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Possible role of Na^+ influx in phorbol ester-induced down-regulation of protein kinase C in HL60 cells

Noriko Takeuchi[a], Eikichi Hashimoto[b], Toru Nakamura[a], Fumito Takeuchi[b], Kiyonao Sada[b] and Hirohei Yamamura[b,c]

[a]*Departments of Internal Medicine and* [b]*Biochemistry, Fukui Medical School, Matsuoka, Fukui, 910-11, Japan* and [c]*Biosignal Research Center, Kobe University, Kobe 657, Japan*

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Amiloride, an inhibitor of Na^+/H^+ exchange, inhibited down-regulation of protein kinase C in HL60 cells induced by tumor-promoting phorbol ester in dose-dependent manner judging from immunoblot analysis. This inhibition was observed with regard to type I (γ), type II (β), and type III (α) isozymes of protein kinase C. On the other hand, monensin, a Na^+ ionophore, accelerated the down-regulation of protein kinase C induced by phorbol ester. When we examined $^{22}Na^+$ uptake by HL60 cells, the higher uptake was observed after stimulation with phorbol ester compared to the control cells and this $^{22}Na^+$ uptake was strongly inhibited by the addition of amiloride. However, monensin further stimulated the $^{22}Na^+$ uptake observed in phorbol ester-treated cells. These data suggest that the increase in intracellular Na^+ concentration may be one of the triggers for the induction of down regulation of protein kinase C.

Protein kinase C; Down-regulation, Na^+/H^+ exchanger; HL60 cell

1. INTRODUCTION

It has been shown that ion channels such as Na^+/H^+ exchanger in plasma membrane are activated by various external signals such as hormones, growth factors and tumor-promoting phorbol esters and activation of these systems is involved in control of intracellular pH and Na^+ concentration [1–5]. In addition, it has been also proposed that intracellular Na^+ increase or pH elevation is important at the initial stage of cell growth [1,2].

On the other hand, it has been well established that protein kinase C (PKC) plays a central role in various metabolic regulations induced by receptor-mediated hydrolysis of inositol phospholipids [6]. It has been also established that PKC is distributed in various mammalian tissues, mainly in their cytosolic fraction [7,8]. When various cells are stimulated with tumor-promoting phorbol esters such as 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA), cytosolic PKC is translocated to plasma membrane and activated, then subjected to proteolytic attacks [9,10]. Moreover, it has been reported that rat liver PKC is activated and translocated to plasma membrane at the initial stage of cellular proliferation, such as regenerating rat liver [11,12].

In the previous reports from our laboratory, it was shown that protease-activated form of PKC (protein kinase M) was released from rat liver plasma membrane under slightly higher ionic strength than physiological level and alkaline pH [13,14]. So we studied the effects of some drugs which change intracellular Na^+ concentration on PKC down regulation using TPA-stimulated HL60 cells.

Correspondence address H. Yamamura, Departments of Biochemistry, Fukui Medical School, Matsuoka, Fukui, 910-11, Japan. Fax: (81) (776) 61-8102.

Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl-phorbol 13-acetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPBS, phosphate-buffered saline containing 0.05% Tween 20.

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

$^{22}NaCl$ (905 mCi/mg) and a monoclonal anti-PKC antibody designated MC-5 (code RPN. 536) were purchased from Amersham. RPMI 1640 culture medium and fetal bovine serum were purchased from Gibco. TPA, ouabain, amiloride, and monensin were from Sigma Monoclonal anti-type I (γ), anti-type II (β), and anti-type III (α) PKC antibodies designated MC-1a, MC-2a, and MC-3a, respectively, were from Seikagaku Kogyo Corp.

2.2. *Cell culture*

Human promyelocytic leukemia cell line HL60 cells were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum, 0.12 mg/ml kanamycin at 37°C in a humidified atmosphere containing 5% CO_2. Cells in the exponential growth phase were used. Down regulation of PKC was induced by addition of 400 nM TPA. Amiloride and monensin were added 30 min before initiation of each experiment except $^{22}Na^+$ uptake experiments. All assay mediums contained 1 mM ouabain to block Na^+/K^+ ATPase activity.

