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Effects of ionophore A23187 and lanthanum on pepsinogen secretion from frog esophageal mucosa in vitro

Jim C. Fong

Institute of Biochemistry, National Yang-Ming Medical College, Taipei, Taiwan (China)

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The role of Ca^{2+} in the mediation of pepsinogen secretion from frog esophagus was investigated by means of ionophore A23187 and $LaCl_3$. The esophageal mucosa from Asian bullfrog *Rana tigerina* was mounted in a double-chamber system to preserve its polarity and was incubated in a medium containing 1.5 mM $CaCl_2$. Pepsinogen secreted was measured and expressed as % of total. The basal secretion averaged 3.5%/h. Bethanechol (25 μM), dibutyryl-cAMP (10 mM), ionophore A23187 (30 μM) and 3-isobutyl-1-methylxanthine (0.1 mM) increased the secretion to 8.7, 7.4, 7.1 and 6.8%, respectively. The stimulatory effect of bethanechol and of dibutyryl-cAMP were not affected by removing the exogenous Ca^{2+} with EGTA. The basal secretion was, however, reduced by 50% when Ca^{2+} in the incubation medium was lowered to 20 μM. At this low Ca^{2+} concentration, ionophore A23187 not only lost its stimulatory effect but also diminished the stimulation caused by bethanechol and dibutyryl-cAMP. While $LaCl_3$ at 1 mM had no effect on basal and bethanechol-stimulated secretion, at 10 mM it abolished the stimulation evoked by bethanechol or dibutyryl-cAMP. The conclusions are: (1) both Ca^{2+} and cAMP are involved in the mediation of pepsinogen secretion from frog esophagus, (2) basal secretion is dependent on extracellular Ca^{2+}, whereas bethanechol-stimulated secretion is not, (3) in the plasma membranes of peptic cells may exist a distinct Ca^{2+} pool (La^{3+}- and ionophore A23187-sensitive) which is involved in the stimulated pepsinogen secretion.

Introduction

Frog esophageal mucosa contains peptic glands which release pepsinogen without attendant acid, thus providing a good model to study the regulatory mechanism of pepsinogen secretion [1]. Several in vivo and in vitro studies employing animals including frog [1], rat [2], rabbit [3,4] and dog [5,6] have indicated that the cholinergic pathway plays an important role in the control of pepsinogen secretion. The chief cells, however, may also be regulated via the peptidergic pathway as evidenced by the observations that secretin [2,7,8], cholecystokinin [2,9] and caerulein [9] stimulate pepsinogen secretion. β-Adrenergic pathway may also be involved [3]. The intracellular mechanism of actions by these stimuli remains unclear, although both Ca^{2+} and cAMP may be involved [3]. Removal of Ca^{2+} from the incubation medium abolished the responses of isolated gastric glands to carbachol [3] and to cholecystokinin [9]. This is consistent with the notion that calcium is the common intracellular messenger for enzyme secretion induced by cholecystokinin or by muscarinic agonists [10–13]. In this respect, frog esophagus

Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; $LaCl_3$, lanthanum chloride; dibutyryl-cAMP, N^6, O^2-dibutyryladenosine 3′,5′-cyclic monophosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

presents an unusual tissue. Recent study with strips of frog esophageal mucosa [1] suggested that bethanechol stimulated pepsinogen release via a cAMP pathway. The stimulation was not affected by removal of extracellular calcium.

In order to investigate further the role of calcium in the mediation of pepsinogen release from the frog esophagus, we have examined the effects of a calcium ionophore, A23187 [14], and a calcium transport inhibitor, lanthanum chloride [15,16], on pepsinogen secretion. The dual-chamber system for mucosa study [17] was employed to preserve the polarity of the mucosa so that serosal side and luminal side effects can be studied separately, to reduce the variation of measurements and to provide an adequate control for each experiment. Part of the work has been published in an abstract form [18].

