Suppression of Capacitative Ca^{2+} Entry by Serine/Threonine Phosphatase Inhibitors in Rat Parotid Acinar Cells

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ABSTRACT—The effects of three serine/threonine protein phosphatase inhibitors, calyculin-A, tautomycin and okadaic acid, on the Ca^{2+} entry across the plasma membrane was studied in Fura-2-loaded rat parotid acinar cells. These protein phosphatase inhibitors did not affect the peak elevation of cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) just after stimulation with the muscarinic agonist carbachol (CCh), but they suppressed the sustained increase in $[\text{Ca}^{2+}]_i$. In the absence of extracellular Ca^{2+} , CCh produced a transient increase in $[\text{Ca}^{2+}]_i$ due to Ca^{2+} release from intracellular Ca^{2+} stores, and this increase in $[\text{Ca}^{2+}]_i$ was unaffected by the phosphatase inhibitors. When Ca^{2+} was added to the external medium after the transient $[\text{Ca}^{2+}]_i$ response, the increase in $[\text{Ca}^{2+}]_i$ in the cells treated with the phosphatase inhibitors was significantly smaller than that in the control cells, indicating that the Ca^{2+} entry was reduced. Similar suppression of Ca^{2+} entry by the phosphatase inhibitors was observed when intracellular Ca^{2+} stores were previously depleted by the microsomal Ca^{2+} -ATPase inhibitor thapsigargin (TG). In addition, the phosphatase inhibitors reduced the Mn^{2+} (Ca^{2+} surrogate) influx following the addition of CCh or TG. The enhancement of Ca^{2+} entry by the protein kinase inhibitor staurosporine was significantly attenuated by the phosphatase inhibitors. These results suggest that the phosphatase inhibitors suppressed the Ca^{2+} entry mechanism activated by depletion of intracellular Ca^{2+} stores in rat parotid acinar cells. The capacitative Ca^{2+} entry may be regulated by protein phosphorylation/dephosphorylation.

Keywords: Capacitative Ca^{2+} entry, Depletion of intracellular Ca^{2+} store, Serine/threonine phosphatase inhibitor, Thapsigargin, Parotid acinar cell

The activation of surface membrane receptors linked to phosphoinositide hydrolysis leads to an increase in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) due to a release of Ca^{2+} from intracellular stores and a sustained entry of Ca^{2+} across the plasma membrane. While it is fully established that the Ca^{2+} release from intracellular stores is mediated by inositol-1,4,5-trisphosphate (InsP_3), a product of phosphoinositide hydrolysis, the mechanism controlling the sustained Ca^{2+} entry is not well-understood.

The agonist-induced Ca^{2+} entry is mimicked by emptying the intracellular Ca^{2+} stores with thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+} -ATPase (1, 2). Based on this finding, Putney (3) has proposed that the Ca^{2+} entry depends on the degree of emptiness of the intracellular Ca^{2+} stores rather than phosphoinositide hydrolysis. This Ca^{2+} entry pathway, which is termed the “capacitative Ca^{2+} entry”, has been demonstrated in a variety of cell types, and the mechanisms linking the

depletion of intracellular Ca^{2+} stores to the activation of Ca^{2+} entry are presently the subject of intensive investigation. Some investigators suggest the involvement of the following molecules in the capacitative Ca^{2+} entry: cyclic GMP (4), small GTP-binding proteins (5), tyrosine kinases (6, 7) and a low molecular weight compound named the “ Ca^{2+} influx factor” (CIF) (8, 9). However, there is no direct evidence showing that these molecules regulate the capacitative Ca^{2+} entry. In addition, another possibility is proposed that there is a direct link between the receptor for InsP_3 and a putative store-operated Ca^{2+} channel at the plasma membrane (10).

