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The inhibitory effects of okadaic acid on platelet function

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Okadaic acid (OA), a potent inhibitor of protein phosphatases type 1 and type 2A, inhibited thrombin-induced platelet aggregation (IC_{50} = 0.8 μM), [^{14}C]serotonin release and increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in the same dose dependence. In the absence of thrombin OA increased the phosphorylation of 50-kDa protein and 20-kDa myosin light chain (MLC20). The 50-kDa protein phosphorylation was accomplished within a shorter time period and at a lower concentration than was the MLC20. OA decreased the thrombin-induced phosphorylation of 47-kDa protein and MLC20, although phosphorylation of MLC20 reincreased at higher concentrations of OA (5–10 μM). Since type 2A phosphatase is more sensitive to OA than type 1, these results suggest that type 2A phosphatases are involved in the regulation of Ca^{2+} signaling in thrombin-induced platelet activation.

Platelet; Phosphatase; Okadaic acid; Myosin light chain

1. INTRODUCTION

Okadaic acid (OA), a potent inhibitor of protein phosphatases type 1 and type 2A [1], is a polyether derivative of a C_{38}-fatty acid which was originally isolated from the marine sponges of the genus *Halichondria okadai* [2]. It is also a potent tumor promoter that is not an activator of protein kinase C [3]. OA inhibits type 2A phosphatase at significantly lower concentrations than type 1 phosphatase; therefore, it serves as a useful probe to investigate the identity of phosphatases responsible for the regulation in vivo. It has been established that OA does not affect enzymes such as protein kinase C, Ca^{2+}/calmodulin-dependent protein kinases, cAMP-dependent protein kinase and inositol 1,4,5-triphosphatase [4,5].

Although activation of platelets leads to the phosphorylation of 47-kDa protein (pleckstrin) and 20-kDa myosin light chain (MLC20), which are mediated by activation of protein kinase C (PKC) and myosin light chain kinase (MLCK), respectively [6,7], little is known about the identity of platelet protein phosphatases or their involvement in controlling platelet function.

In this study, we studied the effects of OA on platelet activation, especially that induced by thrombin. The relationship between the change of phosphorylation with OA exposure and inhibition of platelet function is also discussed.

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2. MATERIALS AND METHODS

2.1. *Materials*

Okadaic acid (OA) was purchased from Wako Pharmaceutical Laboratory (Osaka, Japan). OA was stocked at a concentration of 1 mM in ethanol at −20°C. α-Thrombin was obtained from Mochida Pharmaceutical Ltd. (Tokyo, Japan). [^{32}P]Phosphoric acid was purchased from Amersham Japan (Tokyo, Japan).

2.2. *Platelet function experiments*

Studies of platelet aggregation and serotonin release [8], LDH release [8] and phosphorylation [9] were performed as previously described. $[Ca^{2+}]_i$ increase was measured on CAF 100 (Japan Spectroscopic Co. Ltd., Tokyo, Japan), as reported previously [10].

2.3. *Others*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [11]. The radioactive platelets were directly subjected to SDS-PAGE, stained, dried on filter paper and then exposed to a Kodak X-Omat film. The relative intensity of each band was quantitated by measuring the absorbance at 430 nm using a Shimazu dual-wavelength chromatogram scanner, model CS-910.

3. RESULTS

3.1. *The effect of OA on platelet aggregation*

As shown in Fig. 1, OA is a potent inhibitor of platelet aggregation. Platelet aggregation induced by four agonists (ADP, epinephrine, collagen and thrombin) was inhibited by OA in a dose-dependent manner. Incubation time of platelets with OA was also critical for this inhibition. In the presence of 0.1 μM OA the inhibition was not apparent even with 60-min incubation except in epinephrine. In contrast, about 40% inhibition of the aggregation induced by these agonists was observed even with 2-min incubation in the presence of 10 μM

