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1-(5-ISOQUINOLINESULFONYL)-2-METHYLPIPERAZINE (H-7) IS A SELECTIVE INHIBITOR OF PROTEIN KINASE C IN RABBIT PLATELETS

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Effects of 1-(5-isoquinolinesulfonyl)-2-methylpeperazine (H-7), a potent inhibitor of protein kinase C *in vitro* (1), were investigated with regard to stimulus-induced protein phosphorylation of rabbit platelets. While H-7 inhibited the protein kinase C-mediated phosphorylation in 12-O-tetradecanoylphorbol-13-acetate (TPA)-stimulated platelets, this compound did not block the Ca^{2+}-calmodulin-dependent phosphorylation in Ca^{2+} ionophore A23187-stimulated cells. This selective inhibitor of protein kinase C, in intact cells, will facilitate studies on the biological functions of protein kinase C.

Nishizuka proposed that there are two routes by which extracellular signals are transduced into intracellular events in the Ca^{2+} messenger system. One is mediated by a rise in the cytosolic Ca^{2+} concentration leading to the modulation of calmodulin-dependent reactions. The other is mediated by protein kinase C which is activated by diacylglycerol or tumor promoting phorbol esters, at the basal level of Ca^{2+} (2). The physiological significance of the calmodulin-mediated system has been elucidated both in biochemical and pharmacological studies. Calmodulin antagonists such as naphthalenesulfonamides and phenothiazines have led to acquisition of pertinent data on the biological functions of this system (3,4). Inhibitors of protein kinase C, and which can be used for *in vivo* studies should be potent and selective. We recently synthesized a series of isoquinolinesulfonamide derivatives and characterized one,

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Abbreviations used are: EGTA, ethylene glycol bis (ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate; TPA, 12-O-tetradecanoylphorbol-13-acetate; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine.

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1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7). We found this derivative to be a potent inhibitor of protein kinase C *in vitro* (1). In this communication, we report the effects of H-7 on protein phosphorylation in model systems of cellular activation with TPA and Ca^{2+} ionophore A23187. These latter two agents can activate independently the above mentioned two routes of the Ca^{2+} messenger system (5). As H-7 inhibits the protein kinase C-mediated phosphorylation but not the Ca^{2+}-calmodulin mediated phosphorylation, this agent can serve as a useful tool for clarifying the biological role of the protein kinase C-mediated system.

MATERIALS AND METHODS

Washed rabbit platelets were prepared by differential centrifugation and suspended in Ca^{2+} and phosphate-free Tyrode solution with 1% gelatin. These platelet suspensions were incubated with ^{32}P-orthophosphate at 0.3 mCi/ml for 60 min at 30°C, then sedimented at 1400 g for 5 min. The obtained pellets were resuspended in Ca^{2+}-free Tyrode solution with 1% gelatin to give a platelet count of 5-10 x 10^8/ml. The labelled platelets were preincubated with saline or H-7 in an aggregometer for 2 min at 37°C before stimulation with TPA (100 ng/ml) or A23187 (0.5 μM).

The phosphorylation of platelet endogenous proteins was assayed in the reaction mixture (final 0.2 ml) containing 25 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 1 mM $CaCl_2$ (or 2 mM EGTA), 10 ng phosphatidylserine (or absence), 10 μM [γ-^{32}P]ATP, 0.001% leupeptin and 150 μg of platelet soluble proteins. The soluble fractions of the platelets were prepared by centrifugation (105,000 g, 60 min) after sonication in medium containing 25 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 5 mM EGTA, 50 mM β-mercaptoethanol, 0.01% leupeptin and 75 μg/ml PMSF.

For analysis of phosphoproteins, the reaction was terminated by the addition of the same volume of two fold-concentrated denaturing solution which contained 5% SDS, 3% β-mercaptoethanol, 8% glycerol and 0.1 M Tris-HCl (pH 6.8). The sample was boiled in a waterbath at 100°C for 1 min and subjected to SDS-polyacrylamide gel electrophoresis, under the conditions described by Laemmli (6). The separating and stacking gels contained 14 and 5% acrylamide, respectively. The dried gels were exposed to Kodak X-O mat films and the relative intensity of each band was quantitated by densitometric tracing on the autoradiogram, using a GS 3000 Transmittance/ Refractance Scanning Densitometer.

Protein was estimated by the method of Bradford (7).

Carrier-free [^{32}P]orthophosphate and [γ-^{32}P]ATP were purchased from New England Nuclear and Amersham, respectively. TPA was from LC Service Corp., A23187 was from Calbiochem-Behring Corp., phosphatidylserine (beef brain) was from Serdary Research Laboratory, Inc. All other chemicals were of reagent grade.

