

# Possible role of $\text{Na}^+$ influx in phorbol ester-induced down-regulation of protein kinase C in HL60 cells

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Amiloride, an inhibitor of  $\text{Na}^+/\text{H}^+$  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 ( $\gamma$ ), type II ( $\beta$ ), and type III ( $\alpha$ ) isozymes of protein kinase C. On the other hand, monensin, a  $\text{Na}^+$  ionophore, accelerated the down-regulation of protein kinase C induced by phorbol ester. When we examined  $^{22}\text{Na}^+$  uptake by HL60 cells, the higher uptake was observed after stimulation with phorbol ester compared to the control cells and this  $^{22}\text{Na}^+$  uptake was strongly inhibited by the addition of amiloride. However, monensin further stimulated the  $^{22}\text{Na}^+$  uptake observed in phorbol ester-treated cells. These data suggest that the increase in intracellular  $\text{Na}^+$  concentration may be one of the triggers for the induction of down regulation of protein kinase C.

Protein kinase C; Down-regulation,  $\text{Na}^+/\text{H}^+$  exchanger; HL60 cell

## 1. INTRODUCTION

It has been shown that ion channels such as  $\text{Na}^+/\text{H}^+$  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  $\text{Na}^+$  concentration [1–5]. In addition, it has been also proposed that intracellular  $\text{Na}^+$  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 mem-

brane 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  $\text{Na}^+$  concentration on PKC down regulation using TPA-stimulated HL60 cells.

## 2. MATERIALS AND METHODS

### 2.1. Materials and chemicals

$^{22}\text{NaCl}$  (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 ( $\gamma$ ), anti-type II ( $\beta$ ), and anti-type III ( $\alpha$ ) PKC antibodies designated MC-1a, MC-2a, and MC-3a, respectively, were from Scikagaku 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%  $\text{CO}_2$ . 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  $^{22}\text{Na}^+$  uptake experiments. All assay mediums contained 1 mM ouabain to block  $\text{Na}^+/\text{K}^+$  ATPase activity.

### 2.3. Preparation of protein kinase C

HL60 cells ( $1 \times 10^8$  cells) were harvested by centrifugation, and

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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.

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( $\beta$ -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 1 ml of Buffer A containing 1% (v/v) Triton X-100, and homogenized by 60 strokes with a Dounce homogenizer. The homogenate was stirred at  $0^\circ\text{C}$  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  $1 \mu\text{g}/\text{ml}$  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. Densitometric analyses were performed using Scanning Densitometer GS 300 (Hoefer Scientific Instruments).

#### 2.5. $^{22}\text{Na}^+$ uptake experiments

$1 \mu\text{Ci}/\text{ml}$   $^{22}\text{Na}^+$  was added to HL60 cells suspended at  $1 \times 10^7$  cells/ml in RPMI 1640 medium supplemented with 15% fetal bovine serum and allowed to equilibrate for 15 min at  $37^\circ\text{C}$ . At various times after

the addition of drugs (TPA, amiloride, monensin, and ouabain), 0.5 ml aliquots ( $5 \times 10^6$  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  $^{22}\text{Na}^+$  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  $\text{Na}^+/\text{H}^+$  exchanger, is known to inhibit intracellular  $\text{Na}^+$  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

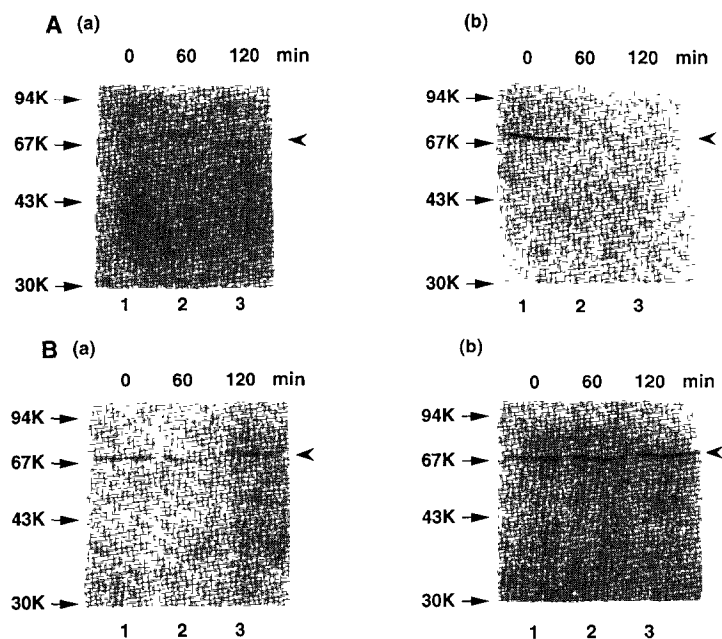


Fig. 1 Effect of amiloride on PKC down-regulation in HL60 cells. HL60 cells were incubated with 400 nM TPA for indicated times in the absence (A) or presence (B) of 0.1 mM amiloride. The samples derived from HL60 cells were prepared as described in section 2. (a) 100  $\mu\text{g}$  of cytosolic proteins and (b) 40  $\mu\text{g}$  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.