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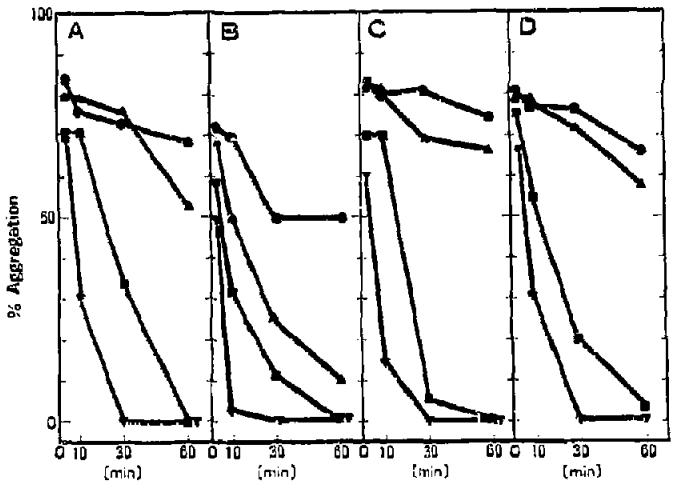

Fig. 1. Inhibition of platelet aggregation by okadaic acid (OA). (A) ADP (10 μM); (B) epinephrine (10 μM); (C) collagen (2 μg/ml); (D) thrombin (0.1 U/ml). PRP (A, B, C) or washed platelets (D) were preincubated with OA for 2 min, 10 min, 30 min or 60 min before the addition of agonists. Three different concentrations (0.1 μM, 0.4 μM and 1 μM) of OA were used. Each plot represents percent maximum aggregation (mean of three experiments). ●—●, control (buffer alone); ▲—▲, 0.1 μM OA; ■—■, 0.4 μM OA; ▼—▼, 1 μM OA.

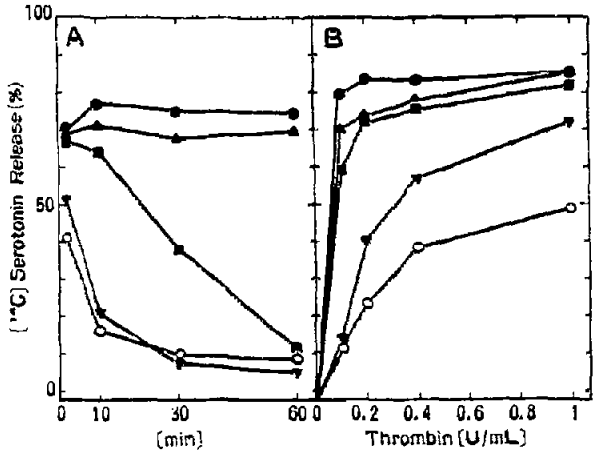

Fig. 2. Inhibition of thrombin-induced [^{14}C]serotonin release by okadaic acid (OA). (A) Washed platelets were preincubated with various concentrations of OA for 2 min, 10 min, 30 min or 60 min and then further incubated with thrombin (0.1 U/ml) for 1 additional min. Each spot represents percent serotonin release (mean of three experiments). ●—●, control (buffer alone); ▲—▲, 0.1 μM OA; ■—■, 0.4 μM OA; ▼—▼, 1 μM OA; ○—○, 5 μM OA. (B) Washed platelets were preincubated with OA for 10 min and then further incubated with various concentrations of thrombin for 1 additional min. ●—●, control (buffer alone); ▲—▲, 0.1 μM OA; ■—■, 0.4 μM OA; ▼—▼, 1 μM OA; ○—○, 5 μM OA.

OA (not shown). No apparent release of LDH was detected with 60-min incubation in the presence of 10 μM OA. IC_{50} of aggregation with 10-min incubation was about 0.5 μM, 0.5 μM, 0.7 μM or 0.8 μM OA for ADP (10 μM), epinephrine (10 μM), collagen (2 μg/ml) or thrombin (0.1 U/ml), respectively. Moreover, the inhibitory effects of OA on these aggregations were diminished with increasing concentrations of agonists (not shown).