Materials and Methods

Animals and reagents. Asian bullfrog (*Rana tigerina*) obtained from Taipei, Taiwan, China in the summer and fall months were used. Chemicals were obtained from the following sources: bethanechol, IBMX, dibutyryl-cAMP, ionophore A23187, EGTA, porcine pepsinogen, bovine serum albumin, bovine hemoglobin, $LaCl_3$, atropine, isoproterenol and Hepes from Sigma Chemicals, St. Louis; Vasoactive intestinal peptide (VIP) from Peninsula Lab., Inc., CA; trifluoperazine from Smith Kline and Beckman, Philadelphia.

Incubation medium was prepared as follows: 70 mM NaCl/5 mM KCl/1.5 mM $CaCl_2$/1 mM $MgCl_2$/11 mM glucose/40 mM Hepes. The medium was adjusted to pH 7.3 by bubbling with 95% O_2/5% CO_2. $CaCl_2$ was omitted in Ca^{2+}-free medium (the free calcium concentration in this medium was estimated to be about 20 μM by atomic absorption measurement).

Pepsinogen secretion in vitro. Fasted frogs were used. After the brain stem was cut and the frog pithed, the abdomen and chest were opened. The esophagus was excised and immediately placed in oxygenated incubation medium. The tissue was cut open longitudinally and mucosa from the distal half was obtained by blunt dissection. The mucosa was then mounted in a dual-chamber with exposed area of 1 cm^2/each chamber. Incubation was maintained at 25°C. 3 ml of incubation medium was recirculated on the serosal side at a rate of 1 ml/min. 1 ml of incubation medium on the luminal side was aspirated in and out several times during each time period. The system was allowed to equilibrate for 90 min before starting the experiment. Reagents were added to the serosal side and secretions from the mucosal side were taken for pepsinogen measurement every 10 min.

Assays. Pepsinogen was measured by the method of Anson and Mirsky modified for the Technicon Auto Analyzer AA-1 (Technicon Instruments Corp., NY) using acidic hemoglobin at pH 2 as substrate [19] and porcine crystalline pepsinogen as the standards. Data are expressed as the percentage of the initial pepsinogen content released into the medium. Protein was measured by the method of Lowry et al. [20] using bovine serum albumin as the standard. Statistical differences were determined by Student's *t*-test.

Results

Esophageal mucosa of Asian bullfrog (*R. tigerina*) contained approx. 80% of the total pepsinogen present in the whole animal (70% in the lower half and 10% in the upper half). The remaining 20% was found in the fundus. The pepsinogen content of mucosa was found to be approx. 160 ± 7 μg/mg dry wt. ($n = 92$). Values are means $\pm$ S.E.

Cholinergic stimulation of pepsinogen release

After 90 min preincubation, the basal secretion came to a steady rate that persisted for at least a 3-h period. The addition of bethanechol (25 μM) increased the pepsinogen release from a basal rate of $3.5 \pm 0.4\%$ ($n = 8$) to $8.7 \pm 1.1\%$ ($n = 8$) during a 60-min period (Fig. 1A). The response reached a peak at 30 min after stimulation, and then started to decline. Bethanechol stimulation of pepsinogen secretion was dose-dependent, with 3 μM producing 50% of the maximal response found at a bethanechol concentration of 25 μM (data not shown). Atropine (1 μM) inhibited the response to bethanechol (25 μM) by 90%, indicating the presence of a muscarinic receptor.