2.3. *Preparation of protein kinase C*

HL60 cells (1×10^8 cells) were harvested by centrifugation, and

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washed twice, then the cell pellet was resuspended in 1 ml of Buffer A (20 mM Tris-HCl at pH 7.5 containing 0.25 M sucrose, 1 mM ethylene glycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 0.4 mM leupeptin). The cells were homogenized by 60 strokes with a Dounce homogenizer. The homogenate was centrifuged for 10 min at $1,500 \times g$, and the supernatant was further centrifuged for 60 min at $100,000 \times g$. The supernatant was employed as the enzyme derived from the cytosolic fraction of HL60 cells. The pellet obtained by centrifugation at $100,000 \times g$ was resuspended in 1ml of Buffer A containing 1% (v/v) Triton X-100, and homogenized by 60 strokes with a Dounce homogenizer. The homogenate was stirred at 0°C for 30 min, then the solubilized sample was sonicated using a Tomy UD-200 ultrasonic disrupter (three 15-s bursts), and centrifuged for 40 min at $100,000 \times g$. The supernatant was employed as the enzyme derived from the particulate fraction of HL60 cells.

2.4. *Immunoblot analysis*

PKC prepared as described above were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [15]. Immunoblot analysis was carried out as described previously [16]. Nitrocellulose membrane was reacted with each antibody. The concentrations of the monoclonal antibodies were approximately 1 μg/ml in the presence of 1 mg/ml bovine serum albumin in phosphate-buffered saline containing 0.05% Tween 20 (TPBS). Mouse immunoglobulin was then reacted with biotin-conjugated rabbit anti-mouse immunoglobulins diluted 1 : 500 in TPBS and then with avidin-conjugated peroxidase diluted 1 : 500 in TPBS. Densitometoric analyses were performed using Scanning Densitometer GS 300 (Hoefer Scientific Instruments).

2.5. *$^{22}Na^+$ uptake experiments*

1 μCi/ml $^{22}Na^+$ was added to HL60 cells suspended at 1×10^7 cells/ml in RPMI 1640 medium supplemented with 15% fetal bovine serum and allowed to equilibrate for 15 min at 37°C. At various times after the addition of drugs (TPA, amiloride, monensin, and ouabain), 0.5 ml aliquots (5×10^6 cells) were removed and placed in 1.5 ml microfuge tubes containing 0.3 ml of denonylphthalate/silicon oil (1:1), and centrifuged at 12,000 rpm for 20 s [17]. The supernatants were removed and washed with 0.5 ml of cold phosphate-buffered saline. The cell pellets were harvested into 0.1 ml of 0.1 N NaOH and the radioactivities present in the pellets were directly counted for $^{22}Na^+$ using a gamma spectrometer (Aloka, ARC-2000).

2.6. *Protein determinations*

Protein was determined by the method of Bradford [18] with bovine serum albumin as a standard.

3. RESULTS

It has been well known that TPA induces down regulation of PKC [9,10]. As shown in Fig. 1A, TPA-induced degradation of PKC was observed within 60 min in both cytosolic and particulate fractions of HL60 cells. At 120 min after stimulation with TPA, PKC was faintly detected in cytosolic fraction, although it remained slightly in particulate fraction.

Amiloride, which is an inhibitor of Na^+/H^+ exchanger, is known to inhibit intracellular Na^+ influx and cytoplasmic alkalinization [19]. We examined the effect of amiloride on PKC down regulation promoted by TPA in HL60 cells. Fig. 1B shows that in the presence of amiloride, PKC was detected in both cytosolic and particulate fractions of HL60 cells until 120 min after the stimulation with TPA. This result indicates that amiloride inhibited PKC down regulation promoted by

