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Effects of Protein Kinase C Modulation on Hepatic Hemodynamics and Glucoregulation

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This study evaluated the effects of PKC activation using phorbol 12-myristate 13-acetate (PMA) and PKC inhibition using the isoquinoline sulfomide derivative H-7 on hemodynamics and glucoregulation in the isolated perfused rat liver. Livers were isolated from fed male Holtzman rats and perfused with Krebs Ringer bicarbonate solution under a constant flow of 50 ml/min at 35°C. Portal vein pressure, glucose and lactate concentrations in the medium and oxygen consumption rates were continuously monitored by a Grass polygraph, YSI glucose and lactate monitors, and a YSI oxygen monitor, respectively. PMA at concentration of 2 to 200 nM increased the portal vein pressure, glucose and lactate production, but decreased oxygen consumption rate in a dose-dependent fashion. H-7 (200 μM) attenuated PMA (50 nM)-induced vasoconstriction (15.1 $\pm$ 1.36 vs 10.56 $\pm$ 1.17 mmHg), glucose production rate (91.3 $\pm$ 6.15 vs 71.8 $\pm$ 2.50 μmoles/g/hr), lactate production rate (72.4 $\pm$ 6.82 vs 53.6 $\pm$ 4.82 μmoles/g/hr) and oxygen consumption rate (33.7 $\pm$ 1.41 vs 27.9 $\pm$ 1.75 μl/g/min). The effects of PMA were blocked either by addition of verapamil (9 μM) or perfusion with Ca^{2+}-free KRB.

These results suggest that the hemodynamic and glucoregulatory changes in the perfused rat liver are mediated by protein kinase C activation and require Ca^{2+} influx from the extracellular fluid.

Key Words: Protein kinase C, PMA, Perfused liver, Glucose output, Protal vein, Hepatocyte

INTRODUCTION

Protein kinase C (PKC) is a calcium-activated and phospholipid-dependent protein kinase which is combined in two functional moieties: a regulatory and a catalytic domain (Nishizuka 1984; Epand & Lester, 1990). It plays a key role in various cellular functions by regulating many different biological processes (Keenan et al, 1998; Nishizawa et al, 1998).

Many PKC activators and inhibitors have been used to elucidate the role of PKC in the cellular functions. Phorbol 12-myristate 13-acetate (PMA), a tumor-promoting phorbol ester, stimulates the PKC activity. Infusion of PMA stimulates glucose production in perfused rat liver (Kimura et al, 1984). Since this glucose output was blocked by verapamil and Ca^{2+} efflux was decreased by PMA, it was suggested that the increase in Ca^{2+} influx into the hepatocytes is the mechanism of PMA-induced glycogenolysis. However, by the fluorescence technique with quin-2, PMA did not alter cytosolic free Ca^{2+} concentration.

Garcia-Sainz and Hernandez-Sotomayor (1985) demonstrated that PMA-stimulated glycogenolysis was blocked by indomethacin. They suggested the involvement of cyclo-oxygenase products on glucose output by PMA. According to Patel (1987), however, PMA did not increase the prostaglandin production. Additionally, in his study PMA did not stimulated the rate of glucose production in hepatocytes isolated from fed rat liver. Thus, the mechanism by which PMA-stimulated glycogenolysis in the perfused liver is not well understood.

PKC was also involved in muscle contraction.

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Phorbol esters have been shown to cause a slowly and sustained contraction of smooth muscle in various animal tissues including rat aorta (Suenaga et al, 1996), porcine carotid arteries (Chatterjee & Tejada, 1986), rabbit trachea (Iizuka et al, 1999), mouse detrusor muscle (Lin et al, 1998). This porbol ester-induced smooth muscle contraction is dependent upon extracellular Ca^{2+} (Rasmussen et al, 1987; Bazan-Perkins et al, 1998), is inhibited by Ca^{2+}-channel blockers, and is facilitated by Ca^{2+} channel agonist (Forder et al, 1985; Lin et al, 1998).

This study evaluated the effects of PKC activation and inhibition on hemodynamics and glucoregulation in the isolated perfused rat liver.

