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MODULATORY EFFECT OF 4 β-PHORBOL 12-MYRISTATE 13-ACETATE (PMA) ON CARBACHOL-INDUCED Ca^{2+} MOBILIZATION IN RAT PAROTID ACINAR CELLS

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Abstract—Treatment of rat parotid acinar cells with 4 β-phorbol 12-myristate 13-acetate (PMA) significantly inhibited an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by carbachol (CCh), a muscarinic agonist. The CCh-induced increase in $[Ca^{2+}]_i$ was also inhibited by another active phorbol ester, 4 β-phorbol 12,13-dibutyrate, but not by 4 α-phorbol 12,13-didecanoate, which does not activate protein kinase C. The treatment with PMA had no effect on increases in $[Ca^{2+}]_i$ evoked by ionomycin and thapsigargin, which do not stimulate phosphoinositide hydrolysis. In contrast, an increase in $[Ca^{2+}]_i$ induced by NaF, a direct activator of GTP-binding proteins, was delayed in the presence of PMA. The formation of inositol phosphates in response to CCh was suppressed significantly by PMA treatment. In radioligand binding assays, PMA did not directly interfere with the specific binding of [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB), a muscarinic antagonist, to plasma membranes. Furthermore, the [^{3}H]QNB binding to plasma membranes prepared from the PMA-pretreated cells was not different from that to the control membranes. These results indicate that PMA attenuated the CCh-induced increase in $[Ca^{2+}]_i$ through inhibition of phosphoinositide hydrolysis. Activation of protein kinase C may play a role in negative-feedback control of the muscarinic pathway in rat parotid acinar cells.

Key words: parotid acinar cells; cytosolic Ca^{2+} concentration; phorbol esters; protein kinase C; phosphoinositide hydrolysis

In rat parotid acinar cells, the activation of muscarinic-cholinergic, substance P and α_1-adrenergic receptors causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate which yields inositol 1,4,5-trisphosphate (1,4,5-IP_3)† and diacylglycerol, an endogenous activator of protein kinase C [1]. The generation of 1,4,5-IP_3 induces Ca^{2+} release from intracellular stores accompanied by the entry of extracellular Ca^{2+}, resulting in a rapid increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) [2–5]. This $[Ca^{2+}]_i$ response is thought to be the crucial trigger in the fluid secretion from rat parotid glands.

The phorbol ester 4 β-phorbol 12-myristate 13-acetate (PMA), which activates protein kinase C, has been shown to suppress receptor-mediated increases in $[Ca^{2+}]_i$ in a variety of cell types, suggesting that protein kinase C plays a role in modulating $[Ca^{2+}]_i$. However, the mechanism of the inhibitory effect of PMA on the $[Ca^{2+}]_i$ response seems to be different in different cell types. In astrocytoma cells [6], PC 12 cells [7], smooth muscle cells [8], and Swiss-mouse 3T3 cells [9], treatment with PMA has been shown to attenuate the agonist-induced Ca^{2+} mobilization by inhibiting the formation of inositol phosphates. In pituitary cells [10], neutrophils [11, 12], and platelets [13], the inhibition of agonist-induced increases in $[Ca^{2+}]_i$ with PMA is suggested to be due to the activation of a Ca^{2+}-efflux mechanism in the plasma membrane. In thyroid FRTL-5 cells [14], PMA inhibits the influx of extracellular Ca^{2+} evoked by the microsomal Ca^{2+}-ATPase inhibitor thapsigargin (ThG), which has no effect on the hydrolysis of phosphoinositide [15, 16], leading to the assumption that protein kinase C is involved in the regulation of Ca^{2+} entry.

In rat parotid acinar cells, it has been shown that substance P-induced IP_3 formation is inhibited by the phorbol esters 4 β-phorbol 12,13-dibutyrate (PDBu) and PMA [17, 18], suggesting that the phosphoinositide hydrolysis is modulated by activation of protein kinase C. In the present study, we examined the effect of PMA on the increase in $[Ca^{2+}]_i$ induced by muscarinic stimulation. The results support the view that there may be negative-feedback regulation of phosphoinositide metabolism through protein kinase C in rat parotid acinar cells.

MATERIALS AND METHODS

Materials. Carbachol (CCh), ionomycin (Iono), ThG, and 4 α-phorbol 12,13-didecanoate (PDD) were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). PMA and PDBu were from Wako Pure Chemical (Osaka, Japan). Fura-2

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† Abbreviations: 1,4,5-IP_3, inositol 1,4,5-trisphosphate; CCh, carbachol; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; PMA, 4 β-phorbol 12-myristate 13-acetate; PDBu, 4 β-phorbol 12,13-dibutyrate; PDD, 4 α-phorbol 12,13-didecanoate; Iono, ionomycin; ThG, thapsigargin; and QNB, quinuclidinyl benzilate.

