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Effects of Extra- and Intracellular Ca Deprivation on ^{45}Ca Efflux and Amylase Release from Perifused Mouse Pancreatic Segments

Kazuo KATOH

Department of Animal Physiology, Tohoku University, Faculty of Agriculture, Tsutsumidori Amamiyamachi, Aoba-ku, Sendai 981, Japan

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Summary

The present experiment was carried out to investigate the role of Ca^{2+} release from intracellular stores and Ca^{2+} entry from extracellular medium on amylase release and $^{45}Ca^{2+}$ efflux in response to stimulation with a submaximal (1.1×10^{-7} M) and a supramaximal (5.5×10^{-6} M) concentration of ACh in mouse pancreatic segments loaded with $^{45}CaCl_2$. To deplete extracellular Ca^{2+}, or extracellular and internally stored Ca^{2+}, the tissue segments were incubated in a Ca^{2+}-free medium containing EGTA (10^{-4} M), or EGTA and a calcium ionophore, A23187 (2×10^{-6} M), for 20 min after loading $^{45}CaCl_2$. ACh at both concentrations significantly ($P<0.05$) caused a sustained increase in amylase release and transient ^{45}Ca efflux. Ca^{2+} depletion with EGTA and A23187 significantly ($P<0.05$) reduced an ACh-induced increase in amylase release and ^{45}Ca efflux. After an extended period, Ca^{2+} depletion with EGTA and A23187 completely abolished increases in amylase release and Ca^{2+} efflux induced by ACh at a submaximal concentration. Application of $CaCl_2$ to increase medium $CaCl_2$ concentration up to 2.56 mM caused a significant ($P<0.05$) and sustained increase in amylase release and ^{45}Ca efflux.

These data suggest that amylase release induced by stimulation with ACh is dependent on Ca^{2+} transiently released from intracellular stores and long-lasting Ca^{2+} entry from the medium in mouse pancreatic acinar cells.

Introduction

It is well established that Ca^{2+} plays a crucial role in stimulus-secretion coupling (1). Agonist-stimulated cytoplasmic Ca^{2+} signals have a complex spatial and temporal regulation, known as Ca^{2+} wave and oscillation, respectively (2).

The stimulus-induced cytoplasmic Ca^{2+} signal is evoked by Ca^{2+} entry through Ca^{2+} channels and/or by release of Ca^{2+} from intracellular Ca^{2+} stores

including those in the endoplasmic reticulum. Furthermore, regulation of Ca^{2+} release from intracellular stores is controlled by two distinct Ca^{2+} channels which are opened by inositol trisphosphate (IP3) and ryanodine, although the Ca^{2+} entry mechanism remains unclear (3).

In the present experiment, dynamic changes of amylase release and $^{45}Ca^{2+}$ efflux from intracellular stores in response to ACh stimulation were investigated to assess the role of extra- and intracellular Ca^{2+} to cause amylase release from mouse pancreatic segments.

Materials and Methods

A calcium ionophore, A23187, was purchased from Sigma (St. Louis, MO). $^{45}CaCl_2$ (1.2 GBq/mg Ca) was purchased from Amersham. Ethylene glycol-O, O′-bis (2-aminoethyl)-N, N, N′, N′-tetraacetic acid (EGTA) and other reagents used were highest grade products of Wako Pure Chem.

The pancreas was isolated from female mice (ddY) and cut into small pieces with a pair of fine scissors at room temperature in an oxygenated Krebs-Henseleit solution (125 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.13 $MgCl_2$, 2.56 mM $CaCl_2$, 2.8 mM D-glucose, 4.9 mM Na pyruvate, 4.9 mM Na glutamate and 2.7 mM Na fumalate) with 5% CO_2 and 95% O_2 gas.

