PANCREATIC ACINAR CELLS: USE OF A Ca^{++} IONOPHORE TO SEPARATE ENZYME RELEASE FROM THE EARLIER STEPS IN STIMULUS-SECRETION COUPLING

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SUMMARY: The Ca^{++} ionophore A23187 had no effect on the release of amylase by mouse pancreas fragments in the absence of Ca^{++} but when Ca^{++} was re-added to the medium amylase release was observed in a pattern which mimicked that produced by normal stimulants. Uptake of $^{45}Ca^{++}$ by pancreatic fragments was increased by A23187. Tetracaine and dinitrophenol at concentrations which block cholinergic stimulated enzyme release blocked ionophore induced release whereas atropine did not. None of the inhibitors studied affected the ionophore induced Ca^{++} uptake.

Secretion of digestive enzymes by the exocrine pancreas conforms in many ways to the stimulus-secretion coupling model in which Ca^{++} acts as an intercellular mediator (1, 2). Extracellular Ca^{++}, however, may not be as important in triggering enzyme release as release of intracellularly sequestered Ca^{++} (3, 4). Since extracellular Ca^{++} can be reduced to below 0.1mM with a normal secretory response, the actual importance of Ca^{++} itself might be questioned. This is especially pertinent as there have been no direct demonstrations of stimulant increased Ca^{++} influx, an increase in intracellular free Ca^{++} or stimulation induced by artificial introduction of Ca^{++}. We have studied this last point by use of the Ca^{++} ionophore A23187 which increases the Ca^{++} permeability of isolated mitochondria and sarcoplasmic reticulum (5-7). This ionophore has recently been applied to studies of stimulus-secretion coupling. Calcium in the presence of A23187 has been shown to induce release of histamine by mast cells (8) and secretion by fly salivary gland (9).

Stimulation of enzyme release by A23187 as reported here not only further establishes the role of Ca^{++} in release of pancreatic enzymes but makes it possible to separate effects on the stimulus-secretion coupling mechanism from effects on the release process, exocytosis (10). Use of the Ca^{++} ionophore allows study of the requirements of the release process itself by bypassing the

steps leading up to an increase in intracellular Ca^{++}.

METHODS: All studies were carried out with male White Swiss mice weighing 18-22 gm which were fasted 14-20 hr prior to use. Animals were stunned, decapitated and the pancreas quickly removed and placed in Krebs-Henseleit bicarbonate solution (KHB) (11) at room temperature. The pancreas was then cut into four peripheral fragments, weighed on a torsion balance, and incubated in 3 ml of KHB at 37°C in 25 ml erlenmyer flasks shaken 90 cycles/min. All solutions were equilibrated with 95% O_2 - 5% CO_2. Alternatively pancreatic fragments were placed in a superfusion apparatus through which 37°C KHB was passed at 2 chamber volumes/min and effluent fractions collected.

Amylase released into the medium was assayed according to the method of Rinderknecht (12) using amylose azure blue (Cal. Biochem.) as the substrate. Amylase is reported in international units based on the reported activity of the α-amylase used as the standard (Sigma Type VI). None of the chemical agents affected the assay procedure at the dilution used.

For determination of pancreatic $^{45}Ca^{++}$ uptake, the pancreas was incubated in medium containing $^{45}Ca^{++}$ and then postincubated 1 hr in a Hepes buffered Ringer containing 10 mM $LaCl_3$. This method, which makes use of the ability of La^{+++} to block transmembrane Ca^{++} flux and "lock-in" intracellular Ca^{++}, was originally described by van Breeman in work on smooth muscle (13) and has recently been adapted for use on the pancreas (15). The pancreas was subsequently blotted and dried overnight at 80° C. Ca^{++} was then extracted for 48 hr in 0.1 N HNO_3 and an aliquot added to a scintillation counting mixture of Triton X-100 and toluene (1:2) containing 4 gm/l of butyl PBD. Quench correction of samples was by the samples channel ratio technique. The Ca^{++} ionophore A23187 was dissolved in ethanol and aliquots or comparable amounts of ethanol alone added to KHB. All other reagents were dissolved directly in KHB.