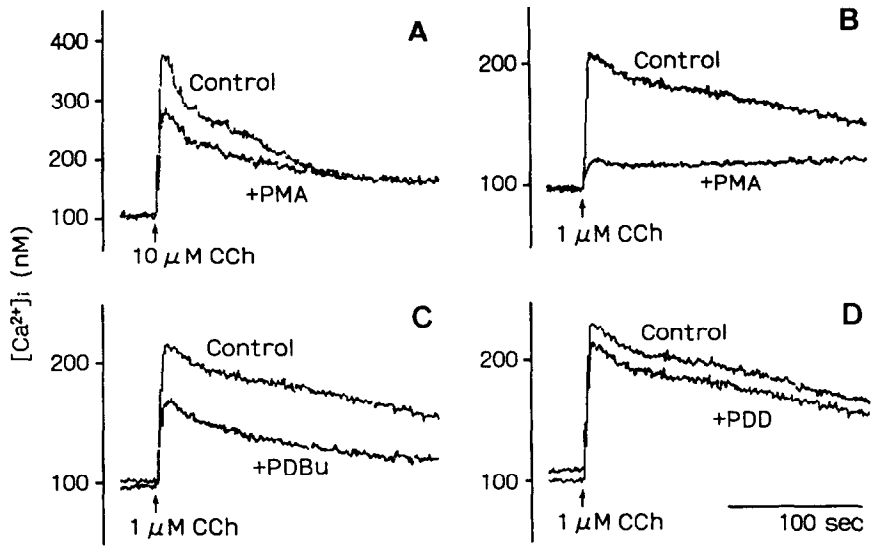

Fig. 1. Effects of phorbol esters on carbachol (CCh)-induced increases in $[Ca^{2+}]_i$. Fura-2-loaded acinar cells were incubated for 5 min with 100 nM PMA (A and B), 200 nM PDBu (C) or 500 nM PDD (D), and 1 or 10 μM CCh was added at the time indicated by the arrow. The control cells were incubated with 0.5% DMSO (vehicle).

acetoxymethyl ester (fura-2/AM) was from Dojin Laboratories (Kumamoto, Japan). *myo*-[2-^{3}H]Inositol was from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) was from New England Nuclear (Boston, MA, U.S.A.). All other reagents were of analytical grade.

Preparation of dispersed parotid acinar cells. Male Wistar-strain rats (300–350 g) were anesthetized with diethyl ether and killed by cardiac puncture. Dissociated acinar cells were prepared by the method of Merritt and Rink [3] with modifications [4]. The cells obtained were suspended in Hanks' balanced salt solution buffered with 20 mM HEPES-NaOH (pH 7.4) (HBSS-H) containing 0.2% BSA.

Measurement of inositol phosphates. Parotid acinar cells were prelabeled with *myo*-[2-^{3}H]inositol (15 μCi/mL), as described elsewhere [19, 20]. The cells were preincubated for 5 min in the presence of 10 mM LiCl and stimulated by CCh for a further 5 min. The reaction was stopped by the addition of $HClO_4$ (final concentration of 4.5%); a portion of the supernatant was then neutralized with 0.5 M KOH/9 mM $Na_2B_4O_7$. Labeled inositol phosphates were separated using a Bio-Rad AG 1-X8 column according to the method of Berridge *et al.* [21].

Determination of $[Ca^{2+}]_i$. Parotid acinar cells were incubated with 2 μM fura-2/AM for 45 min at 37°. The fura-2-loaded cells were washed twice, resuspended in fresh HBSS-H containing 0.2% BSA and kept at room temperature. Just before use, the cells were washed and resuspended in fresh HBSS-H containing 0.2% BSA and transferred into a quartz cuvette. The fluorescence of the fura-2-loaded cells was measured with a Hitachi F-2000 spectrofluorimeter (Hitachi, Tokyo, Japan) with excitation at 340 and 380 nm, and emission at 510 nm. The cuvette was controlled thermostatically at 37°, and the cell suspension was stirred continuously. Maximum fluorescence was determined by lysing the cells with 0.1% Triton X-100, and minimum fluorescence by the addition of 30 mM Tris-base and 5 mM EGTA. The $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence intensities according to Grynkiewicz *et al.* [22].

