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Mediators of Ca^{2+}-Dependent Secretion

by Archana Chaudhry* and Ronald P. Rubin*

Ca^{2+}, an obligatory mediator of the secretory process, acts in concert with other second messengers that further amplify or inhibit the secretory response. In this overview, we will consider the relative roles of diacylglycerol (DAG), arachidonic acid, and cyclic AMP (cAMP) in modulating Ca^{2+}-dependent secretion in nonexcitable cells. DAG, a product of phospholipase C (PLC)-catalyzed breakdown of phosphoinositides, stimulates protein kinase C. Ca^{2+} ionophores and phorbol esters (or DAG analogues) elicit a synergistic secretory response in the exocrine pancreas and parotid gland. These findings suggest that the complete activation of secretion requires stimulation of both Ca^{2+}-dependent and protein kinase C-dependent pathways. Hydrolysis of phospholipids can also lead to the liberation of arachidonic acid in secretory cells. Endogenously generated arachidonic acid inhibits polyphosphoinositide synthesis in exocrine pancreas, leading to inhibition of agonist-induced IP_3 formation, Ca^{2+}-mobilization and amylase secretion. By contrast, arachidonic acid and its metabolites stimulate PLC in the rabbit peritoneal neutrophil, causing Ca^{2+}-mobilization and lysosomal enzyme secretion. Arachidonic acid can thus serve as a positive or negative feedback regulator of secretion induced by Ca^{2+}-mobilizing agonists. Finally, in the parotid gland, stimulation of amylase secretion by norepinephrine, the physiological mediator, which stimulates both the α and β adrenoceptors, requires the interaction of both Ca^{2+} and cAMP pathways to produce a full secretory response. These studies, taken together, indicate that phosphoinositide and cAMP-dependent pathways play coordinate roles in signal transduction, leading to the Ca^{2+}-mediated secretion.

The role of calcium (Ca^{2+}) in stimulus-secretion coupling has been unequivocally established. In electrically excitable cells such as the neuron, adrenal medullary chromaffin cell, the β cell of the endocrine pancreas, and cells of the adeno- and neurohypophysis, the rise in cellular Ca^{2+} following stimulation is derived to a large extent from influx of cation through voltage-sensitive channels (*1*). In nonexcitable secretory cells, such as those of exocrine glands and neutrophils, cellular stores of Ca^{2+} play a more predominant role in regulating secretion, although Ca^{2+} influx through receptor-operated channels also increases cellular Ca^{2+} availability (Fig. 1). The initial secretory response seems dependent on Ca^{2+} released from intracellular stores, but prolonged secretion requires the presence of extracellular Ca^{2+} (*2*).

The concept that increases in intracellular ionic Ca^{2+}stimulate secretion in nonexcitable cells is supported by the following pieces of evidence: *a*) secretagogues evoke increases in cytosolic Ca^{2+} (*3,4*) and cause a rapid efflux of ^{45}Ca from cells (*2*); *b*) Ca^{2+} ionophores which bypass receptors to raise cytoplasmic Ca^{2+} stimulate enzyme secretion (*4*); *c*) depletion of cellular Ca^{2+} inhibits secretion (*2*); and *d*) amylase secretion is stimulated when increasing concentrations of buffered Ca^{2+} are introduced into the cytosol of electropermeabilized cells (*5*).

Ca^{2+}-mobilizing agonists stimulate phospholipase C (PLC), which catalyzes the phosphodiesteratic cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) (Fig. 1). This leads to the formation of 1,4,5-inositol trisphosphate (IP_3) and diacylglycerol (DAG), both of which have important second messenger roles. 1,4,5-IP_3 releases cellular Ca^{2+} by interacting with a specific receptor site on the endoplasmic reticulum (*6*), and DAG activates protein kinase C, a key regulatory enzyme (*7*). Arachidonic acid is also liberated from phosphoinositides during stimulation by secretagogues, and free arachidonic acid and/or its metabolites may also serve as cellular messengers to modulate the secretory response (*8*). Also, some secretory cells possess a signaling system that uses cyclic AMP (cAMP) as a second messenger. In such systems Ca^{2+} and cAMP may act either sequentially or in concert to regulate secretion. Simultaneous changes in the intracellular concentrations of cytosolic Ca^{2+} and cAMP have been reported after stimulation of secretory cells by a variety of secretagogues (*9,10*). However, in contrast to Ca^{2+}, cAMP has not been characterized as a direct mediator of exocytosis.

