Modulation of Ca^{2+} Mobilization by Protein Kinase C in Rat Submandibular Acinar Cells

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Abstract The effects of protein kinase C (PKC) activation and inhibition on the inositol 1,4,5-trisphosphate (IP_3) and cytosolic Ca^{2+} ($[Ca^{2+}]_i$) responses of rat submandibular acinar cells were investigated. IP_3 formation in response to acetylcholine (ACh) was not affected by the PKC activator phorbol 12-myristate 13-acetate (PMA), nor by the PKC inhibitor calphostin C (CaC). The ACh-elicited initial increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} was not changed by short-term (0.5 min) exposure to PMA, but significantly reduced by long-term (30 min) exposure to PMA, and also by pre-exposure to the PKC inhibitors CaC and chelerythrine chloride (ChC). After ACh stimulation, subsequent exposure to ionomycin caused a significantly (258%) larger $[Ca^{2+}]_i$ increase in CaC-treated cells than in control cells. However, pre-exposure to CaC for 30 min did not alter the Ca^{2+} release induced by ionomycin alone. These results suggest that the reduction of the initial $[Ca^{2+}]_i$ increase is due to an inhibition of the Ca^{2+} release mechanism and not to store shrinkage. The thapsigargin (TG)-induced increase in $[Ca^{2+}]_i$ was significantly reduced by short-term (0.5 min), but not by long-term (30 min) exposure to PMA, nor by pre-exposure to ChC or CaC. Subsequent exposure to ionomycin after TG resulted in a significantly (70%) larger $[Ca^{2+}]_i$ increase in PMA-treated cells than in control cells, suggesting that activation of PKC slows down the Ca^{2+} efflux or passive leak seen in the presence of TG. Taken together, these results indicate that inhibition of PKC reduces the IP_3-induced Ca^{2+} release and activation of PKC reduces the Ca^{2+} efflux seen after inhibition of the endoplasmic Ca^{2+}-ATPase in submandibular acinar cells. J. Cell. Biochem. 72:47–55, 1999. © 1999 Wiley-Liss, Inc.

Key words: acetylcholine; thapsigargin; 1,4,5-IP_3, phorbol ester; calphostin C; chelerythrine Cl

An increase in the free Ca^{2+} levels in the cytosol ($[Ca^{2+}]_i$) is an important requirement for the secretion of fluid and electrolytes in salivary cells [Cook et al., 1994; Nauntofte, 1992], as it activates Ca^{2+}-sensitive anion channels in the apical membrane and K^+ channels in the basolateral membrane [Cook et al., 1988; Iwatsuki et al., 1985; Martinez and Cassity, 1986; Nauntofte and Poulsen, 1986], both of which are important in generating the proper ion fluxes associated with secretion. Such an increase is seen after stimulation of muscarinic receptors. This results in the activation of phospholipase C, which catalyzes the formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [Berridge, 1987]. IP_3 initiates a biphasic increase in $[Ca^{2+}]_i$, a transient rise resulting from Ca^{2+} release from an IP_3-sensitive intracellular Ca^{2+} store and a sustained elevation secondary to Ca^{2+} influx across the plasma membrane [Melvin et al., 1991; Merritt and Rink, 1987; Seagrave et al., 1993]. DAG activates protein kinase C (PKC), which phosphorylates a variety of proteins and plays positive as well as negative feedback roles in intracellular signaling pathways [Newton, 1995; Nishizuka, 1995].

Cross-talk between the IP_3-Ca^{2+} and the DAG-PKC arms of this signaling system in salivary cells has not been extensively studied. Activation of PKC with the phorbol ester phorbol 12-myristate 13-acetate (PMA) inhibited carbachol-induced Ca^{2+} release in HSG-PA cells, a submandibular duct cell line [He et al., 1988]. This observation suggests that PKC has an inhibitory effect on Ca^{2+} release from intracellular stores. It is unknown, however, if the effects on Ca^{2+} release are the result of direct regula-

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tion of the Ca^{2+} channels through which this release occurs or are secondary to PKC-induced alterations in the IP_3 response to agonists. We therefore investigated in greater detail how activation and inhibition of PKC affects both IP_3 production and Ca^{2+} mobilization in rat submandibular acinar cells. The results indicate that PKC has an interaction with the IP_3-Ca^{2+} arm of the phosphoinositide signaling pathway in these cells.

MATERIALS AND METHODS

Materials

Acetylcholine (Ach), bovine serum albumin (type V), EGTA, HEPES, hyaluronidase (type V), and ionomycin were from Sigma (St. Louis, MO). Calphostin C (CaC), chelerythrine chloride (ChC), 4a-phorbol-12,13-didecanoate (4αPDD), phorbol 12-myristate 13-acetate (PMA) and thapsigargin (TG) were purchased from CalBiochem (La Jolla, CA). Collagenase (type CLSPA) was purchased from Worthington Biomedical (Malvern, PA). Basal Eagle Medium amino acids (BEM) was from GIBCO (Grand Island, NY). Fura-2/acetoxymethyl ester (fura-2/AM) was from Molecular Probes (Eugene, OR). All other chemicals used were of the highest grade available.

Solutions

The digestion medium consisted of (in mM): 120 NaCl, 15 Hepes, 10 glucose, 4 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1% (w/v) BSA, 1% BEM (v/v), 3 U/mg wet tissue of collagenase, and 10 U/mg wet tissue of hyaluronidase. pH was adjusted to 7.4 with NaOH after gassing with O_2 at 37°C for 45 min. The Krebs-Hepes incubation solution (KHS) contained (in mM): 120 NaCl, 4 KCl, 15 Hepes, 10 glucose, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.0 $CaCl_2$, 1% BME Amino Acids and 0.01% (w/v) BSA. pH was adjusted to 7.4 after gassing with O_2 for 45 min. Determinations of cytosolic free Ca^{2+} were carried out in a low Ca^{2+} (100 nM) KHS solution that contained the same components as KHS, except that Ca^{2+} was omitted and 20–100 μM EGTA were added to adjust free Ca^{2+} concentration to 100 nM.

