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Effect of Phorbol 12-Myristate 13-Acetate on Ca^{2+}-ATPase Activity in Rat Liver Nuclei

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Abstract The effect of phorbol 12-myristate 13-acetate (PMA) on Ca^{2+}-ATPase activity in rat liver nuclei was investigated. Ca^{2+}-ATPase activity was calculated by subtracting Mg^{2+}-ATPase activity from (Ca^{2+}-Mg^{2+})-ATPase activity. The nuclear Ca^{2+}-ATPase activity was significantly increased by the presence of PMA (2–20 μM) in the enzyme reaction mixture; the maximum effect was seen at 10 μM. The PMA (10 μM)-increased Ca^{2+}-ATPase activity was not blocked by the presence of staurosporine (2 μM) or dibucaine (2 and 10 μM), an inhibitor of protein kinase. Meanwhile, vanadate (20 and 100 μM) caused a significant reduction in the nuclear Ca^{2+}-ATPase activity increased by PMA (10 μM). The present finding suggests that PMA has an activating effect on liver nuclear Ca^{2+}-ATPase independent of protein kinase.

Key words: Ca^{2+}-ATPase, phorbol ester, Ca^{2+} transport, nuclear signaling, rat liver nuclei

The role of Ca^{2+} in liver metabolism has been demonstrated in recent investigations [1,2]. Liver metabolism is regulated by Ca^{2+} which is increased in the cytoplasm of liver cells by hormonal stimulation. Recently, there has also been growing evidence that Ca^{2+} plays a role in liver nuclear function [3–7]. Calmodulin, a Ca^{2+}-binding protein which can amplify the effect of Ca^{2+} [8], exists in rat liver nuclei [3]. The existence of an ATP-stimulated Ca^{2+} sequestration system in rat liver nuclei that requires calmodulin and generates a net increase in nuclear matrix free Ca^{2+} concentration has been reported [4]. Calmodulin stimulates DNA synthesis by liver cells [5]. Liver nuclei contain a DNA endonuclease activity dependent upon Ca^{2+} in the submicromolar range, and Ca^{2+} causes extensive DNA hydrolysis [9]. Presumably, Ca^{2+} and calmodulin regulate liver nuclear function. Furthermore, it has been recently demonstrated that regucalcin, a novel Ca^{2+}-binding protein which reverses the effect of Ca^{2+} on many enzymes in liver cells [10,11], can inhibit Ca^{2+}-activated DNA fragmentation in isolated rat liver nuclei [12] and that the protein evokes Ca^{2+} release from the nuclei [13]. Thus, the nuclear Ca^{2+} transport system may be involved in the regulation of liver cell function.

More recent investigations in our laboratory suggest the existence of both Ca^{2+}-sequestering and Ca^{2+}-releasing systems in rat liver nuclei [13–15]. The Ca^{2+}-transporting system may be related to Ca^{2+}-ATPase which exists in liver nuclei [16]. The factors which regulate the nuclear Ca^{2+}-ATPase, however, have not been fully clarified. Therefore, the present study was undertaken to determine whether protein kinase C is involved in the regulation of Ca^{2+}-ATPase activity in rat liver nuclei, because it has been reported that the kinase exists in liver nuclei [17]. The effect of phorbol ester, which can activate protein kinase C [18], on the nuclear Ca^{2+}-ATPase was investigated. It was found that phorbol ester has an activating effect on the nuclear Ca^{2+}-ATPase independent of protein kinase C.

MATERIALS AND METHODS

Chemicals

Adenosine 5′-triphosphate (ATP), phorbol 12-myristate 13-acetate (PMA), dibutyryl cyclic adenosine monophosphate (cAMP), staurosporine, and dibucaine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). $CaCl_2$,

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vanadate, and other reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan). The reagents were dissolved in distilled water and then passed through ion-exchange resin to remove metal ions.