METHODS

Preparation of the isolated perfused rat liver

Male albino rats of the Holtzman strain (330~370 g) were used throughout this study. The rat was anesthetized with pentobarbital sodium (30 mg/kg) via the dorsal vein of the penis. The abdomen was opened widely through midline and midtransverse incisions. The inferior vena cava and portal vein were isolated and 500 USP units of heparin were injected i.v. The abdominal vena cava was ligated above the renal veins and a PE-260 polyethylene catheter was inserted into the portal vein and tied with silk suture (size 2-0). The thorax was opened by a transverse incision just above and along the line of the diaphragm and by a longitudinal cephalad incision. A PE-280 polyethylene catheter was inserted and secured in the thoracic vena cava via penetration of the right atrium. The liver was rapidly excised, transferred onto a liver-platform, covered with saline-moistened gauze and placed in a modified Miller-type perfusion aeration chamber (MRA Corporation, Clearwater, FL).

Measurement of perfusion pressure

This livers were perfused with Krebs Ringer bicarbonate (KRB) solution through the portal vein with a constant flow of 50±1.0 ml/min. Portal vein pressure was measured by a pressure transducer (Gould P23ID) and was recorded by a polygraph (Grass Model 79D). A Gilmont flowmeter F-1300 (accuracy ±2%) was used to adjust and maintain a constant perfusion flow through the portal vein.

Measurements of glucose and lactate production and oxygen consumption rate in the perfused rat liver

Glucose was measured on a Yellow Springs Instrument model 26 glucose monitor. The instrument is designed for the continuous monitoring of glucose using a glucose oxidase enzyme probe. Lactate concentration in the medium was also measured on YSI model 26 monitor by L-lactate oxidase enzyme probe. Glucose and lactate concentration of perfusate were continuously recorded on strip chart recorder (Linear, model 1200). Glucose and lactate production rate (μmoles/g/hr) were calculated from changes in glucose and lactate concentration of perfusate and liver weight. Oxygen tension was measured on a YSI model 53 biological oxygen monitor. Two clark-type oxygen probes were employed-one at the site entering and the other at the site leaving the liver. Oxygen consumption rate (μl/g/min) was calculated from oxygen tension difference between two sites, perfusion flow and liver wet weight.

Solutions

Glucose free Krebs Ringer bicarbonate buffer was used as the perfusion medium. The solution contained (in mM) : NaCl 118, KCl 4.7, $CaCl_2$ 1.92, KH_2PO_4 1.19, $MgSO_4$ 1.44 and $NaHCO_3$ 25. KRB was saturated with an O_2/CO_2 (95:5 v/v) gas mixture and pH was adjust to 7.48 at 35°C. Stock solutions of PMA, H-7 (Isoquinolinyl sulfonyl-methyl piperazine) and 4 α-phorbol were prepared and diluted in dimethyl sulfoxide (DMSO) at a concentration of 80 μM, 68 mM and 80 μM, respectively. Verapamil was prepared in KRB solution at a concentration of 4 mM. DMSO, verapamil and H-7 were injected into the tube 15 cm prior to the liver and PMA and 4 α-phorbol were infused at the same site by infusion pump (Harvard, model 901) at the rate from 0.068 ml/min to 0.136 ml/min for 12 sec to 150 sec.

Statistical analysis of data

Results are presented as mean±standard error of the mean (S.E.M.). Student's paired and unpaired t-tests were used to test the significance of difference between responses. Significance were accepted at $p < 0.05$.

RESULTS

Effect of PMA on the portal vein pressure in the perfused rat liver

The effects of PMA on changes in portal vein pressure under constant perfusion flow were measured after a 35 min stabilization period. PMA increased portal vein pressure; vasoconstrictory responses occurred within 75±5 sec, the portal vein pressure reached a maximum level at 5~8 min after PMA infusion and the pressure subsequently recovered to the basal level at 20 to 30 min. Fig. 1 depicts PMA-dose response on the portal vein pressure. Five different concentrations of PMA were evaluated. As shown in Fig. 1, PMA increased the portal vein pressure (mmHg) from 9.68±0.24 to 9.95±0.26, 12.1±0.55, 15.1±1.36, 18.5±1.94 and 20.8±1.16 in proportion to its concentration of 0, 2, 10, 50, 100 and 200 nM, respectively. After recovery to the basal level, secondary addition of the same dose of PMA did not changes in portal vein pressure (changes: 0.1 ±0.09 mmHg at 2nd 50 nM PMA).

