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ABSTRACT

Fluid and exocrine secretion of mucins by salivary mucous glands is regulated predominantly by parasympathetic activation of muscarinic receptors. A direct role for subsequent putative signaling steps, phospholipase C (PLC), increased intracellular calcium ($[Ca^{2+}]_i$), and isoforms of protein kinase C (PKC) in mediating muscarinic exocrine secretion has not been elucidated, and these are potential therapeutic targets to enhance mucin secretion in hyposalivary patients. We found that muscarinic-induced mucin secretion by rat sublingual tubulo-acini was dependent upon PLC activation and the subsequent increase in $[Ca^{2+}]_i$, and further identified a transient PKC-independent component of secretion dependent upon Ca^{2+} release from intracellular stores, whereas sustained secretion required entry of extracellular Ca^{2+} . Interactions among carbachol, PKC inhibitors, phorbol 12-myristate 13-acetate, and thapsigargin to modulate $[Ca^{2+}]_i$ implicated conventional PKC isoforms in mediating sustained secretion. With increasing times during carbachol perfusion of glands, *in situ*, PKC- α redistributed across glandular membrane compartments and underwent a rapid and persistent accumulation near the luminal borders of mucous cells. PKC- β 1 displayed transient localization near luminal borders, whereas the novel PKCs, PKC- δ or PKC- ϵ , displayed little or no redistribution in mucous cells. Collective results implicate synergistic interactions between diacylglycerol (DAG) and increasing $[Ca^{2+}]_i$ levels to activate cPKCs in mediating sustained muscarinic-induced secretion.

KEY WORDS: salivary glands, muscarinic cholinergic receptors, protein kinase C, mucous cells, exocrine secretion, stimulus-secretion coupling.

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Role of Calcium and PKC in Salivary Mucous Cell Exocrine Secretion

INTRODUCTION

Human salivary glands are diverse in size, location, distribution of secretory cell types, patterns of innervation, and contributions to the fluid and organic components to saliva (Melvin and Culp, 2004). Glands are generally classified by their relative content of histologically distinct secretory cell types, serous (parotid and von Ebner's), mucous (major sublingual and the minor sublingual, labial, buccal, palatine, and lingual mucous), and mixed (submandibular glands with predominantly serous elements and interspersed mucous tubulo-acini) (Pinkstaff, 1980). Mucous glands contribute about 70% of gel-forming mucins in saliva, the rest being contributed by submandibular mucous cells (Milne and Dawes, 1973). As a model for human mucous glands, we study rat sublingual glands, composed of mucous tubulo-acini capped by serous demilune cells. Mucous glands receive predominant parasympathetic innervations, resulting in mucin secretion unresponsive to sympathetic stimulation or adrenergic agonists (Watson and Culp, 1994). Conversely, human submandibular mucous cells are stimulated by adrenergic agonists (McPherson *et al.*, 1985), consistent with robust sympathetic innervation of both human and rodent submandibular and parotid glands (Garrett, 1967; Melvin and Culp, 2004).

In sublingual tubulo-acini, both M1 and M3 muscarinic cholinergic receptor subtypes are coupled to G_q and G_{11} G proteins, which activate phosphatidylinositol 1,4-bisphosphate (PIP_2)-specific phospholipase C (PLC) (Luo *et al.*, 2001). PLC activation generates inositol 1,4,5-trisphosphate (IP_3) (Zhang and Melvin, 1993) to release Ca^{2+} from intracellular stores, followed by an influx of extracellular Ca^{2+} through a store-operated Ca^{2+} entry mechanism to sustain increased intracellular free calcium ($[Ca^{2+}]_i$) (Melvin *et al.*, 1991). Although mechanisms associated with mucous cell ion fluxes (Nguyen *et al.*, 2004) and cell volume (Krane *et al.*, 2001) have been elucidated, roles for PLC and for $[Ca^{2+}]_i$ in mucous cell exocrine secretion are unclear. Moreover, the PLC product, diacylglycerol (DAG), may mediate secretion through activation of protein kinase C (PKC) (Dempsey *et al.*, 2000).