3.2. *The effect of OA on thrombin-induced platelet activation*

Fig. 2A shows the inhibition of thrombin-induced [^{14}C]serotonin release by OA. OA inhibited serotonin release in a dose- and time-dependent manner. As shown in Fig. 2B, the inhibition was decreased with an increase in the concentration of thrombin. OA also inhibited thrombin-induced increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Fig. 3A). The basal level of $[Ca^{2+}]_i$ was lower in the presence of OA (over 1 μM) than in the control. As shown in Fig. 3B, inhibition was observed in both the presence and absence of extracellular Ca^{2+} (in the presence of 2 mM EGTA), suggesting that OA may inhibit both Ca^{2+} influx and Ca^{2+} release from the intracellular Ca^{2+} pool. The extent of inhibition was greater in the presence of extracellular Ca^{2+} (1 mM) than in the absence of Ca^{2+}. Fig. 3C shows the dose and time dependence of the inhibition of $[Ca^{2+}]_i$ increase by thrombin (0.1 U/ml) in the presence and absence of extracellular Ca^{2+}. Platelets incubated with 1 μM OA for 30 min revealed almost complete inhibition of $[Ca^{2+}]_i$ increase in both conditions. The dependence was similar to those for platelet aggregation and serotonin release (see Figs. 1D and 2A).

3.3. *OA induced phosphorylation of platelet protein*

To investigate the relation between the inhibitory effects of OA on platelet function and protein phosphorylation, the effect of OA on platelet protein phosphorylation was examined. ^{32}P-labelled platelets were exposed to OA, and the phosphorylated proteins were analyzed by SDS-PAGE followed by autoradiography. Upon the addition of 1 μM OA for 10 min, several phosphorylated bands were observed; most notable was the prominent increase in 50-kDa and 20-kDa proteins. Fig. 4A shows the time course of phosphorylation of both proteins. The phosphorylation of 50-kDa protein in the presence of OA (1 μM) was increased faster than that of 20-kDa protein, and the phosphorylation level reached a plateau over a 20-min incubation time. In contrast, the phosphorylation of 20-kDa protein in the presence of OA (1 μM) increased much slower and did not reach maximum even at 60 min incubation time.

Fig. 4B shows the extent of phosphorylation of both proteins as a function of OA concentration (see also inset in Fig. 4B). The phosphorylation of 50-kDa protein became apparent over 0.4 μM OA and almost reached a plateau over 1 μM OA, while higher OA concentration was required for the phosphorylation of 20-kDa protein. The 20-kDa protein was immunoprecipitated by rabbit anti-human platelet myosin antibody; therefore, this was concluded to be the 20-kDa myosin light chain (MLC20) (not shown). In contrast,