Animals

Male, 150–200 g Sprague-Dawley strain rats (Sasco Laboratories, Omaha, NB) were used in all experiments. Rats were kept in individual cages in a room with a controlled photoperiod (12:12-h light:dark cycle) and temperature (23°C) and with standard rat chow and water available ad libitum.

Preparation of Dispersed Submandibular Acini

Rat submandibular acini were isolated as previously described [Seagrave et al., 1993; Martinez et al., 1996]. Briefly, the submandibular glands were rapidly minced and incubated in the oxygenated digestion medium at 37°C in a shaking water bath for 60 min. After digestion, the preparation was centrifuged at 50*g* for 2 min and the supernatant was discarded. The preparation was then resuspended in 10 ml of oxygenated washing medium, passed through a four-layer-gauze, washed twice by centrifugation at 50*g* for 2 min, and finally resuspended in fresh incubation medium and kept at room temperature.

Determination of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined using the Ca^{2+} sensitive fluorescent indicator fura-2 as previously described [Zhang et al., 1996; Martinez et al., 1997]. Isolated submandibular acini were loaded with fura-2 by incubation with 1 μM fura-2/AM for 30 min at room temperature. $[Ca^{2+}]_i$ measurements were made using 2 ml of fura-2-loaded acini suspended in Ca^{2+}-free medium in a 4-ml cuvette. Fluorescence was monitored with a PTI Deltascan fluorometer (PTI Inc., S. Brunswick, NJ). The excitation wavelengths used were 340 and 380 nm and emission wavelength was 505 nm.

Calibration of $[Ca^{2+}]_i$ was performed for each measurement trace as previously described [Wells et al., 1997; Zhang et al., 1997]. Briefly, 1 mM $CaCl_2$ and 50 μM ionomycin were added to obtain the limiting ratio for Ca^{2+} saturated form (R_{max}) of fura-2. Then, 0.0005% digitonin and 10 mM EGTA were sequentially added to obtain the limiting ratio for the unbound form (R_{min}) of fura-2. Fluorescence ratios of the 340/380 nm excitation and 505 nm emission were converted to $[Ca^{2+}]_i$ according to Grynkiewicz et al. [1985] using 224 nM as the K_d of fura-2 for Ca^{2+} at 37°C.

Measurement of IP_3

The formation of IP_3 was measured as previously described [Seagrave et al., 1993; Zhang et al., 1997]. Isolated acini were incubated in KHS at 37°C, pretreated with PMA for 30 sec or with CaC for 30 min, and then stimulated with ACh for 30 sec. The reaction was terminated by

adding an equal volume of 1 M ice-cold trichloroacetic acid. The level of IP_3 was measured using a radioligand assay kit (Amersham, Arlington Heights, IL).

Data Presentation and Statistics

All results are presented as means ± SEM of separate determinations using different cell preparations. Comparisons were made using the unpaired Student's *t*-test. *P* values < 0.05 are considered significant.

RESULTS

Effect of PMA, CaC, and ChC on IP_3 Formation

The IP_3 level in unstimulated rat submandibular acinar cells was 0.9 ± 0.3 pmol/mg protein (n = 9). Exposure of the cells to 100 nM phorbol 12-myristate 13-acetate (PMA), a potent PKC activator, for 30 sec or 30 min did not alter the basal IP_3 level (1.0 ± 0.5 pmol/mg, n = 7 and 1.2 ± 0.7 pmol/mg, n = 7, respectively; Fig. 1). Similarly, the IP_3 level after exposure to 500 nM calphostin C (CaC), a membrane-permeable and specific PKC inhibitor [Kobayashi et al., 1989], for 30 min was 1.6 pmol/mg (n = 3; Fig. 1).

Stimulation of submandibular acinar cells with ACh (1 μM) for 0.5 min induced a 589% increase in IP_3 formation (6.2 ± 0.5 pmol/mg, n = 16; *P* < 0.001; Fig. 1). Pretreatment with 100 nM PMA for 0.5 min or 30 min did not change the formation of IP_3 stimulated by ACh (5.4 ± 0.9 pmol/mg, n = 7 and 5.6 ± 0.9 pmol/5 min, n = 7, respectively). Pretreatment with 500 nM CaC for 30 min did not alter the ACh-stimulated IP_3 formation (6.1 ± 1.0 pmol/mg, n = 5) (Fig. 1).

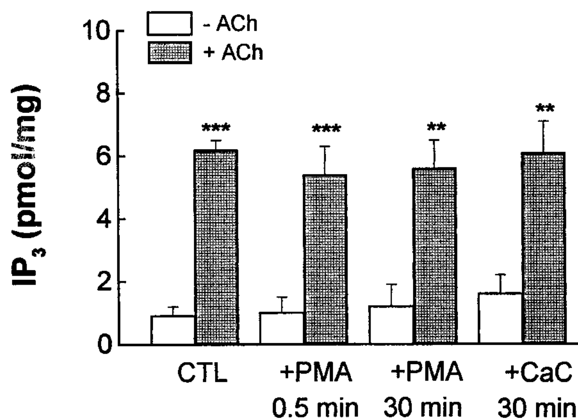

Fig. 1. Effects of PMA and CaC on ACh-stimulated IP_3 formation. Isolated rat submandibular acinar cells were exposed to 100 nM PMA (+*PMA*) for 0.5 or 30 min, to 500 nM CaC (+*CaC*) or to DMSO (*CTL*) for 30 min, then stimulated with 1 μM ACh for 0.5 min. Samples from unstimulated (-*ACh*) and stimulated cells (+*ACh*) were prepared and IP_3 contents were measured. The values presented are means ± SEM of separate experiments (unstimulated: CTL, n = 9; +PMA, 0.5 min, n = 7; +PMA, 30 min, n = 7; +CaC, n = 3; stimulated with ACh: CTL, n = 16; +PMA, 0.5 min, n = 7; +PMA, 30 min, n = 7; +CaC, n = 5). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to unstimulated cells, respectively.