There are 3 families of PKC isoforms, conventional (α , β , β_{II} , γ), novel (δ , ϵ , η , θ), and atypical (ζ , λ/ι). The conventional isoforms (cPKCs) are Ca^{2+} - and phospholipid-dependent and require phosphatidylserine (PS), Ca^{2+} , and DAG or phorbol esters. Novel isoforms (nPKCs) are Ca^{2+} -independent and require DAG and PS. Atypical isoforms (aPKCs) are dependent on PS, inositol lipids, or phosphatidic acid (Dempsey *et al.*, 2000). The expression and exocrine functions of PKC isoforms in mucous cell are unknown. Given the function of salivary gel-forming mucins in hydration and lubrication (Tabak, 1995), elucidation of signaling mechanisms controlling mucous cell exocrine secretion may lead to therapeutic targets for patients with hyposalivary function.

We therefore investigated PLC, $[Ca^{2+}]_i$, and PKC in mediating mucous gland mucin secretion.

MATERIALS & METHODS

Materials

Thapsigargin, Gö6976, calphostin C, phorbol 12-myristate 13-acetate (PMA), 4-O-methyl-PMA, U73122, U73343, 1,2-bis (o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA/AM), and cell-permeable autocamtide-2-related inhibitory peptide II were obtained from Calbiochem (San Diego, CA, USA). Unless indicated, all other materials were from Sigma-Aldrich (St. Louis, MO, USA).

Animals, Preparation of Sublingual Acini, and Measurement of Mucin Secretion

The University of Rochester IACUC committee approved all animal procedures, which were as described previously (Culp *et al.*, 1996). Briefly, tubulo-acini were dispersed enzymatically (collagenase and hyaluronidase) from minced sublingual glands of Wistar rats. Cell mucins were radiolabeled endogenously with $[^3H]$ glucosamine, followed by the distribution of tubulo-acini to culture wells for the times indicated. Tubulo-acini were homogenized, and mucins in the supernatants and homogenates were acid-precipitated and counted. Mucin secretion was normalized as the percentage of the total (supernatant plus pellet) precipitable disintegrations *per* min (dpm) in the supernatant, and expressed as % total precipitable dpm released. Each condition was performed in triplicate. Mucin specificity of this assay was reported previously (Watson *et al.*, 1997). See Appendix for details.

Intracellular Calcium

$[Ca^{2+}]_i$ was the average fluorescent signals captured with a 40x objective from 5 to 8 mucous cells within a tubulo-acinus loaded with the Ca^{2+} -selective dye, fura-2 (Molecular Probes, Eugene, OR, USA) as described previously (Culp *et al.*, 1996). See Appendix for details.

Western Blots

Procedures were similar to those described previously (Fallon *et al.*, 2003). Briefly, freshly excised glands were immediately frozen in liquid nitrogen. Single glands were each pulverized in liquid N_2 and re-suspended in 1 mL of ice-cold TEMPI (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, and 20 μ L protease inhibitor cocktail). Suspensions were sonicated and centrifuged to remove large particulates. Supernatants were run on SDS-PAGE gels (9% running gels with 4% stacking gels), transferred onto Immobilon-PVDF membranes (Millipore Corp., Milford, MA, USA), and blots probed with polyclonal antibodies against PKC isoform-specific C-terminal regions, with detection by $[^{125}I]$ -rProtein A (PerkinElmer Life Sciences, Boston, MA, USA) and autoradiography. See Appendix for details.