Effects of PMA on glucose and lactate production and oxygen consumption rates

Glucose concentration in the perfusate increased rapidly in initial stage of perfusion and then stabilized progressively. Basal glucose production rate (μmoles/g/hr) after stabilization was 34.8±1.67 and was increased following infusion of PMA. As shown in Table 1, the magnitudes of increment were proportional to the PMA concentration. PMA at concentration of 2 to 200 nM also increased the lactate production rate in a dose-dependent fashion. However, hepatic oxygen extraction decreased by PMA infusion. Therefore, oxygen consumption rate (μl/g/min) decreased in proportional to PMA dose. PMA-induced changes in the glucose and lactate production and oxygen consumption occurred significantly from 50 nM of PMA.

Effects of H-7 on PMA-induced changes in portal vein pressure in the perfused rat liver

After 35 min of stabilizing period, basal portal vein pressure (pre-DMSO) was 8.8±0.76 mmHg. Since H-7 was dissolved in DMSO, 0.4 ml of DMSO was injected through the portal vein (post-DMSO). DMSO itself did not affect the portal vein pressure (8.7±

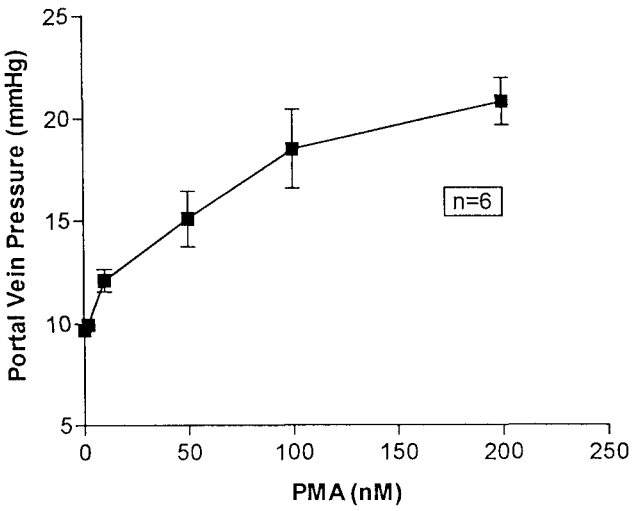

Fig. 1. Dose response of PMA on portal vein pressure. Livers were perfused with KRB under a constant perfusion flow of 50±1.0 ml/min (pH 7.4, 37°C). PMA increased portal vein pressure in dose-dependent manner. Data represents means±S.E.M. for 6 separate experiments.

Table 1. Dose responses of glucose production, lactate production and oxygen consumption rate to PMA in the isolated perfused rat liver

PMA (nM)	Glucose production (μmole/g/hr)	Lactate production (μmoles/hr)	Oxygen consumption (μl/g/min)
0	34.8±1.62	18.0±1.30	36.8±0.64
2	35.5±4.57	19.5±3.95	35.6±1.08
10	44.7±5.47	37.1±8.65	35.8±2.09
50	91.3±6.15*	72.4±6.82*	33.7±1.44*
100	106.1±15.1*	88.5±2.74*	30.0±2.29*
200	141.3±15.9*	98.7±10.8*	24.0±3.30*

Each value represents the mean±S.E.M. from 6 livers. *Denotes $p<0.05$ compared to control (0 nM of PMA).

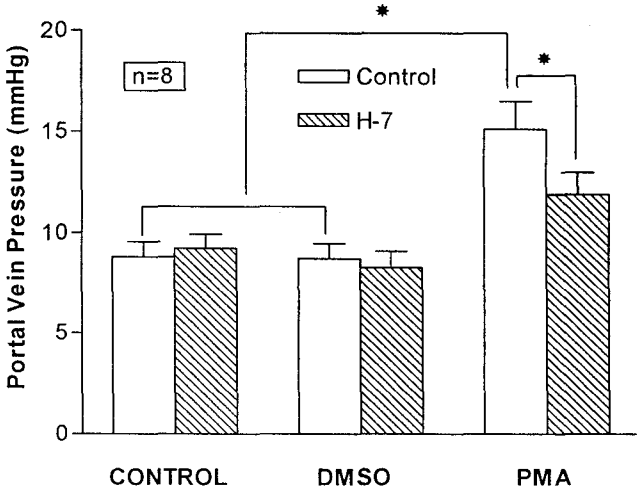

Fig. 2. Effects of H-7 on PMA-induced changes in portal vein pressure. Means±S.E.M. of portal vein pressure for control, DMSO and PMA (50 nM) in the presence or absence of H-7 (200 μM) were presented. Number of experiments is indicated in parentheses. *Denotes $p \leq 0.05$ compared to PMA.

