

BBAGEN 23431

Interactions of intracellular mediators of amylase secretion in permeabilized pancreatic acini

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(Received 27 April 1990)

(Revised manuscript received 10 September 1990)

Key words: Amylase secretion; Permeability; Calcium ion effect; (Mouse pancreatic acinar cell)

Mouse pancreatic acini were permeabilized with streptolysin O to investigate amylase secretion stimulated by various intracellular mediators and the kinetics of secretion as a function of temperature. Amylase secretion was temperature dependent in that the initial rate of Ca^{2+} -stimulated secretion increased with increasing temperature. In addition, there was no enhancement of Ca^{2+} -stimulated secretion by GTP[γ S] at 14°C, while enhancement was maximal at 30°C. GTP[γ S]-mediated enhancement of secretion at a given temperature was mostly due to sustained secretion with a small increase in secretory rate. At 30°C Ca^{2+} -stimulated secretion was also enhanced by cAMP and phorbol ester (TPA) to similar extents as by GTP[γ S]. The maximally effective concentration of cAMP was 1–10 μM in the presence of 0.1 mM isobutylmethylxanthine. The enhancements of Ca^{2+} -stimulated amylase secretion by all combinations of cAMP (100 μM plus 0.1 mM isobutylmethylxanthine), TPA (1 μM), and GTP[γ S] (30 μM) were fully additive. In Ca^{2+} -free buffer, cAMP, TPA or GTP[γ S] individually had no effect on amylase secretion. Together, TPA and GTP[γ S] stimulated Ca^{2+} -independent secretion, which was 187 \pm 38% of basal. Cyclic AMP together with TPA and GTP[γ S] in the absence of Ca^{2+} stimulated 329 \pm 30% of basal secretion. Ca^{2+} -stimulated amylase secretion was decreased about 50% by metabolic inhibition, while the enhancement by cAMP, TPA or GTP[γ S] was totally blocked by metabolic inhibitors. These data demonstrate that amylase secretion in the acinar cell is mediated by multiple intracellular pathways which act in parallel and probably converge at a distal step in the exocytotic process.

Introduction

Pancreatic exocrine secretion is stimulated by the gut hormones cholecystokinin (CCK) and secretin and by the neurotransmitters acetylcholine (ACh) and vasoactive intestinal polypeptide (VIP). CCK and ACh act by stimulating phosphatidylinositol 4,5-bisphosphate breakdown to produce diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), with subsequent activation of protein kinase C (by DAG) and calcium mobilization (by IP_3) [1]. VIP and secretin activate adenylate cyclase to produce cAMP with subsequent activation of

protein kinase A [2]. Intact cells exhibit synergistic stimulation by DAG-, Ca^{2+} - and cAMP-mediated pathways [2,3]. It is difficult, however, in intact cells to investigate the interactions between these pathways because the concentration of cytosolic Ca^{2+} is regulated by the cell and some of the putative intracellular mediators and pharmacologic agents of interest are not permeable to the plasma membrane.

Recently, we developed a permeabilized cell system for pancreatic acini using the bacterial toxin streptolysin O (SLO) [4]. Following permeabilization, amylase secretion was stimulated by submicromolar free Ca^{2+} , and this Ca^{2+} -stimulated amylase secretion was enhanced by the nonhydrolyzable GTP analogue guanosine 5'-[γ -thio]triphosphate (GTP[γ S]) or the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA). In those studies, carried out at 37°C, all regulated secretion took place in the first 10 min and it was difficult to accurately determine the effects of different stimuli on rates of secretion. In the present work, therefore, we evaluated the effects of lower temperatures on the time-course and extent of amylase release. In addition,

Abbreviations: CCK, cholecystokinin; ACh, acetylcholine; VIP, vasoactive intestinal polypeptide; DAG, diacylglycerol; IP_3 , inositol 1,4,5-trisphosphate; GTP[γ S], guanosine 5'-[γ -thio]triphosphate; TPA, 12-O-tetradecanoylphorbol 13-acetate; SLO, streptolysin O; LDH, lactate dehydrogenase.