PKC Immunolocalization and Redistribution *in situ*

A modification of the procedure by Albert and Ford (1999) was used. Rats were sacrificed by CO_2 inhalation, and the vasculature was perfused at 41 mL/min with heated (39°C) and oxygenated (95% O_2 -5% CO_2) modified Krebs Henseleit (KH). After 2 min, the solution was switched to heated and oxygenated KH containing 1.3 mg/L phenol red, $\pm$ 10 μ M carbachol, for increasing intervals. Time zero was when the phenol red entered the glands. One gland was excised and placed in liquid N_2 . The contralateral gland was immediately perfused for 30 min with ice-cold 4% paraformaldehyde in PBS, excised, and incubated for 2 hrs in 4°C fixative; 3- to 4-mm-thick slices were embedded in paraffin. Sections (5 μ m) were probed with anti-PKC antibodies as described previously (Latchney *et al.*, 2004). Frozen contralateral glands were pulverized and sonicated as described above and centrifuged (82,700 $g \times$ 6 min) at 4°C. In a separate experiment, the supernatant was centrifuged at 90,600 $g \times$ 60 min, yielding high-speed pellets. Pellets were re-suspended in TEMPI. Increasing volumes of pellets and supernatants were run on SDS-PAGE gels as described above, stained with Coomassie blue, imaged (ChemImager ready System, Alpha Innotech Corp., San Leandro, CA, USA), and net pixel densities *per* lane determined (ImageJ v.1.41) to equilibrate protein loading in subsequent Western blots, as described previously (Fallon *et al.*, 2003). Identical gels were stained with Coomassie blue as loading controls.

Statistics

Results are expressed as mean $\pm$ SE and compared by the Student's *t* test with $p < 0.05$ as significant.

RESULTS

Relationship between $[Ca^{2+}]_i$ and Carbachol-induced Secretion

The muscarinic agonist, carbachol, induced in tubulo-acinar cells a rapid increase in $[Ca^{2+}]_i$ from an estimated 50 nM basal level to an approximately 5-fold greater sustained level (Fig. 1A). In Ca^{2+} -free medium, the increase in $[Ca^{2+}]_i$ was only transient, whereas in cells loaded with the Ca^{2+} chelator BAPTA, the $[Ca^{2+}]_i$ response was blocked (Fig. 1A). The EC_{50} (1.5 μ M) for the sustained $[Ca^{2+}]_i$ response to carbachol was 5-fold higher than the EC_{50} (0.33 μ M) for mucin secretion (Fig. 1B). Hence, secretion is maximum at a half-maximal $[Ca^{2+}]_i$ response. Both basal and maximal carbachol secretory responses were constant for at least 40 min (Fig. 1C), whereas in Ca^{2+} -free medium secretion was transient (Fig. 1C). Under conditions that block increased $[Ca^{2+}]_i$ (Ca^{2+} -free medium and BAPTA loading), carbachol-induced secretion was abolished, although basal secretion was unaffected (Fig. 1D).

We used thapsigargin to increase $[Ca^{2+}]_i$ by inhibition of the Ca^{2+} -ATPase responsible for sequestering Ca^{2+}_i into intracellular stores (Zhang and Melvin, 1993). Thapsigargin (1 μ M) slowly increased $[Ca^{2+}]_i$, reaching sustained levels equivalent to those of maximal carbachol (Fig. 1E, insert). Nevertheless, maximal thapsigargin-induced secretion was 40% of maximal carbachol