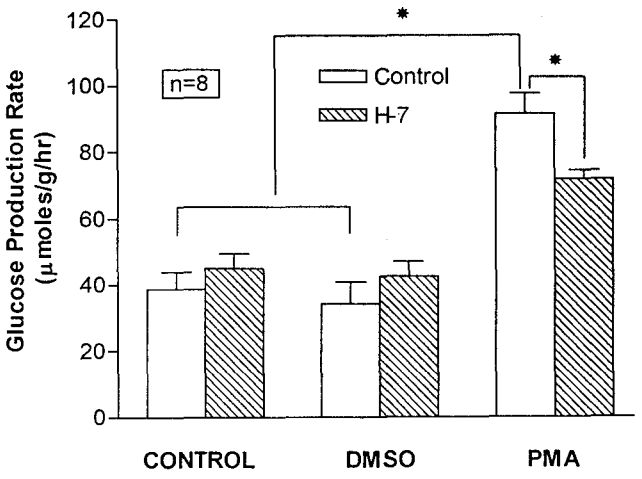

Fig. 3. Effects of H-7 on PMA-induced glucose production rate in the perfused rat liver. Means±S.E.M. of glucose production rate for control, DMSO and PMA (50 nM) in the presence or absence of H-7 (200 μM) were presented. Number of experiments is indicated in parentheses. *Denotes $p \leq 0.05$.

0.76 mmHg). Ten minutes after DMSO injection, 50 nM of PMA was infused through portal vein for 40 sec. Fifty nM of PMA increased the portal vein pressure to 15.1±1.36 mmHg (71.5% to control). In the other hand, pretreatment of H-7 (200 μM) significantly attenuated PMA-induced changes in portal vein pressure ($p < 0.05$). Same dose of PMA increased the portal vein pressure only 28.5% (Fig. 2). The changes in portal vein pressure by H-7 (post H-7), from 9.23 ±0.69 to 8.25±0.82 were not significant. There were some direct diminished effects of H-7.

Effects of H-7 on PMA-induced glucose and lactate production and oxygen consumption rates

Fig. 3 represents the effects of H-7 treatment on PMA-induced glucose production. Basal glucose production rate (μmoles/g/hr) was 38.7±5.12. Fifty nM of PMA increased the glucose production rate from 34.2±6.60 to 91.3±6.15 ($p < 0.05$). In H-7 group, the glucose production rates of control, DMSO and PMA were 44.9±4.62, 42.4±4.59 and 71.8±2.50, respectively. This indicates that H-7 significantly inhibits PMA-induced glucose production ($p < 0.05$). Lactate production was also inhibited by H-7 (Fig. 4). In control group, PMA increased the lactate production 63.3%, from 26.6±5.58 to 72.4±6.82. However, in H-7 group, PMA increased the lactate production only 15.8%, from 34.4±3.37 to 53.6±4.82. Fig. 5

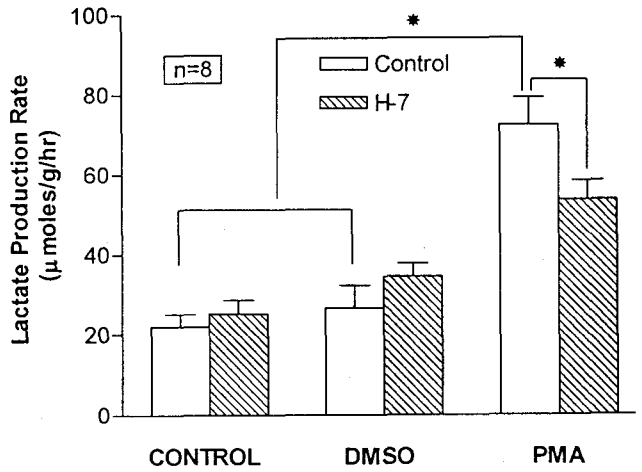

Fig. 4. Effects of H-7 on PMA-induced lactate production rate in the perfused rat liver. Means±S.E.M. of lactate production rate for control, DMSO and PMA (50 nM) in the presence or absence of H-7 (200 μM) were presented. Number of experiments is indicated in parentheses. *Denotes $p \leq 0.05$.