Effects of PMA, CaC, and ChC on the ACh-induced Ca^{2+} Release

The resting level of $[Ca^{2+}]_i$ in submandibular acinar cells was 97 ± 6 nM (n = 18). Exposure of the cells to 100 nM 4α-PDD, a phorbol ester analog commonly used as a negative control for the PKC activators PMA and phobol-12,13-didecanoate [Akiguchi et al., 1993] did not affect basal $[Ca^{2+}]_i$. Similarly, exposure to PMA, CaC, or chelerythrine Cl (ChC), a membrane-permeable and specific PKC inhibitor [Herbert et al., 1990], did not induce an instant increase in $[Ca^{2+}]_i$ (not shown). Exposure of the cells to these agents for 30 min caused a small elevation of the basal $[Ca^{2+}]_i$ level (Fig. 2). After incubation with vehicle (DMSO) for 30 min, the $[Ca^{2+}]_i$ was 101 ± 7 nM (n = 12). The resting levels of $[Ca^{2+}]_i$ after exposure to 4α-PDD, PMA, or ChC for 30 min were 117 ± 10 nM (n = 14), 118 ± 7 nM (n = 15), and 127 ± 13 nM (n = 9), respectively (Fig. 2), which were not significantly different from control. However, exposure to CaC for 30 min induced a significant increase in resting $[Ca^{2+}]_i$ (143 ± 8 nM, n = 17; *P* < 0.001; Fig. 2). The mechanism mediating this increase in resting $[Ca^{2+}]_i$ in the presence

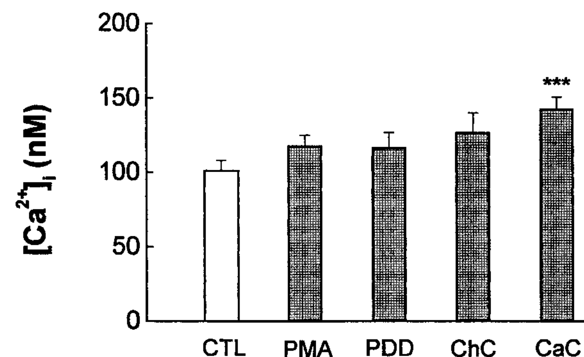

Fig. 2. Effects of PMA, 4α-PDD, CaC, and ChC on $[Ca^{2+}]_i$ in unstimulated cells. Fura-2-loaded submandibular acinar cells were exposed to 100 nM PMA (*PMA*), 100 nM 4α-PDD (*PDD*), 500 nM CaC (*CaC*), or 10 μM ChC (*ChC*), or the same volume of DMSO (*CTL*) for 30 min, and $[Ca^{2+}]_i$ was then measured. $^{***}P < 0.001$ compared to control. Each trace is representative of separate experiments using different cell preparations (CTL, n = 12; PMA, n = 15; 4α-PDD, n = 14; CaC, n = 17; ChC, n = 9).

of CaC is unknown, but could be related to inhibition of the plasma membrane Ca^{2+}-ATPase (Ca^{2+}-pump).

As shown in Figure 3A, stimulation of fura-2-loaded acinar cells with 1 μM ACh triggered a 101 ± 12 nM initial increase in $[Ca^{2+}]_i$ (from 88 ± 6 to 189 ± 12 nM, n = 8). The $[Ca^{2+}]_i$ increase in response to ACh was not affected by a short-term (0.5 min) exposure to 100 nM 4α-PDD. As shown in Figure 3B, the $[Ca^{2+}]_i$ increase induced by ACh in 4α-PDD-treated cells was 96 ± 11 nM (n = 9), which was not significantly different from the increase induced by ACh alone. To examine the effect of a short-term exposure to PMA on the $[Ca^{2+}]_i$ response to muscarinic stimulation, the cells were exposed to 100 nM PMA for 0.5 min, then stimulated with the same concentration of ACh. This manipulation did not alter the initial increase in $[Ca^{2+}]_i$ (net increase: 112 ± 14 nM; from 107 ± 10 nM to 219 ± 10 nM, n = 9; $P > 0.05$; Fig. 3B). Stimulation with ACh (1 μM) of the cells pre-exposed for 30 min to 100 nM 4α-PDD induced a 109 ± 7 nM increase in $[Ca^{2+}]_i$ (n = 8; $P > 0.05$; Fig. 3C), which was not significantly different from the change induced by ACh alone. However, pre-exposure of the cells to 100 nM PMA for 30 min significantly reduced the ACh-induced increase in $[Ca^{2+}]_i$ (net increase: 53 ± 7 nM, n = 13; $P < 0.001$; Fig. 3C).