Figure 1. Exocrine mucin secretion and intracellular calcium ($[Ca^{2+}]_i$) responses to carbachol (Carb) and thapsigargin, and the role of phospholipase C (PLC) and protein kinase C (PKC) in exocrine secretion and intracellular calcium ($[Ca^{2+}]_i$) by dispersed sublingual tubulo-acini. **(A)** $[Ca^{2+}]_i$ responses to carbachol in Ca^{2+} -containing medium (Control) and in Ca^{2+} -free medium with and without pre-loading cells with 2 μM BAPTA/AM. Traces are representative of 4 or 5 separate experiments. **(B)** Exocrine secretion and in response to increasing concentrations of carbachol. Sustained $[Ca^{2+}]_i$ measured 2.5 min after addition of carbachol. Values are means $\pm$ SE from 3 separate paired experiments in both cases. Lines are 2-parameter fits to the logistic function as described previously (Culp *et al.*, 1996). Basal exocrine secretion, 8.6 ± 0.1 ; basal $[Ca^{2+}]_i$, 55.1 ± 3.3 . **(C)** Total secretion vs. time in the absence (Basal) and presence of carbachol by tubulo-acini in Ca^{2+} -containing medium compared with Ca^{2+} -free medium. Values are mean $\pm$ SE from 4 paired experiments. **(D)** Total secretion vs. time in the absence (Basal) and presence of carbachol by tubulo-acini in Ca^{2+} -containing medium compared with Ca^{2+} -free medium with 2 μM BAPTA/AM pre-treatment. Values are mean $\pm$ SE from 3 paired experiments. Lines from regression analysis ($r^2 \geq 0.988$). **(E)** Dose-response curves for secretion and sustained $[Ca^{2+}]_i$ induced by thapsigargin. Sustained $[Ca^{2+}]_i$ measured 5 min after thapsigargin addition. Values are mean $\pm$ SE from 4 paired experiments in both cases. Basal $[Ca^{2+}]_i$, 52.6 ± 3.9 . Basal secretion; 5.5 ± 0.6 . Inset, representative trace of $[Ca^{2+}]_i$ response to thapsigargin. The sustained response was stable for at least 30 min (not shown). **(F)** Time-course of total exocrine secretion in the absence (Basal) or presence of carbachol, thapsigargin, or both secretagogues combined. Values are mean $\pm$ SE from 3 paired experiments. Lines from regression analysis ($r^2 \geq 0.961$). **(G)** Exocrine secretion (left half; means $\pm$ SE of 3 paired experiments) and $[Ca^{2+}]_i$ (right half; means $\pm$ SE of 5 paired experiments) in the absence (Control) or presence of the PLC inhibitor, 10 μM U73122, or the inactive analog, 10 μM U73343. *Values significantly different from basal ($p < 0.05$). †Values significantly different from 0.3 μM carbachol ($p < 0.05$). **(H)** Exocrine secretion induced by phorbol 12-myristate 13-acetate (PMA; mean $\pm$ SE of 4 paired experiments). Secretion was not induced by 10 μM of the weakly active PMA analog, 4-O-methyl-PMA (not shown). Secretion induced by 10 μM carbachol (positive control) was 18.0 ± 0.4 (not shown). **(I)** Time-course of total exocrine secretion (mean $\pm$ SE from 3 paired experiments) under basal conditions and with carbachol $\pm$ Gö6976. **(J)** Time-course of total exocrine secretion (mean $\pm$ SE from 3 paired experiments) under basal conditions and with thapsigargin $\pm$ Gö6976.

Figure 2. Studies of PKC isozymes in sublingual glands. **(A)** Western blots of PKC isozymes in sublingual gland homogenates (S). Positive controls are homogenates of rat brain (B) or heart (H). Panels aligned to 76-kDa marker (far left). See Appendix Fig. 3A for negative controls. **(B)** Redistribution of PKC isoforms between pellets (P; 82,700 $g \times 6$ min) and supernatants (S) from gland homogenates (G). Glands perfused *in situ* with 10 μ M carbachol for times indicated. Control; glands perfused 5 min without carbachol. Results are representative of 2 experiments. See Appendix Figs. 3B and 3C for protein loading controls. **(C)** Redistribution of PKC isoforms after 5 min of gland perfusion *in situ* with (+) and without (-) 10 μ M carbachol. Gland homogenates (G) were fractionated into 2 pellets, a 82,700 $g \times 6$ min pellet (low-speed pellet, L) and a subsequent 90,600 $g \times 60$ min pellet (high-speed pellet, H) plus resultant supernatant (S). See Appendix Fig. 3D for protein loading controls. **(D)** Differential interference contrast images of non-counterstained paraffin sections after immunohistochemical localization of PKC isoforms in glands perfused *in situ* without (Control) and with 10 μ M carbachol for the indicated times. Control glands perfused 5 min. PKC- α in controls displays intense staining of serous demilune cells at the ends of mucous tubulo-acini (arrowheads). Mucous cell staining is concentrated in the lateral (left arrow) and basal/perinuclear regions (right arrow). Striated ducts have moderate staining along the lumen border (black arrow). After 1 min carbachol, PKC- α staining in mucous cells is near apical membranes bordering the lumen (arrows), and staining along the basal borders is more noticeable. Apical membrane staining (arrow) is more intense at 5 min and persists for at least 30 min. PKC- β I staining in controls is intense in serous demilune cells (arrowhead) and moderate in perinuclear regions of mucous cells (arrow). After 1 min, little change is visible. After 5 min, light staining near the apical membrane is apparent (arrow), but absent at 30 min. Note heavy staining of striated duct cells (arrowhead, 5-minute panel). PKC- δ staining in controls is intense along apical membranes of striated duct cells (arrow) with light perinuclear staining of mucous cells (arrowhead). After 5 and 30 min, mucous cells display particulate staining near lateral cell borders (arrowheads) or diffuse light spotty staining in apical regions (arrows). At 30 min, membranes bordering the lumen are negative (black arrow). At all time points, PKC- ϵ staining is intense along the apical border of striated duct cells (arrowheads), but in mucous cells is restricted mostly to the perinuclear region (arrows) with light staining along the basal region of cells. Lower right panel; rabbit non-immune IgG negative control (scale bar = 40 μ m). See Appendix Fig. 4 for Alcian-blue-stained controls and details of gland morphology.