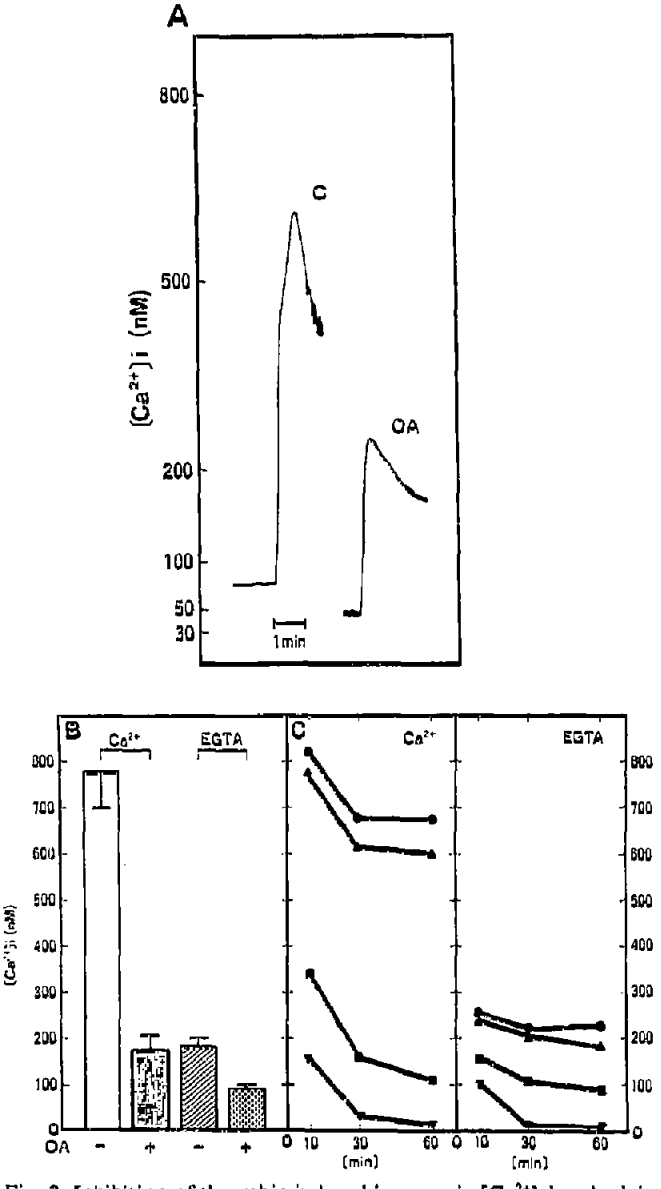

Fig. 3. Inhibition of thrombin-induced increase in $[Ca^{2+}]_i$ by okadaic acid (OA). (A) Thrombin (0.1 U/ml)-induced increase in $[Ca^{2+}]_i$. Control (left); platelets pretreated with OA (5 μM) for 10 min (right). (B) Increase in $[Ca^{2+}]_i$ was monitored in the presence of 1 mM Ca^{2+} or 2 mM EGTA. Washed platelets were preincubated with or without 1 μM OA for 10 min and thrombin (0.1 U/ml) was added. Each value is mean ± SD of five experiments. (C) Washed platelets were preincubated with OA for 10 min, 30 min, or 60 min and increase in $[Ca^{2+}]_i$ was monitored in the presence of 1 mM Ca^{2+} or 2 mM EGTA. Each plot is a mean of three experiments. ●—●, control (buffer alone); ▲—▲, 0.1 μM OA; ■—■, 0.4 μM OA; ▼—▼, 1 μM OA.

the phosphorylation of 47-kDa protein (pleckstrin), which is known to be a substrate of protein kinase C, was not increased in the presence of OA (see lane 8 of inset in Fig. 4B). The phosphorylation of these two proteins was independent of external Ca^{2+}, because the levels of phosphorylation were the same even in the presence of 2 mM EGTA (not shown).