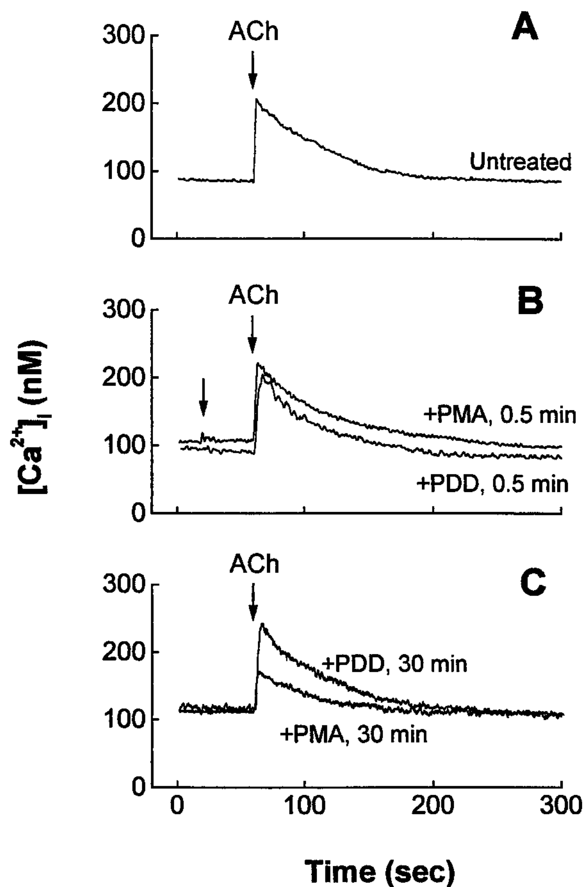

Fig. 3. Effects of PMA and 4α-PDD on ACh-stimulated $[Ca^{2+}]_i$ increase. Fura-2-loaded cells were suspended in Ca^{2+}-free medium and $[Ca^{2+}]_i$ was monitored. Cells were untreated (**A**) or exposed to 100 nM 4α-PDD or 100 nM PMA for 0.5 min (**B**) or to 4α-PDD or 100 nM PMA for 30 min (**C**), and then stimulated with 1 μM ACh (*ACh*) at the time indicated by the arrow. Each trace is representative of separate experiments using different cell preparations (untreated, n = 8; +PDD, 0.5 min, n = 9; +PMA, 0.5 min, n = 9; +PDD, 30 min, n = 9; +PMA, 30 min, n = 9).

Next, we examined the effects of PKC inhibitors on the $[Ca^{2+}]_i$ release seen in response to ACh. Pre-exposure to ChC (10 μM) or CaC (500 nM) for 30 min significantly reduced the $[Ca^{2+}]_i$ increase stimulated by subsequent exposure to 1 μM ACh (ChC: 66 ± 3 nM, n = 5; $P < 0.01$; CaC: 44 ± 10 nM, n = 6; $P < 0.005$; Fig. 4).

To further explore the mechanism mediating this inhibition of the $[Ca^{2+}]_i$ response to ACh by ChC or CaC, we measured the residual Ca^{2+} remaining in the intracellular Ca^{2+} store after ACh stimulation by subsequently adding ionomycin. As shown in Figure 5, addition of ionomycin (1 μM) after ACh induced a 38 ± 4 nM (n = 6) further increase in $[Ca^{2+}]_i$ in untreated (without inhibitor) cells (Fig. 5A), and a significantly larger (136 ± 15 nM, n = 6) increase in CaC-pretreated cells ($P < 0.001$; Fig. 5B). To further clarify whether CaC pretreatment increases the capacity of the intracellular Ca^{2+} store, control cells and CaC-pretreated cells were exposed to ionomycin alone. As shown in Figure 6, the $[Ca^{2+}]_i$ increase induced by ionomycin in control cells was 168 ± 18 nM (n = 11; Fig. 6A) and in CaC-treated cells was 149 ± 16 nM (n = 5; $P > 0.05$; Fig. 6B). These results suggest that inhibition of PKC with CaC does not increase pool capacity but blocks ACh-induced Ca^{2+} release from the IP_3-sensitive intracellular pool, so that a larger amount of Ca^{2+} remains in the pool to be later discharged by ionomycin.

Effects of PMA, CaC, and ChC on the TG-induced Ca^{2+} Release

Ca^{2+} reuptake into the IP_3-sensitive Ca^{2+} store is dependent on an endoplasmic reticulum Ca^{2+}-ATPase. Inhibition of this Ca^{2+}-ATPase with the specific inhibitor thapsigargin (TG) induces passive efflux of Ca^{2+} through a completely separate mechanism from that acti-