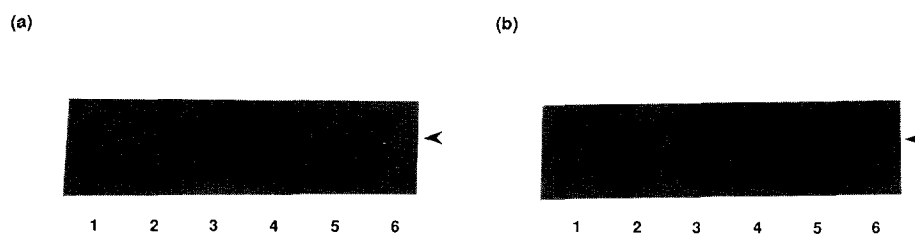


Fig. 2. Dose-dependent inhibitory effect of amiloride on PKC down-regulation in HL60 cells. HL60 cells were incubated with 400 nM TPA in the presence of various concentrations of amiloride for 60 min, then the samples derived from HL60 cells were prepared as described in section 2. (a) 100  $\mu$ g of cytosolic proteins and (b) 40  $\mu$ g 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  $\mu$ M; lane 3, 1  $\mu$ M; lane 4, 10  $\mu$ M; lane 5, 100  $\mu$ M; lane 6, 200  $\mu$ M.

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 ( $\gamma$ ), anti-type II ( $\beta$ ), and anti-type III ( $\alpha$ ) 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  $\text{Na}^+$  influx or pH elevation in HL60 cells.

In the next experiments, we examined the effect of monensin, a  $\text{Na}^+$  ionophore which can induce the elevation of intracellular  $\text{Na}^+$  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 experi-

ments also showed that monensin accelerated the down-regulation of all of type I ( $\gamma$ ), type II ( $\beta$ ), and type III ( $\alpha$ ) PKC (data not shown). These results suggest that the increase in intracellular  $\text{Na}^+$  concentration seems to be important for PKC down-regulation.

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

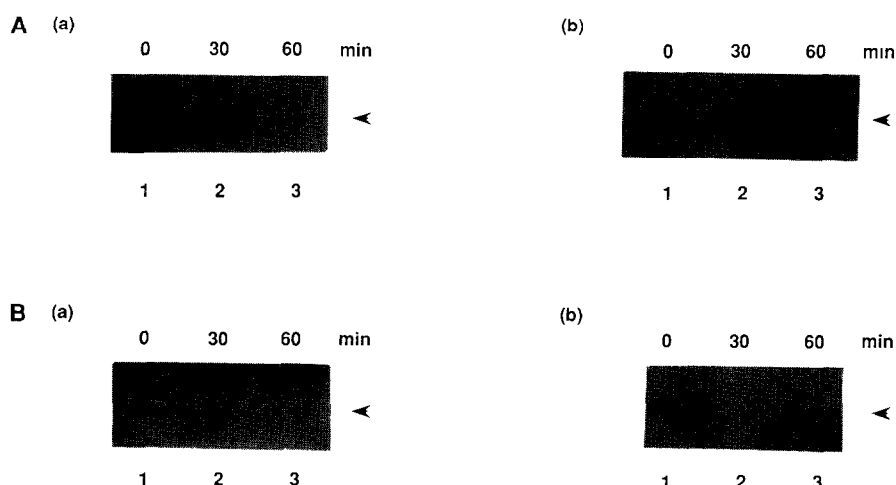


Fig. 3. Effect of monensin on PKC down-regulation in HL60 cells. HL60 cells were incubated with 400 nM TPA for indicated times in the absence (A) or presence (B) of 20  $\mu$ g/ml of monensin, then the samples derived from HL60 cells were prepared as described in section 2. (a) 100  $\mu$ g of cytosolic proteins and (b) 40  $\mu$ g 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.