depicts the effect of H-7 on PMA-induced oxygen consumption. In control, 50 nM of PMA decreased oxygen consumption rate (μl/g/min) from 39.7±1.34 to 33.7±1.41. In H-7 group, PMA also inhibited oxygen consumption rate. Although the oxygen consumption rate by PMA-infusion in H-7 group was lesser than control group ($p < 0.05$), the rates of inhibition

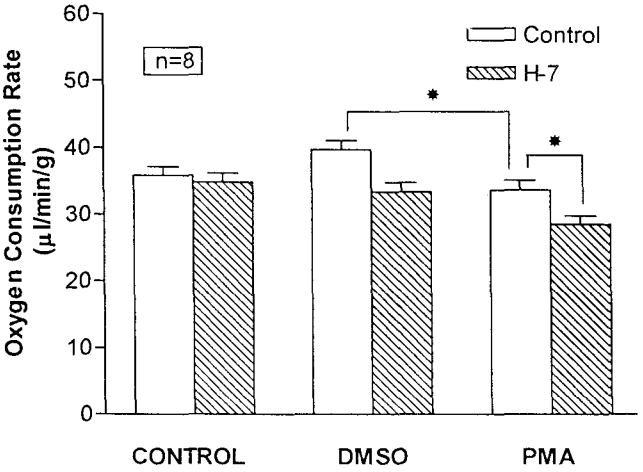

Fig. 5. Effects of H-7 on PMA-induced oxygen consumption rate in the perfused rat liver. Means±S.E.M. of oxygen consumption rate for control, DMSO and PMA (50 nM) in the presence or absence of H-7 (200 μM) were presented. Number of experiments is indicated in parentheses. *Denotes $p \leq 0.05$.

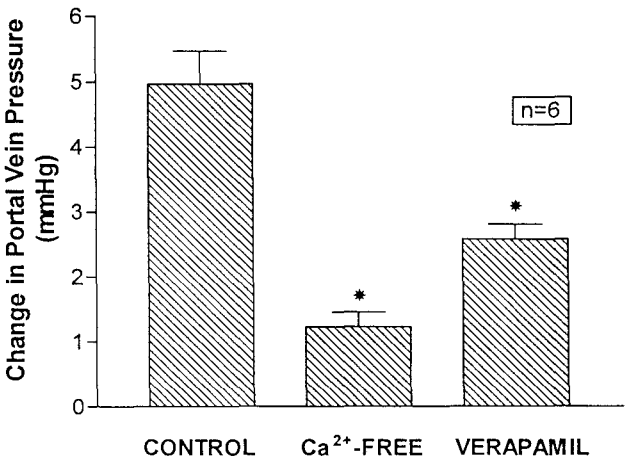

Fig. 6. Effects of verapamil and Ca^{2+}-free on PMA-induced changes in portal vein pressure. Changes in portal vein pressure were induced by addition of 50 nM of PMA. Verapamil (9 μM) was applied to the perfusate 5 min before the addition of PMA. PMA-induced increases in portal vein pressure were attenuated with prior treatment of verapamil and Ca^{2+}-free KRB. *Denotes $p \leq 0.05$.

of oxygen consumption were almost same (17.8% vs 17.2%)

Effects of verapamil and Ca^{2+} free condition on PMA-induced portal vein pressure changes

Fig. 6 represents that the increase of portal vein pressure by PMA was blocked either by addition of verapamil or perfusion with Ca^{2+}-free KRB. Changes in portal vein pressure (ΔmmHg) by 50 nM of PMA in control group was 4.96±0.51. Verapamil (9 μM) inhibited significantly PMA-induced changes in portal vein pressure to 2.58±0.23 ($p<0.05$). In Ca^{2+} free condition (0 mM Ca^{2+} plus 0.5 mM EGTA), the increase of portal vein pressure by PMA was also inhibited. 4 α-phorbol (50 nM), an inactive form of a phorbol ester in PKC stimulation, had no effects on the portal vein pressure, glucose and lactate production rate and oxygen consumption rate (data not shown).