Other stimuli

The pepsinogen release was also stimulated by

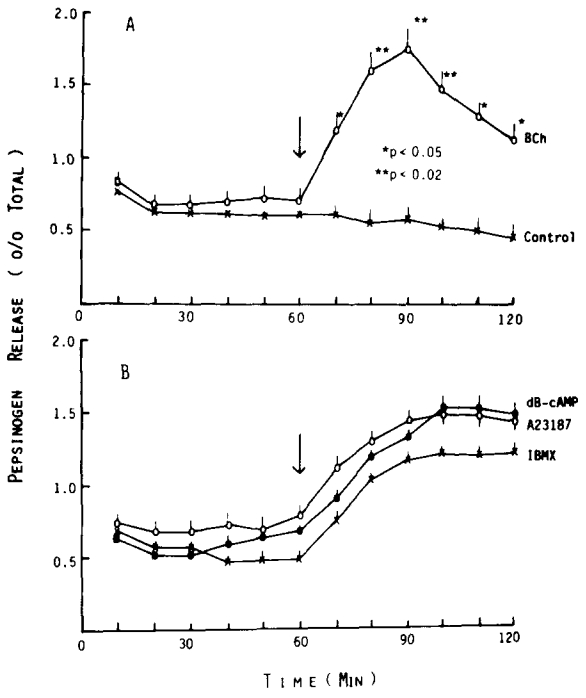

Fig. 1. (A) Time-course for pepsinogen release in response to bethanechol (BCh) (25 μM). Bethanechol was added at arrow indicated. Values are means $\pm$ S.E. ($n = 8$) $^{*}P < 0.05$, $^{**}P < 0.02$ compared with control. (B) Time-course for pepsinogen release in response to dibutyryl-cAMP (dB-cAMP), ionophore A23187 and IBMX. Dibutyryl-cAMP (10 mM), ionophore A23187 (30 μM) or IBMX (0.1 mM) was added at arrow indicated. ($n = 4$)

dibutyryl-cAMP, ionophore A23187 and a phosphodiesterase inhibitor, IBMX [21] (Fig. 1B). At optimal concentration, dibutyryl-cAMP, ionophore A23187 and IBMX each caused a 110%, 87% and 97% increase in pepsinogen release over a

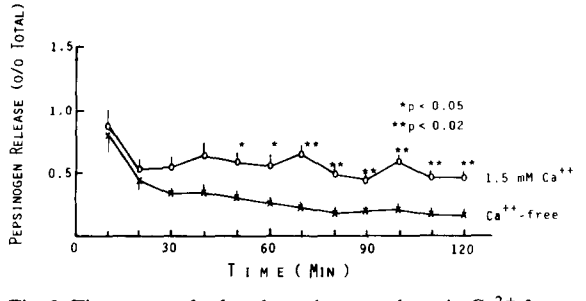

Fig. 2. Time-course for basal pepsinogen release in Ca^{2+}-free or 1.5 mM Ca^{2+} medium ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.02$ for 1.5 mM Ca^{2+} compared with Ca^{2+}-free.

TABLE I

EFFECT OF Ca^{2+} REMOVAL ON BASAL PEPSINOGEN RELEASE AND RELEASE INDUCED BY BETHANECHOL, dibutyryl-cAMP AND IONOPHORE A23187

After preincubation in normal (1.5 mM Ca^{2+}) or Ca^{2+}-free medium for 2 h with a medium change every 30 min, the mucosa was incubated further for 1 h in the same medium or in the same medium added with bethanechol (25 μM), dibutyryl-cAMP (10 mM) or ionophore A23187 (30 μM). Secretions collected from the mucosal side were measured for pepsinogen content. Values are means $\pm$ S.E. ($n = 4$).

	Pepsinogen release (% total)	
	1.5 mM Ca^{2+}	Ca^{2+}-free
Control	4.0 ± 0.4	2.2 ± 0.3 [c]
Bethanechol, 25 μM	8.0 ± 1.1 [b]	5.8 ± 0.7 [b]
Dibutyryl-cAMP, 10 mM	7.2 ± 0.8 [b]	5.6 ± 0.5 [b]
Ionophore A23187, 30 μM	7.1 ± 1.2 [a]	2.1 ± 0.5

[a] $P < 0.05$, [b] $P < 0.02$ compared with corresponding controls, [c] $P < 0.02$ for Ca^{2+}-free control compared with normal control.

60-min period, respectively. The secretions caused by dibutyryl-cAMP, ionophore A23187 and IBMX were not affected by the presence of atropine (1 μM). High KCl concentration (55 mM) evoked pepsinogen release similar in time-course and amplitude to that induced by bethanechol (25 μM). The secretory response to high K^{+} was abolished by the presence of either tetrodotoxin (1 μM) or atropine (1 μM), thus indicating the existence of endogenous cholinergic nerves innervating the peptic glands in the prepared esophageal mucosa. Of other secretagogues tested, neither isopro-