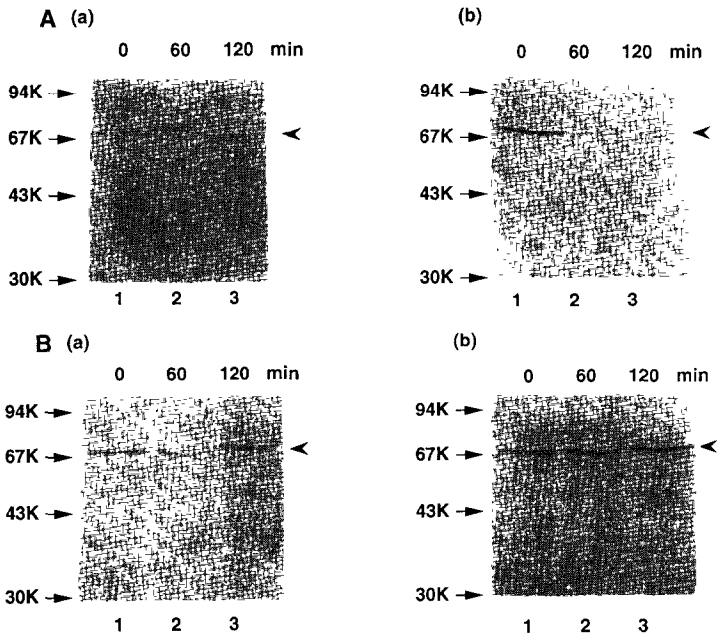

Fig. 1 Effect of amiloride on PKC down-regulation in HL60 cells. HL60 cells were incubated with 400 nM TPA for indicated times in the absence (A) or presence (B) of 0.1 mM amiloride. The samples derived from HL60 cells were prepared as described in section 2. (a) 100 μg of cytosolic proteins and (b) 40 μg of particulate proteins were employed for immunoblot analyses with anti-PKC antibody (MC-5). Numbers with arrows indicate the molecular mass markers (K, kDa), phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa). The arrowheads indicate the position of PKC. Incubation times of HL60 cells with TPA were as follows: lane 1, 0 min; lane 2, 60 min; lane 3, 120 min.

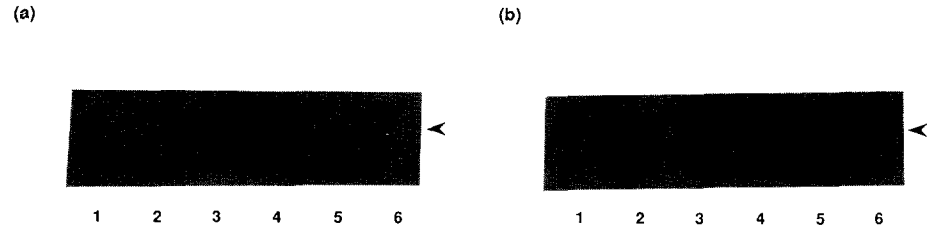

Fig. 2. Dose-dependent inhibitory effect of amiloride on PKC down-regulation in HL60 cells. HL60 cells were incubated with 400 nM TPA in the presence of various concentrations of amiloride for 60 min, then the samples derived from HL60 cells were prepared as described in section 2. (a) 100 μg of cytosolic proteins and (b) 40 μg of particulate proteins were employed for immunoblot analyses with anti-PKC antibody (MC-5) The arrowheads indicate the position of PKC. Lane 1, control cells. Amiloride concentrations were as follows: lane 2, 0 μM; lane 3, 1 μM; lane 4, 10 μM; lane 5, 100 μM; lane 6, 200 μM.

TPA. Fig. 2 shows that amiloride inhibited PKC down regulation in dose-dependent manner in both cytosolic and particulate fractions of HL60 cells. When we examined the effect of this drug on PKC isozymes using anti-type I (γ), anti-type II (β), and anti-type III (α) PKC antibodies, amiloride inhibited the down regulation of all of three PKC isozymes (Table I). So we supposed that this inhibitory effect of amiloride on PKC down-regulation might be caused by interfering with intracellular Na^+ influx or pH elevation in HL60 cells.

In the next experiments, we examined the effect of monensin, a Na^+ ionophore which can induce the elevation of intracellular Na^+ concentration, on PKC down-regulation promoted by TPA in HL60 cells. As shown in Fig. 3, in the presence of monensin PKC was not detected at 30 min after the stimulation by TPA in both cytosolic and particulate fractions of HL60 cells, although PKC was still detected at that time after the stimulation by TPA in the absence of monensin. This result suggests that monensin accelerated PKC down regulation promoted by TPA. PKC isozyme experiments also showed that monensin accelerated the down regulation of all of type I (γ), type II (β), and type III (α) PKC (data not shown). These results suggest that the increase in intracellular Na^+ concentration seems to be important for PKC down regulation.