In this brief overview, we will consider the concept that the second messengers DAG, arachidonic acid,

*Department of Pharmacology and Toxicology, Medical College of Virginia, Box 524-MCV Station, Richmond, VA 23298.

Address reprint requests to R. Rubin, Department of Pharmacology and Toxicology, Medical College of Virginia, Box 524-MCV Station, Richmond, VA 23298.

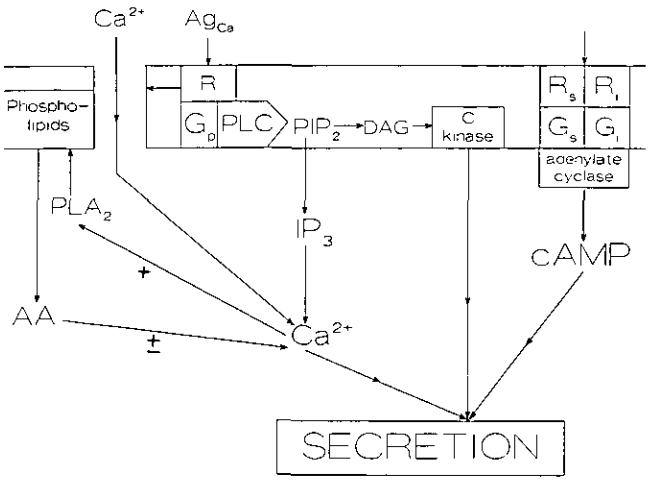

FIGURE 1. A hypothetical model depicting the various second messengers that can regulate cellular secretion. Occupation of receptors (R) by calcium-mobilizing agonists (Ag_{Ca}) activates phospholipase C (PLC) through a G protein (G_p). PLC-mediated breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) leads to the formation of inositol trisphosphate (IP_3) and diacylglycerol (DAG), both of which act as second messengers—IP_3 by mobilizing intracellular Ca^{2+} and DAG by activating protein kinase C. Ca^{2+} and protein kinase C act synergistically to promote a full secretory response. The increase in intracellular Ca^{2+} levels brought about by 1,4,5-IP_3, or via influx through receptor-operated calcium channels, can stimulate phospholipase A_2, and lead to arachidonic acid release (AA). Arachidonic acid and its metabolites can either amplify or inhibit agonist-induced rises in cytosolic Ca^{2+} thus modulating secretion. Agonists that cause secretion by modulating cyclic AMP (Ag_{cAMP}) express their actions via stimulatory and inhibitory G proteins (G_s and G_i) to activate and inhibit adenylate cyclase, respectively. Cyclic AMP can act in concert with Ca^{2+} to promote secretion, perhaps by enhancing Ca^{2+} availability.

and cAMP interact with Ca^{2+} to modulate the secretory response (Fig. 1). We will employ the parotid and pancreatic acinar cells, as well as the rabbit neutrophil, to offer evidence to support this thesis.

A large body of evidence suggests that in many tissues optimal secretion requires both Ca^{2+} and DAG (Fig. 1). Nishizuka and his colleagues (*7*) first demonstrated that DAG activates a phospholipid-dependent kinase (protein kinase C) by increasing the affinity of the kinase for Ca^{2+}. Thus, in the presence of DAG, protein kinase C can be maximally stimulated at submicromolar concentrations of Ca^{2+}. The interactions between Ca^{2+} and protein kinase C in cellular secretion have been probed by using calcium ionophores (which bypass receptors to raise cytoplasmic Ca^{2+}) and phorbol esters (which substitute for DAG) to activate the Ca^{2+}-dependent and protein kinase C-dependent pathways separately. In isolated pancreatic acini, phorbol 12,13-dibutyrate (PDBu) when added together with a threshold concentration of the Ca^{2+} ionophore, ionomycin, causes a synergistic potentation of amylase secretion, with no further elevation in cytoplasmic Ca^{2+} than the one elicited by ionomycin alone (Fig. 2). Diacylglycerols containing unsaturated fatty acids also stimulate amylase secretion and exhibit synergistic effects on secretion in combination with ionomycin (*4*). These results suggest that complete activation of amylase secretion by the pancreas requires stimulation of both Ca^{2+}-dependent and protein kinase C-dependent pathways. Similar synergistic effects of ionophores and phorbol esters have been reported in other model secretory systems (*11,12*).