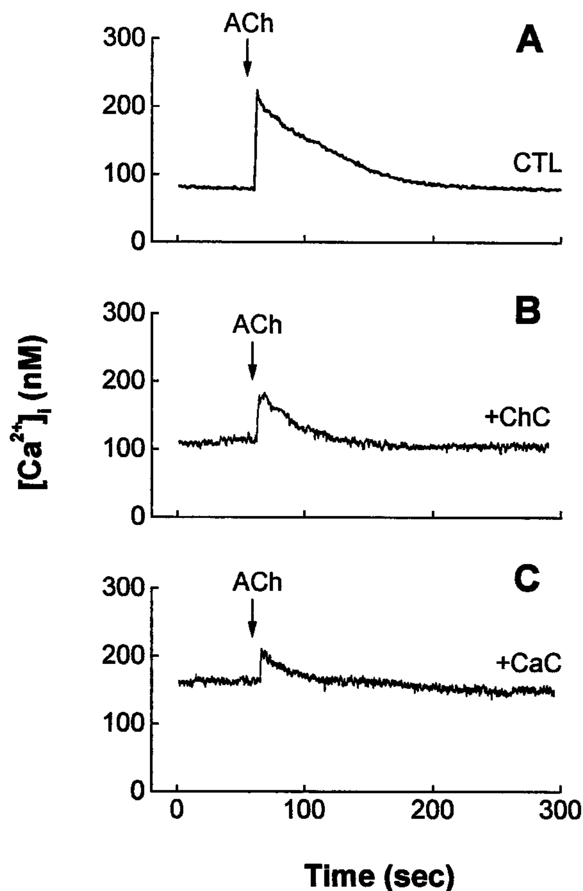

Fig. 4. Effects of CaC and ChC on ACh-stimulated $[Ca^{2+}]_i$ increase. Fura-2-loaded cells were suspended in Ca^{2+}-free medium and $[Ca^{2+}]_i$ was monitored. Cells were exposed to DMSO (*CTL;* **A**), 10 µM ChC (*+ChC;* **B**), or 500 nM CaC (*+CaC;* **C**) for 30 min, and then stimulated with 1 µM ACh (*ACh*) at the time indicated by the arrow. Each trace is representative of separate experiments using different cell preparations (CTL, n = 8; +ChC, n = 5; +CaC, n = 6).

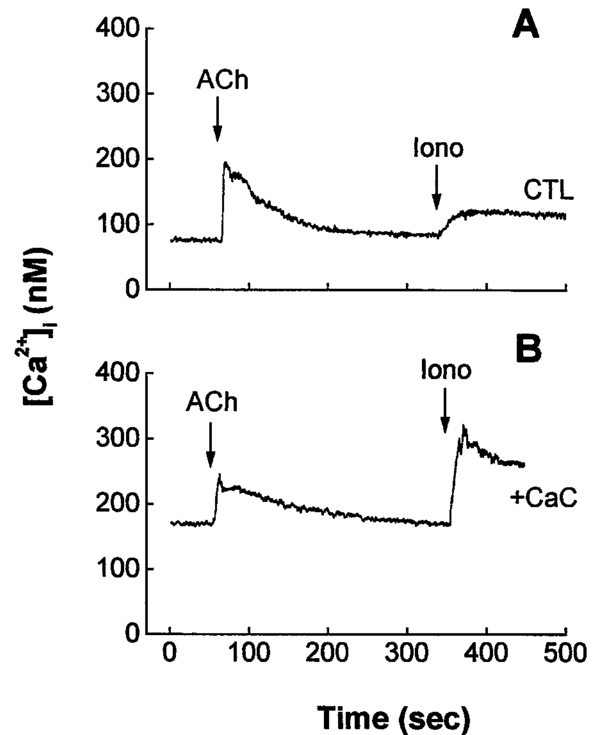

Fig. 5. Effect of CaC on residual Ca^{2+} in the IP_3-sensitive store after ACh stimulation. Fura-2-loaded rat submandibular acinar cells were exposed to vehicle (*CTL;* **A**) or 500 nM CaC (*+CaC;* **B**) in physiological salt solution containing 1 mM Ca^{2+} for 30 min, then suspended in Ca^{2+}-free medium ($[Ca^{2+}]_0$ = 100 nM), and $[Ca^{2+}]_i$ was monitored. At the time indicated by the arrow, 1 µM ACh (*ACh*) and 1 µM ionomycin (*Iono*) were sequentially added. Each trace is representative of separate experiments using different cell preparations (CTL, n = 6; +CaC, n = 6).

vated by IP_3 [Thastrup, 1990; Thastrup et al., 1989]. To test whether PKC modulates this Ca^{2+} efflux, we investigated the effects of PKC activation and inhibition on the TG-induced increase in $[Ca^{2+}]_i$. As shown in Figure 7A, exposure of the cells to TG (3 µM) induced a 131 ± 17 nM increase in $[Ca^{2+}]_i$ (from 98 ± 4 to 229 ± 32 nM, n = 10). This response was not altered by a short-term (0.5 min) or longer-term (30 min) pretreatment with 4α-PDD (0.5 min: 134 ± 21 nM, n = 5; 30 min: 124 ± 13 nM, n = 6; Fig. 7B and C). However, pre-exposure to 100 nM PMA for 0.5 min significantly reduced the TG-induced increase in $[Ca^{2+}]_i$ (63 ± 13 nM, n = 6; $P < 0.005$; Fig. 7B), and a longer pretreatment with PMA (30 min) only slightly reduced it (95 ± 11 nM, n = 5; Fig. 7C). The latter effect was not statistically different from that in control (4α-PDD-treated) cells.

In contrast, the TG-induced Ca^{2+} release was not altered by CaC or ChC. The $[Ca^{2+}]_i$ increase in cells exposed to TG alone was 132 ± 14 nM (n = 8; Fig. 8A). Pretreatment with 10 µM ChC for 30 min did not alter the TG-induced increase in $[Ca^{2+}]_i$ (net increase: 131 ± 13 nM, n = 5; $P > 0.05$; Fig. 8B). A slightly smaller $[Ca^{2+}]_i$ increase in response to TG was observed in cells pre-exposed to 500 nM CaC for 30 min (net increase: 89 ± 13 nM; n = 6; Fig. 8C). However, this difference was not statistically significant ($P > 0.05$). These results indicate that inhibition of PKC does not affect the TG-induced Ca^{2+} release.