Animals

Male Wistar rats, weighing 100–120 g, were used. They were obtained commercially from Japan SLC, Inc. (Hamamatsu, Japan). Animals were fed commercial laboratory chow (solid) containing 57.5% carbohydrate, 1.1% calcium, and 1.1% phosphorus at a room temperature of 25°C and were allowed distilled water freely. After 1 week on the diet animals were killed by bleeding.

Isolation of Nuclei

Liver nuclei were isolated by the procedure of Jones et al. [9] with a minor modification. Rats were killed by cardiac puncture, and the livers were perfused with approximately 10 ml of ice-cold TKM solution (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$) to remove blood. Livers were then removed, cut into small pieces, and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle in 40 ml of the same solution containing 0.25 M sucrose and 1.0 mM ethyleneglycol-bis-(aminoethylether)N′,N′-tetraacetic acid (EGTA). The homogenate was filtered through three layers of cheesecloth. The nuclei were pelleted by centrifugation at 700*g* for 10 min. The pellets were homogenized (five strokes) in 40 ml of the same solution and centrifuged again at 700*g* for 10 min. The pellet was resuspended in 24 ml of the same solution by homogenization (five strokes), and 6 ml was added to each of four tubes containing 12 ml of TKM including 2.3 M sucrose. The tubes were gently mixed, and a 6 ml cushion (TKM containing 2.3 M sucrose) was carefully layered on the bottom of each tube. The tubes were centrifuged at 37,000*g* for 30 min. The upper layer and the sucrose cushion were removed with an aspirator. The resulting pellet of highly purified nuclei was resuspended in the incubation medium (125 mM KCl, 2 mM potassium phosphate, 25 mM Hepes, 4 mM $MgCl_2$, pH 7.0) by hand homogenization. Assay of marker enzymes, as reported previously [13], showed that there was less than 5% contamination by microsomes, plasma membranes, or mitochondria. DNA content in the nuclei was determined using the diphenylamine reaction [19].

Ca^{2+}-ATPase Assay

Basal, Mg^{2+}-, and Ca^{2+} + Mg^{2+}-ATPase activities were determined by modification [16] of the method of Zhang et al. [20]. The incubation medium (2.5 ml) contained 100 mM KCl, 20 mM Hepes, and 0.4 mg/ml nuclear protein. In the Mg^{2+}- and Ca^{2+} + Mg^{2+}-ATPase assay, either 5 mM $MgCl_2$, 40 μM $CaCl_2$, or both were added to the incubation media, respectively. The reaction was started by adding 2 mM ATP. After incubation at 37°C for 10 min, the ice-cold trichloroacetic acid was added to a final concentration of 7% (w/v) to stop the reaction. The protein-free supernatant was obtained after centrifugation at 5,000*g* for 5 min and assayed for inorganic phosphate concentration [21]. Protein concentration was measured as described by Lowry et al. [22] using bovine serum albumin as standard. Enzyme activity was expressed as nanomoles of inorganic phosphate released per minute per milligram of protein. Ca^{2+}-ATPase activity was calculated by subtracting Mg^{2+}-ATPase activity from (Ca^{2+}-Mg^{2+})-ATPase activity.

Ca^{2+} Transport Assay

Ca^{2+} uptake and release were determined with a Ca^{2+} electrode [13,14]. A reaction mixture (5.0 ml) composed of 100 mM KCl, 20 mM Hepes, pH 6.8, 5 mM $MgCl_2$, and other desired reagents was used with the electrode (model EA 940; Orion, Cambridge, MA). Nuclei used contained approximately 100 μg DNA/ml and 1.7 mg protein/ml of the reaction mixture. Ca^{2+} concentration was adjusted to the desired Ca^{2+} level (about 40 μM) with 10 mM $CaCl_2$, and uptake was initiated by the addition of 1.0 M ATP (neutralized with KOH) to a final concentration of 2.0 mM at 37°C. The Ca^{2+} electrode was calibrated using Ca^{2+}-EGTA buffers of known ionized Ca^{2+} concentrations, which were prepared and standardized using a Ca^{2+} standard solution purchased from Orion Associates Inc. Ca^{2+} release was monitored, as Ca^{2+} uptake was completely saturated by the addition of 2.0 mM ATP. Various reagents were added to the incubation medium 6 min after the addition of 2.0 mM ATP, and then Ca^{2+} release was measured for 10 min. Ca^{2+} uptake and release are expressed as nanomoles of total Ca^{2+} per milligram protein of the nuclei.