[^{3}H]*QNB binding assays.* Plasma membranes were prepared from the homogenates of glands or dispersed acinar cells, according to the method of Dai *et al.* [23]. Membranes (50 or 100 μg protein) were incubated for 70 min at 37° in 1.0 mL of 50 mM Tris–HCl buffer, pH 7.4, containing 10–500 pM [^{3}H] QNB, a muscarinic antagonist. Nonspecific binding was determined in the presence of 10 μM atropine. After incubation, samples were filtered rapidly through Toyo membrane filters (TM-1, 0.45 μm) presoaked in 0.1% polyethylenimine. The filters were washed three times with 2.5 mL of ice-cold buffer, dried and transferred into scintillation vials. The radioactivity was determined by liquid scintillation counting. Membrane protein concentration was determined by the Bio-Rad protein assay kit.

RESULTS

Effects of phorbol esters on CCh-induced increases in $[Ca^{2+}]_i$. The capacity of PMA to modulate changes in $[Ca^{2+}]_i$ induced by CCh, a muscarinic agonist, was examined using fura-2-loaded parotid acinar cells. This phorbol ester itself did not affect resting $[Ca^{2+}]_i$. When the cells were preincubated with 100 nM PMA for 5 min, the subsequent rise in $[Ca^{2+}]_i$ evoked by 10 μM CCh, a concentration producing

Table 1. Effects of phorbol esters on carbachol (CCh)-induced increase in $[Ca^{2+}]_i$

Stimulation	Treatment	Basal $[Ca^{2+}]_i$	Peak $[Ca^{2+}]_i$ (nM)
10 μM CCh	DMSO	97.9 ± 6.9	304.9 ± 27.0
	100 nM PMA	97.0 ± 7.6	254.6 ± 18.7
1 μM CCh	DMSO	96.7 ± 2.1	221.3 ± 8.4
	100 nM PMA	93.1 ± 2.9	135.8 ± 12.5*
	200 nM PDBu	88.0 ± 4.5	176.8 ± 16.4†
	500 nM PDD	94.7 ± 2.9	215.0 ± 10.0

Fura-2-loaded cells were incubated for 5 min in the presence of PMA, PDBu or PDD, and then stimulated with 1 or 10 μM CCh. The control cells were incubated with 0.5% DMSO (vehicle). Basal $[Ca^{2+}]_i$ was measured immediately prior to the addition of CCh. Values are means ± SEM of five experiments.

*,† Significantly different from control (Student's *t*-test): *$P < 0.001$ and †$P < 0.05$.

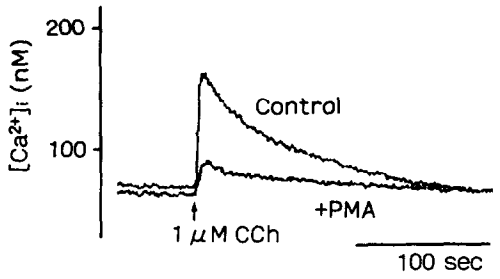

Fig. 2. Effect of PMA on CCh-induced increases in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. Cells were suspended in Ca^{2+}-free medium containing 1 mM EGTA, incubated for 5 min with 100 nM PMA or 0.5% DMSO (vehicle), and then stimulated with 1 μM CCh.

nearly maximum response [3, 5], was attenuated modestly (Fig. 1A), but the inhibition was not statistically significant (Table 1). There is the possibility that relatively high concentrations of CCh overcome the inhibitory effect of the PMA treatment on the $[Ca^{2+}]_i$ response, and the concentration of CCh applied to cells was decreased to 1 μM. Stimulation with 1 μM CCh caused about half the increase in $[Ca^{2+}]_i$ induced by 10 μM CCh, and pretreatment for 5 min with 100 nM PMA strongly suppressed the increase in $[Ca^{2+}]_i$ in response to 1 μM CCh (Fig. 1B). The increase in $[Ca^{2+}]_i$ in the presence of PMA was 34% of that in the control cells (Table 1). Another active phorbol ester, PDBu (200 nM), also significantly inhibited the increase in $[Ca^{2+}]_i$ induced by 1 μM CCh (Fig. 1C and Table 1), although this phorbol ester was not as effective as PMA. In contrast, the phorbol ester PDD (500 nM), which lacks the ability to activate protein kinase C, did not produce any modulation of the CCh-stimulated $[Ca^{2+}]_i$ response (Fig. 1D and Table 1).