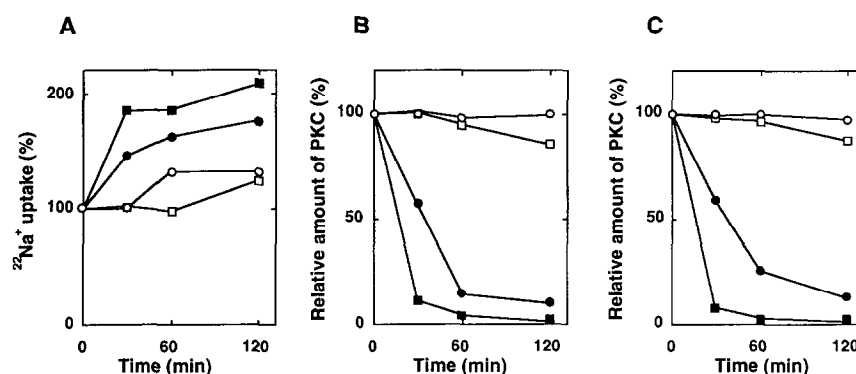


Fig. 4. Effect of amiloride and monensin on TPA-induced  $^{22}\text{Na}^+$  uptake and PKC down-regulation in HL 60 cells. HL60 cells equilibrated with  $^{22}\text{Na}^+$  were incubated with 400 nM TPA for indicated times in the absence or presence of 0.1 mM amiloride or 20  $\mu\text{g}/\text{ml}$  monensin, then intracellular  $^{22}\text{Na}^+$  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  $\mu\text{g}/\text{ml}$  monensin, then the samples derived from HL 60 cells were prepared as described in section 2. One-hundred  $\mu\text{g}$  of cytosolic proteins (B) and 40  $\mu\text{g}$  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  $\text{Na}^+/\text{H}^+$  exchanger, inhibited PKC down-regulation promoted by TPA in HL60 cells, but monensin, a  $\text{Na}^+$  ionophore, accelerated it. Amiloride decreased intracellular  $^{22}\text{Na}^+$  concentration in TPA-treated HL60 cells,

but monensin increased it. These results strongly suggest that the changes in intracellular  $\text{Na}^+$  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].

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  $\text{Na}^+/\text{H}^+$  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  $\text{Na}^+/\text{H}^+$  exchanger in plasma membrane of various mammalian cells and these events cause increase in intracellular  $\text{Na}^+$  concentration and cytoplasmic alkalinization [4,5,21]. This increase in intracellular  $\text{Na}^+$  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  $\text{Na}^+$  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  $\text{Na}^+$  concentration may induce PKC down regulation in HL60 cells. Further studies seem to be necessary for full understanding the role of

Table 1

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  $\mu\text{g}$  of cytosolic proteins and 40  $\mu\text{g}$  of particulate proteins were employed for immunoblot analyses with anti-type I ( $\gamma$ ), anti-type II ( $\beta$ ), and anti-type III ( $\alpha$ ) 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.

activation of  $\text{Na}^+/\text{H}^+$  exchanger in the process of cell proliferation including the mechanism of down regulation of PKC.

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## REFERENCES

- [1] Schuldiner, S. and Rozengurt, E. (1982) *Proc. Natl. Acad. Sci. USA* 79, 7778–7782.
- [2] Moolenaar, W.H., Yarden, Y., de Laat, S.W. and Schlessinger, J. (1982) *J. Biol. Chem.* 257, 8502–8506.
- [3] Cassel, D., Rothenberg, P., Zhuang, Y.-X., Deuel, T.F. and Glaser, L. (1983) *Proc. Natl. Acad. Sci. USA* 80, 6224–6228.
- [4] Rosoff, P.M., Stein, L.F. and Cantley, L.C. (1984) *J. Biol. Chem.* 259, 7056–7060.
- [5] Grinstein, S., Cohen, S., Goetz, J.D., Rothstein, A. and Gelfand, E.W. (1985) *Proc. Natl. Acad. Sci. USA* 82, 1429–1433.
- [6] Nishizuka, Y. (1988) *Nature* 334, 661–665.
- [7] Kuo, J.F., Anderson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, I., Brackett, N.L., Katoh, N., Shoji, M. and Wrenn, R.W. (1980) *Proc. Natl. Acad. Sci. USA* 77, 7039–7043.
- [8] Kikkawa U., Takai, Y., Minakuchi, R., Inohara, S. and Nishizuka, Y. (1982) *J. Biol. Chem.* 257, 13341–13348.
- [9] Rodriguez-Pena, A. and Rozengurt, E. (1984) *Biochem. Biophys. Res. Commun.* 120, 1053–1059.
- [10] Nishizuka, Y. (1989) *Cancer* 63, 1892–1903.
- [11] Buckley, A.R., Putnam, C.W., Evans, R., Laird II, H.E., Shah, G.N., Montgomery, D.W. and Russel, D.H. (1987) *Life Sci.* 41, 2827–2834.
- [12] Okamoto, Y., Nishimura, K., Nakayama, M., Nakagawa, M. and Nakano, H. (1988) *Biochem. Biophys. Res. Commun.* 151, 1144–1149.
- [13] Hashimoto, E., Sakanoue, Y., Mizuta, K. and Yamamura, H. (1986) *FEBS Lett.* 200, 63–66.
- [14] Hashimoto, E. and Yamamura, H. (1989) *J. Biochem.* 106, 1041–1048.
- [15] Laemmli, U.K. (1970) *Nature* 227, 680–685.
- [16] Nakamura, S. and Yamamura, H. (1989) *J. Biol. Chem.* 264, 7089–7091.
- [17] Smith, R.L., Macara, I.G., Levenson, R., Housman, D. and Cantley, L. (1982) *J. Biol. Chem.* 257, 773–780.
- [18] Bradford, M.M. (1976) *Ann. Biochem.* 72, 248–254.
- [19] Moolenaar, W.H., Boonstra, J., van der Saag, P.T. and de Laat, S.W. (1981) *J. Biol. Chem.* 256, 12883–12887.
- [20] Woodgett, J.R. and Hunter, T. (1987) *Mol. Cell. Biol.* 7, 85–96.
- [21] Moolenaar, W.H., Tertoolen, L.G.J. and de Laat, S.W. (1984) *Nature* 312, 371–374.