Statistical Methods

The significance of differences between values was estimated by using Student's t-test; P values of less than 0.05 were considered to indicate statistically significant differences.

RESULTS

The effect of PMA on Ca^{2+}-ATPase activity in rat liver nuclei is shown in Figure 1. PMA with various concentrations (2–20 μM) was added into the enzyme reaction mixture containing the nuclei immediately after 2 mM ATP addition. When the nuclei were incubated with PMA, the Ca^{2+}-ATPase activity was significantly increased by the presence of 5 μM PMA. The maximum effect of PMA was seen at 10 μM. Meanwhile, the presence of dibutyryl cAMP (20, 50, and 100 μM) did not have an appreciable effect on Ca^{2+}-ATPase activity in the nuclei (data not shown).

Ca^{2+} uptake by liver nuclei was negligible in the absence of ATP, as reported previously [13]. In the presence of ATP (0.5–2 mM), the nuclear Ca^{2+} uptake was seen markedly [13]. PMA (2–20 μM) had an appreciable effect on the net increase in Ca^{2+} taken by the nuclei in the presence of 2 mM ATP (Table I). However, in the absence of ATP, PMA had no effect on nuclear Ca^{2+} uptake (data not shown). Meanwhile, Ca^{2+} release from the Ca^{2+}-loaded nuclei was not altered by the presence of PMA (2–20 μM), although it is known that the presence of various factors (zinc, copper, NAD^+, and arachidonic acid), which can inhibit Ca^{2+}-ATPase activity in liver nuclei, causes a clear Ca^{2+} release from liver nuclei [13–16]. Presumably, the effect of PMA on the nuclear Ca^{2+}-ATPase and Ca^{2+} uptake was not based on nonspecific effects like alteration of nuclear membrane structure by high concentrations of PMA.

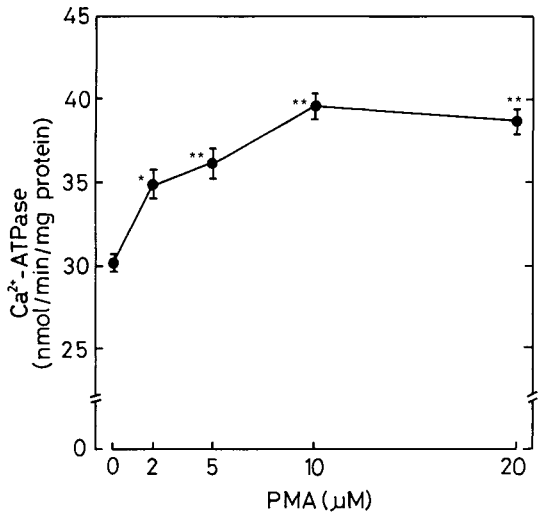

Fig. 1. Effect of phorbol 12-myristate 13-acetate (PMA) on Ca^{2+}-ATPase activity in rat liver nuclei. The enzyme activity was measured for 10 min in the reaction mixture containing the nuclei (400 μg protein/ml) and PMA (2, 5, 10, and 20 μM) in the presence of either 5 mM $MgCl_2$ or 5 mM $MgCl_2$ plus 40 μM $CaCl_2$. Ca^{2+}-ATPase activity was calculated by subtracting Mg^{2+}-ATPase activity from (Ca^{2+}-Mg^{2+})-ATPase activity. In the absence of PMA, Mg^{2+}-ATPase and (Ca^{2+}-Mg^{2+})-ATPase activities were 364.8 ± 8.9 and 400.9 ± 7.9 (nmol/min/mg protein), respectively. With 10 μM PMA, Mg^{2+}-ATPase and (Ca^{2+}-Mg^{2+})-ATPase activities were 365.6 ± 9.1 and 407.3 ± 8.0 (nmol/min/mg protein), respectively. Each value represents the mean ± SEM of five separate experiments using different preparations of liver nuclei. *$P < 0.05$ and **$P < 0.01$, as compared with the control value.