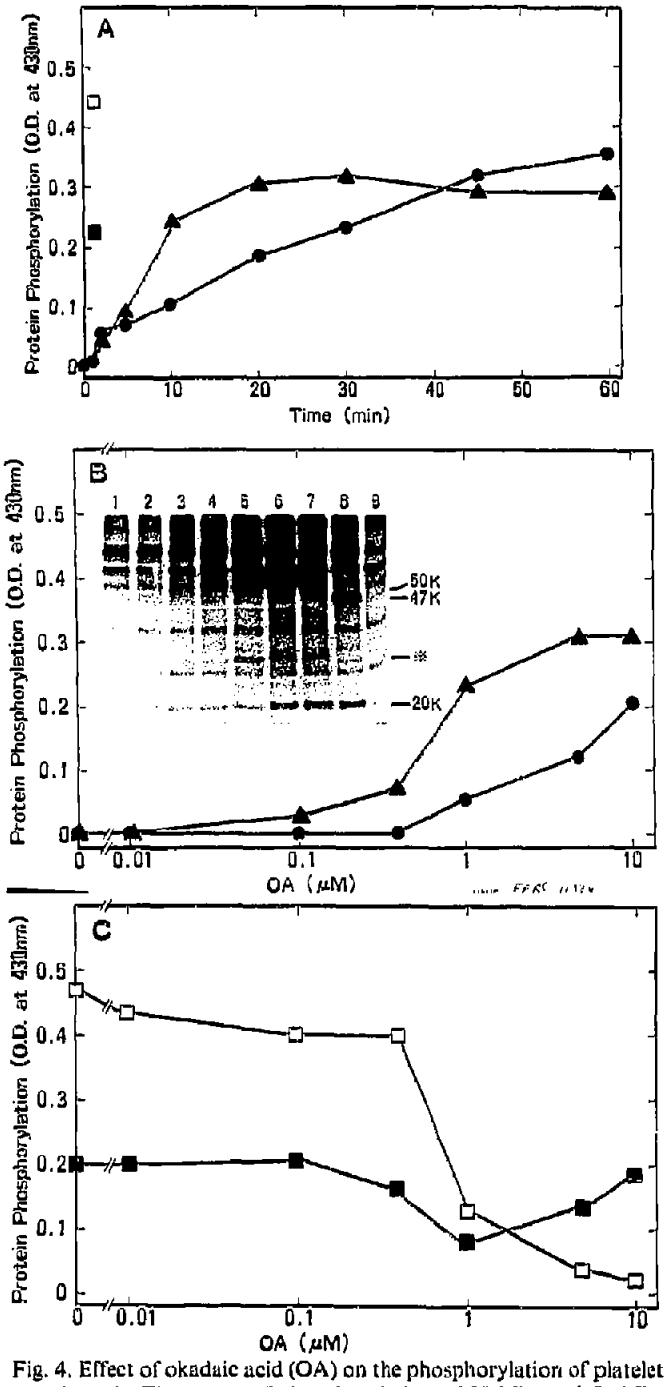

Fig. 4. Effect of okadaic acid (OA) on the phosphorylation of platelet proteins. (A) The extent of phosphorylation of 20-kDa and 50-kDa proteins with various incubation times of OA (1 μM). ▲—▲, 50-kDa protein; ●—●, 20-kDa protein. □ and ■, 47-kDa and 20-kDa proteins phosphorylated by thrombin (0.1 U/ml), respectively. (B) The extent of phosphorylation of 20-kDa and 50-kDa proteins with various concentrations of OA. The incubation time was 10 min. ▲—▲, 50-kDa protein; ●—●, 20-kDa protein. Inset shows autoradiogram. (Lane 1 and 9) buffer alone; (lane 2) 0.01 μM OA; (lane 3) 0.1 μM OA; (lane 4) 0.4 μM OA; (lane 5) 1 μM OA; (lane 6) 5 μM OA; (lane 7) 10 μM OA; (lane 8) 0.1 U/ml thrombin for 1 min. The band of 27-kDa protein (*) was also detected. (C) Inhibition of thrombin-induced 47-kDa protein phosphorylation by OA. Platelets were preincubated with various concentrations of OA for 10 min and further incubated with thrombin (0.1 U/ml) for 1 more min. □—□, 47-kDa protein; ■—■, 20-kDa protein.

Pretreatment with OA led to the inhibition of phosphorylation of 20-kDa and 47-kDa proteins induced by thrombin. As shown in Fig. 4C, phosphorylation of 47-kDa protein by thrombin (0.1 U/ml) was inhibited intensively with exposure of over 1 μM OA for 10 min in a dose-dependent manner. Moreover, the inhibitory effects of OA on this phosphorylation were diminished with increased concentration of thrombin (not shown). The phosphorylation of MLC20 was also decreased with exposure of OA, but at higher concentrations of OA (5–10 μM) the phosphorylation reincreased.

4. DISCUSSION

Although some phosphatases of human platelets, including type 1 and type 2A, have been partially purified [12,13], the role of phosphatases in the platelet function remains unknown. The goal of this study is to shed light on the role of phosphatases in the platelet activation. The probe we employed is okadaic acid (OA), a potent inhibitor of phosphatases type 1 and type 2A [1]. It is known that OA inhibits type 2A phosphatase at relatively lower concentration and type 1 at relatively higher concentration [14].

OA inhibited platelet aggregation induced by four agonists: ADP, epinephrine, collagen and thrombin (Fig. 1), as well as serotonin release and increase in $[Ca^{2+}]_i$ induced by thrombin (Figs. 2 and 3). For thrombin, these inhibitions were observed in the same dose- and time-dependent manner.

Hence we examined the change in the phosphorylation of intrinsic platelet proteins induced by OA exposure. OA predominantly induced the phosphorylation of proteins at two molecular weight regions, 50-kDa and 20-kDa (MLC20) (Fig. 4A). While this study was in process, Lorea reported [15] that the 50-kDa band is composed of three basic polypeptides (pI 6.5–7.0), although the function of these proteins is unknown.