We recently showed that in rat parotid acinar cells, the protein kinase inhibitor staurosporine enhances the Ca^{2+} entry induced by depletion of intracellular Ca^{2+} stores (11). This result suggests that the Ca^{2+} entry pathway may be regulated by a protein phosphorylation/dephosphorylation mechanism. As the basal levels of protein phosphorylation reflect a balance of

protein kinase and phosphatase activities, inhibition of serine/threonine protein phosphatase would be expected to induce or enhance serine/threonine protein phosphorylation. The protein phosphatase inhibitors have been demonstrated to accelerate phosphorylation of some proteins in rat parotid acinar cells (12, 13), although the physiological significance of the phosphorylation is at present unclear. If protein phosphorylation is involved in the Ca^{2+} entry induced by depletion of intracellular Ca^{2+} stores, the phosphatase inhibitors would affect the capacitative Ca^{2+} entry. Here we examined the effects of three serine/threonine protein phosphatase inhibitors, calyculin-A (14, 15), tautomycin (16) and okadaic acid (17, 18), on the Ca^{2+} entry in response to the muscarinic receptor agonist carbachol (CCh) and thapsigargin (TG).

MATERIALS AND METHODS

Materials

Fura-2 acetoxymethyl ester (fura-2/AM), EGTA and HEPES were purchased from Dojin Laboratories (Kumamoto). Calyculin-A, tautomycin, okadaic acid and

4 β -phorbol 12-myristate 13-acetate (PMA) were from Wako Pure Chemical (Osaka). Staurosporine, carbachol, thapsigargin, trypsin (type III), trypsin inhibitor (type II-S), collagenase (type II) and bovine serum albumin (BSA) were from Sigma Chemical Co. (St Louis, MO, USA). 1-Norokadaone was from Alomone Labs. Ltd. (Jerusalem, Israel). Other reagents were of analytic grade.

Cell preparation

Dispersed acinar cells were prepared from the parotid glands of male Wistar-strain rats (300–350 g) using trypsin and collagenase, as described elsewhere (19, 20). After dispersion, the cells were washed and resuspended in a Hanks' balanced salt solution buffered with 20 mM HEPES (pH 7.4) (HBSS-H) containing 0.2% BSA.

Measurement of $[\text{Ca}^{2+}]_i$

Parotid acinar cells were incubated for 40 min with 2 μM fura-2/AM in HBSS-H containing 0.2% BSA at 37°C. The fura-2-loaded cells were washed twice, resuspended in fresh HBSS-H containing 0.2% BSA and stored at room temperature until use. Fura-2 fluorescence

Fig. 1. Effects of the phosphatase inhibitors on carbachol (CCh)-induced increases in $[\text{Ca}^{2+}]_i$ in the presence of extracellular Ca^{2+} . A–C: Fura-2-loaded cells were preincubated for 5 min with 50 nM calyculin-A (Caly-A), 1 μM tautomycin (Tauto) or 2.5 μM okadaic acid (Okada) and then stimulated with 10 μM CCh. D: Fura-2-loaded cells were stimulated with 10 μM CCh, and 50 nM Caly-A was added at the time indicated by the arrow. The data are representative traces from 3–6 experiments.

was measured at 37°C in a magnetically stirred cuvette in a Hitachi F-2000 spectrofluorimeter (Hitachi, Tokyo) with excitation at 340 and 380 nm and emission at 510 nm. The $[\text{Ca}^{2+}]_i$ was calculated from the ratio of fluorescence intensities as described by Grinkiewicz et al. (21).

Determination of Mn^{2+} influx

Fura-2-loaded cells were prepared by the same protocol as that for the $[\text{Ca}^{2+}]_i$ measurements. The Mn^{2+} entry was monitored as the quenching of fura-2 fluorescence, with the excitation and emission wavelength set at 360 and 510 nm, which is insensitive to changes in $[\text{Ca}^{2+}]_i$ (22).

Statistical analyses

The statistical significance of differences between values was determined by Student's *t*-test.