The procedure for the measurement of ^{45}Ca efflux was, in principle, similar to that previously reported by Matthews *et al.* (4) and Case and Clausen (5). The segments were perifused after being loaded with $^{45}CaCl_2$ as described below. That is, tissue segments of about 250 mg were incubated for 1 hr at 37°C in 10 ml Krebs-Henseleit solution containing $^{45}CaCl_2$ (740 KBq). The segments were then dipped in "cold" Krebs-Henseleit solution on a sheet of gauze to wash out the isotope, and placed into a flow chamber (1.0 ml) which was perifused with a Krebs-Henseleit solution or a modified solution (Ca-free solution containing EGTA (10^{-4} M) and/or A23187 (2×10^{-6} M)) using a peristaltic tube pump at a flow rate of 1 ml/min. After 15-min pre-perifusion, effluent samples were collected at 1 min intervals using a fraction collector. Stimulation was made by superfusing the tissue segments with a solution containing ACh at a sub-(1.1×10^{-7} M) or a supramaximal (5.5×10^{-6} M) concentration. At the end of each experiment, tissue segments were weighed and solubilized for 2 days in 1 ml solubilizing solution (NCS, Amersham).

^{45}Ca specific activity (cpm) in effluent samples or solubilized tissues was measured with a liquid scintillation counter (Aloka, LSC-751). ^{45}Ca efflux (rate coefficient) was calculated from the following formula:

$$^{45}Ca \text{ efflux } (min^{-1}) = \Delta X/\Delta t \cdot Xt$$

where ΔX represents ^{45}Ca (cpm) released in the time interval Δt, and Xt the tissue

^{45}Ca content (cpm) at the mid-point of interval Δt (1 min).

Amylase concentration was determined by the method previously reported (6) using 100 μl of effluent samples, and amylase release (u/g/min) was calculated from amylase concentration, flow rate of perifusion (1 ml/min) and wet weight of tissue segments used.

The results are represented as mean $\pm$ S.E. (n=3). Statistical analysis was made by Student's *t*-test.

Results

The effects of deprivation of extra- and intracellular Ca^{2+} on amylase release and ^{45}Ca efflux from pancreatic segments loaded with $^{45}CaCl_2$ are depicted in Fig. 1 and 2.

As shown in Fig. 1A, stimulation with a submaximal concentration of ACh (1.1×10^{-7} M) for 5 min caused a rise in amylase release in the control solution from 29.9 ± 1.0 (at 0 min) to 60.9 ± 2.2 (the peak value at 4 min post-stimulation) u/g/min, in a Ca-free solution containing EGTA (10^{-4} M) from 39.3 ± 1.9 to 64.1 ± 1.6 (at 3 min post-stimulation) u/g/min and a Ca-free solution containing EGTA and A23187 (2×10^{-6} M) from 31.2 ± 2.0 to 35.9 ± 0.9 (at 3 min post-stimulation) u/g/min, respectively. The increment in amylase release stimulated with ACh in a Ca-free solution containing EGTA and A23187 to decrease both extra- and intracellular concentrations was suppressed to 15.2% of the control, being statistically significant ($P < 0.01$). The increment in amylase release stimulated with ACh in a Ca-free solution containing EGTA to decrease mainly the extracellular concentration was suppressed to 80.0% of the control, but was not statistically significant ($P > 0.05$).

Stimulation with a submaximal concentration of ACh concurrently caused a rise in ^{45}Ca efflux (rate coefficient) (Fig. 1B), time reaching a peak value after stimulation which coincided with that of the amylase increment. ^{45}Ca efflux in the control solution increased from 0.0233 ± 0.0008 (at 0 min) to 0.0395 ± 0.0017 (at 4 min post-stimulation)/min, in a Ca-free solution containing EGTA from 0.0242 ± 0.0007 to 0.0369 ± 0.0011 (at 3 min post-stimulation)/min and in a Ca-free solution containing EGTA and A23187 from 0.0303 ± 0.0012 to 0.0330 ± 0.0024 (at 3 min post-stimulation)/min, respectively. The increment in ^{45}Ca efflux stimulated with ACh in a Ca-free solution containing EGTA and A23187 to decrease both extra- and intracellular concentrations was suppressed to 16.5% of the control, which was statistically significant ($P < 0.01$). On the other hand, the increment in ^{45}Ca efflux stimulated with ACh in a Ca-free solution containing EGTA to decrease mainly the extracellular concentration, was suppressed to 77.4% of the control, but was not statistically significant ($P > 0.05$).