RESULTS: The ability of the Ca^{++} ionophore A23187 to allow Ca^{++} to stimulate pancreatic amylase release is shown in Fig. 1. The ionophore did not increase amylase release but the reintroduction of Ca^{++} to the medium rapidly increased

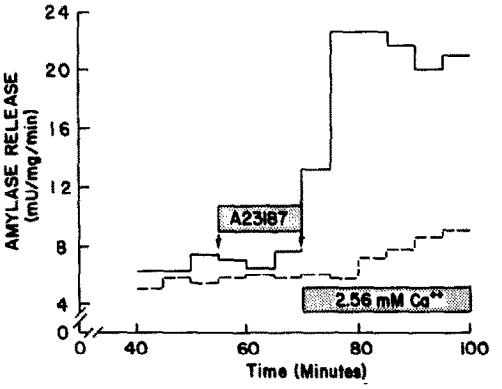

Fig. 1. Effect of Ca^{++} in the presence of the Ca^{++} ionophore A23187 on amylase release from mouse pancreatic fragments in vitro. Two halves of pancreas were superfused with 0 Ca^{++}, 0 Mg^{++} KHB. From 55-70 min 10 μM A23187 in 1% ethanol (solid line) or 1% ethanol alone (dashed line) was added to the medium. At 70 min 2.56 mM Ca^{++} was reintroduced into both chambers.

amylase release in a manner similar to that seen with cholinergic stimulants (3). In 5 experiments the increase in amylase release over the subsequent 30 min varied from 126 to 223%, values comparable to the increase brought about by maximal bethanechol stimulation after 60 min superfusion in either KHB or Ca^{++} free KHB (3, unpublished data). When A23187 was added directly to the KHB superfusate (containing Ca^{++}) the onset of amylase release was much more gradual requiring 15 min to reach a maximum suggesting that about this time is required for the ionophore to be incorporated into the cell membrane. When uptake of $^{45}Ca^{++}$ was determined 15 min after exposure to stimulants, A23187 increased the 2 min $^{45}Ca^{++}$ uptake (Table 1). No other divalent cation tested (Mg^{++}, Sr^{++}, Ba^{++}, Mn^{++} and Co^{++}) was able to stimulate amylase release comparably to Ca^{++}.

Since it was desired to study the effect of tetracaine on A23187-induced amylase its effects on bethanechol-stimulated release were first studied. Fig. 2 shows the concentration-inhibition curve for tetracaine on amylase release. The curves show a biphasic inhibition-stimulation sequence as the concentration of tetracaine is increased, characteristic of the action of membrane stabilizers. Maximal inhibition of amylase release was seen at 0.6 - 1.0 mM tetracaine.

Table 1: Effects of stimulators and inhibitors of pancreatic amylase release on $^{45}Ca^{++}$ uptake by mouse pancreas *in vitro*.

	$^{45}Ca^{++}$ uptake (nMoles/mg/2 min)	
Agent	Basal	A23187
None (KHB)	0.34±.03 (19)	0.62±0.06 (19)
Tetracaine (1mM)	0.51±.05 (10)	0.84±0.07 (10)
Dinitrophenol (0.2mM)	0.52±0.06 (8)	0.87±0.08 (8)
Atropine (3μM)	0.37±0.03 (13)	0.61±0.04 (13)

Pancreatic fragments were preincubated 30 min in KHB with the specified agent and then incubated 17 min in the same medium with A23187 (10μM). $^{45}Ca^{++}$ uptake was carried out over the last 2 min of the incubation period followed by postincubation in non-radioactive medium containing $LaCl_3$ (10mM) to retain intracellular $^{45}Ca^{++}$ while extracellular Ca^{++} is washed out. All values are the mean ± SEM of the number of pancreases shown in parenthesis.

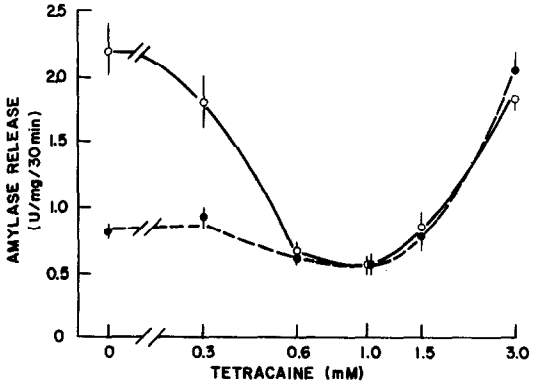

Fig. 2. Effect of tetracaine on basal and bethanechol stimulated amylase release by mouse pancreatic fragments *in vitro*. Tissue was preincubated 30 min in the specified concentration of tetracaine and then incubated in similar medium with (0—0) or without (0—0) 30 μM bethanechol. All values are the mean ± SEM of 4-6 pancreases.