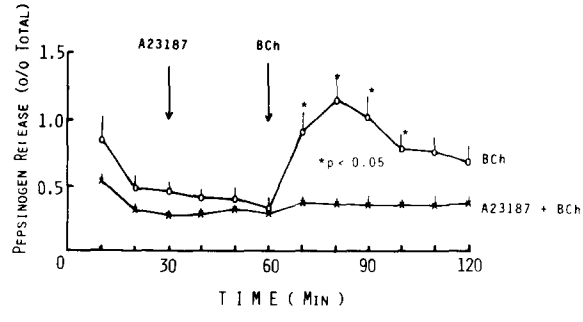

Fig. 3. Effect of ionophore A23187 (30 μM) on pepsinogen release stimulated by bethanechol (BCh) (25 μM) in Ca^{2+}-free medium ($n = 3$). $^{*}P < 0.05$ for bethanechol compared with ionophore A23187 + bethanechol.

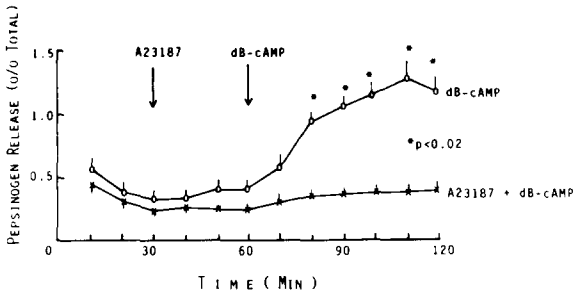

Fig. 4. Effect of ionophore A23187 (30 μM) on pepsinogen release stimulated by dibutyryl-cAMP (dB-cAMP) (10 mM) in Ca^{2+}-free medium ($n = 3$). *$P < 0.02$ for dibutyryl-cAMP compared with ionophore A23187 + dibutyryl-cAMP.

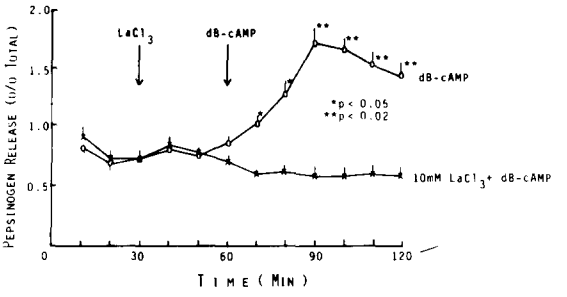

Fig. 6. Effect of $LaCl_3$ (10 mM) on pepsinogen release stimulated by dibutyryl-cAMP (dB-cAMP) (10 mM) ($n = 2$). *$P < 0.05$, **$P < 0.02$ compared with 10 mM $LaCl_3$ + dibutyryl-cAMP.

terenol at 10^{-9}–10^{-6} M nor VIP at 10^{-11}–10^{-6} M caused any stimulation of pepsinogen release.

Calcium effects

Basal secretion. When the free calcium concentration in the preincubation or incubation medium was only 20 μM (in Ca^{2+}-free medium), the basal pepsinogen release was reduced to about 50% of that observed in the presence of 1.5 mM Ca^{2+} (Fig. 2 and Table I). Only after 90 min of extensive washing with preincubation medium and another 30 min equilibration in the presence of incubation medium, was the calcium effect on basal secretion observed (Fig. 2). This finding may explain why in earlier studies with strips of frog esophageal mucosa no difference in basal release with or without Ca^{2+} in the incubation medium was observed [1].

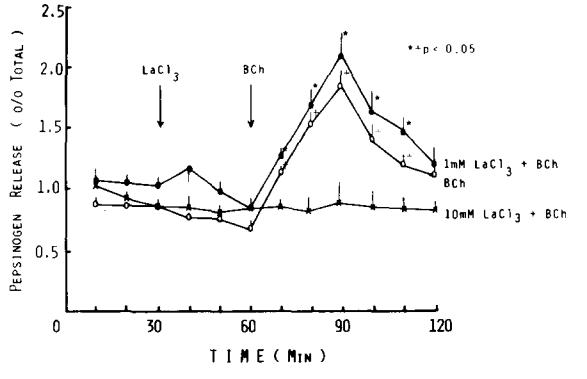

Fig. 5. Effect of $LaCl_3$ (1 and 10 mM) on pepsinogen release stimulated by bethanechol (BCh) (25 μM) ($n = 2$). *$P < 0.05$ compared with 10 mM $LaCl_3$ + bethanechol.