RESULTS

Effect of phosphatase inhibitors on the Ca^{2+} entry induced by muscarinic receptor stimulation

From the preliminary experiments, 50 nM calyculin-A, 1 μM tautomycin and 2.5 μM okadaic acid were chosen as the concentrations for producing submaximal effects in this study. The phosphatase inhibitors themselves did not alter the resting level of $[\text{Ca}^{2+}]_i$. Figure 1A shows a typical change in $[\text{Ca}^{2+}]_i$ induced by the muscarinic receptor agonist CCh in the presence or absence of calyculin-A. The addition of 10 μM CCh induced a rapid increase in $[\text{Ca}^{2+}]_i$, which then slowly declined to a sustained level of $[\text{Ca}^{2+}]_i$. When the cells were preincubated for 5 min in the presence of 50 nM calyculin-A, the return of the peak $[\text{Ca}^{2+}]_i$ to the sustained level was more rapid than that in the control cells, and the sustained elevation (mean $\pm$ S.E.: 148 ± 6 nM, $n=6$) of $[\text{Ca}^{2+}]_i$ was significantly suppressed ($P < 0.01$) compared with that (178 ± 7 nM, $n=6$) in the control cells. The preincubation with 1 μM tautomycin or 2.5 μM okadaic acid also attenuated the sustained elevation of $[\text{Ca}^{2+}]_i$ in response to CCh (Fig. 1: B and C), although the effect was more modest than that of calyculin-A. The suppression of the $[\text{Ca}^{2+}]_i$ response was also observed when 50 nM calyculin-A was added at the plateau phase of the CCh-induced increase in $[\text{Ca}^{2+}]_i$

Fig. 2. Effects of the phosphatase inhibitors on Ca^{2+} entry following stimulation with carbachol (CCh) in Ca^{2+} -free medium. Fura-2-loaded cells were preincubated for 5 min with 50 nM calyculin-A (Caly-A) (A), 1 μM tautomycin (Tauto) (B) or 2.5 μM okadaic acid (Okada) (C) in Ca^{2+} -free medium containing 1 mM EGTA and then stimulated with 10 μM CCh. Where indicated, 2.5 mM CaCl_2 was added to the external medium. D: Fura-2-loaded cells were preincubated in the presence or absence of 50 nM Caly-A, and 2.5 mM CaCl_2 was added without CCh stimulation. The data are representative traces from 3–8 experiments.

(Fig. 1D). The $[Ca^{2+}]_i$ slowly decreased with a delay of 1–2 min after the addition of calyculin-A.

To determine whether the phosphatase inhibitors affected the release of Ca^{2+} from intracellular Ca^{2+} stores or Ca^{2+} entry across the plasma membrane, the effects of the phosphatase inhibitors on the CCh-induced increase in $[Ca^{2+}]_i$ were examined in a Ca^{2+} -free medium containing 1 mM EGTA. Stimulation with 10 μ M CCh induced a transient increase in $[Ca^{2+}]_i$ that then returned to the resting level of $[Ca^{2+}]_i$ (Fig. 2: A, B and C). Neither of 50 nM calyculin-A, 1 μ M tautomycin nor 2.5 μ M okadaic acid modified the transient elevation of $[Ca^{2+}]_i$ (Fig. 2: A, B and C; Table 1), indicating that the phosphatase inhibitors did not affect the mobilization of Ca^{2+} from intracellular stores. Following the transient $[Ca^{2+}]_i$ response, 2.5 mM $CaCl_2$ was added to the external medium to examine the effect of phosphatase inhibitors on Ca^{2+} entry. The increase in $[Ca^{2+}]_i$ evoked by the addition of Ca^{2+} was significantly reduced by preincubation with the phosphatase inhibitors (Fig. 2: A, B and C; Table 1). When the cells were not previously stimulated with CCh, the small increase in $[Ca^{2+}]_i$ following addition of external Ca^{2+} was unaffected by the phosphatase inhibitors (Fig. 2D).

1-Norokadaone, a structural analogue of okadaic acid unable to inhibit protein phosphatases (23), was also tested as a control for okadaic acid. The cells were preincu-

bated with 2.5 μ M 1-norokadaone in a Ca^{2+} -free medium, and Ca^{2+} was added to the medium following CCh stimulation. The preincubation with the analogue did not affect the elevation of $[Ca^{2+}]_i$ induced by the addition of Ca^{2+} (data not shown). The elevated $[Ca^{2+}]_i$ was 200 ± 18 nM (mean $\pm$ S.E., $n=3$) and 197 ± 13 nM ($n=3$) in the presence and absence of 1-norokadaone, respectively.