DISCUSSION

There are three major membrane generated second messengers including cAMP, calcium-calmodulin and phosphoinositides (IP_3). It is widely accepted that catecholamine and glucagon-induced hepatic glycogenolysis is mediated with cAMP dependent protein kinase (PKA). The other protein kinase system which induces glycogenolysis is Ca^{2+}-activated, phospholipid dependent protein kinase (PKC). In this system, agonist binding to its receptor lead to release of phosphatidylinositol bisphosphate from plasma membrane. This converts to diacylgylcerol and inositol trisphosphate. Diacylglycerol activates protein kinase C by intensifying its binding to Ca^{2+}. PKC then activates a variety of intracellular enzymes via phosphorylation (Blackshear et al, 1988; Nishizuka 1992; Ikebe & Brozovich, 1996).

It is recognized that in muscle PKC activates phosphorylase kinase and the kinase converts inactive phosphorylase b to active phosphorylase a. This catalyzes glycogen breakdown. However, in hepatocyte, it is not clear. Kimura et al (1984) reported that 12-0-tetradeconoylphorbol-13 acetate (TPA), protein kinase C activator, stimulated glycogenolysis in the perfused rat liver. However, in the study they did not focalize on hemodynamic changes which affect the glycogenolysis. Phorbol ester-dependent glycogenolysis in the perfusion rat liver was also reported by Patel (1987). However, in his study, PMA did not stimulate glycogenolysis in the isolated hepatocyte. Therefore, he suggested that phorbol ester-stimulated glycogenolysis is not a result of a direct effect on paren-

chymal cells.

In present study, PMA also increased glucose production in the perfused rat liver. Two mechanisms for the increased glycogenolysis by PMA are probable. First is direct effect of PMA on hepatic glycogenolysis. According to Fain et al (1984), PMA increased glycogen phosphorylase activity of the rat hepatocytes in the presence of Ca^{2+} Ionophore (A23187). However, in the other study (Patel, 1987) PMA failed to increase glucose output in hepatocytes isolated from rat. Therefore, the direct effect of PMA on hepatic glycogenolysis is controversal.

Second, vasoconstriction of portal vasculature by PMA may indirectly produce glucose and lactate production. Even though livers were perfused with a constant flow, if redistribution of perfusion flow occured within the liver, this may lead to reduction of oxygen consumption and result in glycogenolysis. Livers has heterogeniety in glycogen content (Deane, 1944), mitochondrial number and size (Loud, 1968) and enzyme distribution (Shank et al, 1959) in different hepatic zones. The peripheral region is associated with high oxygen consumption. Daniel and Prichard (1951, a & b) demonstrated uneven distribution of perfusion flow by certain stimuli using serial angiography. Administration of adrenaline shifted to the portal perfusion flow through central deep part due to constriction of peripheral vessel. They also observed that when intra-hepatic blood flow is shift in this manner, the portal blood reached the inferior vena cava more rapidly than the liver is perfused throughout. If PMA constricted small vessels of the portal vasculature, the perfusate would flow readly through the central zone which is less oxygen consumption region, then the perfusate would retain a high oxygen content. Actually after infusion of PMA the oxygen content of the perfusate at the site leaving the liver increased for a while. During the period of PMA action, hepatocytes in the less-perfusion region (peripheral zone) might undergo a hypoxic condition. Since hepatic glycogenolysis is very sensitive to the oxygen tension, if oxygen delivery to the liver is limited, hepatic glycogenolysis is accelerated (Hems et al, 1975).