Stimulated secretion. The pepsinogen release stimulated by bethanechol or dibutyryl-cAMP was not affected by incubating the mucosa in a Ca^{2+}-free medium (Table I) or in a Ca^{2+}-free medium to which EGTA had been added (0.1 mM) (data not shown). The effectiveness of ionophore A23187 in stimulating pepsinogen release, nevertheless, was eliminated in the Ca^{2+}-free medium (Table I). Furthermore, in Ca^{2+}-free medium (which contained about 20 μM free Ca^{2+}), the addition of ionophore A23187 before a subsequent addition of bethanechol or dibutyryl-cAMP diminished the effectiveness of both secretagogues in inducing pepsinogen release (Figs. 3 and 4). Ionophore A23187, by itself, had no effect on basal secretion.

Effect of lanthanum chloride. Lanthanum is a rare earth element which can displace Ca^{2+} in the plasma membrane and block the Ca^{2+} fluxes across the membranes [15,22,23]. Thus, lanthanum chloride was used in this study because this compound may modify the effects of bethanechol and dibutyryl-cAMP differently. While La^{3+} at 1 mM had no effect on pepsinogen release, at a concentration of 10 mM it abolished the stimulation induced by bethanechol or by dibutyryl-cAMP (Figs. 5 and 6). At either concentration, La^{3+} did not change the basal secretion.

Discussion

The results in this study prove that the pepsinogen secretion from esophagus of Asian bullfrog (*R. tigerina*) can be included by ionophore

A23187, bethanechol, dibutyryl-cAMP and IBMX. Thus, it seems that both cAMP and Ca^{2+} may be involved in the intracellular regulation of pepsinogen secretion in this tissue. The divalent cation ionophore A23187 probably increases the pepsinogen release by inducing a rise in cytosolic free calcium, whereas dibutyryl-cAMP and IBMX may control the zymogen secretion viai the cAMP pathway. Since IBMX can cause a stimulation of pepsinogen release comparable to those induced by ionophore A23187 or dibutyryl-cAMP (Fig. 1B), it seems that the activities of phosphodiesterase and adenylate cyclase in this particular tissue may be very high.

Our findings that both bethanechol and dibutyryl-cAMP stimulated pepsinogen release could be affected in the same manner by the two agents with opposite actions on Ca^{2+} fluxes (i.e., ionophore A23187 and $LaCl_3$) and that the stimulatory effects of bethanechol and dibutyryl-cAMP were not additive (data not shown) seem to suggest that both bethanechol and dibutyryl-cAMP may act via a common pathway and in the intracellular control of pepsinogen release calcium may be involved in a step distal to cAMP formation. Process of exocytosis in luminal membranes seems not likely to be this step for the following reasons: (1) in the present study, chambered mucosa was used and the polarity of the mucosa was preserved. In this arrangement, lanthanum added to the serosal side of the mucosa could only bind to the basolateral plasma membranes on the same side. Since lanthanum has been shown to bind to Ca^{2+}-binding sites at the cell surface without entering the cell [24] and not to pass through the junctional complexes sealing off the lumen from the intercellular space [25], the possibility that La^{3+} might bind to the luminal membranes is very slim. (2) Trifluoperazine, as inhibitor of the calmodulin-Ca^{2+} complex [26], did not show any effect on basal or bethanechol-stimulated secretion (data not shown). However, in esophageal peptic cells, calmodulin may not be involved in the final process of exocytosis as has been shown in other tissues [27].

Thus, the argument discussed above may lead to the suggestion that in the basolateral plasma membranes of peptic cells in the esophagus may exist a calcium pool which controls the stimulated pepsinogen secretion in a step distal to cAMP production. This calcium pool may be associated with some common Ca^{2+}-dependent enzymatic reactions essential for stimulated secretion, or may play a role similar to the 'trigger Ca^{2+} pool' as defined by Stolze and Schulz [28]. They have proposed that the trigger Ca^{2+} pool in or near the plasma membranes may release Ca^{2+} upon stimulation into the cytosol, thereby increasing the cytosolic free calcium ions which activate a variety of processes leading to increased release of enzymes [24,28]. If the same is true for the esophageal peptic cells, it is possible that cAMP may cause an increase in cytosolic free calcium by releasing Ca^{2+} from a pool in the plasma membranes.