The results in Figures 7 and 8 suggest that activation of PKC inhibits the passive Ca^{2+} efflux induced by inhibition of the Ca^{2+}-pump present in the IP_3-sensitive Ca^{2+} store. To further elucidate whether the reduction of the

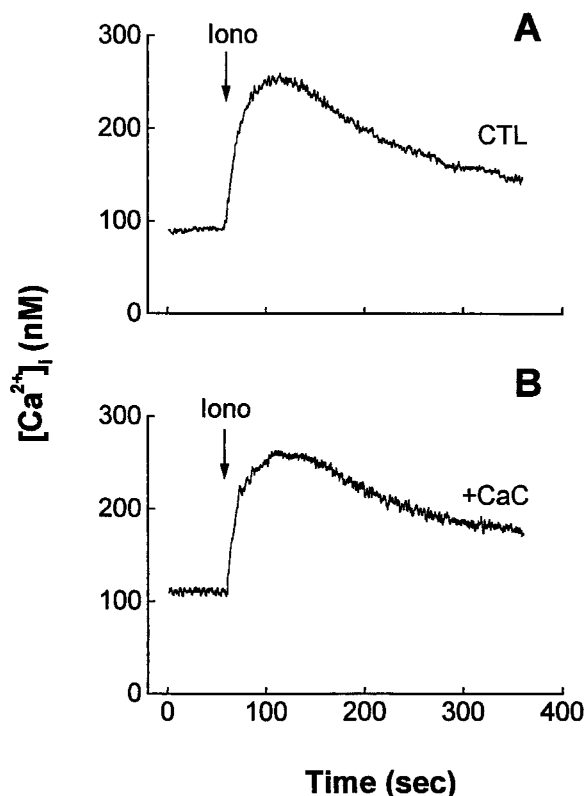

Fig. 6. Effect of CaC pre-exposure on the capacity of Ca^{2+} Stores. Fura-2-loaded rat submandibular acinar cells were exposed to vehicle (*CTL;* **A**) or 500 nM CaC (*+CaC;* **B**) in physiological salt solution containing 1 mM Ca^{2+} for 30 min, then suspended in Ca^{2+}-free medium ($[Ca^{2+}]_0$ = 100 nM), and $[Ca^{2+}]_i$ was monitored. At the time indicated by the arrow, 1 μM ionomycin (*Iono*) was added. Each trace is representative of separate experiments using different cell preparations (CTL, n = 11; +CaC, n = 5).

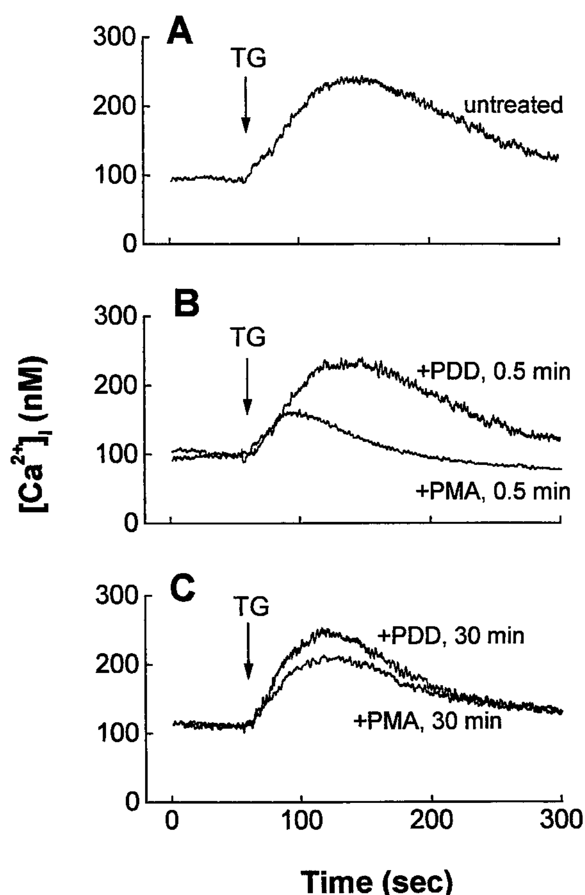

Fig. 7. Effects of PMA on TG-induced $[Ca^{2+}]_i$ increase. Fura-2-loaded cells were suspended in Ca^{2+}-free medium ($[Ca^{2+}]_0$ = 100 nM) and $[Ca^{2+}]_i$ was monitored. Cells were untreated (**A**) or exposed to 100 nM 4α-PDD or 100 nM PMA for 0.5 min (**B**) or to 4α-PDD or 100 nM PMA for 30 min (**C**), and then exposed to 3 μM TG (*TG*) at the time indicated by the arrow. Each trace is representative of separate experiments using different cell preparations (untreated, n = 10; +PDD, 0.5 min, n = 5; +PDD, 30 min, n = 6; +PMA, 0.5 min, n = 6; + PMA, 30 min, n = 5).

TG-induced increase in $[Ca^{2+}]_i$ by PKC is due to effects on the Ca^{2+} efflux mechanism, we also measured the $[Ca^{2+}]_i$ response to ionomycin after TG. As shown in Figure 9A, exposure to ionomycin (1 μM) after TG resulted in a 109 ± 9 nM further increase in $[Ca^{2+}]_i$ in control (i.e., not pre-exposed to other test substances) cells (n = 5). In contrast, the increase in $[Ca^{2+}]_i$ in response to ionomycin after TG was 185 ± 8 nM (n = 5) in PMA-pretreated cells (Fig. 9B). This was significantly greater than that seen in control cells ($P < 0.001$). These results suggest that activation of PKC with PMA reduces or slows down the TG-induced passive Ca^{2+} efflux from the IP_3-sensitive intracellular pool. Alternatively, activation of PKC may decrease the sensitivity of the Ca^{2+}-ATPase to the inhibitor TG.