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we investigated the effects of cAMP on amylase secretion from permeabilized acini and the interactions between cAMP, Ca^{2+} , TPA and GTP[γ S].

Materials and Methods

Chemicals. Streptolysin O (SLO) was purchased from Wellcome Diagnostics (Greenville, NC); guanosine 5'-[γ -thio]triphosphate (GTP[γ S]) and 3-isobutyl-1-methyl xanthine (IBMX) from Calbiochem Corporation (La Jolla, CA); cyclic adenosine-3',5'-m-nophosphate (cAMP) from Boehringer Mannheim Biochemicals (Indianapolis, IN); chromatographically purified collagenase from Worthington Biochemical Corporation (Freehold, NJ); bovine serum albumin (fraction V) from ICN Immunobiologicals (Lisle, IL); and minimal essential amino acids from GIBCO (Grand Island, NY). All other chemicals were obtained from Sigma Chemicals (St. Louis, MO).

Preparation of isolated acini. Pancreatic acini were prepared by the modified method of Williams et al. [5,6] from 19 to 21 g male White Swiss mice that had been fasted overnight. Briefly, pancreatic tissue was digested by purified collagenase and dispersed into individual acini by pipetting through polypropylene pipettes of decreasing diameters. Acini were purified by centrifugation through 4% bovine serum albumin. The isolated acini were then allowed to recover at 37°C for 30 min in Hepes-buffered Ringer solution supplemented with 11.1 mM glucose, minimal essential amino acids, 5 mg/ml bovine serum albumin, 0.1 mg/ml soybean trypsin inhibitor, and equilibrated with 100% O_2 . Acini were then pelleted and resuspended in Pipes-buffered Ringer solution containing 137 mM NaCl, 2.7 mM KCl, 20 mM Pipes (pH 7.0), 5.6 mg/ml glucose, 1 mg/ml bovine serum albumin and 0.1 mg/ml soybean trypsin inhibitor.

Membrane permeabilization and amylase secretion. In a typical secretion experiment, 1 ml of acini in Pipes-buffered Ringer solution were added to 1 ml of permeabilizing buffer to obtain final concentrations of 0.4 IU/ml SLO, 5 mM ethylene glycolbis(β -aminoethyl ether) N,N,N',N' -tetraacetic acid (EGTA), 1 mM adenosine 5'-triphosphate magnesium salt (MgATP), 1 mM free Mg^{2+} , and various concentrations of free Ca^{2+} . Acini were incubated at 30°C for 30 min, unless otherwise noted, and a 1 ml aliquot from each sample was then separated from the medium by centrifugation at $10000 \times g$ for 15 s in an Eppendorf microcentrifuge. The supernatant was taken for measurement of amylase and lactate dehydrogenase (LDH). Basal release is defined as the release occurring at a free [Ca^{2+}] of less than 10^{-9} M, in the absence of other stimulatory substances. Amylase activity was determined using procion yellow starch as a substrate [7]. LDH was measured by a colorimetric method using a commercial kit (Sigma).

Secretion of amylase or leakage of LDH is expressed as a percentage of the total content at the beginning of the incubation.

Determination of free Ca^{2+} concentration. Different free Ca^{2+} concentrations were obtained by altering the Ca^{2+} /EGTA ratio. Free Ca^{2+} concentrations were determined with a computer program by taking into account the binding of Ca and Mg ions to EGTA and ATP [8]. In the nominally Ca^{2+} -free buffer containing 5 mM EGTA (without addition of Ca^{2+}), the free Ca^{2+} concentration was less than 10^{-9} M. The expression ' pCa ' is used to denote the negative logarithm of the concentration of free Ca^{2+} .

Statistics. All determinations were carried out in duplicate or triplicate, and experiments were repeated at least three times with independent preparations of acini, except where otherwise noted. Statistical analysis was performed by analysis of variance (ANOVA) followed by Newman-Keuls test to determine the differences among means. $P < 0.05$ was considered to be significant.