Stimulation with a supramaximal concentration of ACh (5.5×10^{-6} M) for 5

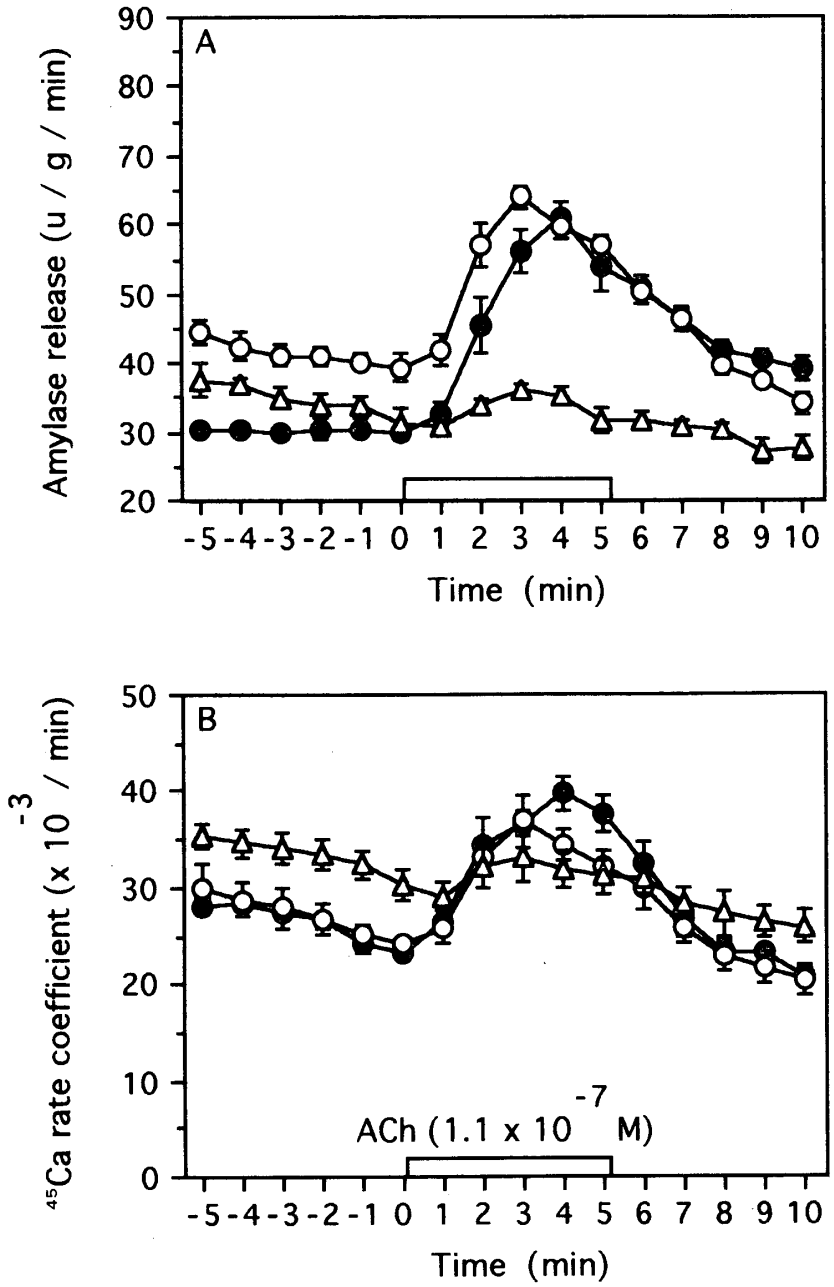

FIG. 1. Effects of ACh stimulation (1.1×10^{-7} M) on amylase release (A) and ^{45}Ca efflux (rate coefficient) (B) from $^{45}CaCl_2$-loaded mouse pancreatic segments in the control solution containing 2.56 mM $CaCl_2$ (●), in a Ca-free solution containing EGTA (10^{-4} M) (○), or in a Ca-free solution containing EGTA (10^{-4} M) and A23187 (2×10^{-6} M) (△). ACh stimulation period (5 min) is shown as a rectangle in B. The results are represented as mean±S.E. (n=3).