The increase in the phosphorylation of the 50-kDa protein reached maximum within 20 min while the phosphorylation of the MLC20 kept increasing even after 60 min of preincubation with 1 μM OA (Fig. 4A). This suggests that a great deal of time is required for OA to penetrate the membranes as shown by Klumpp et al. [16], and that higher OA may be required for the phosphorylation of the MLC20 than for that of the 50-kDa protein. We recently suggested that the phosphatase which dephosphorylated smooth muscle myosin is type 1-like phosphatase and is inhibited by OA at relatively high concentration [17], and that platelet myosin has a functional property similar to that of smooth muscle myosin [18]. Therefore, it is understandable that the phosphorylation of platelet MLC20 was observed with exposure to higher concentration of OA (1–10 μM) (Fig. 4B). Because the free calmodulin concentration is thought to be greater than 10 μM [19] and the K_d of MLCK for Ca^{2+}/calmodulin is less than 1 nM [20], if type 1 phosphatase is inhibited by OA, there is enough MLCK activity to phosphorylate MLC20 even in the resting state: $[Ca^{2+}]_i$ is $0.5–1 \times 10^{-7}$ M.

On the other hand, the increase in the phosphorylation of the 50-kDa protein may be due to the inhibition of type 2A phosphatases, which are more sensitive to OA, since the increase in the phosphorylation was accomplished within a shorter time period (Fig. 4A) and at a lower concentration than in the case of the MLC20 (Fig. 4B). However, it is still not clear whether type 2A phosphatase dephosphorylates the 50-kDa protein and therefore OA treatment increases this phosphorylation, or whether type 2A phosphatase inactivates putative protein kinase, which phosphorylates the 50-kDa proteins. The kinase responsible for the phosphorylation of the 50-kDa protein is unlikely to be Ca^{2+}-activated kinase since $[Ca^{2+}]_i$ was reduced when the cells were treated with OA alone (Fig. 3A).

It has been known that the MLC20 and 47-kDa protein (pleckstrin) are phosphorylated during the platelet activation induced by thrombin [6,7]. In this study, we found that OA inhibited thrombin-induced platelet aggregation, serotonin release and increase in $[Ca^{2+}]_i$, and that the phosphorylation of both proteins decreased with preincubation with OA (higher than 1 μM) for 10 min (Fig. 4C). These effects of OA are probably due to the inhibition of $[Ca^{2+}]_i$ increase, since it is known that the phosphorylations of both proteins are Ca^{2+}-dependent and that the increase of $[Ca^{2+}]_i$ is prerequisite for the platelet activation. OA inhibited the increase in $[Ca^{2+}]_i$ both in the presence and absence of the extracellular Ca^{2+} (Fig. 3), therefore, the inhibition seems to be attributed both to Ca^{2+} mobilization from extracellular space and intracellular store; that is, the inhibitory effect of OA on $[Ca^{2+}]_i$ increase may be due to the modulation of Ca^{2+} channels (surface membrane Ca^{2+} channels and endoplasmic reticulum Ca^{2+} channels). However, we cannot neglect the possibility that OA inhibits the phosphoinositide-signaling cascade which decreases the production of inositol triphosphate. The phosphatases responsible for the change in Ca^{2+} signaling are likely to be type 2A phosphatases since the modulation of Ca^{2+} mobilization occurred at the lower OA concentration than that required for the increase in the 20-kDa phosphorylation which is due to the inhibition of type 1 phosphatases. At higher concentrations of OA, phosphorylation of MLC20 by thrombin re-increased (Fig. 4C), although $[Ca^{2+}]_i$ was decreased (Fig. 3C). This can be explained by the fact that type 1-like phosphatase, which is responsible for the dephosphorylation of MLC20, is inhibited at relatively higher OA concentrations. On the other hand, the phosphorylation of 47-kDa protein was not increased even at higher OA concentrations, suggesting that the phosphatase responsible for the dephosphorylation of the 47-kDa protein is neither type 1 nor type 2A.

It is possible that the 50-kDa protein is involved in

the regulation of platelet activation either directly or via changing $[Ca^{2+}]_i$, since the inhibition of platelet activation by OA treatment and 50-kDa protein phosphorylation occurred at the same dose and with the same time dependence (Figs. 1, 2, 3 and 4). However, further studies are required to determine whether or not the phosphorylation of the 50-kDa proteins is involved in the regulation of platelet function.