In the last experiments, we examined the correlation between $^{22}Na^+$ uptake by HL60 cells and PKC down-regulation in the presence of various drugs employed above (Fig. 4). $^{22}Na^+$ uptake in phorbol ester-treated cells was constantly higher than that of control cells and the extent of stimulation in this $^{22}Na^+$ uptake was similar to that previously reported [4]. However, amiloride strongly inhibited the $^{22}Na^+$ uptake detected in phorbol ester-stimulated cells. In contrast, monensin further accelerated the $^{22}Na^+$ uptake observed with the treatment by phorbol ester. In correspondence to the changes in $^{22}Na^+$ uptake, PKC down-regulation induced by TPA was strongly inhibited by amiloride, but accelerated by monensin in both cytosolic and particulate fractions. These results strongly suggest that the down-regulation of PKC is correlated with the Na^+ influx.

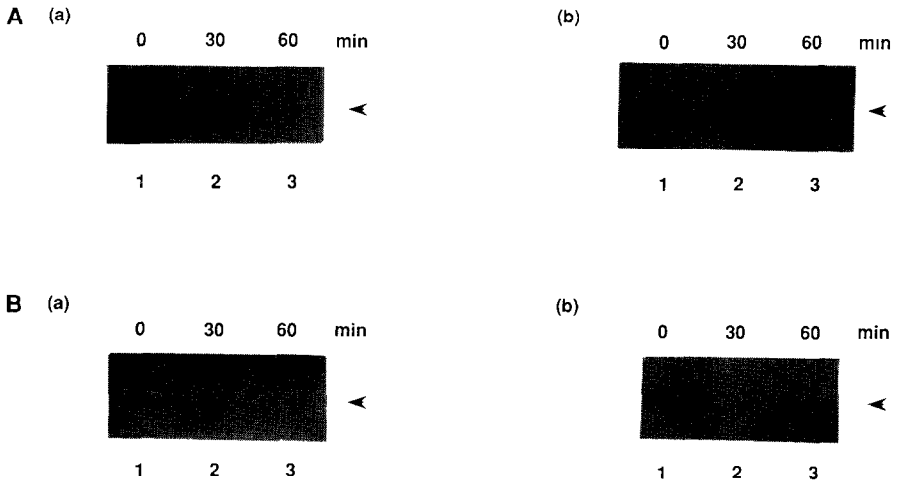

Fig. 3. Effect of monensin on PKC down-regulation in HL60 cells. HL60 cells were incubated with 400 nM TPA for indicated times in the absence (A) or presence (B) of 20 μg/ml of monensin, then the samples derived from HL60 cells were prepared as described in section 2. (a) 100 μg of cytosolic proteins and (b) 40 μg of particulate proteins were employed for immunoblot analyses with anti-PKC antibody (MC-5). The arrowheads indicate the position of PKC. Incubation times of HL60 cells with TPA were as follows: lane 1, 0 min; lane 2, 30 min; lane 3, 60 min.