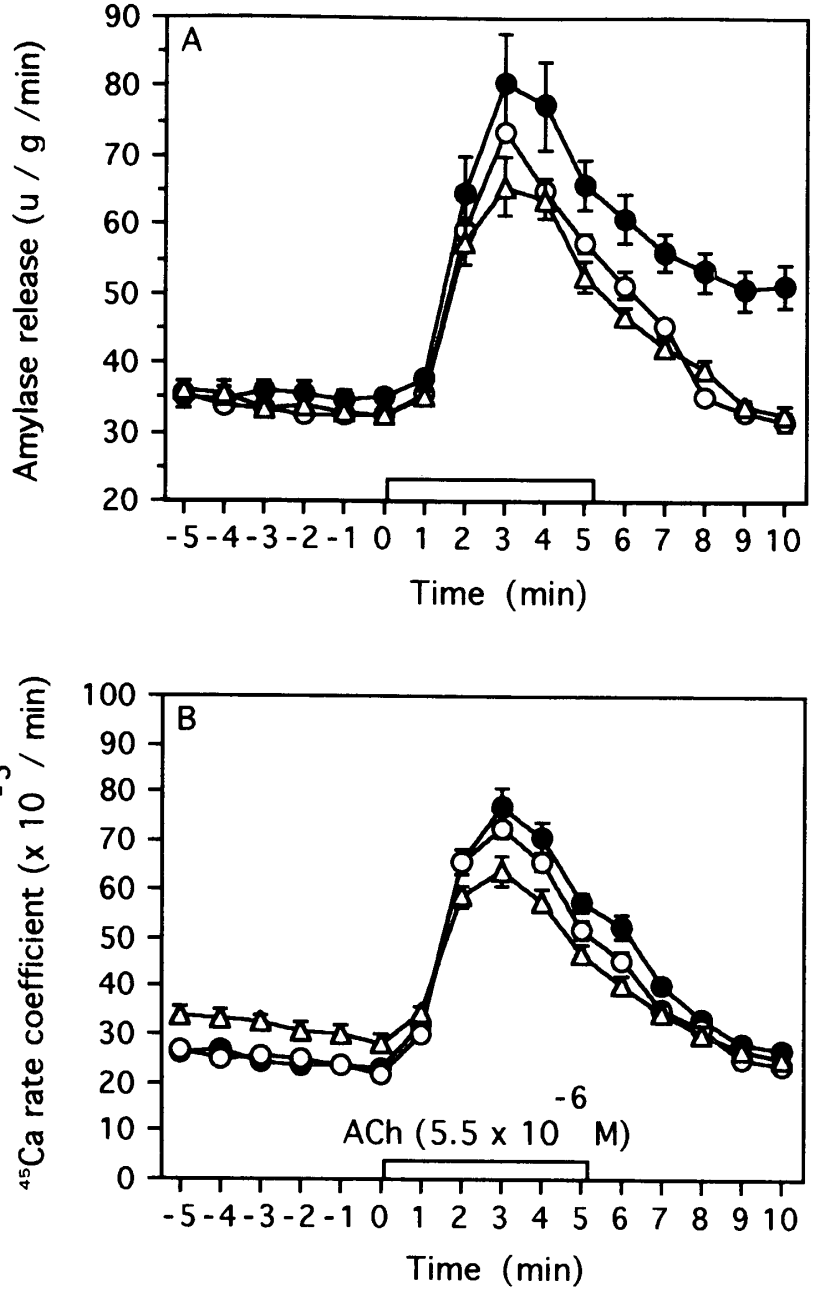

FIG. 2. Effects of ACh stimulation (5.5×10^{-6} M) on amylase release (A) and ^{45}Ca efflux (rate coefficient) (B) from $^{45}CaCl_2$-loaded mouse pancreatic segments in the control solution containing 2.56 mM $CaCl_2$ (●), in a Ca-free solution containing EGTA (10^{-4} M) (○), or in a Ca-free solution containing EGTA (10^{-4} M) and A23187 (2×10^{-6} M) (△). ACh stimulation period (5 min) is shown as a rectangle in B. The results are represented as mean±S.E. (n=3).