The effect of PMA on the CCh-induced increase in $[Ca^{2+}]_i$ was examined in Ca^{2+}-free medium containing 1 mM EGTA. In control cells, stimulation with 1 μM CCh transiently increased $[Ca^{2+}]_i$ from a resting level of 66.0 ± 1.5 nM (N = 6) to a peak of 153.1 ± 12 nM (N = 3), but in the cells pretreated for 5 min with 100 nM PMA the peak $[Ca^{2+}]_i$ was suppressed to 87.4 ± 6.9 nM (N = 3; $P < 0.001$, as compared with the control cells) (Fig. 2).

Effects of PMA on changes in $[Ca^{2+}]_i$ *induced by Iono, ThG and NaF.* To test whether PMA affects an increase in $[Ca^{2+}]_i$ evoked by mechanisms independent of phosphoinositide metabolism, the fura-2-loaded cells were stimulated by the Ca^{2+} ionophore Iono and the microsomal Ca^{2+}-ATPase inhibitor ThG in the presence and absence of PMA. Addition of 0.2 μM Iono and 0.02 μM ThG slowly increased $[Ca^{2+}]_i$ from a resting level of 108.6 ± 7.7 nM (N = 6) to plateau levels of 193.2 ± 26.4 nM (N = 3) and 191.7 ± 19.1 nM (N = 3), respectively, and pretreatment for 5 min with 100 nM PMA did not affect the changes in $[Ca^{2+}]_i$ (Fig. 3, A and B). Unlike Iono and ThG, NaF has been shown to increase $[Ca^{2+}]_i$ by stimulating phosphoinositide hydrolysis in rat parotid acinar cells [24–26], and we examined whether PMA modifies the NaF-induced increase in $[Ca^{2+}]_i$. As shown in Fig. 3C, the onset of the increase in $[Ca^{2+}]_i$ following addition of 10 mM NaF was delayed significantly in cells pretreated with 100 nM PMA, although the $[Ca^{2+}]_i$ finally reached the maximum (204.8 ± 15.4 nM; N = 4) observed in the control cells (212.0 ± 16.5 nM; N = 4). The time required to reach half the maximum increase in $[Ca^{2+}]_i$ was 3.12 ± 0.06 min (N = 4) in the control cells, whereas in the PMA-treated cells it took 4.31 ± 0.16 min (N = 4; $P < 0.001$, as compared with the control cells).

Effect of PMA on inositol phosphate formation stimulated by CCh. To obtain direct evidence that pretreatment with PMA affects the phosphoinositide breakdown, inositol mono-, bis- and trisphosphates (IP_1, IP_2 and IP_3, respectively) produced by CCh stimulation were measured in the presence or absence of 100 nM PMA (Table 2). PMA alone decreased the basal levels of IP_1 and IP_3 somewhat. Stimulation of cells for 5 min with 1 μM CCh elicited a substantial increase in IP_1, IP_2, and IP_3; this increase was strongly suppressed when 100 nM PMA was added 5 min before stimulation with CCh (Table 2); the formation of IP_3 in the PMA-treated cells was 32% of that in the control cells. Pretreatment with PMA also inhibited the formation of inositol phosphates stimulated by a higher concentration (10 μM) of CCh, but the inhibition was relatively modest compared with that of cells stimulated by 1 μM CCh.

Effect of PMA on the binding of [^{3}H]*QNB to muscarinic receptors.* We tested the possibility that PMA inhibits the $[Ca^{2+}]_i$ response and inositol phosphate formation by directly interfering with the binding of CCh to muscarinic receptors. Plasma membranes prepared from tissue homogenates were incubated for 70 min at 37° with various concentrations of [^{3}H]QNB (10–500 pM) in the presence or absence of 100 nM PMA. The specific [^{3}H]QNB binding was saturable with the increase in