Apart from interacting with the protein kinase C pathway, Ca^{2+} may interact with the arachidonic acid messenger system to modulate secretion (*13*). Mammalian phospholipids are enriched in arachidonic acid, and Ca^{2+}-mobilizing agonists liberate free arachidonic acid either through activation of phospholipase A_2, the sequential activation of PLC and DAG lipase, or phosphatidate-specific phospholipase A_2 (*13*). For example, the mucarinic agonist carbachol elevates free arachidonate levels in pancreatic acinar cells (*14*). The time course of this event parallels that of other cellular responses to carbachol, including IP_3 accumulation and amylase

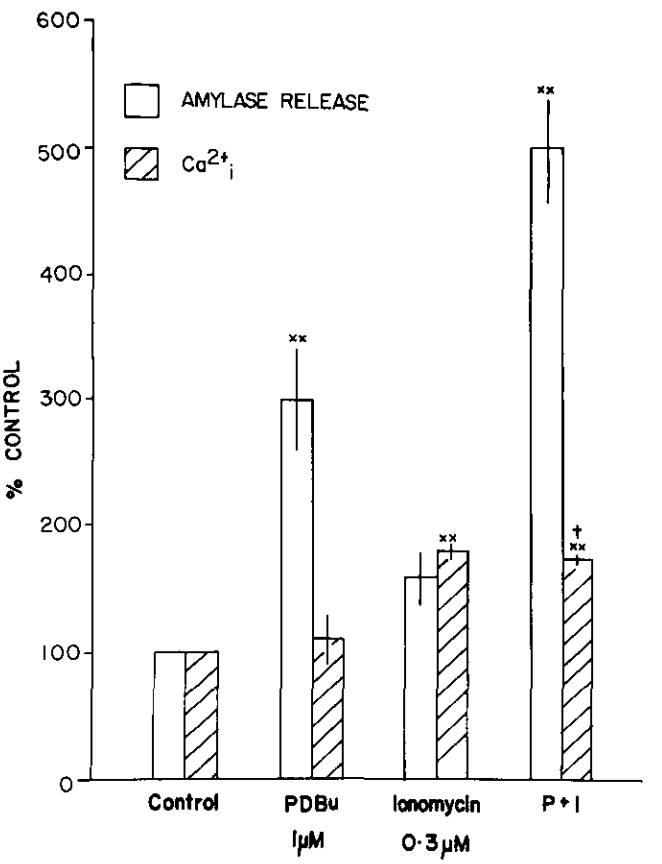

FIGURE 2. Potentiation by phorbol dibutyrate of ionomycin-induced amylase secretion without a further rise in $[Ca^{2+}]_i$. Rat pancreatic acini were incubated in the presence of either phorbal dibutyrate (PDBu) (1 μM), or ionomycin (0.3 μM), or PDBu plus ionomycin (P+I). $[Ca^{2+}]_i$ (Quin-2 fluorescence) and amylase secretion were determined after 10 and 30 min, respectively. Double asterisks (**) indicate significantly different from control value ($p < 0.05$). Modified from Merritt and Rubin (*4*).

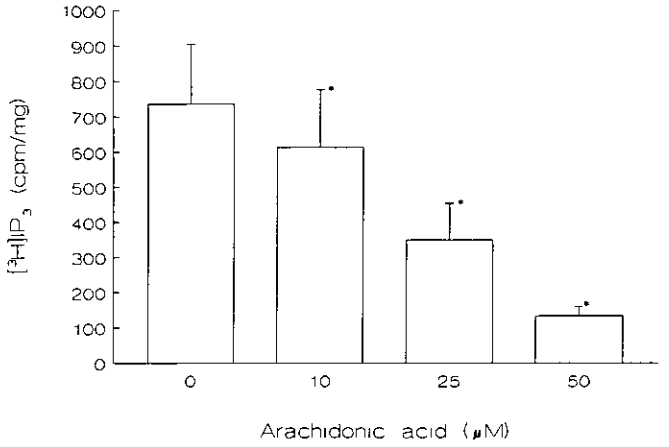

FIGURE 3. Inhibition of carbachol-induced $[^3H]IP_3$ formation by arachidonic acid in rat pancreatic acinar cells. Cells prelabeled with *myo*-[^{3}H]inositol (*14*) were pretreated for 5 min with various concentrations of arachidonic acid and exposed to carbachol (10 μM) for an additional 5 min. Carbachol-induced accumulation of $[^3H]IP_3$ is shown after subtracting the radioactivity in untreated control samples. Each value is mean ± SE ($n = 5$). Asterisk (*) indicates significantly different from samples treated with carbachol alone ($p < 0.05$).