min caused a rise in amylase release in the control solution (Fig. 2A) from 35.0±0.4 (at 0 min) to 80.7±7.1 (the peak value at 3 min post-stimulation) u/g/min, in a Ca-free solution containing EGTA from 32.5±0.3 to 73.5±0.9 (at 3 min post-stimulation) u/g/min and a Ca-free solution containing EGTA and A23187 from 32.7±1.6 to 65.6±4.2 (at 3 min post-stimulation) u/g/min, respectively. The value for amylase release at 10 min post-stimulation (51.1±3.2 u/g/min) was still significantly ($P<0.05$) greater than that at 0 min, although a transient increment in ^{45}Ca efflux ceased. The increment in amylase release stimulated with ACh in a Ca-free solution containing EGTA, or both EGTA and A23187 was suppressed to 89.7 or 72% of the control, the latter being statistically significant ($P<0.05$).

On the other hand, stimulation with a supramaximal concentration of ACh concurrently caused a transient rise in ^{45}Ca efflux (rate coefficient) (Fig. 2B), time reaching a peak value at 3 min post-stimulation which coincided with that of the amylase increment. ^{45}Ca efflux in the control solution increased from 0.0229±0.0004 (at 0 min) to 0.0770±0.0039 (at 3 min post-stimulation)/min, in a Ca-free solution containing EGTA from 0.0218±0.0002 to 0.0724±0.0018/min and in a