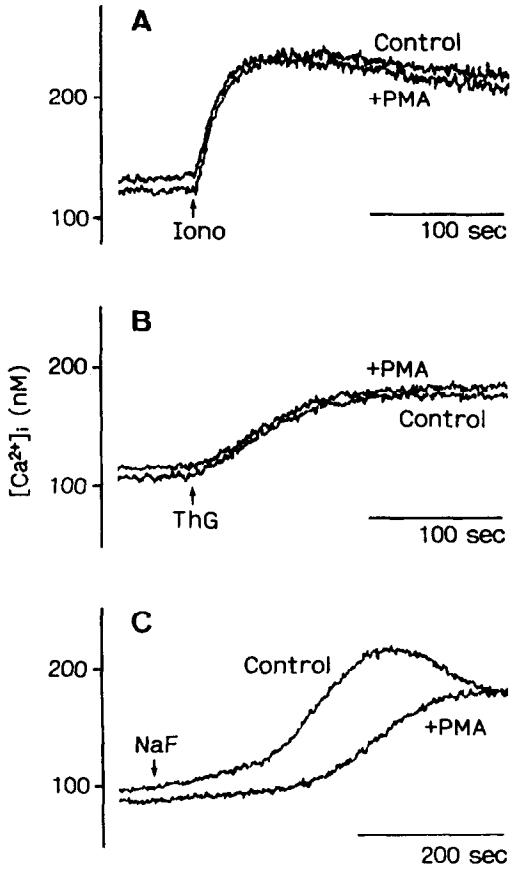

Fig. 3. Effects of PMA on increases in $[Ca^{2+}]_i$ induced by ionomycin (Iono), thapsigargin (ThG) and NaF. Cells were incubated for 5 min with 100 nM PMA or 0.5% DMSO (vehicle), and 0.2 μM Iono (A), 0.02 μM ThG (B) or 10 mM NaF (C) was added at the time indicated by the arrow.

Table 2. Effect of PMA on CCh-induced formation of inositol phosphates

CCh (μM)	Treatment	dpm/tube		
		IP_1	IP_2	IP_3
0	DMSO	3270 ± 130	1030 ± 20	300 ± 20
	PMA	2620 ± 140*	1030 ± 60	180 ± 30*
1	DMSO	5290 ± 140	3100 ± 170	1630 ± 190
	PMA	3550 ± 170†	1710 ± 120†	610 ± 60†
10	DMSO	8070 ± 300	8310 ± 1300	3260 ± 450
	PMA	5550 ± 220†	4570 ± 370*	2210 ± 280

[^{3}H]Inositol-labeled cells were incubated for 5 min in the presence of 100 nM PMA or 0.5% DMSO (vehicle) and stimulated for a further 5 min with 1 or 10 μM CCh. Values are means ± SEM of four experiments.

*,† Significantly different from the control (DMSO) (Student's t-test): *$P < 0.05$, and †$P < 0.001$.

[^{3}H]QNB concentration, and the addition of PMA did not affect this specific binding (Fig. 4).

Furthermore, to test the possibility that activation of protein kinase C alters the affinity of muscarinic receptors, plasma membranes were prepared from the acinar cells previously treated for 15 min with 100 nM PMA, and the [^{3}H]QNB binding was examined using the plasma membranes. When plasma membranes were incubated with 100 or 300 pM [^{3}H]QNB, there was no significant difference in the specific binding between the PMA-treated and untreated membranes (Table 3).

DISCUSSION

In the present study, CCh-induced increases in $[Ca^{2+}]_i$ were attenuated significantly by treatment with PMA. There are several possible explanations for this inhibitory effect of PMA. One is that protein kinase C activated by PMA may stimulate Ca^{2+} extrusion from the cells. In several cell types, PMA has been shown to cause a depression of $[Ca^{2+}]_i$ elevated by the Ca^{2+} ionophores Iono and A23187 [11–13], and this inhibitory effect has been interpreted as evidence that protein kinase C stimulates Ca^{2+} extrusion from the cells by activating the Ca^{2+} pump in the plasma membrane. In our study, however, the addition of PMA did not affect the increase in $[Ca^{2+}]_i$ induced by Iono or ThG, and it seems unlikely that PMA accelerates Ca^{2+} extrusion from rat parotid acinar cells. Another explanation is that activated protein kinase C decreases the influx of extracellular Ca^{2+}. However, this possibility may be ruled out as: (1) the inhibitory effect of PMA on CCh-induced increases in $[Ca^{2+}]_i$ was observed even in the absence of extracellular Ca^{2+}, and (2) PMA did not inhibit the rise in $[Ca^{2+}]_i$ evoked by ThG, which is thought to mimic receptor-mediated Ca^{2+} entry [15].

Thus, the most plausible explanation for the inhibition of the CCh-induced increase in $[Ca^{2+}]_i$ by PMA is that Ca^{2+} release from intracellular stores is inhibited by activation of protein kinase C. In permeabilized pancreatic acinar cells, pretreatment with PMA has been shown to decrease Ca^{2+} release from intracellular stores induced by the addition of 1,4,5-IP_3 [27], suggesting that protein kinase C depresses the function of 1,4,5-IP_3-operated Ca^{2+} channels of the stores. Although this possibility cannot be excluded, the present study demonstrated that the inhibitory effect of PMA on the CCh-induced increase in $[Ca^{2+}]_i$ was accompanied by a suppression of inositol phosphate formation. This result suggests that PMA attenuates the CCh-induced Ca^{2+} release through a decrease in phosphoinositide hydrolysis rather than direct action on intracellular Ca^{2+} stores.