secretion (Fig. 1F). Moreover, the EC_{50} (0.1 μ M) for sustained $[Ca^{2+}]_i$ induced by thapsigargin was equivalent to that for exocrine secretion (Fig. 1E), and maximal thapsigargin (1 μ M) did not alter the secretory (Fig. 1F) or sustained $[Ca^{2+}]_i$ (not shown) responses to maximal carbachol.

Inhibitors of PLC and PKCs

Inhibition of PLC with 10 μ M U73122 abolished carbachol secretory and $[Ca^{2+}]_i$ responses, whereas U73343 (10 μ M; inactive analog) was without effects (Fig. 1G). Furthermore, PLC-independent/ Ca^{2+} -dependent signaling *via* Ca^{2+} /calmodulin kinase II did not mediate secretagogue-induced secretion (see Appendix Table 2). Because $[Ca^{2+}]_i$ alone partially induces secretion, we hypothesized that PLC-derived DAG is additionally required for maximal muscarinic secretion. The DAG mimic, phorbol 12-myristate 13-acetate (PMA), stimulated secretion with an EC_{50} of about 0.1 μ M, but the maximal response was one-third that of maximal carbachol (Fig. 1H). In control experiments, PMA did not affect $[Ca^{2+}]_i$ levels under basal or stimulated conditions (see Appendix Figs. 1A and 1B). We tested PKC inhibitors, calphostin C, selective for cPKC and nPKC isoforms (Kobayashi *et al.*, 1989), and Gö6976, selective for cPKC isoforms (Martiny-Baron *et al.*, 1993). Gö6976 did not reduce secretion induced by maximal carbachol or thapsigargin over the initial 5 min, whereas secretion was subsequently reduced to basal levels (Figs. 1I and 1J). Calphostin C produced similar results (see Appendix Figs. 2A and 2B). Transient secretion resistant to both inhibitors is reminiscent of secretion in Ca^{2+} -free medium (Fig. 1C), suggesting an initial transient phase of secretion dependent only on intracellular Ca^{2+} release, whereas the sustained second phase is PKC-dependent and apparent at 5 min. Indeed, both inhibitors were without effects on transient carbachol- or thapsigargin-induced secretion in the absence of extracellular Ca^{2+} (Table, study 1), further suggesting that PKC-mediated secretion requires Ca^{2+} influx. We found $[Ca^{2+}]_i$ under basal or carbachol-stimulated conditions unaffected by either inhibitor (see Appendix Table 3). Both inhibitors were very effective in counteracting secretion induced by PMA (Table, studies 2 and 3).

Synergistic Secretion

Because thapsigargin and PMA each induced secretion to less than the half-maximal carbachol response, we tested for additive or synergistic effects of the 2 secretagogues. Half-maximal PMA (0.1 μ M) with increasing thapsigargin (25%, 50%, and 100% effective concentrations) produced synergistic secretion. Synergism was also obtained with maximal PMA (1 μ M) and submaximal levels of thapsigargin, and maximal synergistic secretion was equivalent to the maximal carbachol response (Table, study 4). We further found synergism with half-maximal carbachol (0.3 μ M) plus low (0.01 μ M), but not maximal, thapsigargin (1.0 μ M) (Table, study 5).