RESULTS

Activation of protein kinase C and mobilization of Ca^{2+} are induced separately in intact cells by the treatment with TPA and Ca^{2+} ionophore A23187, respectively (5). When rabbit platelets are stimulated by TPA, this

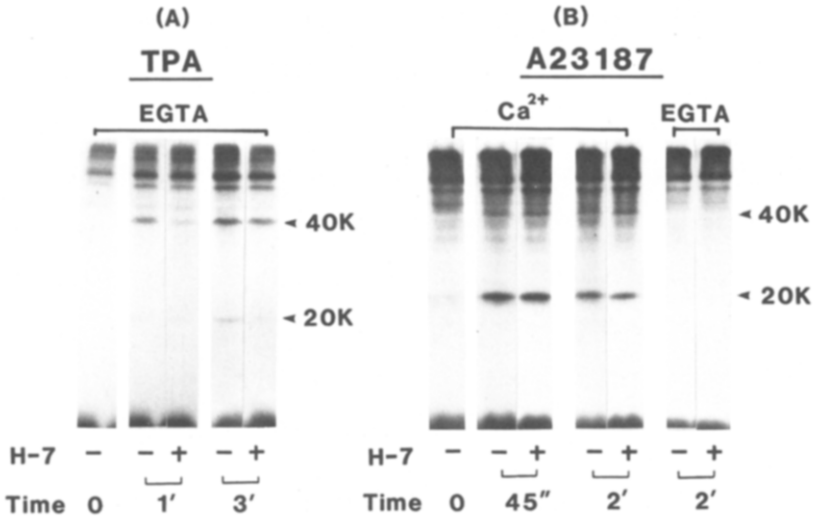

Fig. 1. TPA-or A23187-induced protein phosphorylation in platelets and the effects of the protein kinase C inhibitor H-7. The ^{32}P-labelled rabbit platelets were preincubated for 2 min at 37°C, with or without 50 μM H-7 and then stimulated with 100ng/ml TPA in the presence of 2 mM EGTA (A) or 0.5 μM A23187 in the presence of 0.1 mM $CaCl_2$ or 2 mM EGTA (B) for the various periods of time as indicated. Protein phosphorylation was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

agent may be intercalated into the membrane phospholipid and directly activate protein kinase C (8).We found that TPA induces a rapid and intensive phosphorylation of an endogenous protein with a molecular weight of about 40,000 (40K protein), reaches the maximum level within 3 min and induces phosphorylation of the 20,000-dalton protein (20K protein), at a slower rate (Fig. 1-A). The identity and function of 40K protein has not yet determined, however, circumstantial evidence suggests that this phosphorylation is catalyzed by protein kinase C (8). The 20K protein was identified as myosin light chain and can be phosphorylated by both protein kinase C and the Ca^{2+}-calmodulin-dependent enzyme, myosin light chain kinase (MLC Kinase) (9). Although TPA sometimes elicits mobilization of Ca^{2+} (10), leading to activation of Ca^{2+}-calmodulin-dependent reactions, if extracellular Ca^{2+} is chelated by EGTA, TPA induces little mobilization of Ca^{2+}, in rabbit platelets. Rabbit platelets, unlike human platelets, contain little intracellular Ca^{2+} so that the amount of this ion required for cellular activation is readily manipulated by the extracellular

concentrations of Ca^{2+} (11). Judging from analysis of the two-dimensional phosphopeptide mapping of 20K protein, (a useful method to identify the enzyme catalyzing the myosin phosphorylation (9)), the role of MLC kinase in 20K protein phosphorylation stimulated by TPA in the presence of the sufficient amount of EGTA is less than 10% (data not shown). On the other hand, when the rabbit platelets were stimulated with A23187 in the presence of Ca^{2+}, the 20K protein was rapidly phosphorylated with little phosphorylation of the 40K protein and the maximum level was reached within 1 min. A23187 elicited no detectable protein phosphorylation in the absence of extracellular Ca^{2+} (Fig. 1-B). Two-dimensional phosphopeptide mapping of the 20K protein revealed that almost all of this phosphorylation is catalyzed by MLC kinase (data not shown). Thus, under the given experimental conditions, TPA and A23187 induces a separate activation of protein kinase C and Ca^{2+} mobilization, respectively. The 40K protein phosphorylation induced by TPA and the 20K protein phosphorylation induced by A23187 are reliable indices for activation of the protein kinase C system and of the Ca^{2+}-calmodulin system, respectively, even though they are not sole targets for these systems.

To determine the selectivity of the H-7 action in intact platelets, we compared the effects of H-7 on protein phosphorylation in the TPA-stimulated cells with that seen in the A23187-stimulated ones. As shown in Fig. 1, pretreatment of the platelets with 50 μM H-7 followed by TPA activation resulted in a marked reduction of radioactive phosphate incorporation into the 40K protein and also into 20K protein. As evidenced by the data on 40K protein phosphorylation given in Fig. 2, the extent of the inhibition depends on the concentration of H-7. Inversely, H-7 produced no remarkable inhibition of 20K protein phosphorylation in the A23187-stimulated cells, in doses up to 50 μM (Figs. 1 and 2).