The effect of staurosporin or dibucaine on the PMA (10 μM)-increased Ca^{2+}-ATPase activity in rat liver nuclei is shown in Table II. Staurosporin or dibucaine is known to be an inhibitor of protein kinase [23,24]. These inhibitors had no effect on the PMA-increased Ca^{2+}-ATPase activity. Also, staurosporin (2 μM) and dibucaine (10 μM) were not altered by the basal activity of Ca^{2+}-ATPase.

The effect of vanadate on Ca^{2+}-ATPase activity in rat liver nuclei is shown in Table III. Vanadate can inhibit the phosphorylation of the enzyme by ATP [20,25]. The presence of vanadate (20 and 100 μM) caused a significant decrease in the nuclear Ca^{2+}-ATPase activity. Also, vanadate (20 and 100 μM) blocked clearly the PMA (10 μM)-induced increase in nuclear Ca^{2+}-ATPase activity.

TABLE I. Effect of PMA on Ca^{2+} Uptake and Release in Rat Liver Nuclei†

Treatment	Ca^{2+} uptake (nmol/mg protein)	Ca^{2+} release (nmol/mg protein)
Control	18.0 ± 0.6	0.8 ± 0.3
PMA (2 μM)	18.3 ± 0.9	1.0 ± 0.3
PMA (5 μM)	21.9 ± 0.6*	0.8 ± 0.3
PMA (10 μM)	22.8 ± 0.6*	1.0 ± 0.4
PMA (20 μM)	23.9 ± 1.0**	0.9 ± 0.3

†Ca^{2+} uptake and release were determined with a Ca^{2+} electrode. The Ca^{2+} uptake took 6 min after addition of 2.0 mM ATP in the presence of PMA. PMA was added into incubation medium at 6 min after 2.0 mM ATP addition, and then the Ca^{2+} release was measured for 10 min. Each value represents the mean ± SEM of five separate experiments using different preparations of liver nuclei.
*$P < 0.05$ compared to the control value.
**$P < 0.01$ compared to the control value.

TABLE II. Effect of Protein Kinase Inhibitors on PMA-Increased Ca^{2+}-ATPase Activity in Rat Liver Nuclei†

Treatment	Ca^{2+}-ATPase (nmol/min/mg protein)	% of control
Control	30.4 ± 1.0	100
PMA (10 μM)	38.7 ± 1.3*	127
Staurosporine (2 μM)	31.3 ± 1.2	103
PMA + staurosporine (2 μM)	36.7 ± 0.6*	121
Dibucaine (2 μM)	33.3 ± 0.9	110
PMA + dibucaine (2 μM)	38.6 ± 1.3*	127
Dibucaine (10 μM)	32.5 ± 1.1	107
PMA + dibucaine (10 μM)	37.9 ± 0.2*	125

†Ca^{2+}-ATPase activity was calculated by subtracting Mg^{2+}-ATPase activity from (Ca^{2+}-Mg^{2+})-ATPase activity. Each value represents the mean ± SEM of five separate experiments using a different preparation of liver nuclei.
*$P < 0.01$ compared to the control value.