PKC Isoforms and Their Redistribution

Western blots demonstrated that cPKC isoforms α and β_1 , nPKC isoforms δ and ϵ , and the atypical isoform η are expressed in sublingual glands (Fig. 2A). Because PKC isoforms display little

Figure 3. Working model of the intracellular signaling pathway in the activation of mucous cell exocrine secretion by muscarinic cholinergic receptors. (**Step 1** of Fig.) Salivary mucous cells express roughly equivalent amounts of muscarinic cholinergic M1 and M3 receptor subtypes that are coupled to the G proteins, G_q and G_{11} (Culp *et al.*, 1996). Binding of the parasympathetic neurotransmitter acetylcholine (A) to either receptor subtype (**step 2**) initiates the exchange of GTP for GDP binding to the α -subunits of G_q and G_{11} (**step 3**), resulting in dissociation of the G proteins (**step 4**) and the subsequent interaction of GTP-bound α -subunits to phosphatidylinositol 1,4-bisphosphate (PIP_2)-specific phospholipase C (PLC) (Luo *et al.*, 2001) (**step 5**). The subsequent activation of PLC generates inositol 1,4,5-trisphosphate (IP_3) (Zhang and Melvin, 1993) and diacylglycerol (DAG) (Melvin *et al.*, 2005) (**step 6**). Interaction of IP_3 with its cognate receptors (**step 7**) results in the release of Ca^{2+} from intracellular stores (**step 8**), associated presumably with the endoplasmic reticulum (Melvin *et al.*, 1991). The initial transient release of Ca^{2+} from intracellular stores promotes the release of a fusion-competent pool of secretory granules at the apical membrane (**step 9**) and further induces the influx of extracellular Ca^{2+} through a store-operated Ca^{2+} entry mechanism (**step 10**), resulting in a sustained increase in intracellular free Ca^{2+} (Melvin *et al.*, 1991). DAG in concert with the sustained increase in cell Ca^{2+} activates cPKC isoforms in a synergistic manner (**step 11**), possibly through DAG lowering the Ca^{2+} requirement of cPKCs (Oancea and Meyer, 1998). One or more cPKC isoforms may be responsible for activating mechanisms (e.g., granule docking and priming) required for the latent (post-5 min in time-course experiments) and sustained release of the pool of secretory granules that are initially fusion-incompetent at the time of M1/M3 receptor activation (**step 12**). PKC- α is an attractive candidate in mediating one or more of the cPKC-dependent mechanisms regulating secretion, as indicated by its rapid and persistent activation upon muscarinic stimulation and continual accumulation in apical membrane regions.

substrate specificity, *in vitro*, specificity is largely conferred by enzyme translocation to a target substrate (Dempsey *et al.*, 2000). Redistribution of PKC to different cellular compartments (e.g.,

Table. Exocrine Secretion: Protein Kinase C Inhibitors and PMA-Thapsigargin Synergism