Results

Effects of temperature on the kinetics of amylase secretion.

We have previously shown that Ca^{2+} stimulates amylase secretion from SLO-permeabilized acini incubated at 37°C with a half-maximal effect at 0.4 μM and a maximal effect at 1 μM free Ca^{2+} [4]. At this temperature Ca^{2+} -stimulated secretion occurred primarily within the first 10 min of stimulation. This rapid time-course made it difficult to examine the effects of mediators which enhance Ca^{2+} -stimulated secretion. Therefore, we evaluated the effects of temperature on amylase secretion from SLO-permeabilized acini to obtain more accurate values for differences in rates of secretion. The time-courses of amylase release were examined using medium containing $< 10^{-9}$ M Ca^{2+} , 1 μM Ca^{2+} and 1 μM Ca^{2+} plus 30 μM GTP[γ S] at 14, 22 and 30°C (Fig. 1). Amylase secretion from permeabilized acini was temperature-dependent. When incubated at 14°C, Ca^{2+} -stimulated amylase secretion after 60 min was only 3% of the total (Fig. 1A). There was an increase in Ca^{2+} -stimulated amylase secretion with increasing temperature. The initial rate of secretion (the linear portion) was greater at higher temperatures (Fig. 1B and C), but the longevity of stimulated secretion was shorter as temperature increased.

The other observed effect of temperature was that GTP[γ S] failed to enhance Ca^{2+} -stimulated secretion at 14°C (Fig. 1A), while it enhanced secretion at 22°C and more strongly at 30°C (Fig. 1B and C). The major effect of GTP[γ S], at a given temperature, was to increase the longevity of secretion rather than affecting

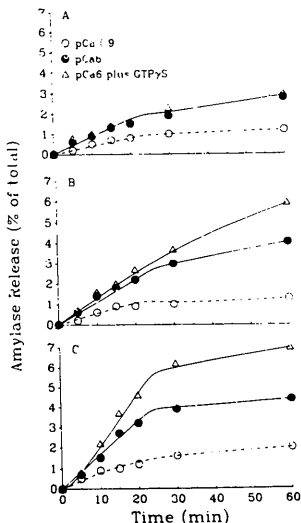


Fig. 1. Temperature-dependence of kinetics of amylase secretion from permeabilized acini stimulated by Ca^{2+} and $\text{GTP}[\gamma\text{S}]$. Acini were incubated with 0.4 IU/ml SLO in Ca^{2+} -free ($p\text{Ca} < 9$), $1 \mu\text{M} \text{Ca}^{2+}$ ($p\text{Ca} 6$) or $1 \mu\text{M} \text{Ca}^{2+}$ with $30 \mu\text{M} \text{GTP}[\gamma\text{S}]$ ($p\text{Ca} 6$ plus $\text{GTP}[\gamma\text{S}]$) at 14°C (A), 22°C (B) or 30°C (C). Amylase secreted into the medium was determined at the indicated times. Data are means of duplicate determinations from a representative of two independent experiments. \circ , $p\text{Ca} < 9$; \bullet , $p\text{Ca} 6$; and Δ , $p\text{Ca} 6$ plus $\text{GTP}[\gamma\text{S}]$.

the secretory rate. However, at 30°C , $\text{GTP}[\gamma\text{S}]$ also slightly increased the secretory rate (Fig. 1C).

Ca^{2+} -stimulated amylase secretion at 30°C was sustained for 20–30 min and the $\text{GTP}[\gamma\text{S}]$ -enhancement was maximal and comparable to that at 37°C [4]. The kinetics of secretion at 30°C were examined in greater detail in further experiments (see below). The basal secretion was lower at 22°C than at 30°C , but the $\text{GTP}[\gamma\text{S}]$ -enhancement of secretion was less pronounced at 22°C . Therefore, we chose 30°C as the incubation temperature for the remainder of these experiments.