We also examined the effects of H-7 on the 40K protein phosphorylation in a crude cell-free system. Since both 40K protein and protein kinase C are mostly distributed in the soluble cytosolic fraction when the cellular

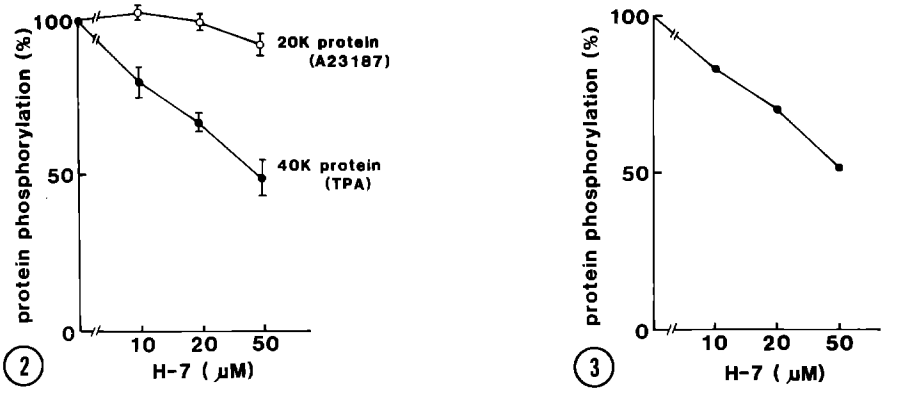

Fig. 2. Effects of H-7 on the TPA-induced 40K protein phosphorylation and the A23187-induced 20K protein phosphorylation. The ^{32}P-labelled rabbit platelets were preincubated for 2 min at 37°C in various concentrations of H-7 and then stimulated with 100 ng/ml TPA for 2 min in the presence of 2 mM EGTA or 0.5 μM A23187 for 45 sec in the presence of 0.1 mM $CaCl_2$. The phosphorylation of 40K protein in TPA-stimulated cells (•—•) and that of 20K protein in A23187-stimulated cells (○—○) was analyzed as described under the "Materials and Methods". The data are expressed as a percentage of the control, and shown as mean ± S.E.M. (n=3).

Fig. 3. Effects of H-7 on the Ca^{2+}-phospholipid-dependent phosphorylation of 40K protein. The crude soluble rabbit platelet proteins were incubated with [γ-^{32}P]ATP in the presence of Ca^{2+} and phosphotidylserine with various concentrations of H-7. Phosphorylation of 40K protein was analyzed as described under the "Materials and Methods". The data are expressed as a percentage of the control.

Ca^{2+} is chelated by EGTA (12,13), we used the 100,000 g supernatant of the sonicated platelets as enzyme and substrate sources. Phosphorylation of the 40K protein *in vitro* depends on phospholipid and Ca^{2+}. As shown in Fig. 3, H-7 inhibited this Ca^{2+} and phospholipid-dependent phosphorylation of 40K protein, in a dose-dependent manner.

Thus, H-7 inhibits protein kinase C-mediated protein phosphorylation in intact cells, while this compound has little effect on the Ca^{2+}-calmodulin-mediated reaction.

DISCUSSION

Since it has been demonstrated that the activation of protein kinase C is coupled with the stimulus-induced breakdown of inositol phospholipid in membrane and may be related to biochemical mechanisms that underlie the diverse cellular effects of tumor promoters (2), much attention has been directed to the role of protein kinase C in cellular activities. Despite a

recent surge of interest, it remains difficult to determine the physiological function of the protein kinase C-mediated phosphorylation in biological systems. Pharmacological antagonism of protein phosphorylation should be one approach at resolution. Antipsychotic drugs and polypeptide cytotoxins act as inhibitors of protein kinase C in vitro (14-16), and most have primary effects on the enzyme activating process by phospholipids but not on the enzyme catalytic action. H-7, an inhibitor of protein kinase C was synthesized in our laboratory. This compound inhibits protein kinase activity via a direct interaction on the catalytic site of the enzyme (1). Though there is variation in enzyme activating conditions in different cells or with different stimuli, protein kinase C-mediated phosphorylation is inhibited by H-7, as the action of this inhibitor is independent of enzyme activators such as phospholipid. Furthermore, natural stimuli which activate protein kinase C with inositol phospholipid breakdown usually elevate the levels of cytosolic Ca^{2+}. Nonetheless, H-7 produces a selective inhibition of protein kinase C-mediated reaction without inhibiting Ca^{2+}-calmodulin-dependent enzymes such as myosin light chain kinase. This H-7 selective inhibitor of protein kinase C has considerable potential merit for studying the role of this enzyme in a wide range of cells.

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