Effect of phosphatase inhibitors on the Ca^{2+} entry induced by TG

The addition of TG depletes Ca^{2+} from the $InsP_3$ -sensitive intracellular Ca^{2+} stores by inhibition of the endoplasmic reticulum Ca^{2+} pumps, resulting in the activation of the capacitative Ca^{2+} entry (2). To show that the suppression of Ca^{2+} entry by the phosphatase inhibitors is evoked by mechanisms independent of phosphoinositide metabolism, we examined the Ca^{2+} entry activated by TG in the presence and absence of the phosphatase inhibitors. In a Ca^{2+} -free medium containing 1 mM EGTA, the addition of 0.5 μ M TG produced a transient increase in $[Ca^{2+}]_i$ due to a release of Ca^{2+} from intracellular Ca^{2+} stores. Preincubation for 5 min with 50 nM calyculin-A did not affect the transient response of $[Ca^{2+}]_i$, but significantly suppressed the elevation of $[Ca^{2+}]_i$ induced by subsequent addition of Ca^{2+} to the cell suspension (Fig. 3A, Table 2). Similar attenuation of the TG-activated Ca^{2+} entry was observed in cells pre-

Table 1. Effects of the phosphatase inhibitors on the CCh-induced $[Ca^{2+}]_i$ response in the absence of extracellular Ca^{2+}

Stimulation	Inhibitor	$[Ca^{2+}]_i$ (nM)		n
		Peak after stimulation	After Ca^{2+} addition	
10 μ M CCh	50 nM Caly-A			
	—	147 $\pm$ 12	217 $\pm$ 17	8
	+	141 $\pm$ 14	135 $\pm$ 19**	8
	1 μ M Tauto			
	—	148 $\pm$ 16	204 $\pm$ 20	4
	+	144 $\pm$ 22	167 $\pm$ 15*	4
	2.5 μ M Okada			
	—	145 $\pm$ 20	188 $\pm$ 6	3
	+	140 $\pm$ 20	132 $\pm$ 8*	3

Cells were preincubated for 5 min in the presence or absence of calyculin-A (Caly-A), tautomycin (Tauto) or okadaic acid (Okada) in Ca^{2+} -free medium containing 1 mM EGTA, and stimulated with 10 μ M carbachol (CCh). External $CaCl_2$ (2.5 mM) was added to the cells after transient increases in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ was monitored at the transient peak after CCh stimulation and the maximally elevated level of control cells after addition of Ca^{2+} . In the inhibitor-treated cells, the $[Ca^{2+}]_i$ after the addition of Ca^{2+} was measured at the time corresponding to the maximal level in the control cells. Values are means $\pm$ S.E. n: numbers of experiments. * $P < 0.01$, ** $P < 0.001$, compared with the values in the absence of the inhibitors.

Table 2. Effects of the phosphatase inhibitors on the TG-induced $[Ca^{2+}]_i$ response in the absence of extracellular Ca^{2+}

Stimulation	Inhibitor	$[Ca^{2+}]_i$ (nM)		n
		Peak after stimulation	After Ca^{2+} addition	
0.5 μ M TG	50 nM Caly-A			
	—	120 $\pm$ 17	213 $\pm$ 7	4
	+	116 $\pm$ 14	146 $\pm$ 12**	4
	1 μ M Tauto			
	—	118 $\pm$ 9	235 $\pm$ 16	5
	+	120 $\pm$ 10	190 $\pm$ 18*	5
	2.5 μ M Okada			
	—	126 $\pm$ 10	255 $\pm$ 14	4
	+	121 $\pm$ 11	153 $\pm$ 6*	4

Cells were preincubated for 5 min in the presence or absence of calyculin-A (Caly-A), tautomycin (Tauto) or okadaic acid (Okada) in Ca^{2+} -free medium containing 1 mM EGTA and then stimulated with 0.5 μ M thapsigargin (TG). External $CaCl_2$ (2.5 mM) was added to the cells after transient increases in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ was monitored at the transient peak after TG stimulation and the maximally elevated level of control cells after addition of Ca^{2+} . In the inhibitor-treated cells, the $[Ca^{2+}]_i$ after the addition of Ca^{2+} was measured at the time corresponding to the maximal level in the control cells. Values are means $\pm$ S.E. n: numbers of experiments. * $P < 0.01$, ** $P < 0.001$, compared with the values in the absence of the inhibitors.

incubated with 1 μM tautomycin or 2.5 μM okadaic acid (Fig. 3: B and C; Table 2).