DISCUSSION

Activation of phospholipase C by stimulation of muscarinic receptors causes formation of both IP_3 and DAG which are critical signal transduction molecules mediating various cellular events in a variety of cells, including salivary acinar cells [Berridge, 1987]. The IP_3-Ca^{2+} arm of this signal transduction pathway has been extensively studied and a great deal of progress has been made in our understanding of the mechanisms by which Ca^{2+} is mobilized and by which Ca^{2+} mobilization regulates the function of salivary and other secretory cells. However, the physiological and regulatory roles of the other arm of the pathway, the DAG-PKC arm, are

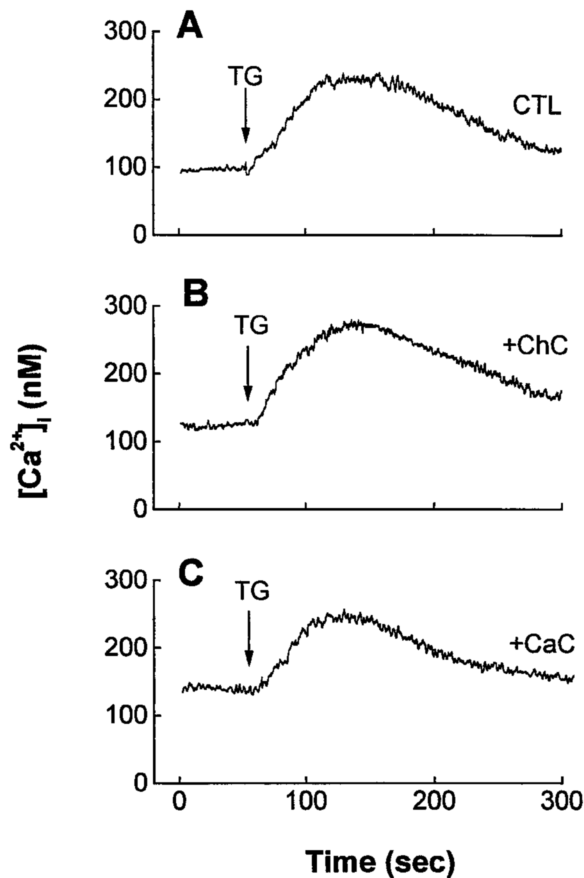

Fig. 8. Effects of CaC and ChC on TG-stimulated $[Ca^{2+}]_i$ increase. Fura-2-loaded cells were suspended in Ca^{2+}-free medium and $[Ca^{2+}]_i$ was monitored. Cells were pre-exposed to DMSO (*CTL;* **A**), 10 μM ChC (*+ChC;* **B**), or 500 nM CaC (*+CaC;* **C**) for 30 min, and then exposed to 3 μM TG (*TG*) at the time indicated by the arrow. Each trace is representative of separate experiments using different cell preparations (CTL, n = 8; +ChC, n = 5; +CaC, n = 6).

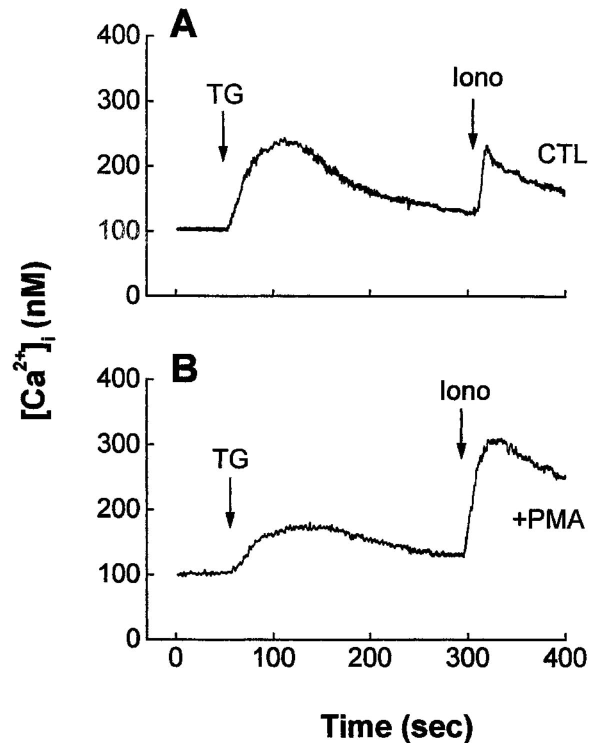

Fig. 9. Effect of PMA on residual Ca^{2+} in the TG-sensitive store. Fura-2-loaded cells were suspended in Ca^{2+}-free medium ($[Ca^{2+}]_o$ = 100 nM), then exposed to DMSO (*CTL;* **A**) or 100 nM PMA (*+PMA;* **B**) for 30 sec. At the time indicated by the arrows, 3 μM TG (*TG*) or 1 μM ionomycin (*Iono*) was added. Each trace is representative of separate experiments using different cell preparations (CTL, n = 5; +PMA, n = 5).

much less well understood in salivary cells. Furthermore, the interaction or cross-talk between the two arms of the pathway remains poorly understood. The results of this study demonstrate that PKC is involved in the regulation of cytosolic Ca^{2+} levels in these cells by modulating Ca^{2+} release from intracellular stores.

A study on pancreatic acinar cells found that activation of PKC with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) for 5 min inhibited cholecystokinin-evoked IP_3 formation by decreasing the affinity state of the receptor [Willems et al., 1995]. However, this is not the case in submandibular acinar cells. The results of the present study show that IP_3 formation in response to ACh stimulation in our cells was not affected by either short term (0.5 min), or longer term (30 min) pretreatment with PMA. Similar results have been reported by Berrie and Elliott [1994], who showed that pretreatment of rat submandibular acinar cells with TPA for 5 min did not alter the ACh-induced IP_3 formation. We did not observe, furthermore, any effect of PKC inhibition with CaC on ACh-induced IP_3 formation. Thus, our findings do not suggest that PKC directly modulates the activity of PLC or phosphoinositide turnover in submandibular acinar cells. PKC may affect the coupling between the ACh receptor and membrane phosphoinositides, but the effect may be a permissive one and not a true regulatory one. Since many factors can contribute to phosphoinositide turnover, further investigation is required to elucidate a possible modulation of the coupling mechanism by PKC.