secretion (*15,16*). Additionally, exogenous arachidonic acid causes a concentration-dependent reduction in the steady-state levels of $[^{32}P]PIP_2$ (*14*). The decrease in the PIP_2 pool is not due to PLC-stimulated PIP_2 hydrolysis, since arachidonic acid alone does not promote the accumulation of IP_3. In fact, pretreatment of [^{3}H]*myo*-inositol labeled cells with arachidonic acid abolishes subsequent IP_3 accumulation in response to carbachol (Fig. 3), and arachidonic acid blocks the enhanced incorporation of [^{3}H]*myo*-inositol into phospholipids elicited by carbachol (*14*). These findings indicate that in exocrine pancreas, arachidonic acid inhibits the synthesis of the polypholphoinositide pool used by Ca^{2+}-mobilizing agonists.

To confirm the inhibitory role of endogenously released arachidonic acid on phosphoinositide turnover and, consequently, on agonist-mediated Ca^{2+} mobilization as well as amylase secretion, we utilized tetrahydrocannabinol (THC) to increase endogenous levels of unesterified arachidonate. THC is an inhibitor of acyl-CoA transferase and stimulates arachidonic acid release from cells by activating phospholipase A_2 (*17–19*). In acinar cells prelabeled with both [^{3}H]arachidonic acid and $[^{32}P]P_i$, THC causes a significant increase in levels of free [^{3}H]arachidonic acid that correlates with a corresponding decrease in the steady-state levels of PIP_2 (Fig. 4). The effects of THC on arachidonate release and $[^{32}P]P_i$ levels are dose-related over the concentration range of 1 to 20 μM (unpublished observations). Pretreatment with THC causes a dose-related inhibition of $[^3H]IP_3$ accumulation (unpublished observations), as well as cytoplasmic Ca^{2+} and amylase secretion (Table 1) elicited by cerulein in the exocrine pancreas. These results indicate that endogenously generated arachidonic acid and/or its metabolites can serve as a negative feedback regulator of phosphoinositide turnover and, thus, inhibit agonist-induced rises in cytosolic [Ca^{2+}] and amylase secretion.

By contrast, in the rabbit neutrophil, the lipooxygenase metabolite leukotriene B_4 (LTB_4) stimulates IP_3 accumulation, Ca^{2+} mobilization, and enzyme degranulation (*20,21*). Thus, in the neutrophil, arachidonate metabolites may amplify the agonist-