Study # (n)	Secretagogue	Inhibitor	Net Secretion		% Inhibition
			-Inhibitor	+ Inhibitor	
1 (n = 3)	Basal	—	3.9 ± 0.2	—	—
	10 μ M carbachol	0.5 μ M calphostin C	4.6 ± 0.4	†4.3 ± 0.6	0
	10 μ M carbachol	5 μ M Gö6976		†4.6 ± 0.6	0
	1 μ M thapsigargin	0.5 μ M calphostin C	1.6 ± 0.1	†1.5 ± 0.1	0
	1 μ M thapsigargin	5 μ M Gö6976		†1.6 ± 0.2	0
2 (n = 4)	Basal	—	8.8 ± 0.9	—	—
	0.3 μ M carbachol	0.5 μ M calphostin C	7.4 ± 0.8	1.4 ± 0.4	81 ± 7
	10 μ M carbachol	0.5 μ M calphostin C	21.0 ± 2.6	5.5 ± 0.2	74 ± 4
	1 μ M PMA	0.5 μ M calphostin C	9.1 ± 2.4	2.2 ± 0.7	76 ± 8
	0.25 μ M PMA	0.5 μ M calphostin C	6.4 ± 1.1	*0.6 ± 0.4	100
3 (n = 3)	Basal	—	5.9 ± 0.3	—	—
	0.3 μ M carbachol	5 μ M Gö6976	5.9 ± 0.6	1.7 ± 0.2	71 ± 4
	10 μ M carbachol	5 μ M Gö6976	16.8 ± 0.3	6.2 ± 0.4	63 ± 2
	1 μ M PMA	5 μ M Gö6976	6.0 ± 0.8	2.2 ± 0.3	63 ± 2
	0.25 μ M PMA	5 μ M Gö6976	4.5 ± 0.5	*0.4 ± 0.4	100
Study # (n)	Secretagogue 1	Secretagogue 2	Net Secretion	Additive Sum	
4 (n = 4)	Basal	—	6.4 ± 0.2	—	
	10 μ M carbachol	—	18.8 ± 1.1	—	
	0.03 μ M thapsigargin	—	1.9 ± 0.1	—	
	0.1 μ M thapsigargin	—	4.6 ± 0.6	—	
	1 μ M thapsigargin	—	9.8 ± 0.8	—	
	0.1 μ M PMA	—	2.2 ± 0.4	—	
	0.03 μ M thapsigargin	0.1 μ M PMA	†6.4 ± 0.4	4.0 ± 0.7	
	0.1 μ M thapsigargin	0.1 μ M PMA	†12.6 ± 1.4	6.8 ± 0.9	
	1 μ M thapsigargin	0.1 μ M PMA	†19.8 ± 1.4	12.0 ± 1.2	
	1 μ M PMA	—	4.5 ± 0.8	—	
	0.03 μ M thapsigargin	1 μ M PMA	†10.5 ± 0.8	6.4 ± 0.7	
	0.1 μ M thapsigargin	1 μ M PMA	†14.2 ± 0.9	9.2 ± 1.3	
5 (n = 4)	Basal	—	7.3 ± 0.5	—	
	10 μ M carbachol	—	16.9 ± 1.9	—	
	0.3 μ M carbachol	—	7.2 ± 0.6	—	
	0.01 μ M thapsigargin	—	0.6 ± 0.2	—	
	1.0 μ M thapsigargin	—	7.2 ± 1.4	—	
	0.01 μ M thapsigargin	0.3 μ M carbachol	†12.2 ± 0.5	7.8 ± 0.5	
	1.0 μ M thapsigargin	0.3 μ M carbachol	15.1 ± 1.4	14.2 ± 1.8	

Results for all studies are mean ± SE of n separate experiments and expressed as percentage total precipitable dpm released (see Appendix for details). In all cases, total secretion for each secretagogue alone is significantly different ($p < 0.05$, paired t test) from the corresponding basal secretion. In study #1, secretion was measured over 10 min with tubulo-acini in Ca^{2+} -free medium. Studies #2 through #5 were for 30 min with extracellular Ca^{2+} . Net Secretion, total secretion minus basal secretion. % Inhibition, inhibition with respect to net secretion. Additive Sum, sum of net secretion for each secretagogue alone. *Net secretion is not significantly different from basal. †Net secretion is not significantly different from stimulated total secretion in the absence of inhibitor. ‡Net secretion is significantly different ($p < 0.05$, paired t test) from the corresponding additive sum. PMA, phorbol 12-myristate 13-acetate.

plasma membrane, endoplasmic reticulum, the nuclear region, and the cytoskeleton) therefore serves as a marker of enzyme activation. We initially followed a protocol to monitor PKC translocation used in heart (Albert and Ford, 1999) and intestinal epithelium (Mammen *et al.*, 2005) to assess activation of glandular cPKC and nPKC isoforms. Glands were perfused *in situ* with and without carbachol for increasing intervals, followed by homogenation and fractionation into medium-speed particulate pellets and supernatants.

PKC- α redistribution to the supernatant was apparent 1 min after stimulation, peaked at 5 min, and then decreased to a persistent level above the control (Fig. 2B). Other PKC isoforms displayed no changes with carbachol. In a separate experiment, supernatants from glands perfused for 5 min, when PKC-mediated secretion is apparent, were further fractionated to produce high-speed pellets and supernatants. As before, only PKC- α underwent redistribution, from the medium-speed to high-speed pellets (Fig. 2C).