In examining the basal secretion under various conditions, it seems contradictory to find that the Ca^{2+} flux inhibitor lanthanum at a concentration which inhibited the stimulated secretion did not affect the basal release, while the basal release was reduced due to lack of sufficient Ca^{2+} in the extracellular medium. However, the reason for this finding may be the existence of two independent control mechanisms for basal and stimulated secretion. Both mechanisms are Ca^{2+}-dependent and are subject to perturbation by Ca^{2+}-regulating agents. Thus, lanthanum may interfere with these two mechanisms to a differential extent. The basal release, on the other hand, may be dependent on the passive Ca^{2+} influx for which the driving force is the concentration gradient caused by a higher Ca^{2+} concentration in the extracellular compartment compared to the intracellular compartment. Lanthanum may have little influence on this part. On the other hand, the stimulated secretion may be related to the calcium pool in the plasma membranes as suggested above, which is more susceptible to interference by lanthanum. This hypothesis is in agreement with the finding of Wakasugi et al. [24]. They found that in pancreatic acinar cells, lanthanum at a concentration of 5 mM or more blocked the Ca^{2+} uptake as well as the secretagogue-induced Ca^{2+} release from an intracellular pool in or near plasma membranes, whereas at a lower concentration (1 or 2 mM), lanthanum inhibited only the latter.

To examine further the role of Ca^{2+} in the control of pepsinogen secretion, we have looked into the effect of a calcium ionophore A23187 on

basal or stimulated secretion in Ca^{2+}-free medium. The Ca^{2+}-free medium actually contained approx. 20 μM free Ca^{2+} as determined by atomic absorption measurement. This small amount of Ca^{2+} probably is contaminant of reagents used in preparing the medium. At this low Ca^{2+} concentration, although higher than the intracellular free calcium concentration which is assumed to be less than 1 μM, ionophore A23187 not only lost its stimulatory effect on pepsinogen release, but also diminished the stimulation caused by bethanechol and dibutyryl-cAMP. The loss of stimulation by ionophore A23187 at low extracellular Ca^{2+} may be due to a lack of sufficient Ca^{2+} influx to increase the cytosolic free calcium concentration. However, the loss of stimulation by bethanechol or dibutyryl-cAMP after exposure to ionophore A23187 cannot be explained in the same way. Both bethanechol and dibutyryl-cAMP remain effective in stimulating pepsinogen release even without extracellular Ca^{2+}. Thus, there should be another explanation. Earlier studies with mitochondria have shown that ionophore A23187 in a Ca^{2+}-free medium may uncouple mitochondria; this uncoupling could be prevented by a calcium-chelating agent but not by Ca^{2+}. In fact, Ca^{2+} aggravated the uncoupling [14]. If mitochondria in the peptic cells of frog esophagus have a matrix concentration of Ca^{2+} of about 60 μM (as reported in mitochondria in hepatocytes [29]), the addition of ionophore A23187 to a medium containing only 20 μM Ca^{2+}, may cause the release of calcium from mitochondria. However, loss of calcium from mitochondria by itself is not sufficient to cause uncoupling of mitochondria [14]. The observation that in a medium containing 1.5 mM Ca^{2+}, the addition of ionophore A23187 actually stimulated pepsinogen release disproves the possibility that in a low Ca^{2+} medium, ionophore A23187 might uncouple the mitochondria. Recent studies with hepatocytes [30] or with pancreatic acinar cells [28] have shown that in a medium containing either high or low Ca^{2+}, ionophore A23187 was able to release intracellular calcium from some intracellular pool into the medium and this loss of calcium interfered with the hormonal action. All information together leads to the suggestion that in a medium of low calcium concentration, ionophore A23187 may deplete of calcium from an intracellular pool which plays a role in controlling the stimulated secretion. In addition, this ionophore A23187-sensitive Ca^{2+} pool may be the same La^{3+}-sensitive Ca^{2+} pool located in or near the basolateral plasma membranes.

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