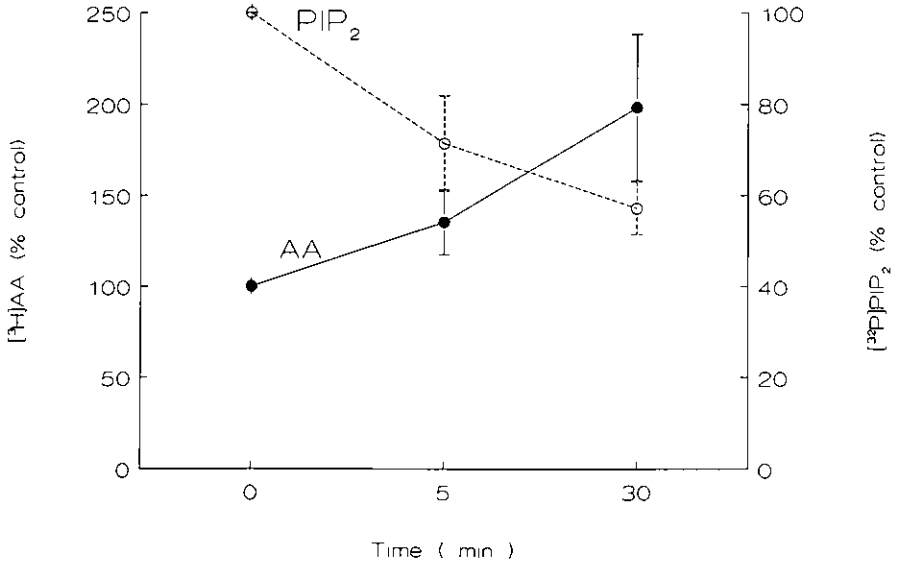

FIGURE 4. Effect of THC on [^{3}H]arachidonic acid (AA) and $[^{32}P]PIP_2$ levels in pancreatic acinar cells. Cells were simultaneously incubated with [^{3}H]AA (10 μCi/mL), $[^{32}P]P_i$ (10-25 μCi/mL), and carbachol (10 μM) for 15 min, at which time atropine (100 μM) was added. After 75 min, the cells were resuspended in medium containing [^{32}P]Pi (10-25 μCi/mL), and exposed to THC (20 μM) for various times. Analysis of [^{3}H]AA acid and $[^{32}]PIP_2$ was carried out as previously described (*14,18*). Values are expressed as percent of the radioactivity in control samples.

Table 1. Inhibitory effects of THC on cerulein-stimulated increases in cytosolic Ca^{2+} and amylase secretion in rat exocrine pancreas.[a]

	$[Ca^{2+}]_i$ nM	Amylase release, % total tissue content
Cerulein, 0.1 μM	294 ± 54	9.5 ± 0.5
Cerulein + THC, 5 μM	158 ± 30*	7.4 ± 0.1*

[a]Fura-2 loaded acinar cells were exposed to cerulein in the presence and absence of THC and $[Ca^{2+}]_i$ was calculated as previously described (*25*). Amylase secretion was determined in acini incubated for 30 min with cerulein in the presence and absence of THC. Basal values for $[Ca^{2+}]_i$ and amylase secretion have been subtracted from the data that is shown here as mean ± SE (n = 3–5).

*Significantly different from samples treated with cerulein alone ($p < 0.05$).

induced breakdown of polyphosphoinositides by being exported from the cell and, subsequently, acting as receptor agonists on neighboring cells to stimulate PLC and mobilize cellular Ca^{2+}. A positive feedback mechanism also appears operative in the blood platelet (*22*) and rat corpus luteum (*23*). Thus it appears that agonist-induced liberation of arachidonic acid can either amplify Ca^{2+}-induced secretion or inhibit it, depending upon the secretory system under scrutiny.

Recent evidence suggests that Ca^{2+} can also interact with the cAMP-dependent pathway to enhance the secretory responses of secretagogues. Cyclic AMP generated through beta adrenoceptor action appears to be a critical modulator of amylase secretion by the rat parotid gland. By contrast, Ca^{2+}-mobilizing agonists which express their actions through muscarinic and α-adrenergic receptors cause a predominance of water and electrolyte release (*24*). Norepinephrine (NE), the physiological neurotransmitter for salivary amylase secretion, which stimulates both β and α adrenoceptors, requires participation of both the cAMP and Ca^{2+} pathways to produce a full secretory response. Figure 5 shows that amylase secretion induced by NE is greater than the sum of the release

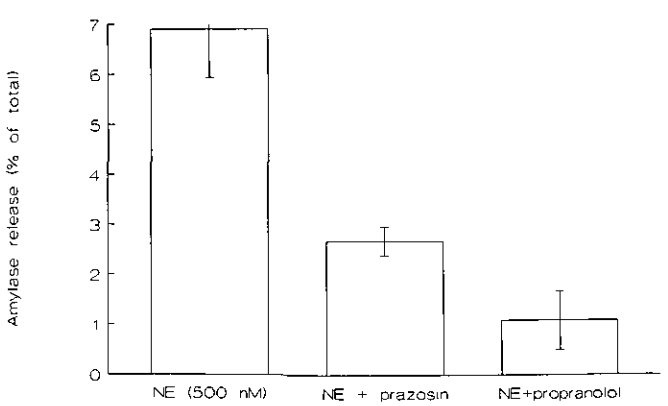

FIGURE 5. Effect of prazosin or propranolol on norepinephrine (NE)-induced salivary amylase release. Rat parotid acinar cells were exposed for 15 min to 500 nM NE in the presence and absence of either 100 nM prazosin or 1 μM propranol. Values are means ± SE with basal release subtracted (n = 3).