TABLE III. Effect of Vanadate on PMA-Increased Ca^{2+}-ATPase Activity in Rat Liver Nuclei†

Treatment	Ca^{2+}-ATPase (nmol/min/mg protein)	% of control
Control	30.3 ± 0.9	100
PMA (10 μM)	40.0 ± 0.7*	132
Vanadate (20 μM)	24.3 ± 1.6*	80
PMA + vanadate (20 μM)	30.7 ± 1.2**	101
Vanadate (100 μM)	19.5 ± 1.1*	64
PMA + vanadate (100 μM)	24.5 ± 1.4**	81

†Ca^{2+}-ATPase activity was calculated by subtracting Mg^{2+}-ATPase activity from (Ca^{2+}-Mg^{2+})-ATPase activity. Each value represents the mean ± SEM of five separate experiments using a different preparation of liver nuclei.
*$P < 0.01$ compared to the control value.
**$P < 0.01$ compared with the PMA alone.

DISCUSSION

In recent years, there has been growing evidence that Ca^{2+} plays a role in liver nuclear function [3–7]. The existence of an ATP-stimulated Ca^{2+} sequestration system in rat liver nuclei that requires calmodulin and generates a net increase in nuclear matrix free Ca^{2+} concentration has been reported [4]. More recently, it has been demonstrated that Ca^{2+}-stimulated Mg^{2+}-ATPase (Ca^{2+}-ATPase) exists in the nuclei isolated from rat liver and that the nuclear Ca^{2+}-sequestering system is involved in Ca^{2+}-ATPase [16]. To clarify a regulating factor for the nuclear Ca^{2+}-ATPase, the effect of PMA, which can activate protein kinase C, on the Ca^{2+}-ATPase activity was investigated.

PMA increased significantly Ca^{2+}-ATPase activity in rat liver nuclei. This increase was seen at the concentration of PMA (2–20 μM), which activates protein kinase C [25]. However, the effect of PMA to increase the nuclear Ca^{2+}-ATPase activity was not altered appreciably by the presence of staurosporine or dibucaine, which is an inhibitor of protein kinase [23] or Ca^{2+}/calmodulin-dependent protein kinase [24]. This result indicates that PMA increases the nuclear Ca^{2+}-ATPase activity independent of protein kinase C. Also, staurosporine or dibucaine did not have an appreciable effect on the nuclear Ca^{2+}-ATPase in the absence of PMA, suggesting that Ca^{2+}-ATPase is not regulated by the effect of protein kinases. Now, it is unlikely that major quantities of this Ca^{2+}-ATPase activity originate from contamination of the microsomes besides the nuclei in liver, because ryanodine (10 μM) and thapsigargin (10 μM), which are known to have effects on Ca^{2+} transport in sarcoplasmic reticulum and liver microsomes [26,27], did not have an appreciable effect on liver nuclear Ca^{2+}-ATPase activity (unpublished observations). Also, ryanodine and thapsigargin (10 μM) did not have a significant effect on Ca^{2+} uptake and release in rat liver nuclei, as reported previously [28].

Vanadate can inhibit the phosphorylation of Ca^{2+}-ATPase by ATP in the microsomes of rat liver [20]. Previous investigation showed that vanadate (100 μM) can cause a significant inhibition of Ca^{2+} uptake by rat liver nuclei [13], although it was not reported whether vanadate can inhibit the nuclear Ca^{2+}-ATPase. In the present study, vanadate (20 and 100 μM) caused a significant decrease of Ca^{2+}-ATPase activity in rat liver nuclei. Moreover, PMA (10 μM) could not increase the nuclear Ca^{2+}-ATPase activity in the presence of vanadate. Presumably, PMA increases the nuclear Ca^{2+}-ATPase activity due to influence on the process of phosphorylation of the enzyme by ATP. Thus, PMA had an activating effect on Ca^{2+}-ATPase in rat liver nuclei. This may be useful as a pharmacological tool in the estimation of the Ca^{2+}-sequestering mechanism in liver nuclei.

In conclusion, it has been demonstrated that PMA can increase Ca^{2+}-ATPase activity independent of protein kinase C in rat liver nuclei. PMA may have an activating effect on Ca^{2+}-ATPase in the nuclei.

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