Effect of phosphatase inhibitors on Mn^{2+} entry

Mn^{2+} has been extensively used as a probe for the capacitative Ca^{2+} entry pathway in a variety of cell types including rat parotid acinar cells (11, 24, 25). To obtain more direct evidence that the phosphatase inhibitors

reduce the Ca^{2+} entry across the plasma membrane, we monitored the quenching of fura-2 fluorescence following the addition of Mn^{2+} . Fura-2-loaded cells were preincubated for 5 min with or without 50 nM calyculin-A and then stimulated with 10 μM CCh. In the presence of calyculin-A, the rate of the quenching initiated by subsequent addition of 300 μM Mn^{2+} was significantly reduced compared with that in the control cells (Fig. 4A), indicating that Mn^{2+} influx was suppressed. A similar suppression of Mn^{2+} influx was observed in cells preincubated with 1 μM tautomycin or 2.5 μM okadaic acid (data not shown). In addition, the phosphatase inhibitors also decreased the Mn^{2+} influx induced by addition of 0.5 μM TG. A typical pattern of the quenching of fura-2 fluorescence in the presence or absence of 50 nM calyculin-A is shown in Fig. 4B.

Effect of calyculin-A on the Ca^{2+} entry enhanced by staurosporine

The protein kinase inhibitor staurosporine was found to enhance the Ca^{2+} entry activated by depletion of intracellular Ca^{2+} stores in rat parotid acinar cells (11).

Fig. 3. Effects of the phosphatase inhibitors on Ca^{2+} entry following stimulation with thapsigargin (TG) in Ca^{2+} -free medium. Fura-2-loaded cells were preincubated for 5 min with 50 nM calyculin-A (Caly-A) (A), 1 μM tautomycin (Tauto) (B) or 2.5 μM okadaic acid (Okada) (C) in Ca^{2+} -free medium containing 1 mM EGTA and stimulated with 0.5 μM TG. Where indicated, 2.5 mM CaCl_2 was added to the external medium. The data are representative traces from 4–5 experiments.

Fig. 4. Effects of calyculin-A (Caly-A) on carbachol (CCh)- and thapsigargin (TG)-induced Mn^{2+} entry. Fura-2-loaded cells were preincubated for 5 min in the presence or absence of 50 nM Caly-A and stimulated with 10 μM CCh (A) or 0.5 μM TG (B) in Ca^{2+} -free medium without EGTA. Mn^{2+} (300 μM) was added at the times indicated by the arrows. The data are representative traces from 3 or 5 experiments.

If the enhancement of Ca^{2+} entry by staurosporine is due to inhibition of protein phosphorylation, the effect of staurosporine would be expected to be diminished by protein phosphatase inhibitors which are able to accelerate protein phosphorylation. When the cells were preincubated for 5 min with a maximally effective concentration (100 nM) of staurosporine and stimulated with 10 μM CCh (trace a in Fig. 5A), the sustained elevation of $[\text{Ca}^{2+}]_i$ (261 ± 10 nM, $n=4$) following a rapid peak was significantly higher than that (178 ± 7 nM, $n=6$) observed in the absence of staurosporine. The high sustained $[\text{Ca}^{2+}]_i$ reflects an enhanced Ca^{2+} influx (11). When 50 nM calyculin-A was added at the plateau phase of the CCh-induced $[\text{Ca}^{2+}]_i$ elevation, the sustained level of $[\text{Ca}^{2+}]_i$ enhanced by staurosporine was slowly attenuated (trace b in Fig. 5A). In addition, the enhancement of the sustained $[\text{Ca}^{2+}]_i$ elevation was not observed in cells preincubated for 5 min with a combination of staurosporine and calyculin-A (Fig. 5B).