Leakage of the cytosolic enzyme lactate dehydrogenase (LDH) was used as a measure of cell permeabilization as previously described [4]. The rate of permeabilization at either 22°C or 30°C was similar to that at 37°C [4], but was somewhat slower at 14°C (data not shown). A partial explanation for the decreased secretory rate at 14°C may be the decreased rate of permeabilization.

Effects of cAMP on amylase secretion

An additional intracellular messenger that stimulates secretion in the acinar cell is cAMP [1–3], and its effects on secretion from permeabilized acini were determined in this study. Cyclic AMP in the absence of Ca^{2+} , like $\text{GTP}[\gamma\text{S}]$ and TPA [4], had no effect on amylase secretion (Fig. 2). In the presence of $1 \mu\text{M} \text{Ca}^{2+}$, cAMP enhanced amylase secretion with a maximally effective cAMP concentration of 0.1–1 mM. Addition of the phosphodiesterase inhibitor IBMX, decreased the maximally effective [cAMP] to 1–10 μM , but did not further increase the extent of amylase secretion (Fig. 2). IBMX alone or with Ca^{2+} had no effect on amylase secretion (data not shown). Maximal Ca^{2+} -stimulated amylase secretion enhanced by cAMP was $431 \pm 23\%$ of that in Ca^{2+} -free medium, compared to $254 \pm 16\%$ in the presence of $1 \mu\text{M} \text{Ca}^{2+}$ alone ($n = 3$, $P < 0.01$ by post-hoc Newman-Keuls test).

Comparison of the kinetics of secretion enhanced by cAMP, TPA or $\text{GTP}[\gamma\text{S}]$

We studied the time-courses of amylase secretion at 30°C stimulated by $1 \mu\text{M} \text{Ca}^{2+}$, and by $1 \mu\text{M} \text{Ca}^{2+}$ plus cAMP (100 μM), TPA (1 μM) or $\text{GTP}[\gamma\text{S}]$ (30 μM) (Fig. 3). These experiments were similar to those in Fig. 1, except that the maximum time used was 30 min, with more time points taken in the early phase of secretion. The more detailed time-courses allowed more accurate evaluation of secretory rates when calcium was supplemented by other intracellular mediators. All three compounds enhanced Ca^{2+} -stimulated secretion to similar extents (Fig. 3 A–C). The major effect of cAMP and TPA was that the longevity of secretion was prolonged in their presence, while the initial rate of secretion seemed to be relatively unaffected. $\text{GTP}[\gamma\text{S}]$ increased the longevity of the secretory response and also slightly

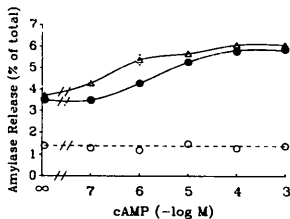


Fig. 2. Effects of cAMP on amylase secretion from permeabilized acini. Acini were incubated for 30 min at 30°C with 0.4 IU/ml SLO in Ca^{2+} -free ($p\text{Ca} < 9$) or $1 \mu\text{M} \text{Ca}^{2+}$ ($p\text{Ca} 6$) plus the indicated concentrations of cAMP, with or without 0.1 mM IBMX. Data are means \pm S.E.M. from three independent experiments. Error bars not shown are smaller than the symbols. \circ , $p\text{Ca} < 9$; \bullet , $p\text{Ca} 6$; and Δ , $p\text{Ca} 6$ plus IBMX.

