lane 2) of nonradioactive dexamethasone and immunoprecipitated with anti-GR serum. Lanes 3 and 4, respectively, are from the samples incubated in the absence and in the presence of nonradioactive dexamethasone and immunoprecipitated with the nonimmunized serum. GR, glucocorticoid receptor; St, standard molecular weight markers (myosin, 200 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21 kDa; lysozyme, 14 kDa).

unknown. In order to elucidate the exact role of PKC on the expression of glucocorticoid action, we used H-7, an inhibitor of PKC, to study the involvement of PKC in phosphorylation of the glucocorticoid receptor.

Figure 1 shows that H-7 reduces significantly the specific nuclear binding (dpm/100 μg DNA) of [^{3}H]dexamethasone in rat liver slices and indicates that PKC is involved in the nuclear binding of glucocorticoid. This is in agreement with our earlier report that H-7 inhibits the accumulation of glucocorticoid–receptor

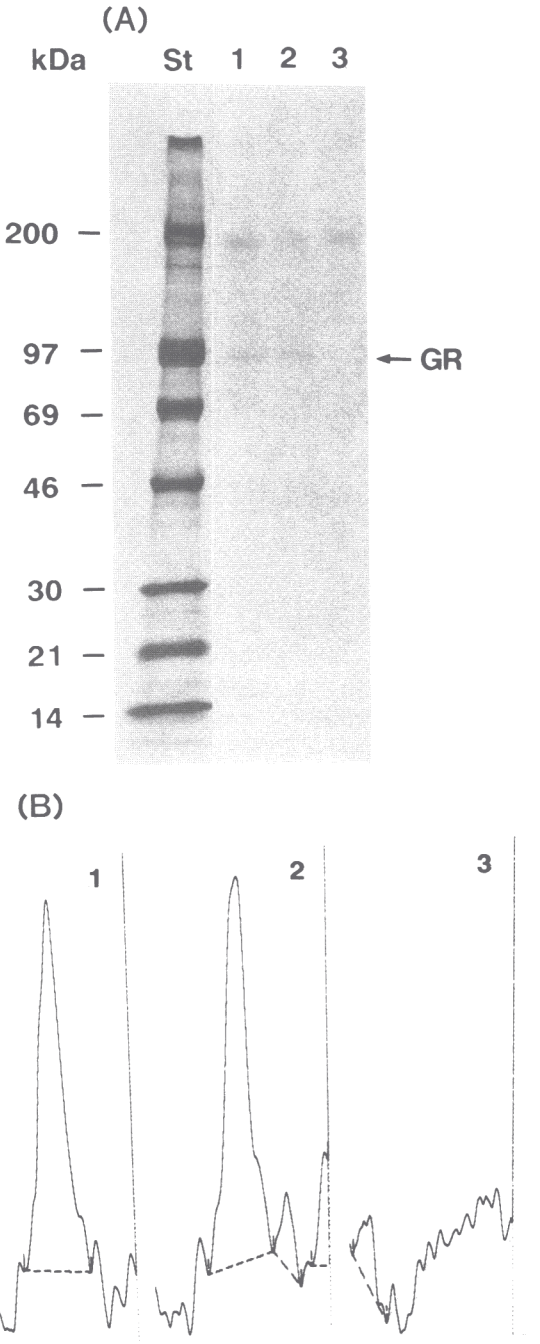

FIG. 4. The effect of H-7 on the *in vivo* phosphorylation of glucocorticoid receptor in rat liver slices. Experimental procedures are as mentioned under Materials and Methods. (A) Autoradiogram of the phosphorylated receptor in the absence (lane 1) or the presence (lane 2) of H-7. Lane 3 shows the immunoprecipitate of the phosphorylated receptor sample with the nonimmunized serum. (B) Densitometric scan of the receptor band from A. Panels 1, 2, and 3 are of the receptor bands from lanes 1, 2, and 3, respectively. The relative intensity of the glucocorticoid receptor peaks is expressed in arbitrary units. GR, glucocorticoid receptor; St, standard molecular weight markers.

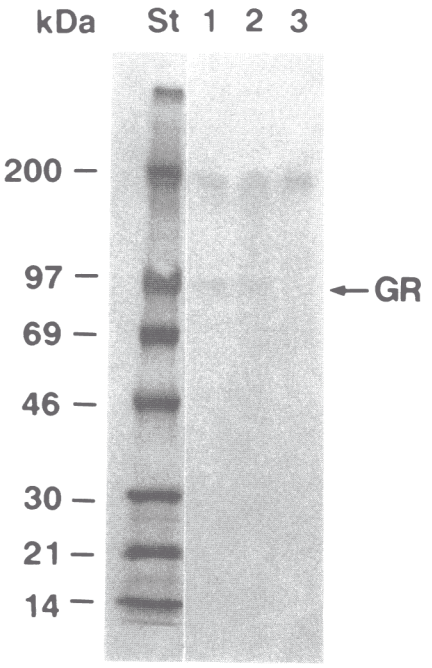

FIG. 5. The effect of H-7 on the *in vivo* phosphorylation of the glucocorticoid receptor in the presence of dexamethasone. Dexamethasone (200 nM) was added 30 min before the completion of incubation at 37°C. The remainder of the experimental procedures are as those described in the legend of Fig. 4A.

complexes in the nuclear fraction of rat hepatocytes (7). H-7 did not affect the incorporation of glucocorticoid in hepatocytes. Based on these findings, the involvement of PKC was attributed to the translocation of glucocorticoid–receptor complexes into nuclei. Whether this involvement of PKC is directly at the receptor and/or on some other factor(s) involved in the mechanism of glucocorticoid action, we investigated the *in vivo* phosphorylation of the glucocorticoid receptor by PKC.

We have raised an antibody against an epitope (aa 407–423) of the rat glucocorticoid receptor (GR). Figure 2 shows the specificity of our antibody against glucocorticoid–receptor complexes. Addition of anti-GR serum to the labeled cytosol immunoprecipitates the hormone–receptor complexes, whereas nonimmunized serum does not immunoprecipitate the labeled receptor. Using this antibody, the covalent affinity-labeled [^{3}H]dexamethasone mesylate receptor gave a single band on SDS–PAGE at a molecular weight of 94 kDa (Fig. 3; lane 1). We did not observe any band in the presence of the excess cold dexamethasone (lane 2). Nonimmunized serum does not show any immunoprecipitation of affinity-labeled glucocorticoid receptor complexes (lanes 3, 4). Figure 4A shows that H-7 does not affect glucocorticoid receptor phosphorylation. The intensity of receptor bands was the same in the control (lane 1) and in the H-7-treated (lane 2) slices as evident from the densitometric scans of the receptor band (Fig. 4B). We also

observed a phosphorylated protein band of molecular weight (200 kDa) in this experiment (Fig. 4A). This protein may be a nonspecific contaminant and not the aggregate of glucocorticoid receptors since this protein was also found immunoprecipitated with the nonimmunized serum. In addition, we have studied the effect of H-7 on the phosphorylation of the glucocorticoid receptor in the presence of added dexamethasone (Fig. 5). H-7 did not affect the extent of phosphorylation even in the presence of dexamethasone. These findings rule out the possibility that PKC phosphorylates the glucocorticoid receptor resulting in enhancement of the nuclear accumulation.

Further experiments are needed to identify the protein(s) phosphorylated by PKC which induces translocation of the glucocorticoid receptor into the nuclei and/or stimulates the binding of the glucocorticoid receptor to DNA.

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