Preincubation with 1 μM tautomycin or 2.5 μM okadaic acid failed to suppress the enhancement of CCh-induced $[\text{Ca}^{2+}]_i$ elevation by 100 nM staurosporine (data not shown). However, when the concentration of staurosporine applied to cells was decreased to 20 nM, a concentration producing submaximum effect (11), the enhancement of the sustained increase in $[\text{Ca}^{2+}]_i$ was reduced by preincubation with 1 μM tautomycin and 2.5 μM okadaic acid (Fig. 5: C and D).

Effect of phorbol ester on the CCh-induced Ca^{2+} entry

To test the possibility that the phosphatase inhibitors suppress the Ca^{2+} entry through phosphorylation of a substrate protein(s) for protein kinase C, we examined the effect of the protein kinase C activator PMA on the capacitative Ca^{2+} entry. In a Ca^{2+} -free medium, the cells were preincubated for 5 min with or without 100 nM PMA prior to stimulation with 10 μM CCh, and Ca^{2+} was added to the external medium following the transient

Fig. 5. Effects of the phosphatase inhibitors on the Ca^{2+} entry enhanced by staurosporine (Stauro) in the presence of extracellular Ca^{2+} . **A:** Fura-2-loaded cells were preincubated for 5 min in the presence of 100 nM Stauro and stimulated with 10 μM carbachol (CCh). In trace b, 50 nM calyculin-A (Caly-A) was added at the time indicated by the arrow. **B:** Fura-2-loaded cells were preincubated for 5 min in the presence of 100 nM Stauro or a combination of 100 nM Stauro and 50 nM Caly-A. **C:** Fura-2-loaded cells were preincubated for 5 min in the presence of 20 nM Stauro or a combination of 20 nM Stauro and 1 μM tautomycin (Tauto). **D:** Fura-2-loaded cells were preincubated for 5 min in the presence of 20 nM Stauro or a combination of 20 nM Stauro and 2.5 μM okadaic acid (Okada). The data are representative traces from 3–4 experiments.

increases in $[\text{Ca}^{2+}]_i$. In the PMA-treated cells, the $[\text{Ca}^{2+}]_i$ (187 ± 8 nM, $n=3$) elevated by the addition of Ca^{2+} was similar to that (188 ± 6 nM, $n=3$) in the control (Fig. 6).

DISCUSSION

The present study shows that three protein phosphatase inhibitors, calyculin-A, tautomycin and okadaic acid, effectively suppressed the Ca^{2+} entry induced by muscarinic receptor stimulation or by inhibition of the endoplasmic reticulum Ca^{2+} pumps. This effect of the phosphatase inhibitors is unlikely to be evoked by inhibition of phosphoinositide hydrolysis, because the compounds suppressed the TG-induced Ca^{2+} entry which is not accompanied by formation of inositol phosphates (2, 26). The phosphatase inhibitors attenuated the enhancement of the capacitative Ca^{2+} entry by the protein kinase inhibitor staurosporine. These results suggest that the phosphatase inhibitors suppress the Ca^{2+} entry activated by depletion of intracellular Ca^{2+} stores and that the Ca^{2+} entry is regulated by protein phosphorylation/dephosphorylation.

In thyroid FRTL-5 cells (27), *Xenopus* oocytes (28) and rabbit neutrophils (29), protein kinase C has been suggested to be involved in the regulation of capacitative Ca^{2+} entry. In this study, however, PMA, an activator of protein kinase C, failed to suppress the Ca^{2+} entry induced by the store-depletion, and the protein(s) involved in the regulation of the Ca^{2+} entry is probably different from the substrates for protein kinase C. Our earlier study has shown that the enhancement of Ca^{2+} entry by staurosporine is not attenuated by addition of PMA (11),

which is compatible with the conclusion that protein kinase C is unlikely to play a major role in regulating the capacitative Ca^{2+} entry in rat parotid acinar cells. Although staurosporine is a potent inhibitor of protein kinase C (30), it also inhibits other kinases such as cyclic AMP-dependent kinase and tyrosin kinase (31–33).