Several investigators reported that phorbol esters desensitize muscarinic receptors [28–30], and it is possible that inhibition of phosphoinositide hydrolysis by PMA results from a decrease in the affinity or the number of muscarinic receptors. In the present study, however, pretreatment of acinar cells with PMA did not affect [^{3}H]QNB binding to the plasma membrane fraction, suggesting that PMA inhibits CCh-induced phosphoinositide hydrolysis at

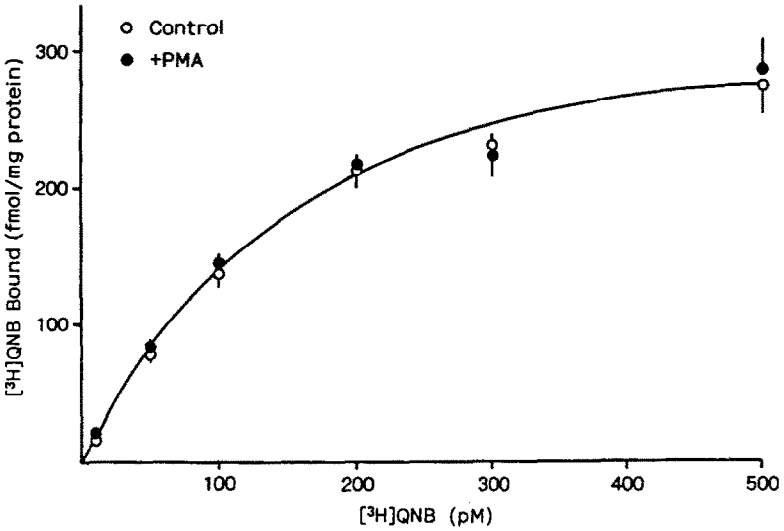

Fig. 4. Effect of PMA on [^{3}H]QNB binding to plasma membranes. Plasma membranes were prepared from tissue homogenates by the method of Dai *et al.* [23], and the aliquots (100 μg protein/tube) were incubated for 70 min at 37° with various concentrations (10–500 pM) of [^{3}H]QNB. Nonspecific binding was determined in the presence of 10 μM atropine. Values are means ± SEM of four experiments.

Table 3. [^{3}H]QNB binding to plasma membranes prepared from PMA-treated cells

[^{3}H]QNB (pM)	Specific [^{3}H]QNB bound (fmol/mg protein)	
	DMSO (control)	100 nM PMA
100	161.4 ± 10.2	162.7 ± 4.3
300	290.1 ± 58.2	326.1 ± 10.4

Acinar cells were preincubated for 15 min with PMA or 0.5% DMSO (vehicle), and plasma membranes were prepared by the method of Dai *et al.* [23]. Membranes (50 μg protein/tube) were incubated for 70 min at 37° with 100 or 300 pM [^{3}H]QNB. Nonspecific binding was determined in the presence of 10 μM atropine. Values are means ± SEM of three or four experiments.

a step after activation of muscarinic receptors. Earlier studies [17, 18] demonstrated that treatment of rat parotid acinar cells with phorbol esters inhibited substance P-induced IP_3 formation without affecting substance P binding to the cell surface, and concluded that the action site of protein kinase C is distal to the substance P receptors. This inhibition of IP_3 formation probably involves a mechanism similar to that reported in our study.

We found that PMA delays an increase in $[Ca^{2+}]_i$ in response to NaF. Fluoride is well known to be a direct activator for the GTP-binding proteins (G-proteins) [31, 32]. Earlier works have shown that NaF causes inositol phosphate formation and Ca^{2+} mobilization in rat parotid acinar cells [24–26], and this effect is thought to be associated with activation of the G-protein coupled to phospholipase C. The finding that PMA affected the rise in $[Ca^{2+}]_i$ evoked by NaF supports the view that the site of action of protein kinase C lies between G-protein activation and 1,4,5-IP_3 production. It has been demonstrated that protein kinase C can phosphorylate phospholipase C and G-proteins [33, 34]. However, further studies are necessary to determine the exact site of phosphorylation involved in inhibition of phosphoinositide hydrolysis by PMA.

Stimulation of rat parotid acinar cells with muscarinic agonists causes a rapid release of K^+, which is thought to reflect ionic transport during fluid secretion [35], and an increase in $[Ca^{2+}]_i$ is believed to be the major intracellular signal for the K^+ release [36]. We have found that the CCh-induced K^+ release is inhibited significantly by PMA [37]. It is reasonable to assume that the inhibitory effect of PMA on CCh-induced K^+ release is due to an attenuated $[Ca^{2+}]_i$ response.