It is still not clear how resting $[Ca^{2+}]_i$ decreases with OA treatment (Fig. 3A). However, modification of Ca^{2+} signaling by phosphorylation has been suggested. Witcher et al. [21] recently reported that the activity of Ca^{2+} release channels of the cardiac sarcoplasmic reticulum (SR) is inhibited by Ca^{2+}-dependent phosphorylation. We found previously [22] that the microinjection of Ca^{2+}/calmodulin-dependent kinase inhibitor peptide into single isolated smooth muscle cells markedly enhances the transient Ca^{2+} increase after the depolarization. Recently we also found that calmodulin-dependent kinase may modulate the smooth muscle surface membrane Ca^{2+} channel [23]. These results suggest that protein kinase–protein phosphatase systems modulate the Ca^{2+} homeostasis. Therefore, our results can be explained by the fact that OA inhibits the platelet phosphatase activity so as to increase the phosphorylation of the Ca^{2+} channel which results in a decrease in the $[Ca^{2+}]_i$.

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REFERENCES

[1] Cohen, P., Holmes, C.F.B. and Tsukitani, Y. (1990) Trends Biochem. Sci. 15, 98–102.
[2] Tachibana, K., Shever, P.J., Tsykitani, Y., Kikuchi, H., Van Eugen, D., Clardy, J., Giopichand, Y. and Schmitz, F.J. (1981) J. Am. Chem. Soc. 103, 2469–2471.
[3] Suganuma, M. (1988) Proc. Natl. Acad. Sci. USA 85, 1768–1777.
[4] Bialojan, C. and Takai, A. (1988) Biochem. J. 256, 283–290.
[5] Haystead, T.A.J., Sim, A.T.R., Carling, P., Honnor, R.C., Tsukitani, Y., Cohen, P. and Hardie, D.G. (1990) Nature 337, 78–81.
[6] Tyers, M., Rachubinski, R.A. and Stewart, M.I. (1989) Nature 333, 470–472.
[7] Daniel, J.L., Molish, I.R., Rigmaiden, M. and Steward, G. (1984) J. Biol. Chem. 259, 9826–9831.
[8] Higashihara, M., Maeda, H., Shibata, Y., Kume, S. and Ohashi, T. (1985) Blood 65, 382–391.
[9] Higashihara, M., Maeda, H., Yatomi, Y., Takahata, K., Oka, H. and Kume, S. (1985) Biochem. Biophys. Res. Commun. 133, 306–313.
[10] Yatomi, Y., Higashihara, M., Ozaki, Y., Kume, S. and Kurokawa, K. (1990) Biochem. Biophys. Res. Commun. 171, 109–115.
[11] Laemmli, U.K. (1970) Nature 227, 680–685.
[12] Tallant, E.A. and Wallace, R.W. (1985) J. Biol. Chem. 260, 7744–7751.
[13] Sakon, M., Kambayashi, J., Kajikawa, Y., Uemura, Y., Shiba, E., Kawasaki, T. and Mori, T. (1990) Biochem. Int. 22, 149–161.
[14] Cohen, P., Klumpp, S. and Schelling, D.L. (1989) FEBS Lett. 250, 596–600.
[15] Lorea, K.M. (1991) Biochemistry 30, 6819–6824.
[16] Klumpp, S., Cohen, P. and Schultz, J.E. (1990) EMBO J. 9, 685–689.
[17] Mitsui, M., Inagaki, M. and Ikebe, M. (1992) J. Biol. Chem. (in press).
[18] Higashihara, M., Takahata, K. and Kurokawa, K. (1991) Blood 78, 3224–3231.
[19] Hartshorne, D.J., in: Physiology of Gastrointestinal Tract, 2nd edn. (Johnson, L.R. ed.) pp. 432–482, Raven Press, New York.
[20] Conti, M.A. and Adelstein, R.S. (1981) J. Biol. Chem. 256, 3178–3181.
[21] Witcher, D.R., Kovacs, R.J., Schulman, H., Cefali, D.C. and Jones, L.R. (1991) J. Biol. Chem. 266, 11144–11152.
[22] Ito, T., Ikebe, M., Kargacin, G., Hartshorne, D.J., Kemp, B.E. and Fay, F.S. (1989) Nature 338, 164–167.
[23] McCarron, J.G., McGeown, J.G., Reardon, S., Ikebe, M., Fay, F.S. and Walsh Jr., J.V. (1992) Nature 357, 74–77.