While treatment of acinar cells with TPA for 5 min did not affect the ACh-induced increase in $[Ca^{2+}]_i$ [Berrie and Elliot, 1994], treatment

with PMA for 5 min significantly reduced the carbachol-induced initial $[Ca^{2+}]_i$ increase in HSG-PA cells, a cell line of ductal origin [He et al., 1988]. In the present study, we observed that short-term pre-exposure of submandibular acinar cells to PMA did not alter the Ca^{2+} release elicited by ACh. Our results agree therefore with those of Berrie and Elliot [1994] but not with those He et al. [1988]. The difference between our results and those reported by He et al. [1988] is most likely due to the different cell types involved. He et al. used the human duct cell line HSG-PA which has different functional characteristics from the freshly isolated rat acinar cells [Patton and Welner, 1993]. It is not surprising that $[Ca^{2+}]_i$ responses to PKC activation differ in acinar and duct cells. Not only are the Ca^{2+} stores probably different in the two types of cells, but the Ca^{2+} increase seen upon stimulation regulates different ion transport mechanisms. Moreover, we have also recently observed that as in HSG-PA cells, exposure of another salivary duct cell line (A253) to PMA for short-term (0.5, 5, or 10 min) strikingly reduces Ca^{2+} release in response to ATP, a P_2-purinergic agonist (Sugita et al., unpublished data).

Longer-term (30 min) pretreatment with PMA significantly inhibited Ca^{2+} release stimulated by ACh. The mechanism mediating this inhibition is unclear. It is possible that a 30 min exposure to PMA actually downregulates PKC. The time course of PKC downregulation varies among different cell types. The fastest downregulation was reported in GH_4C_1 cells [Kiley et al., 1991]. Stimulation of these cells with thyrotropin-releasing hormone (TRH) for 15 sec induced the activation of PKC, as judged by the redistribution (translocation) of PKCs from the soluble (cytosolic) fraction to the insoluble (membrane) fraction. This redistribution of PKCs disappeared after 10 min due to downregulation of the enzymes.

Inhibition of PKC with CaC or ChC had no effect on IP_3 formation but reduced the Ca^{2+} response to ACh. The results in Figures 5 and 6 indicate that inhibition of PKC does not affect the capacity of the IP_3-sensitive Ca^{2+} store, but reduces or slows down the process of Ca^{2+} release. These results further indicate that PKC plays a role in regulating Ca^{2+} release through the IP_3 receptors. Taken together, these findings suggest that PKC may be directly involved in the modulation of the opening state of the Ca^{2+} channels in the endoplasmic reticulum. These channels are receptors for IP_3 and PKC is likely to phosphorylate the channel protein and alter its affinity for IP_3. Both PKC and the cyclic AMP-dependent protein kinases (PKA) can phosphorylate IP_3 receptors in various cell types [Bygrave and Roberts, 1995]. We have recently shown that activation of PKA in rat submandibular acinar cells inhibited the Ca^{2+} release induced by ACh [Martinez and Zhang, 1998]. Thus, our data suggest that the endoplasmic Ca^{2+} channels in submandibular cells may be selectively phosphorylated by both kinases, with opposite changes in their affinity for IP_3.

Our results also show that the Ca^{2+} signal induced by inhibition of the endoplasmic Ca^{2+}-ATPase by TG is also under PKC modulation. It is well established that inhibition of the endoplasmic Ca^{2+} ATPase unmasks a passive Ca^{2+} efflux from the store [Kraus-Friedmann, 1990]. This efflux is believed to be mediated by the reversal of the Ca^{2+}-ATPase [Webb and Anders, 1985; Jencks, 1989; Kraus-Friedmann, 1990]. Since the Ca^{2+} concentration inside the Ca^{2+} store is up to 10,000 times higher than in the cytosol, a large amount of energy is stored in the electrochemical Ca^{2+} gradient. When the Ca^{2+}-ATPase is inhibited, the driving force maintaining the gradient is reduced or disappears, resulting in a passive Ca^{2+} efflux and ATP synthesis [Webb and Anders, 1985; Jencks, 1989; Kraus-Friedmann, 1990]. Activation of PKC by PMA reduced the Ca^{2+} release induced by TG. A reasonable explanation of this finding is that PKC phosphorylates the endoplasmic Ca^{2+}-ATPase responsible for Ca^{2+} re-uptake and renders the ATPase less sensitive to TG so that TG has a smaller inhibitory effect when added to the cell suspensions. Alternatively, PKC activation may inhibit the passive Ca^{2+} efflux mechanism which occurs from the store by mechanisms other than IP_3 receptors [Thastrup et al., 1989] or it may potentiate the activity of the Ca^{2+} pump in the endoplasmic reticulum so that there is more Ca^{2+} re-uptake even in the presence of TG. These possibilities are currently being further investigated.

In summary, our results demonstrate interactions between the DAG-PKC and the IP_3-Ca^{2+} arms of the phosphoinositide pathway in rat submandibular acinar cells. PKC influences Ca^{2+} release via IP_3 receptors and the passive

Ca^{2+} efflux which occurs upon inhibition of the endoplasmic Ca^{2+}-ATPase.

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