The suppression of Ca^{2+} entry by serine/threonine phosphatase inhibitors has also been described in human platelets (34), HeLa cells (35) and human neutrophils (36). Therefore, it seems that modulation of the capacitative Ca^{2+} entry through phosphorylation/dephosphorylation is a widespread mechanism present in many cell types. However, a recent study using astrocytoma cells shows that okadaic acid and calyculin-A potentiate the submaximal elevation of $[\text{Ca}^{2+}]_i$ by low doses of CCh, TG or CIF (putative Ca^{2+} influx factor) (37). The authors suggest that the phosphatase inhibitors potentiated the capacitative Ca^{2+} entry by preventing the degradation of CIF. This is contrary to our conclusion that protein phosphorylation induced by the phosphatase inhibitors results in attenuation of the capacitative Ca^{2+} entry. The discrepancy may be attributed to differences in the experimental protocols, since the concentrations (< 500 nM) of okadaic acid, which can enhance the Ca^{2+} entry in astrocytoma cells, are very low compared with the concentration ($2.5 \mu\text{M}$) used in our study. We tested the effect of a low concentration of okadaic acid on the Ca^{2+} entry in rat parotid acinar cells. However, preincubation with 100 nM okadaic acid had no effect on the submaximal elevation of $[\text{Ca}^{2+}]_i$ induced by $1 \mu\text{M}$ CCh or 1 nM TG (our unpublished data).

There was no essential difference in the effect on Ca^{2+} entry among calyculin-A, tautomycin and okadaic acid, suggesting that these phosphatase inhibitors act on a common target protein. The phosphatase inhibitors used here have been described as potent inhibitors of type-1 and -2A serine/threonine protein phosphatases (14–18), but the potencies of these inhibitors vary in inhibiting the phosphatases. In a cell-free system, calyculin-A inhibits protein phosphatase type-1 with a lower value (2 nM) of IC_{50} than that (60–500 nM) for okadaic acid, while both inhibitors inhibit protein phosphatase type-2A with almost identical values (0.5–1 nM) of IC_{50} (38). Tautomycin is less potent than calyculin-A (39). In the present study, calyculin-A most effectively suppressed the capacitative Ca^{2+} entry, leading to the possibility that the phosphatase inhibitors may attenuate the Ca^{2+} entry through inhibition of protein phosphatase type-1 rather than type-2A. The concentrations of the phosphatase inhibitors required to suppress the Ca^{2+} entry are relatively high compared with those used in cell-free systems (15–18, 38). This is probably due to the difference in accessibility of the phosphatase inhibitors to target en-

Fig. 6. Carbachol (CCh)-induced Ca^{2+} entry in the presence of 4 β -phorbol 12-myristate 13-acetate (PMA). Fura-2-loaded cells were preincubated for 5 min with or without 100 nM PMA in Ca^{2+} -free medium containing 1 mM EGTA and stimulated with $10 \mu\text{M}$ CCh. Where indicated, 2.5 mM CaCl_2 was added to the external medium. The data are representative traces from 3 experiments.

zymes. The concentrations used here are comparable with those able to accelerate protein phosphorylation in intact or permeabilized parotid acinar cells (12, 13).

Although our data suggest that the capacitative Ca^{2+} entry is regulated by protein phosphorylation/dephosphorylation, the target protein(s) or site(s) for phosphorylation remains to be elucidated. One possibility is that the protein phosphatase inhibitors may phosphorylate Ca^{2+} channels in the plasma membrane. In this case, the phosphorylation would result in inactivation of the Ca^{2+} channels. However, in non-excitabile cells including parotid acinar cells, the plasma-membrane Ca^{2+} channels are still not identified as protein molecules, and it is at present difficult to show whether or not the phosphatase inhibitors phosphorylate the Ca^{2+} channels. Another possibility is that the protein phosphatase inhibitors may phosphorylate the enzyme involved in degradation of the putative messenger, such as CIF (8), linking intracellular Ca^{2+} stores and plasma-membrane Ca^{2+} channels. The phosphorylated enzyme would accelerate degradation of the messenger, leading to inactivation of the surface membrane Ca^{2+} channels. These two possibilities are rather speculative, and the molecular mechanisms by which the protein phosphatase inhibitors modulate the Ca^{2+} entry should be further explored.

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