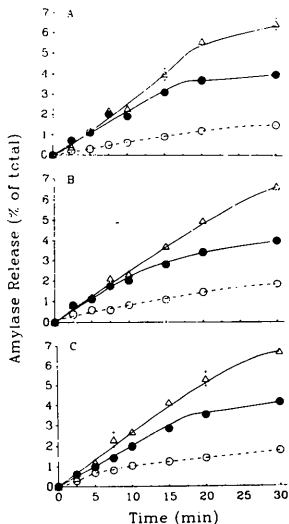


Fig. 3. Kinetics of amylase secretion from permeabilized acini stimulated by Ca^{2+} plus cAMP, TPA or GTP[γ S]. Acini were incubated at 30°C for the indicated times with 0.4 IU/ml SLO in Ca^{2+} -free ($p\text{Ca} < 9$), $1 \mu\text{M}$ Ca^{2+} ($p\text{Ca} 6$) or $1 \mu\text{M}$ Ca^{2+} plus $100 \mu\text{M}$ cAMP plus 0.1 mM IBMX ($p\text{Ca} 6$ plus cAMP), $1 \mu\text{M}$ TPA ($p\text{Ca} 6$ plus TPA) or $30 \mu\text{M}$ GTP[γ S] ($p\text{Ca} 6$ plus GTP[γ S]). Data are means \pm S.E.M. from three independent experiments. Error bars not shown are smaller than the symbols. \circ , $p\text{Ca} < 9$; \bullet , $p\text{Ca} 6$; and Δ , $p\text{Ca} 6$ plus (A) cAMP, (B) TPA and (C) GTP[γ S].

increased the rate of secretion (Fig. 3C). These data suggest that Ca^{2+} is important in the initiation of amylase secretion, and the other intracellular mediators act to sustain secretion.

Interactions between Ca^{2+} , cAMP, TPA and GTP[γ S]

It is known that different intracellular mediators have synergistic effects in intact acinar cells [1-3]. The permeabilized cell provides a situation where the concentrations of such intracellular mediators can be precisely controlled and membrane-impermeant compounds can be used. The interactions between maximally-stimulatory concentrations [3] of Ca^{2+} , cAMP, TPA, and GTP[γ S] were studied (Fig. 4). To evaluate levels of secretion stimulated by the different combinations of agents, the values were compared to the $p\text{Ca} < 9$ alone (basal) or $p\text{Ca} 6$ alone values. This was done, rather than a comparison of each $p\text{Ca} < 9$ to $p\text{Ca} 6$ value pairwise, because some combinations of agents

stimulated calcium-independent secretion (see below). As noted above, cAMP, TPA and GTP[γ S] individually enhanced Ca^{2+} -stimulated amylase secretion to similar extents (Figs. 3 and 4). Combinations of these three agents in the presence of $1 \mu\text{M}$ Ca^{2+} yielded values for secretion that amounted to levels of secretion equal to the sums of the individual enhancements. The enhancement of Ca^{2+} -stimulated amylase secretion by cAMP plus TPA was $106 \pm 6\%$; by cAMP plus GTP[γ S] was $90 \pm 4\%$; and by TPA plus GTP[γ S] was $92 \pm 3\%$ of the respective sums of the enhancements observed with two of the compounds individually (Fig. 4). The enhancement of Ca^{2+} -stimulated amylase secretion by all three compounds was $97 \pm 4\%$ of the sum of the enhancements observed with each compound individually.

In Ca^{2+} -free buffer, cAMP, TPA and GTP[γ S] had no effect on amylase secretion individually. With combinations of two compounds, only TPA plus GTP[γ S] stimulated Ca^{2+} -independent amylase secretion, which was $187 \pm 38\%$ of basal (Fig. 4). The combination of cAMP plus TPA plus GTP[γ S], demonstrated Ca^{2+} -independent secretion to a greater extent, which was $329 \pm 30\%$ of basal. The enhancement of secretion due to the additional presence of calcium with all three agents is of the same magnitude as the enhancement observed in the presence of calcium and any pair of agents. Thus, the higher level of secretion with all three agents plus calcium is not due to a novel interaction of calcium with the combination of the three agents, but