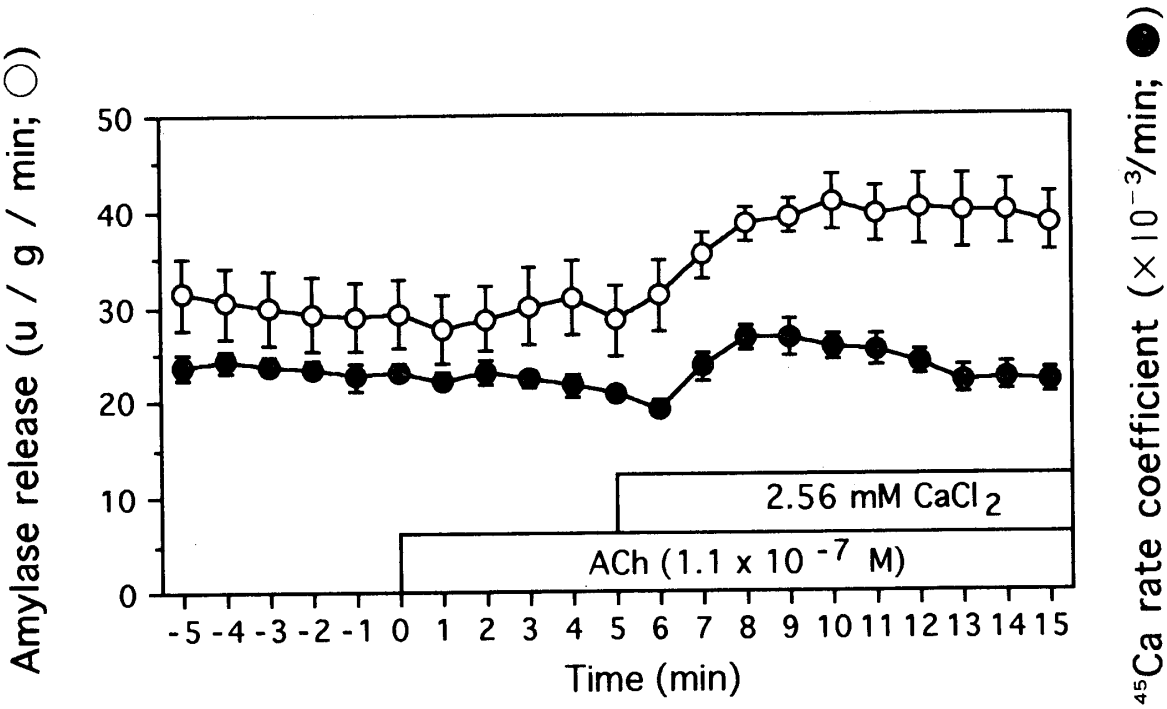

FIG. 3. Effects of extra- and intracellular Ca^{2+} deprivation and addition of 2.56 mM $CaCl_2$ into a Ca-free solution on amylase release (○) and ^{45}Ca efflux (rate coefficient) (●) in response to ACh stimulation (1.1×10^{-7} M) in $^{45}CaCl_2$-loaded mouse pancreatic segments. The tissue segments had been incubated for 50 min in a Ca-free solution containing EGTA (10^{-4} M) and A23187 (2×10^{-6} M) before ACh stimulation after $^{45}CaCl_2$ loading. The segments were stimulated with ACh in a Ca-free solution containing EGTA and A23187, then the $CaCl_2$ concentration of the medium was raised to 2.56 mM. The results are represented as mean±S.E. (n=3).

Ca-free solution containing EGTA and A23187 from 0.0281 ± 0.0018 to 0.0636 ± 0.0033/min, respectively. The increment in ^{45}Ca efflux stimulated with ACh in a Ca-free solution containing EGTA, or both EGTA and A23187 was suppressed to 93.5 or 65.6% of the control, the latter being statistically significant ($P < 0.05$).

The effects of Ca application to a medium on amylase release and ^{45}Ca efflux in response to ACh stimulation was investigated in tissue segments deprivated of extra- and intracellular calcium by incubation in a Ca-free solution containing EGTA and A23187 for an extended period (50 min before stimulation). In this experiment, a submaximal ACh concentration was used because an increment in ^{45}Ca efflux was more easily suppressed in response to the submaximal ACh concentration than the supramaximal concentration as shown in Figs. 1 and 2. Stimulation with ACh (1.1×10^{-7} M) in the tissue segments incubated in a Ca-free solution containing EGTA and A23187 did not cause a rise in both amylase release and ^{45}Ca efflux (Fig. 3). After 5 min in the presence of ACh stimulation, the application of $CaCl_2$ into a medium to increase Ca^{2+} concentration up to 2.56 mM, which caused a significant ($P < 0.05$) and sustained increase in amylase release from 28.5 ± 3.7 (at 5 min) to 40.9 ± 2.8 (at 10 min) u/g/min and ^{45}Ca efflux from 0.0208 ± 0.006 to 0.0267 ± 0.0013/min (at 8 min post-stimulation).

Discussion

It has been reported that digestive enzyme release and fluid secretion depend on the existence of Ca^{2+} in a medium (7-10). As described in the **Introduction**, an increase in intracellular Ca^{2+} concentration is caused both by Ca^{2+} entry from a medium and by release from intracellular stores. It was Petersen and Ueda (7) who showed the important role of Ca^{2+} entry for amylase release stimulated with ACh in rat pancreatic segments. Furthermore, an increase in $^{45}Ca^{2+}$ efflux means the release of Ca^{2+} from intracellular stores in response to agonists, and also coincides with digestive enzyme release (4, 5).

We previously showed that increases in amylase release and $^{45}Ca^{2+}$ efflux induced by a variety of agonists, except VIP and secretin which activate the cyclic-AMP system, were dose-dependent and both are well coincided in mouse pancreatic segments (11). Furthermore, amylase increment is sustained while that of $^{45}Ca^{2+}$ efflux is transient as shown in Fig. 2 and our previous report (11). The increased amylase release is sustainable because of the dependence on Ca^{2+} released from stores and entry through the plasma membrane.

The present data show that a small decrease of extracellular Ca^{2+} rarely affects Ca^{2+} release induced by ACh stimulation from intracellular stores, although Ca^{2+} decreases in both the medium and intracellular stores by incubation for an extended period in Ca^{2+}-free solution containing EGTA and A23187, completely abolished ACh-induced responses. Application of Ca^{2+} into a

medium caused a sustained amylase release, which coincides with previous results by Petersen and Ueda (7). However, Ca^{2+} efflux was also concurrently increased with amylase release. The reason for the result is not clear at present, but suggents that a part of the Ca^{2+} stored intracellularly might be exchangeable with a new portion of Ca^{2+} in the medium, or Ca^{2+} bound to digestive enzymes is released in response to ACh stimulation.

The present data show that amylase release induced by stimulation with ACh is dependent on Ca^{2+} transiently released from intracellular stores and long-lasting Ca^{2+} entry from a medium.

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