Hepatic glycogenolysis in the hypoxic condition is due mainly to changes in intracellular metabolite concentration (Hirasawa et al, 1978). Glycogenolysis is accelerated by AMP and inhibited by ATP and glucose-6 phosphate. Therefore, in a normal state of oxygen tension, hepatic glycogenolysis is suppressed due to high level of intracellular ATP and low level of AMP. However, in hypoxic condition intracellular ATP concentration decreases while intracellular AMP increases (Faupel et al, 1972). It is not clear whether the hepatic glycogenolysis induced by hypoxia is related to activation of PKC. It may be possible. Because a rapid and profound increase of phosphorylase in the liver was induced by hypoxia (Theen et al, 1982) and PMA also increased glycogen phosphorylase activity of the hepatocyte (Fain et al, 1984).

The porbol ester-induced contractile responses were proved in various tissue including rat aorta (Gleason & Flaim, 1986; Suenaga et al, 1996), rabbit ear arteries (Forder et al, 1985), rabbit tracheal smooth muscle (Iizuka et al, 1999), rat messenteric artery (MacKay & Cheung, 1987) and isolated renal artery (Scholz & Kurtz, 1990). The present study also demonstrated that PMA induced vascular contraction in the isolated perfused rat liver (Fig. 1). In the experiment the vasoconstrictory responses by PMA initiate slowly and maintained for long period (20~30 min). According to a concept of smooth muscle contraction, it has two phases-initial phase and sustained phase. The initial phase of contraction is mediated by rising in intracellular Ca^{2+} and calmodulin-dependent activation of myosin light chain kinase. This initial phase is followed by a sustained phase in which may associate with protein kinase C (Rasmussen et al, 1987).

After recovery from the PMA-induced contraction, secondary addition of PMA did not induce changes in portal vein pressure. This represents a down-regulation of PKC activity. PMA-induced down regulation was also proved in insulin stimulation of glucose transport in fat cells (Cherqui, 1987).

To elucidate the involvement of PKC on smooth muscle contraction, various PKC inhibitors have been employed. Most of isoquistline sulfonamaid inhibit protein kinase including cGMP-dependent protein kinase, cAMP-dependent protein kinase, myosin light chain kinase and protein kinase C. Among them H-7 is relatively specific inhibitor of PKC and H-8 inhibits more marked cGMP-dependent and cAMP-dependent protein kinase (Hidaka et al, 1984; Inaba et al, 1995). The other PKC inhibitors are staurosporine (Davis et al, 1989; Murtha et al, 1999), polymixin B (Stutchfiel et al, 1986) and Sangivamycin (Loomis & Bell, 1988; Renau et al, 1996). In present study effects of H-7 on PMA-induced portal vein pressure changes were observed. H-7 attenuated

PMA-induced increase in portal vein pressure (Fig. 2). This suggests that certain role of PKC on contractility of portal vein smooth muscle. According to Scholz and Kurtz (1990) angiotensin II-induced vasoconstriction in the isolated perfused kidney was prevented by staurosporine and H-7. This also indicates that PKC involves hormone-dependent vasoconstriction.

H-7 also inhibited PMA-induced glucose and lactate production but it did not affect non-stimulated (pre-H-7) response. It is not clear whether these inhibition by H-7 is due to direct inhibition of glycogenolysis in hepatocyte (↓ PKC - ↓ phosphorylase), indirect inhibition via vasoconstriction (↓ PKC - ↓ Ca^{2+} influx - ↓ vasoconstriction - ↓ hypoxia - ↓ phosphorylase), or both effects. PMA decreased oxygen consumption rate but H-7 did not prevent PMA-induced decrease in oxygen consumption. This indicate that the lower oxygen consumption is not sole mechanism which causes glucose and lactate production.

The exact mechanism of PKC role in contraction of vascular smooth muscle is not well understood. There are some evidences indicating that PKC may participate in the regulation of Ca^{2+} entry into the smooth muscle cell. For instance, PMA and PDB (phorbol 12, 13-dibutyrate) promote calcium influx in vascular smooth muscle cells (Forder et al, 1985; Gleason & Flaim, 1986). Litten et al (1988) also suggested that PKC may augment Ca^{2+} influx through Ca^{2+} channel by phosphorylation. In present study the effects of PMA on portal vein pressure changes were blocked either by addition of verapamil or perfusion with Ca^{2+}-free KRB (Fig. 6). This indicate that hepatic vasoconstriction may be mediated by protein kinase C activation and require Ca^{2+} influx from the extracellular fluid.

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