In conclusion, the present study indicates that PMA exerts inhibitory action on phosphoinositide hydrolysis and Ca^{2+} mobilization in response to CCh, suggesting that activation of protein kinase C plays a role in a negative-feedback control of the muscarinic responses in rat parotid acinar cells. Although the molecular mechanism causing this feedback regulation is not fully established, the site of action of protein kinase C is probably distal to the muscarinic receptors.

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REFERENCES

1. Sugiya H and Furuyama S, The activation of Ca^{2+}-mobilizing receptors in salivary gland. *Biomed Res* **10**: 111–121, 1989.

2. Takemura H, Changes in cytosolic free calcium concentration in isolated rat parotid cells by cholinergic and β-adrenergic agonists. *Biochem Biophys Res Commun* **131**: 1048–1055, 1985.
3. Merritt JE and Rink TJ, Regulation of cytosolic free calcium in fura-2-loaded rat parotid acinar cells. *J Biol Chem* **262**: 17362–17369, 1987.
4. Tanimura A, Matsumoto Y and Tojyo Y, Evidence that isoproterenol-induced Ca^{2+}-mobilization in rat parotid acinar cells is not mediated by activation of β-adrenoceptors. *Biochim Biophys Acta* **1055**: 273–277, 1990.
5. Tojyo Y, Matsui S, Tanimura A and Matsumoto Y, Relationship between cytosolic Ca^{2+} concentration and amylase release in rat parotid acinar cells following muscarinic stimulation. *Biochim Biophys Acta* **1134**: 278–284, 1992.
6. Orellana SA, Solski PA and Brown JH, Phorbol ester inhibits phosphoinositide hydrolysis and calcium mobilization in cultured astrocytoma cells. *J Biol Chem* **260**: 5236–5239, 1985.
7. Vicentini LM, Virgilio FD, Ambrosini A, Pozzan T and Meldolesi J, Tumor promoter phorbol 12-myristate, 13-acetate inhibits phosphoinositide hydrolysis and cytosolic Ca^{2+} rise induced by the activation of muscarinic receptors in PC 12 cells. *Biochem Biophys Res Commun* **127**: 310–317, 1985.
8. Brock TA, Rittenhouse SE, Powers CW, Ekstein LS, Gimbrone MA Jr and Alexander RW, Phorbol ester and 1-oleoyl-2-acetylglycerol inhibit angiotensin activation of phospholipase C in cultured vascular smooth muscle cells. *J Biol Chem* **260**: 14158–14162, 1985.
9. Brown KD, Blakeley DM, Hamon MH, Laurie MS and Corps AN, Protein kinase C-mediated negative-feedback inhibition of unstimulated and bombesin-stimulated polyphosphoinositide hydrolysis in Swiss-mouse 3T3 cells. *Biochem J* **245**: 631–639, 1987.
10. Drummond AH, Bidirectional control of cytosolic free calcium by thyrotropin-releasing hormone in pituitary cells. *Nature* **315**: 752–755, 1985.
11. Rickard JE and Sheterline P, Evidence that phorbol ester interferes with stimulated Ca^{2+} redistribution by activating Ca^{2+} efflux in neutrophil leucocytes. *Biochem J* **231**: 623–628, 1985.
12. McCarthy SA, Hallam TJ and Merritt JE, Activation of protein kinase C in human neutrophils attenuates agonist-stimulated rises in cytosolic free Ca^{2+} concentration by inhibiting bivalent-cation influx and intracellular Ca^{2+} release in addition to stimulating Ca^{2+} efflux. *Biochem J* **264**: 357–364, 1989.
13. Rink TJ and Sage SO, Stimulated calcium efflux from fura-2-loaded human platelets. *J Physiol (Lond)* **393**: 513–524, 1987.
14. Törnquist K, Modulatory effect of protein kinase C on thapsigargin-induced calcium entry in thyroid FRTL-5 cells. *Biochem J* **290**: 443–447, 1993.
15. Takemura H, Hughes AR, Thastrup O and Putney JW Jr, Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. Evidence that intracellular calcium pool, and not an inositol phosphate, regulates calcium fluxes at the plasma membrane. *J Biol Chem* **264**: 12266–12271, 1989.
16. Tojyo Y, Tanimura A, Matsui S and Matsumoto Y, Effect of thapsigargin on cytosolic Ca^{2+} level and amylase release in rat parotid acinar cells. *Cell Struct Funct* **17**: 223–227, 1992.
17. Sugiya H, Obie JF and Putney JW Jr, Two modes of regulation of the phospholipase C-linked substance-P receptor in rat parotid acinar cells. *Biochem J* **253**: 459–466, 1988.