Table 2: Effects of inhibitors on basal and A23187 stimulated pancreatic amylase release from mouse pancreas in vitro.

		Amylase Release (U/mg/30 min)	
	Agent	Basal	A23187
Experiment 1:	None (KHB)	0.44±0.04	0.81±0.08
	Tetracaine (1mM)	0.39±0.06	0.44±0/05
	Atropine (3μM)	0.55±0.06	0.81±0.07
Experiment 2:	None (KHB)	0.49±0.04	0.90±0.10
	Dinitrophenol (0.2mM)	0.28±0.04	0.37±0.07
	Cycloheximide (0.5mM)	0.50±0.06	0.84±0.08

Pancreatic fragments were preincubated 30 min with the specified agent and then incubated 45 min in the same medium with or without A23187 (10μM). Amylase release was measured over the last 30 min of the incubation period. All values are the mean ± SEM of 8-10 pancreases.

The effects on A23187-induced amylase release of tetracaine and several other known inhibitors of cholinergic stimulated pancreatic enzyme release is shown in Table 2. Tetracaine and dinitrophenol almost completely inhibited Ca^{++} induced amylase release while atropine was without effect. Cycloheximide was studied to show that the inhibitory effects of tetracaine and dinitrophenol were not due to inhibition of protein synthesis.

Since inhibition of A23187-induced amylase release can be taken as indicative of an effect on the release process only if the agent used does not interfere with ionophore induced Ca^{++} uptake this was studied and the results shown in Table 1. In every case A23187 induced an increase of about 0.3 nMoles/mg in the 2 min Ca^{++} uptake. Somewhat surprisingly both DNP and

tetracaine increased $^{45}Ca^{++}$ uptake in contrast to their inhibition of amylase release.

DISCUSSION: Release of hormones, enzymes and neurotransmitters stored intracellularly as granules or vesicles is generally felt to take place by exocytosis with Ca^{++} acting as the intracellular control (1, 15). Ca^{++} which activates the release process is generally believed to enter from the external medium as a result of an increased membrane permeability to Ca^{++} (16, 17). Release of digestive enzymes from the pancreas occurs by exocytosis (10) but is much less sensitive to the external concentration of divalent cations (17). An intracellular source of Ca^{++} has been suggested based on pancreatic stimulators increasing $^{45}Ca^{++}$ efflux from slowly exchanging cellular stores (3, 4, 18). The Ca^{++} ionophore A23187, however, allows direct entry of Ca^{++} into the cell by artificial means and demonstrates that Ca^{++} can completely duplicate the secretory response to cholinergic agonists. These findings both emphasize the importance of Ca^{++} in control of secretion and allow study of the release process independent of the steps by which normal stimulants increase intracellular Ca^{++}. Thus dinitrophenol and tetracaine both inhibited Ca^{++} stimulated release indicating a requirement for metabolic energy and a functioning cell membrane in the release process per se. Tetracaine also blocks the increased influx of Ca^{++} into adrenal chromaffin cells stimulated by acetylcholine (19). The present work, however, shows that inhibition of secretion by tetracaine can not be taken alone as indicative of stimulus induced increase in Ca^{++} permeability. In fact tetracaine increased Ca^{++} uptake at the same concentration which blocks amylase release.

The present findings should not be taken as necessarily implicating an influx of Ca^{++} in release of pancreatic enzymes induced by normal stimulants. When measured in the same way bethanechol at a concentration which maximally stimulates amylase release failed to increase ^{45}Ca uptake (14). Recent electrophysiologic studies also show no increase in pancreatic acinar cell Ca^{++} permeability upon cholinergic stimulation (20). If instead Ca^{++} is released

from intracellular compartments there must be a means by which the normal stimulus acting on the cell membrane brings this process about. The Ca^{++} ionophore allows this process to be studied since any condition or agent affecting cholinergic-stimulated but not A23187-stimulated amylase release must be affecting this coupling mechanism. A23187 thus appears a useful tool to further study the mechanism of stimulus secretion coupling.

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