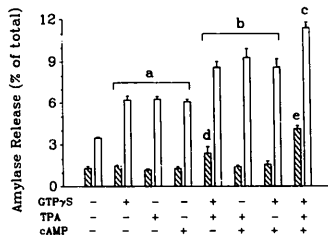


Fig. 4. Combined effects of cAMP, TPA, GTP[γ S] and Ca^{2+} on amylase secretion from permeabilized acini. Acini were incubated for 30 min at 30°C with 0.4 IU/ml SLO in Ca^{2+} -free buffer ($p\text{Ca} < 9$) or $1 \mu\text{M}$ Ca^{2+} buffer containing the indicated agents: GTP[γ S] ($30 \mu\text{M}$), TPA ($1 \mu\text{M}$) or cAMP ($100 \mu\text{M}$ cAMP + 0.1 mM IBMX). Data are means \pm S.E.M. from three independent experiments except for $p\text{Ca} < 9$ plus GTP[γ S] plus TPA, where six independent experiments were performed. Statistical analysis by analysis of variance and post-hoc Newman-Keuls: (a) All values at $p\text{Ca} 6$ were greater than at $p\text{Ca} < 9$ in the absence of the second agent, $P < 0.01$. (b) All values at $p\text{Ca} 6$ were greater than the corresponding values marked (a), $P < 0.01$. (c) Value was greater than all $p\text{Ca} < 9$ values marked (b), $P < 0.01$. (d) Value greater than all $p\text{Ca} < 9$ values except (c), $P < 0.01$. (e) Value greater than (d), $P < 0.01$. Striped bars, $p\text{Ca} < 9$; and open bars, $p\text{Ca} 6$.

TABLE 1

Specificity of agents that stimulate Ca^{2+} -independent secretion

Acini were incubated with 0.4 IU/ml SLO at 30°C for 30 min with Ca^{2+} -free medium ($pCa < 9$) containing the indicated agents: cAMP (100 μ M cAMP plus 0.1 mM IBMX), GTP[γ S] (30 μ M), TPA (1 μ M), 4 α -phorbol (1 μ M 4 α -phorbol 12,13-didecanoylate), GDP[β S] (30 μ M guanosine 5'-[β -thioldiphosphate), AMP (100 μ M adenosine 3', monophosphate). Data are means \pm S.E.M. from three independent experiments. Statistical analysis by ANOVA and post-hoc Newman-Keuls, N.S., not significant compared to corresponding value in the absence of the analogue.

	Amylase secretion (% of total)	Significance
$pCa < 9$	1.37 \pm 0.12	
plus cAMP + TPA + GTP[γ S]	4.37 \pm 0.07	$P < 0.05$
plus :AMP + GTP[γ S]	1.53 \pm 0.09	N.S.
plus cAMP + GTP[γ S] + 4 α -phorbol	1.67 \pm 0.07	N.S.
plus cAMP + TPA	1.33 \pm 0.03	N.S.
plus cAMP + TPA + GDP[β S]	1.23 \pm 0.03	N.S.
plus TPA + GTP[γ S]	2.60 \pm 0.06	N.S.
plus TPA + GTP[γ S] + AMP	2.53 \pm 0.09	N.S.

rather to the calcium-independent interaction of cAMP, TPA and GTP[γ S] and the same degree of calcium-enhancement of secretion seen with pairs of the agents. Ca^{2+} -independent amylase secretion was not observed when each of the compounds was individually replaced by chemically similar, inactive analogs (Table 1), demonstrating the specificity of each agent.

ATP dependence of amylase secretion stimulated by the different agents

In intact acinar cells, metabolic inhibitors block amylase secretion [9,10]. To study whether secretion stimulated by interactions between the different intracellular mediators was ATP-dependent, acini were treated with metabolic inhibitors (10 μ M antimycin A, 6 mM 2-deoxyglucose and 0.5 mM 1,2-dinitrophenol) and ATP was omitted from the medium. Addition of metabolic inhibitors had no effect on basal amylase secretion ($pCa < 9$). Metabolic inhibitors decreased 1 μ M Ca^{2+} -stimulated secretion by 46 \pm 8% (Fig. 5). The enhancements of Ca^{2+} -stimulated secretion by cAMP, TPA or GTP[γ S], however, were completely abolished by metabolic inhibitors. Similarly, Ca^{2+} -independent amylase secretion stimulated by combined cAMP, GTP[γ S] and TPA was inhibited by 75 \pm 9%. These data suggest that in permeabilized pancreatic acinar cells, Ca^{2+} -dependent amylase secretion is partially ATP-dependent, but the enhancements by GTP[γ S], TPA or cAMP are absolutely ATP-dependent. The failure of metabolic inhibition to fully block secretion stimulated in the absence of Ca^{2+} by cAMP plus TPA plus GTP[γ S] suggests that the interaction of these