18. Sugiya H and Putney JW Jr, Protein kinase C-dependent and -independent mechanisms regulating the parotid substance P receptor as revealed by differential effects of protein kinase C inhibitors. *Biochem J* **256**: 677–680, 1988.
19. Sugiya H, Tennes KA and Putney JW Jr, Homologous desensitization of substance-P-induced inositol polyphosphate formation in rat parotid acinar cells. *Biochem J* **224**: 647–653, 1987.
20. Tanimura A, Matsumoto Y and Tojyo Y, Mastoparan increases membrane permeability in rat parotid cells independently of action on G-proteins. *Biochem Biophys Res Commun* **177**: 802–808, 1991.
21. Berridge MJ, Dawson RMC, Downes CP, Heslop JP and Irvine RF, Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem J* **212**: 473–482, 1983.
22. Grynkiewicz G, Poenie M and Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260**: 3440–3450, 1985.
23. Dai Y, Ambudkar IS, Horn VJ, Yeh C-K, Kousvelari EE, Wall SJ, Li M, Yasuda RP, Wolfe BB and Baum BJ, Evidence that M_3 muscarinic receptors in rat parotid gland couple to two second messenger systems. *Am J Physiol* **261**: C1063–C1073, 1991.
24. Taylor CW, Merritt JE, Putney JW Jr and Rubin RP, A guanine nucleotide-dependent regulatory protein couples substance P receptors to phospholipase C in rat parotid gland. *Biochem Biophys Res Commun* **136**: 362–368, 1986.
25. Mertz LM, Horn VJ, Baum BJ and Ambudkar IS, Calcium entry in rat parotid acini: Activation by carbachol and aluminum fluoride. *Am J Physiol* **258**: C654–C661, 1990.
26. Tojyo Y, Tanimura A, Matsui S, Matsumoto Y, Sugiya H and Furuyama S, NaF-induced amylase release from rat parotid cells is mediated by PI breakdown leading to Ca^{2+} mobilization. *Am J Physiol* **260**: C194–C200, 1991.
27. Willems PHGM, van Den Broek BAM, van Os CH and De Pont JJHHM, Inhibition of inositol 1,4,5-trisphosphate-induced Ca^{2+} release in permeabilized pancreatic acinar cells by hormonal and phorbol ester pretreatment. *J Biol Chem* **264**: 9762–9767, 1989.
28. Liles WC, Hunter DD, Meier KE and Nathanson NM, Activation of protein kinase C induces rapid internalization and subsequent degradation of muscarine acetylcholine receptors in neuroblastoma cells. *J Biol Chem* **261**: 5307–5313, 1986.
29. Xu J and Chuang D-M, Muscarinic acetylcholine receptor-mediated phosphoinositide turnover in cultured cerebellar granule cells: Desensitization by receptor agonists. *J Pharmacol Exp Ther* **242**: 238–244, 1987.
30. Eva C, Gamalero SR, Genazzani E and Costa E, Molecular mechanisms of homologous desensitization and internalization of muscarinic receptors in primary cultures of neonatal corticostriatal neurons. *J Pharmacol Exp Ther* **253**: 257–265, 1990.
31. Sternweis PC and Gilman AG, Aluminum: A requirement for activation of the regulatory component of adenylate cyclase by fluoride. *Proc Natl Acad Sci USA* **79**: 4888–4891, 1982.
32. Katada T, Northup JK, Bokoch GM, Ui M and Gilman AG, The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. Subunit dissociation and guanine nucleotide-dependent hormonal inhibition. *J Biol Chem* **259**: 3578–3585, 1984.
33. Katada T, Gilman AG, Watanabe Y, Bauer S and Jakobs KH, Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in

hormonal inhibition of adenylate cyclase. *Eur J Biochem* **151**: 431–437, 1985.
34. Rhee SG, Suh P-G, Ryu S-H and Lee SY, Studies of inositol phospholipid-specific phospholipase C. *Science* **244**: 546–550, 1989.
35. Mangos JA, McSherry NR and Barber T, Dispersed rat parotid acinar cells. III. Characterization of cholinergic receptors. *Am J Physiol* **229**: 566–569, 1975.
36. Putney JW Jr, Identification of cellular activation mechanisms associated with salivary secretion. *Annu Rev Physiol* **48**: 75–88, 1986.
37. Tojyo Y, Tanimura A, Matsui S and Matsumoto Y, Carbachol-induced potassium release in rat parotid acini: Comparison of the roles of cytosolic Ca^{2+} and protein kinase C. *Jpn J Pharmacol* **63**: 439–446, 1993.