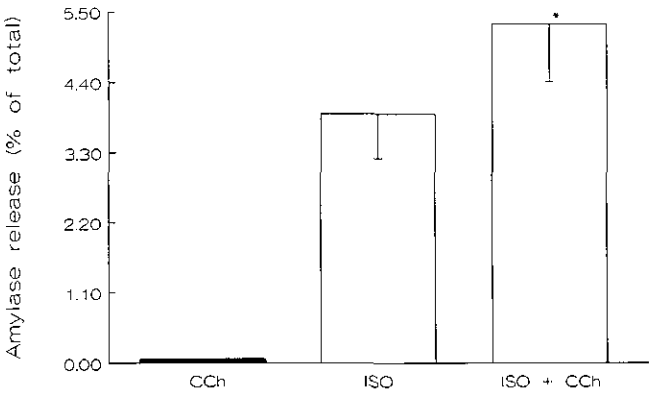

FIGURE 6. Enhancement of isoproterenol-induced amylase release by carbachol in parotid acinar cells. Cells were incubated for 15 min in the presence or absence of 1 μM carbachol (CCh) and 200 nM isoproterenol (ISO). Each value is mean ± SE (n = 3) with basal release subtracted. Asterisk (*) indicates $p < 0.05$.

obtained when NE is used as an α adrenoceptor agonist (in the presence of propranolol) and a β adrenoceptor agonist (in the presence of prazosin).

Further support for an interaction between the Ca^{2+} and cAMP pathways comes from our observation that a subthreshold concentration of carbachol causes a significant enhancement of isoproterenol-induced secretion (Fig. 6). The site of interaction between the two transduction systems is distal to the catalytic site of adenylate cyclase because carbachol failed to elevate isoproterenol-stimulated cAMP levels (unpublished observations). One possible explanation for the above findings is that there is an enhancement of Ca^{2+} *availability produced by the coordinate interactions* of the two pathways. Parotid acinar cells exposed to

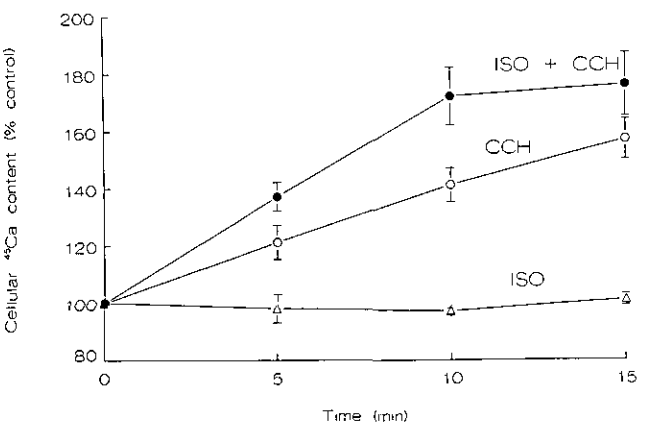

FIGURE 7. Effect of carbachol (CCh) and isoproterenol (ISO), alone and in combination, on the steady-state accumulation of ^{45}Ca in rat parotid acinar cells. Cells were incubated with ^{45}Ca (8 μCi/mL) for 60 min and then exposed to either CCh (100 μM) or ISO (200 nM) for various times. A third group of cells was pretreated for 2 min with 200 nM ISO prior to exposure to CCh. The incubation was terminated by filtering the cells through Millipore filters. The filters were counted, and the cellular ^{45}Ca content of each sample was calculated as a percent of its corresponding control value. Each value is mean ± SE (n = 3–8).

^{45}Ca under steady-state conditions show an enhanced ^{45}Ca accumulation in cells exposed to carbachol, but not to isoproterenol (Fig. 7). However, the combination of carbachol plus isoproterenol produces a further elevation in cellular ^{45}Ca content. The above findings, therefore, suggest that the Ca^{2+}-mediated pathway interacts with the cAMP-mediated pathway to regulate amylase secretion by increasing Ca^{2+} availability.

In conclusion, this brief account has dealt with the concept that phosphoinositide- and cAMP-dependent pathways play coordinate roles in signal transduction leading to the activation of Ca^{2+}-mediated exocytotic secretion. While Ca^{2+} appears to be a *sine qua non* for activation of the secretory apparatus, there is convincing evidence that DAG, arachidonic acid, and cAMP serve to modulate this pivotal action of Ca^{2+}. In secretory cells it appears as though controls are arranged in an integrative system in which information from several levels or sources may influence the final Ca^{2+}-dependent message that is transmitted to the secretory apparatus. Continued intensive experimentation will be required to define the full scope of the close association, if not tight coupling, between Ca^{2+} and these messengers systems. The current intensity of attention to this subject seems likely to generate additional paradigms for this aspect of cell activation.

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