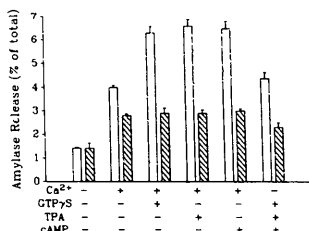


Fig. 5 Effects of ATP depletion on amylase secretion from permeabilized acini. Acini were pretreated with metabolic inhibitors (10 μ M antimycin A, 6 mM 2-deoxyglucose and 0.5 mM 1,2-dinitrophenol) for 3 min before permeabilization, and ATP was omitted from the incubation medium. Acini were permeabilized with 0.4 IU/ml SLO at 30°C for 30 min in Ca^{2+} -free ($pCa < 9$), or 1 μ M Ca^{2+} buffer containing the indicated agents with or without the same concentrations of metabolic inhibitors and without or with 1 mM ATP. (cAMP: 100 μ M, cAMP plus 0.1 mM IBMX; GTP[γ S]: 30 μ M GTP[γ S]; TPA: 1 μ M TPA). Data are means \pm S.E.M. from three independent experiments. Open bars, control; and striped bars, metabolic inhibitors.

mediators also stimulates the ATP-independent pathway to some extent.

Discussion

In the current work we examined the effects of temperature on the kinetics of secretion from streptolysin O permeabilized pancreatic acini and the interactions between the three major intracellular messenger pathways (Ca^{2+} , DAG and cAMP) and the G-protein modulator, GTP[γ S]. Secretion was found to be temperature-dependent in two respects. In addition to slowing the rate of Ca^{2+} -stimulated secretion, lower temperatures also decreased or abolished the enhancement of secretion by GTP[γ S] seen at 37 or 30°C. At 30°C, the longevity of secretion is more than 2-times that at 37°C [4] and the enhancement of Ca^{2+} stimulated secretion by other intracellular mediators is about 2-fold over Ca^{2+} alone, similar to that observed at 37°C [4].

The kinetics of secretion at 30°C showed that enhancement of Ca^{2+} -stimulated secretion by cAMP or TPA was due to increasing the longevity of secretion, rather than an obvious change in the initial rate of secretion. With GTP[γ S] there was also a small increase in the secretory rate in addition to increased longevity of the secretory response. If GTP[γ S] is directly stimulating exocytosis through G_q , as postulated in Ref. 19, it may be expected to act more rapidly than agents which have their effects indirectly such as cAMP which activates cAMP-dependent protein kinase.

In a recent study, using perfused electrically permeabilized islet cells, the effect of cAMP and phorbol ester on Ca^{2+} -stimulated insulin secretion was reported to be an increase in the duration of the secretory response [11], similar to what we observed. Since secretory responsiveness is rapidly lost from unstimulated SLO-permeabilized acini [4], our data suggest that activation of the various pathways immobilizes, and thus preserves, some activity that is soluble in the resting cell. It has been demonstrated that permeabilized cells do lose cytosolic components necessary for exocytosis [12,13]. Alternatively, it may be that the activated state can persist even after the effector (e.g., cAMP-dependent protein kinase or protein kinase C) has diffused out of the permeabilized cell and thus sustain Ca^{2+} -stimulated secretion. We cannot at this time distinguish between these two possibilities.

The effectiveness of cAMP to stimulate amylase secretion from permeabilized pancreatic acini has been examined in two previous studies. In one of these studies, rat pancreatic acinar cells were permeabilized by electric field discharge [14], and little effect of cAMP on Ca^{2+} -stimulated amylase release was observed. In the other study, rat acini were permeabilized with saponin [15], and cAMP was found to about double protein secretion stimulated by $100 \mu\text{M}$ Ca^{2+} . Our results, using streptolysin O permeabilized mouse acini and lower concentrations of Ca^{2+} , agree with the latter study, in that we found cAMP enhanced Ca^{2+} -stimulated secretion about 2-fold over Ca^{2+} alone.