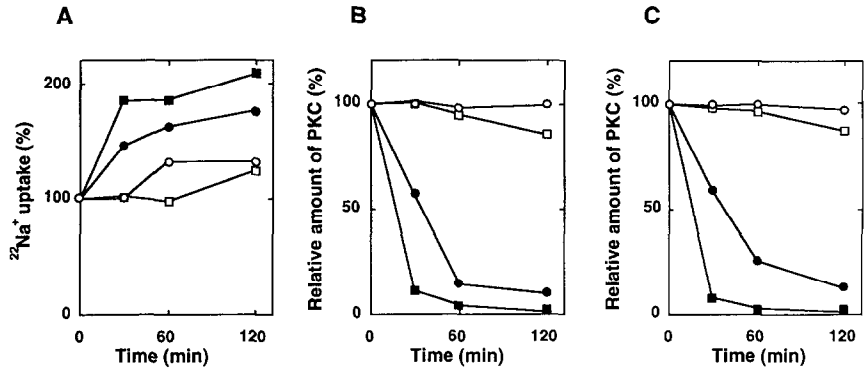

Fig. 4. Effect of amiloride and monensin on TPA-induced $^{22}Na^+$ uptake and PKC down-regulation in HL 60 cells. HL60 cells equilibrated with $^{22}Na^+$ were incubated with 400 nM TPA for indicated times in the absence or presence of 0.1 mM amiloride or 20 μg/ml monensin, then intracellular $^{22}Na^+$ was counted using a gamma-counter as described in section 2 (A). HL60 cells were also incubated with 400 nM TPA for indicated times in the absence or presence of 0.1 mM amiloride or 20 μg/ml monensin, then the samples derived from HL 60 cells were prepared as described in section 2. One-hundred μg of cytosolic proteins (B) and 40 μg of particulate proteins (C) were employed for immunoblot analyses with anti-PKC antibody (MC-5), then the PKC bands were subjected to densitometric analyses as described in section 2. The results represent percentages taking that at 0 time as 100%. A typical result from two independent experiments was presented. (○) None (control); (●) TPA; (□) TPA + amiloride; (■) TPA + monensin.

4. DISCUSSION

In this report, we showed that amiloride, an inhibitor of Na^+/H^+ exchanger, inhibited PKC down-regulation promoted by TPA in HL60 cells, but monensin, a Na^+ ionophore, accelerated it. Amiloride decreased intracellular $^{22}Na^+$concentration in TPA-treated HL60 cells, but monensin increased it. These results strongly suggest that the changes in intracellular Na^+ concentration are important for PKC down regulation. The PKC bands recognized by the monoclonal anti-PKC antibody (MC-5) were often shown as double bands. We supposed that some modifications such as phosphorylation changed their mobilities on SDS-PAGE as previously described [20].

Table I

Effect of amiloride on down-regulation of PKC isozymes in HL60 cells

Subtype	Amiloride	Fraction	Relative amount of PKC (%)		
			0 min	60 min	120 min
I	−	cytosolic	100	14	9
	−	particulate	100	28	23
	+	cytosolic	100	77	62
	+	particulate	100	81	85
II	−	cytosolic	100	18	32
	−	particulate	100	32	23
	+	cytosolic	100	78	65
	+	particulate	100	95	69
III	−	cytosolic	100	53	21
	−	particulate	100	56	29
	+	cytosolic	100	96	88
	+	particulate	100	87	72

HL60 cells were incubated with 400 nM TPA for indicated times in the absence or presence of 0.1 mM amiloride. The samples derived from HL60 cells were prepared as described in section 2. One-hundred μg of cytosolic proteins and 40 μg of particulate proteins were employed for immunoblot analyses with anti-type I (γ), anti-type II (β), and anti-type III (α) PKC antibodies then the PKC bands were subjected to densitometric analyses as described in section 2. The results represent relative amounts of PKC taking that at 0 min in each experiments as 100%. A typical result from two independent experiments was presented.

It has been well known that PKC is activated by receptor-mediated hydrolysis of inositol phospholipids induced by various external signals such as hormones, neurotransmitters and some growth factors and plays important roles in controlling many cellular functions and proliferation [6]. It has been also shown that Na^+/H^+ exchanger is activated by various external signals such as hormones, growth factors and TPA [1–5]. There have been many reports suggesting that PKC phosphorylates and activates Na^+/H^+ exchanger in plasma membrane of various mammalian cells and these events cause increase in intracellular Na^+ concentration and cytoplasmic alkalinization [4,5,21]. This increase in intracellular Na^+ concentration and alkalinization are thought to be important at the initial stage of cell growth but their physiological meanings are still unknown [1,2].

We suppose that this increase in intracellular Na^+ concentration and alkalinization may affect the proteolytic degradation of PKC. Indeed, our previous reports indicated that PKC was easily subjected to proteolytic attacks under slightly higher ionic strength than physiological level and alkaline pH in vitro [13,14]. In this study, we presented the evidence suggesting that the increase in intracellular Na^+ concentration may induce PKC down regulation in HL60 cells. Further studies seem to be necessary for full understanding the role of

activation of Na^+/H^+ exchanger in the process of cell proliferation including the mechanism of down regulation of PKC.

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