As a complementary and more cell-specific approach, we studied carbachol-induced translocation of PKC isoforms by immunohistochemistry (see Fig. 2D). PKC- α in resting mucous cells was confined to the lateral and basal/perinuclear regions, but was detected in apical regions bordering the lumen after 1 min stimulation, which persisted for at least 30 min. PKC- β I displayed a transient light staining near the apical membrane at 5 min. PKC- δ developed particulate staining along basal regions of mucous cells at 1 min that persisted at 30 min. At 5 and 30 min, some mucous cells displayed particulate staining along their lateral cell borders, whereas other cells had spotty diffuse apical staining. PKC- ϵ mucous cell staining was perinuclear and basal, with no changes upon stimulation.

DISCUSSION

Our collective evidence indicates that muscarinic-induced exocrine secretion by sublingual mucous cells is dependent upon PLC activation and the resultant increase in $[Ca^{2+}]_i$. We further identified two phases of secretion, an initial transient phase dependent upon Ca^{2+} release from intracellular stores and a second phase dependent upon extracellular Ca^{2+} and PKC. We propose that the initial transient phase represents Ca^{2+} -dependent release of previously primed and fusion-competent apical granules (Liu *et al.*, 2010). Moreover, coupling of both sustained fluid (Melvin *et al.*, 1991) and exocrine secretion upon muscarinic stimulation may serve to flush the viscous primary saliva into the oral cavity. Key evidence further implicates cPKCs in sustained muscarinic-induced exocrine secretion. For example, the synergistic interactions between PMA and $[Ca^{2+}]_i$ induced by either submaximal carbachol or thapsigargin are consistent with DAG lowering the Ca^{2+} requirement for cPKC isoforms (Oancea and Meyer, 1998). Furthermore, calphostin C (a nPKC and cPKC selective inhibitor) has no further inhibitory effects than the cPKC selective inhibitor, Gö6976.

PKC- α undergoes a rapid and persistent carbachol-induced activation, as evidenced in both gland fractionation and immunohistochemical studies. Its accumulation to apical membrane regions of mucous cells suggests that its activation is obligatory for the maturation of non-fusion-competent granules (*e.g.*, granule priming or docking). Gland fractionation experiments were unable to detect activation of PKC- β I and nPKC isoforms. Their translocation in mucous cells may have been masked by abundant ductal cell expression of these isoforms and/or requires a more specific subcellular fractionation scheme. Nevertheless, the short-lived localization of PKC- β I near the luminal border detected at 5 min, when cPKC-mediated sustained secretion is apparent, may represent a transient role in triggering a mechanism necessary for granules to become fusion-competent. A working model of signaling events in muscarinic-induced exocrine secretion by mucous cells is shown in Fig. 3. Steps 9, 11, and 12 highlight our current results supporting initial transient PLC- and Ca^{2+} -dependent, but PKC-independent, exocrine secretion (step 9), with sustained secretion conditional upon activation of cPKC isoforms (PKC- α and possibly PKC- β I) by DAG and extracellular Ca^{2+} influx.

In airway surface goblet cells, mucin secretion activated by P2Y₂ purinoceptors is similarly mediated by PLC, DAG, and Ca^{2+} , but utilizes PKC- ϵ (Davis and Dickey, 2008). Conversely,

there is evidence for a role for PKC- δ (Park *et al.*, 2007). PKC- ϵ and Ca^{2+} are also implicated in colonic goblet cell secretion (Hong *et al.*, 1997). Interestingly, we find that PKC- ϵ and PKC- δ are not obligatory for mucous cell secretion, indicating different roles for PKC isoforms between surface goblet cells and glandular mucous cells. In parotid acinar cells, muscarinic-induced fluid secretion involves cPKC-mediated phosphorylation of Na^+/K^+ -ATPase (Soltoff *et al.*, 2010), whereas PKC- δ functions downstream of PKA to promote β -adrenergic-induced amylase release (Satoh *et al.*, 2009). PKC- δ and PKC- α may also function in parotid cell apoptosis (Reyland *et al.*, 2000). PKC isoforms thus perform different cell-specific functions, dependent upon their subcellular localization and mechanisms targeting them to specific substrates upon activation. Elucidation of additional roles of PKC isoforms in mucin granule priming, docking, and release may define potential therapeutic targets to enhance mucin levels in patients suffering from hyposalivation.

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