We had previously noted that the enhancements of Ca^{2+} -stimulated secretion by TPA and GTP[γ S] were additive, suggesting that the pathways mediated by these two agents were distinct from each other [4]. In the present work, we studied the effects of the other major intracellular messenger, cAMP, in various combinations with the other intracellular mediators. Like TPA and GTP[γ S], cAMP enhanced Ca^{2+} -stimulated secretion. Furthermore, enhancement of Ca^{2+} -stimulated secretion by cAMP was additive with either TPA or GTP[γ S] as well as the combination of both. These data demonstrate that the three pathways mediated by cAMP, TPA and GTP[γ S] are parallel and probably converge at a distal step in the exocytotic process. These different pathways also interact since they stimulate Ca^{2+} -independent secretion in certain combinations, but not individually (see below). The data also suggest that each pathway activated to its full extent does not stimulate the maximum secretory activity possible. Thus, the capacity of the exocytotic machinery is greater than any single pathway can activate. This type of arrangement provides for a graded secretory response in situ, where combinations of hormones and neurotransmitters differentially stimulate a $[\text{Ca}^{2+}]_i$ increase, DAG production and cAMP generation [1-3].

In intact cells metabolic activity is necessary for secretion [9]. However, in permeabilized cells, metabolism (or the addition of exogenous ATP) is not so clearly required for secretion [16-19]. In our system, we found that Ca^{2+} -stimulated secretion was partially ATP-dependent, while about 50% secretory response remained with metabolically poisoned cells in ATP-free medium. By contrast, the enhancements of secretion by cAMP, TPA or GTP[γ S] were fully ATP-dependent. This is expected for cAMP and TPA, which are believed to act by stimulation of protein kinase A (cAMP-dependent protein kinase) and protein kinase C, respectively. Why the GTP[γ S]-induced enhancement should be ATP-dependent awaits the discovery of the mechanism by which G proteins act in exocytosis (see Ref. 19 for a current review of G-proteins in exocytosis). The failure of metabolic inhibition to fully block Ca^{2+} -independent secretion stimulated by the combination of cAMP, TPA and GTP[γ S] is not clear at this time, but this result suggests that the interaction of these three intracellular mediators stimulates both ATP-dependent and ATP-independent mechanisms of exocytosis.

Of the intracellular messengers we used, only Ca^{2+} was able to stimulate amylase secretion by itself. However, in Ca^{2+} -free medium, the combination of TPA and GTP[γ S] significantly stimulated secretion, which was further enhanced by the additional presence of cAMP. This finding of calcium-independent secretion in permeabilized cells has implications for the biochemical mechanism of exocytosis. It had been widely believed from studies on intact cells that exocytosis was Ca^{2+} -dependent (Refs. 2, 10 and 20; for a current review the role of Ca^{2+} in exocytosis see Ref. 21). In the permeabilized cell, the free $[\text{Ca}^{2+}]_i$ can easily be lowered to less than 1 nM, which is, physiologically speaking, Ca^{2+} free. The fact that a combination of intracellular messengers can elicit secretion in Ca^{2+} -free medium demonstrates that the exocytotic mechanism in the pancreatic acinar cell under certain conditions is not a totally Ca^{2+} -dependent process. Calcium-independent secretion has been reported in a variety of secretory cells studied by permeabilization techniques. For example, exocytosis can be stimulated without Ca^{2+} by cAMP in parotid acini [22], by phorbol ester plus GTP[γ S] in HL60 promyelocytic cells [23], and by TPA in GH₃ pituitary cells [24]. The actual exocytotic mechanism remains unknown, but it is clear that exocytosis may be activated and modulated by multiple pathways in a variety of cell types.

Acknowledgments

This work was supported by NIH grants DK 41122 (J.A.W.) and GM 41388 (R.C.D.), and a Lilly International Fellowship (M.K.).

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